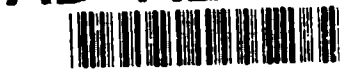
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CONTRACT NO: DAMD17-86-C-6234

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TITLE: SYNTHETIC VACCINES FOR THE CONTROL OF ARENAVIRUS INFECTIONS

PRINCIPAL INVESTIGATOR: Michael J. Buchmeier

CONTRACTING ORGANIZATION: Scripps Clinic and Research Foundation
10666 N. Torrey Pines Road
La Jolla, California 92037

REPORT DATE: March 31, 1992

TYPE OF REPORT: Annual and Final Report

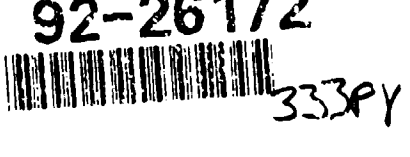
PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

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92-26172



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# REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | 31 March 1992 | Annual and Final Report |

**4. TITLE AND SUBTITLE**

Synthetic Vaccines for the Control of Arenavirus Infections

**5. FUNDING NUMBERS**

Contract No.
DAMD17-86-C-6234

62787A
3M162787A871.AB.356

**6. AUTHOR(S)**

Michael J. Buchmeier

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Scripps Clinic and Research Foundation
10666 N. Torrey Pines Road
La Jolla, California 92037

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**

U.S. Army Medical Research and Development Command
Fort Detrick
Frederick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

Annual Report covers the period of time 8/1/91 - 2/29/92;
Final Report covers the period of time 8/1/86 - 2/29/92

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for public release; distribution unlimited

**12b. DISTRIBUTION CODE**

**13. ABSTRACT** *(Maximum 200 words)*

**Arenaviruses are endemic on both the African and South American continents and represent significant public health hazards. Prophylactic immunization, precise diagnostic methods, and effective treatment protocols are not currently available. We are using genetic cloning methods to develop an effective vaccine against arenaviruses. Developmental studies have been carried out and techniques established with the prototype arenavirus, lymphocytic choriomeningitis virus (LCMV), and these methods can now be applied for rapid development and evaluation of vaccines against the pathogenic arenaviruses Lassa, Junin, and Machupo. Using techniques of peptide and immunochemistry we have identified and mapped the gene products of the L and S-RNA segments of LCMV and mapped the important immunogenic regions of the viral glycoproteins. The LCMV genomic RNAs have been cloned and primary sequences of the RNAs and their gene products are being completed.**

**14. SUBJECT TERMS**

Mice; Lab animals; RAI; BD; Arenavirus; Glycoprotein; Peptide; Vaccine; Biotechnology; C DNA; Lassa; Plasmids

**15. NUMBER OF PAGES**

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

## Summary

This contract proposal has been targeted toward development, in an experimental model of arenavirus infection, a strategy appropriate to direct development of vaccines to human arenaviruses of clinical importance. We have explored and developed several approaches, including recombinant LCMV proteins expressed in vaccinia and baculovirus vectors, anti-idiotypic vaccines, and most recently, passive humoral protection employing monoclonal antibodies. The latter strategy has proven very effective, not only in the short term goal of achieving protection against acute and persistent LCMV infection, but judicious use of these monoclonals has defined epitopes on GP-1 which should serve as important targets for active immunization. Most important is the fundamental observation that a preexisting antibody titer to GP-1 epitopes virtually guarantees resistance to lethal challenge infection. Thus the long-term goal of derivation of useful information to target specific structural determinants of LCMV for vaccine development has been achieved.



We would like to thank the Department of Defense for support of this effort and to thank Drs. Peter Jahrling, Joel Dalrymple, Tom Monath, Connie Schmaljohn and C. J. Peters for valuable collaborative and intellectual input into this project.



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## FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practives Supplements.

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## Summary Progress Report

### A. Introduction and Overview

The results described in the following summary progress report cover the five and one-half year term of this contract, 1 Aug 1986 through 29 Feb 1992 inclusive. During this time we have made substantial progress toward defining a strategy applicable to protective vaccination and immunotherapy of arenavirus infections in man. Moreover, we have defined posttranslational events in the arenavirus life cycle which are important considerations in vaccine development, and early events involved in viral entry which may prove useful targets for immuno- or pharmacological intervention. Finally, research funded by this contract has facilitated development of a working model of the structure of the viral spike which will guide future efforts to develop effective immunogens. One goal described in the original proposal proved inaccessible as the contract was structured. It was originally proposed to grow and purify viral RNA for Lassa and Mozambique viruses at USAMRIID and transport them to La Jolla for cloning and sequencing. Three trips to USAMRIID were made by Drs. Southern, Fazakerley, and myself, but none of these trips were productive for reasons beyond our control. As the efforts of Dr. Peter Jahrling were redirected toward SIV and Ebola research, by mutual agreement and consultation with Dr. Peters and Dr. Jahrling it was decided to focus on other goals. Despite this setback the project has been extraordinarily successful, resulting in 23 published articles.

Research highlights will be described in the summary progress report, and the reader is referred to the published articles or manuscripts for detailed description of the experimental design and results. These papers are listed in Appendix I and for convenience copies of each have been supplied with the report.

B. Molecular cloning of LCMV and comparison with other arenaviruses

We have completed and extended our nucleotide sequencing studies of the genomic S RNA from lymphocytic choriomeningitis virus (Armstrong strain). Coding regions within the S RNA were identified using the synthetic peptide approach and we have generated several computer comparisons between the predicted amino acid sequences for the LCMV structural proteins and other arenavirus structural proteins (Southern et al., 1987; Southern and Bishop, 1987). Conserved and divergent regions can be readily identified; the extent of conservation is significantly higher in GP-2 than in GP-1 for the sequences currently available (Pichinde, Lassa, LCM WE, LCM Arm).

We have now identified several cDNA clones from the genomic L RNA segment of LCMV. Currently, the clone nucleotide sequence information covers about 4 kb, representing approximately 50% of the L segment. We have predicted parts of the amino acid sequence for L-encoded proteins and have synthesized short peptides corresponding to these potential viral proteins. Anti-peptide antibodies have then been used in Western blotting experiments to identify a high molecular L protein that is present both in purified virions and intracellular viral RNP complexes (Singh et al., 1987). The anti-peptide antibodies have provided the first mono-specific reagents to study the distribution of L proteins during infection.

In order to understand the molecular events associated with the progression from acute to persistent arenavirus infection, we have studied the temporal relationship of LCMV replication and transcription during acute infection. At early times, NP mRNA and progeny genomic sense RNAs begin to accumulate simultaneously; the GP mRNA accumulates more slowly within the cells (Fuller-Pace and Southern, 1988). In other experiments, we have established an in vitro assay for the viral RNA-dependent RNA polymerase activity. We anticipate combining all of this information to allow systematic comparisons of intracellular RNAs, proteins and polymerase activities in acute and persistent infection to explore the molecular basis of persistent arenavirus infection.

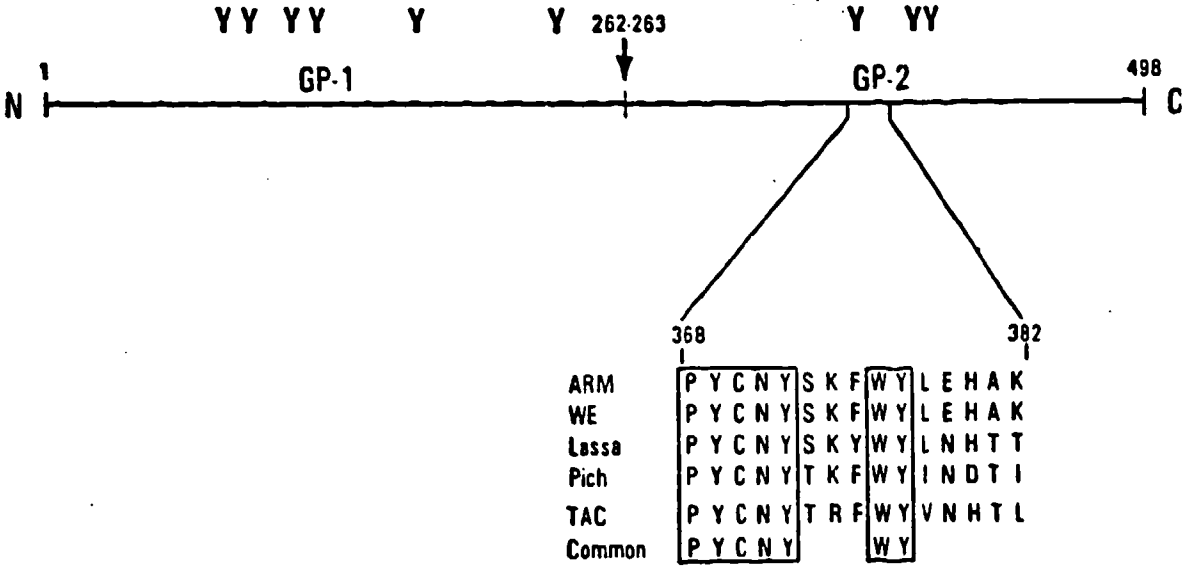
During our visit to Ft. Detrick in June 1987, Drs. Southern and Buchmeier performed RNA extractions of Lassa viral genomic RNA for purposes of molecular cloning. The extracted RNA was agarose gel purified, safety tested and when proven non-infectious was sent to California where an initial round of cDNA cloning was done. In that effort insufficient product was obtained to clone.

C. Precise mapping of antigenic determinants conserved among arenavirus glycoproteins

Toward the goal of precisely identifying antigenic determinants conserved among arenaviruses, we have localized and characterized a major antigenic site on the GP-2 glycoprotein of LCMV (Weber

and Buchmeier, 1987). Lymphocytic choriomeningitis virus S RNA encodes an intracellular precursor glycoprotein GP-C (76k) which is posttranslationally cleaved to yield the structural glycoproteins GP-1 (44k) and GP-2 (35k). Previous work from this laboratory has demonstrated that the GP-1 bears a single conformationally sensitive epitope against which neutralizing monoclonal and polyclonal antibodies are directed. GP-2, in contrast, contains an immunodominant epitope which is resistant to denaturation and a subset of antibodies to this epitope react broadly with heterologous arenaviruses of both the Old World (LCM, Lassa, Mopeia) and New World (Tacaribe complex) subgroups. We have mapped, to the level of amino acid sequence, the conserved epitope on GP-2 and find immunoreactivity of poly- and monoclnoal antibodies against this protein confined to a synthetic peptide representing 13 amino acids spanning residues 370-382 of GP-C. Specificity was demonstrated by direct ELISA binding assays to the synthetic peptide, and by inhibition of binding of monoclonal and polyclonal antibodies to both naitve and denatured GP-2 by peptide. Comparisons of GP-C sequences of LCMV, Pichinde and Lassa viruses (Fig. 1) revealed a high degree of homology among these viruses in residues 370-377 toward the amino terminus of the peptide wherein 6 of 8 amino acids are identical among these viruses. In contrast the 5 carboxyl terminal amino acids of this peptide are not conserved. The epitope appears to be of biological significance since polyclonal antisera against LCMV, Junin, and Lassa viruses all react with peptide 370-382.

Fig. 1 Identification of a Conserved Antigenic Site on LCMV GP-2




We have completed fine mapping of this conserved GP-2 epitope. Briefly, the binding site of promiscuous GP-2 specific monoclonals (33.6 and 83.6), that is, those which react broadly with all arenaviruses, as well as MAb 9-7.9 which reacts with

LCMV and Mopeia virus only, have been mapped to a stretch of 9 amino acids spanning residues 370-378 of GP-C (Fig. 2):

Fig. 2

| | 370 | | | | | | | 378 |
|---|---|---|---|---|---|---|---|---|
| LCM | Cys | Asn | Tyr | Ser | Lys | Phe | Trp | Tyr | Leu |
| Lassa | . | . | . | . | . | Tyr | . | . | . |
| Pic | . | . | . | Thr | . | . | . | . | . |
| Tac | . | . | . | Thr | Arg | . | . | . | Tyr |

As is evident from the sequences shown, this region is highly conserved across both Old and New World arenaviruses. From assays of polyclonal human and experimental animal antisera we have found that the same epitope accounts for up to 70% of the reactivity of a polyclonal antiserum against GP-2. LCM convalescent hyperimmune guinea pig and Junin late convalescent human sera were assayed for binding activity against GP-2 by Western blotting. We found that preincubation with the synthetic peptide in solution reduced by more than 70% the binding of these polyclonal sera against GP-2.

The molecular basis of limited specificity of MAb 9-7.9 was also elucidated. This MAb, which recognizes LCMV and Mopeia but not Lassa virus, was assayed for reactivity against the following peptide sequences corresponding to LCMV and Lassa respectively:

Fig. 3

| | 370 | 382 |
|---|---|---|
| LCM | CNYSKFWYLEHAK | |
| Lassa | CNYSKYWYLEHAK | |

While the broadly reactive MAb 33.6 reacted with both sequences equally well, 9-7.9 reacted only with the LCM sequence (EIA titer of >1:10,000 vs <1:160). Thus substitution of Phe 375 with Tyr rendered the antibody nonreactive. These findings demonstrate the molecular identity of a native epitope on the LCMV GP-2 glycoprotein and the precise nature of the sequence difference between LCMV and Lassa conferring species specificity to the 9-7.9 monoclonal. Furthermore the existence of two epitopes within a 9 amino acid segment is proven.

D. Identification of the neutralizing antigenic site on LCMV

We focused our attention on efforts to map the major neutralizing domain of LCMV-GP-1 glycoprotein using neutralization escape mutants (Wright, Salvato and Buchmeier, 1989). Two selections for escape mutants were done using the monoclonal antibody 2-11.10 (epitope GP-1D) (Parekh and Buchmeier, 1986). The results of those selections are summarized in Table 1 below.

Table 1

Selection of Antigenic Variants with Monoclonal 2-11.10: Reactivity of Selected Strains with LCMV Monoclonals

| Monoclonal (epitope) | Reactivity[a] Positive/Total Expt. 1 | Expt. 2 |
|---|---|---|
| 2-11.10 (GP-1D) | 7/14 | 4/9 |
| WE 6.4 (GP-1A) | 14/14 | 9/9 |
| 9-7.5 (GP-2B) | 14/14 | 9/9 |
| 1-1.3 (NP-A) | 14/14 | 9/9 |

[a]Reactivity with GP-1 specific antibodies was judged by ELISA, indirect immunofluorescence and by virus neutralization. Antibodies to GP-2 and NP were evaluated by ELISA and by indirect immunofluorescence.

It is evident from this selection that approximately 40-50% of the plaques selected were true escape mutants with regard to MAb 2-11.10, but showed no difference in binding of MAb WE 6.2. Other antibodies to the GP-1A site, including WE197.1, WE258.4 and WE36.1, behaved like WE 6.2 in their recognition of these strains. Four strains, two escape mutants (2-11.10 negative) and two representing the wild type phenotype (2-11.10 positive) were selected for detailed mapping studies. These strains and their 2-11.10 binding characteristics are summarized in Table 2 below.

Table 2

Antibody Binding Profiles of Four Strains of LCMV-Armstrong

| Strains Isolate | Reactivity[a] with MAb 1-1.3 (NP) | 33.6 (GP-2) | WE258.4 (GP-1A) | 2-11.10 (GP-1D) |
|---|---|---|---|---|
| ARM-3 | + | + | + | - |
| ARM-4 | + | + | + | + |
| ARM-5 | + | + | + | - |
| ARM-10 | + | + | + | + |

[a]Reactivity in ELISA and indirect immunofluorescence assays.

These strains were subjected to direct RNA sequencing by the dideoxy chain termination method of Sanger, and mutation was observed at a single codon (nucleotides 594-596) of the GP-C precursor corresponding to amino acid 173 of the GP-1 posttranslational product. This mutation is detailed below in Table 3.

Table 3

Sequence Comparison and Antibody Reactivity of LCMV Arm Isolates

| LCMV Isolate | GP-C Sequence[a] nucleotides 582-620 amino acids 169-181 | Epitope[b] A | Epitope[b] D |
|---|---|---|---|
| ARM-5 | CAATACAACTTGACATTCTCAGATCGACAAAGTGCTCAG<br>GlnTyrAsnLeuThrPheSerAspArgGlnSerAlaGln | + | - |
| ARM-4 | GCA<br>----------Ala---------- | + | + |
| ARM-3 | ACA<br>----------Thr---------- | + | - |
| ARM-10 | AAA<br>----------Lys---------- | + | + |

[a]Sequences were determined by primer extension.
[b]Reactivity with MAbs to epitope A (MAbs 197-1 and 6.2) and epitope D (2-11.10) is scored. + indicates a positive reaction.

These results established that mutants which contained the sequence Asn-Leu-Thr at amino acids 171-173 of GP-1 failed to be recognized by MAb 2-11.10, suggesting that the mechanism of escape was insertion of a glycosylation site of the form (Asn x Ser/Thr) at this position. We therefore compared glycosylation of these strains and found that as predicted, the MAb 2-11.10 resistant strains carried an additional sixth oligosaccharide chain on GP-1 relative to those strains which bound 2-11.10. Moreover, we observed heterogeniety at position 173 between the antibody binding strains ARM-4 and ARM-10. The former had the sequence Asn-Leu-Ala at this position, while the latter was Asn-Leu-Lys, suggesting that limited heterogeniety was tolerable. Neither of these isolates showed any increased or decreased avidity of binding of MAb 2-11.10.

Further experiments were done to define the role of glycosylation and disulfide bond formation in the structure of this important epitope, and these results are described in detail in a manuscript publication (Wright, Salvato and Buchmeier, 1989).

Briefly, we found that the major neutralizing epitope was conformational in nature, that it required native protein folding for expression, and that this folding required prior N-linked glycosylation of the GP-C polypeptide chain and intrachain disulfide bond formation. The epitope was expressed on monomeric (n = 1) and homopolymeric (n = 1-4) forms of GP-1 but was destroyed by heating and by reducing agents.

Based on this data we attempted to synthesize the GP-1D epitope chemically with limited success. It was reasoned that a peptide containing the 171-173 sequence and spanning the adjacent flanking cysteine residues might form a hairpin or loop structure containing the GP-1D epitope. To this end three peptides were synthesized as shown below:

| | | |
|---|---|---|
| GP-C peptide | 160-185 | CDFNNGITIQYNLAFSDEQSAQSQC |
| | 170-185 | YNLAFSDEQSAQSQC |
| | 176-185 | DEQSAQSQC |

These peptides were used to coat ELISA plates and tested for reactivity with a large panel of 22 mouse and rat monoclonals to LCMV as well as normal and immune guinea pig antisera to LCMV. Binding was observed as described in Table 4 below.

Table 4

| Antibody | Binding to Substrate Containing Native Virus | 160-185 | 170-185 | 176-185 |
|---|---|---|---|---|
| 2-11.10 | >1:10,000 | 1:160 | <1:10 | <1:10 |
| WE 258.4 | 1:5,600 | 1:80 | <1:10 | <1:10 |
| eE 197.4 | ND | 1:160 | <1:10 | <1:10 |
| WE 36.1 | ND | <1:10 | <1:10 | <1:10 |
| WE 6.2 | ND | 1:40 | <1:10 | <1:10 |
| WE 327.3 | ND | 1:160 | <1:10 | <1:10 |
| WE 18.7 | ND | <1:10 | <1:10 | <1:10 |
| WE 67.8 | ND | <1:10 | <1:10 | <1:10 |
| Rat MAb #8-12 | ND | <1:10 | <1:10 | <1:10 |
| #8-13 | ND | <1:10 | <1:10 | <1:10 |
| #8-14 | ND | <1:10 | <1:10 | <1:10 |
| #8-21 | ND | <1:10 | <1:10 | <1:10 |
| #8-22 | ND | <1:10 | <1:10 | <1:10 |
| #8-24 | ND | <1:10 | <1:10 | <1:10 |
| #8-25 | ND | 1:20 | <1:10 | <1:10 |
| #8-26 | ND | <1:10 | <1:10 | <1:10 |
| #8-29 | ND | <1:10 | <1:10 | <1:10 |
| #8-32 | ND | 1:20 | <1:10 | <1:10 |
| #8-35 | ND | <1:10 | <1:10 | <1:10 |
| #8-40 | ND | <1:10 | <1:10 | <1:10 |
| #8-50 | ND | <1:10 | <1:10 | <1:10 |
| #8-55 | ND | <1:10 | <1:10 | <1:10 |
| Normal guinea pig | <1:20 | 1:20 | <1:10 | <1:10 |
| Guinea pig immune serum | ND | 1:20 | <1:10 | <1:10 |

ND = not determined. Qualitative determinations by indirect immunofluorescence established titers of >1:100.

The results suggested very low avidity specific binding of the GP-1 antibodies to peptide 160-185, however the titer was extremely low in proportion to the titer of the same monoclonals against native virus, hence this approach was not aggressively pursued pending availability of pertinent structural details of the GP-1 molecule to allow a more accurate prediction to be made.

E. In vivo protective efficacy of Vaccinia constructs containing LCMV NP and GP-C genes

We have obtained Vaccinia vectors containing various constructions of the LCMV GP-C and NP genes and have performed two pilot experiments to test their protective efficacy in guinea pigs against an LCMV WE challenge inoculation. The results of these experiments are summarized in Table 5.

Table 5

Protective Efficacy of vaccinia LCMV Constructs Against LCMV-WE Challenge in Guinea Pigs

Experiment 1

| Immunize with | N | Challenge | Mortality |
|---|---|---|---|
| LCMV Arm ($10^4$ pfu ip) | 3 | WE ($10^4$ pfu) | 0/3 |
| $VV_{SC11}$ (without insert) ($10^8$ pfu Id) | 3 | WE | 3/3 |
| $VV_{B5}$ (full length WE, GP-C insert) ($10^8$ pfu Id) | 8 | WE | 8/8 |

Experiment 2

| Immunize with | N | Challenge | Mortality |
|---|---|---|---|
| LCMV Arm ($10^4$ pfu ip) | 2 | WE | 0/2 |
| $VV_{SC11}$ | 3 | WE | 3/3 |
| $VV_{NP}$ (full length NP) ($10^8$ pfu Id) | 8 | WE | 8/8 |
| $VV_{NP}$ & $VV_{GP}$ ($5 \times 10^7$ pfu each Id) | 4 | WE | 4/4 |

In neither case did we see evidence of significant protection against in vivo challenge with WE.

The results of these experiments were disappointing to us and forced a reconsideration of our vaccinia approaches. We examined the expression of LCMV GP-C by these constructs in connection with studies of protein transport and folding. These results are described below (Sections F, P).

F. Detailed studies of the biosynthesis folding, transport and intracellular processing of GP-C to GP-1 and GP-2.

Two lines of evidence suggested to us that a full understanding of the intracellular events in biosynthesis and processing of GP-C to form GP-1 and GP-2 was an important short-term goal of this project. First, as described above, native folding of the GP-C polypeptide chain as indicated by acquisition of reactivity with conformation dependent neutralizing monoclonal antibodies requires both prior glycosylation and intrachain disulfide bond formation. This observation must be taken into account in the design of any vaccine which is intended to mimic the relevant native epitope. Second, in an attempt to use the baculovirus expression system to produce glycoprotein for study, we obtained two vectors containing the full length LCMV NP and GP-C genes from Dr. David Bishop, NERC Insect Virus Unit, Oxford, U.K. These viruses, termed YON (NP) and YOG (GP-C), were grown in Spodoptera frugiperda cells and lysates prepared at the time of maximum CPE. These lysates were coated on microtiter wells in a standard ELISA format and several LCMV monoclonals were assayed for reactivity. The results of this assay are shown in Table 6 below:

Table 6

Reactivity of LCMV Monoclonal Antibodies with NP and GP-C Expressed by Baculovirus

| Antibody | Specificity | Titer vs. Substrate[a] YON (NP) | YOG (GP-C) |
|---|---|---|---|
| Guinea pig anti LCMV | GP+NP | >1:10,000 | 1:1000 |
| 1-1.3 | NP | >1:10,000 | <1:10 |
| WE258.4 | GP-1 | <1:10 | <1:10 |
| WE6.2 | GP-1 | <1:10 | <1:10 |
| WE33.6 | GP-2 | <1:10 | 1:100 |
| 9-7.9 | GP-2 | <1:10 | 1:100 |

[a]Substrate was 2 ug protein/well of Spodoptera lysate from cells infected with the indicated vector. Titer expressed as the highest dilution of antiserum scoring 2 times the background optical density.

These vectors clearly directed the synthesis of GP-C and NP, however neither of the conformation dependent, neutralizing MAb to site GP-1A recognized those proteins produced in insect cells. Moreover, Bishop has observed that the YOG-infected cells accumulate the GP-C polypeptide in the endoplasmic reticulum with little or no transport to the plasma membrane, again suggesting a defect in folding. Antisera produced in Bishop's laboratory by immunization of rabbits with YOG or YON lysates failed to recognize native GP-1 and did not neutralize viral infectivity. These results taken together suggest a defect in folding or processing of GP-C in the insect cell. We therefore sought to define the normal sequence of events in biosynthesis and processing of GP-C (Wright et al., 1990).

We first used a series of glycosylation inhibitors to define the minimal glycosyl chain structure required for proper folding of the chain to express the 2-11.10 epitope. We found that fully deglycosylated GP-C and GP-1 produced either by digestion of virions with N-glycanase (which removes complex sugar side chains) or by growth in the presence of tunicamycin (which blocks the en-bloc addition of the initial high mannose sugar chain to the growing polypeptide chain) were non-reactive with neutralizing antibodies. In contrast, addition of the most elementary high mannose oligosaccharide chain as expressed in the presence of the inhibitors castantospermine or 1-NM-deoxynojirimycin was sufficient to allow appropriate folding and acquisition of conformational epitopes. Significantly, intracellular transport via the normal secretory pathways was also dependent upon glycosylation.

By pulse chase and temperature blocking studies we showed that terminal glycosylation occurs in the medial to trans-golgi and precedes cleavage of GP-C at the GP-1/GP-2 junction.

G. Mechanism and Specificity of Antibody Mediated Protection from Acute LCMV Disease

It is highly desirable in the design of a vaccine to have prior knowledge of the protective mechanisms operative in acquired resistance. In the case of arenavirus disease, anecdotal evidence suggested that the protective role of humoral antibody had been largely underestimated. Therefore we investigated the mechanism of monoclonal antibody (MAb) mediated protection agaist LCMV-induced acute CNS disease. The basic protocol was to passively administer the MAb under study intraperitoneally from 1 day before to 2 days after intracerebral (ic) challenge with 500 pfu (ca. >1,000 $LD_{50}$) of LCMV-Arm. Virgin mice challenged in this way always died by the sixth to seventh day post-infection. Table 7 illustrates the experimental data for *in vivo* protective effect of antibody in acute LCM disease. Note from the data that 3 MAbs, 2-11.10, 258.2 and 67.2, protected mice against lethal virus challenge. All were GP-1 specific, but significantly, only two of these MAb neutralize virus *in vitro*. MAb 2-11.10 was effective when administered as late as two days (20% mortality) after infection, indicating an inhibition of spread of virus

infection. Table 8 illustrates the observation that protected mice show 2 log or greater reductions in virus burden in their brains, and do not become virus carriers, as indicated by clearance at days 14 and 30. Table 9 illustrates that relative to their non-antibody treated cohorts, the antibody protected mice showed lower levels of CTL activity, which may account for lower inflammatory responses and survival of these mice.

These findings are important in that they establish that a pre-existing humoral antibody to LCM virus is protective, and that epitopes on GP-1 are crucial. Protection did not require *in vitro* virus neutralizing activity however. The latter finding may be particularly important since the presence of neutralizing antibody to Lassa virus in convalescent patients and animals is difficult to demonstrate.

Table 7

Protection against intracranial challenge with LCMV by transfer of monoclonal antibodies

| MAb | Specificity | ELISA | Neut. Titre | Day of Transfer | %Mortality (n) |
|---|---|---|---|---|---|
| 211.10 | GP-1 | 204,000 | + | -1,0 | 4 (24) |
| 258.2 | GP-1 | 12,800 | + | -1,0 | 24 (15) |
| 67.2. | GP-1 | 3,200 | - | -1,0 | 0 (11) |
| 9-7.9 | GP-2 | 800 | - | -1,0 | 90 (10) |
| 1-1.3/ 10-7.5 | NP | 204,000 | - | -1,0 | 94 (16) |
| MHV MAb | - | | - | -1,0 | 90 (18) |
| 211.10 | GP-1 | | + | 0 | 0 ( 5) |
| | | | | +1 | 0 (10) |
| | | | | +2 | 20 ( 5) |
| | | | | +3 | 80 ( 5) |
| Saline | | | - | 0 | 88 ( 9) |

Female 4-6 week old Balb/c ByJ mice were given 0.2 ml ascites intraperitoneally (i.p.) on the specified days, where day 0 is the day of challenge with 500 PFU LCMV, strain Armstrong intracranially (i.e.). All mice were observed for at least 30 days. 500 PFU is equal to 263 LD50 of the Arm stock used in these experiments.

Table 8

Virus clearance from brains of mice challenged with LCMV i.c.

| MAb | log10 PFU per g brain | | | | |
|---|---|---|---|---|---|
| | day 1 | day 4 | day 6/7 | day 14 | day 30 |
| Expt 1 | | | | | |
| 2.11.10 | <2.0 | <2.0 | n.d. | <2.0 | <2.0 |
| | <2.0 | <2.0 | | <2.0 | <2.0 |
| | <2.0 | 4.3 | | | <2.0 |
| | <2.0 | 4.3 | | | <2.0 |
| | <2.0 | 3.2 | | | |
| 10-7.5 | <2.0 | 7.4 | n.d. | | |
| | 4.0 | 5.8 | | | |
| | 3.5 | 6.0 | | | |
| | 4.5 | 5.7 | | | |
| | 4.3 | 6.3 | | | |
| Expt 2 | | | | | |
| 2.11.10 | <2.0 | 4.4 | <2.0 | | |
| | <2.0 | 4.7 | <2.0 | | |
| | <2.0 | 4.8 | <2.0 | | |
| | <2.0 | 4.9 | <2.0 | | |
| | <2.0 | 5.1 | 4.5 | | |
| 9-7.9 | <2.0 | 6.9 | <2.0 | | |
| | <2.0 | 6.9 | <2.0 | | |
| | <2.0 | 6.4 | 6.6 | | |
| | <2.0 | 5.8 | 6.4 | | |
| | <2.0 | 5.4 | 3.7 | | |

Groups of 4-6-week old female Balb/c mice were given 0.2 ml ascites intraperitoneally, then challenged with 1000 pfu (Expt 1) or 500 pfu (Expt 2) i.c. 24 hrs later. Brains were collected at specified times, frozen and assayed for infectious virus on Vero cell monolayers. Unprotected mice were usually dead by day 7.

Table 9

Cytotoxic T-cell responses to LCMV in protected mice

| Effector | | Percent $^{51}$Chromium Release $H\text{-}2^d$-LCMV | $H\text{-}2^d$ Mock | $H\text{-}2^b$-LCMV | |
|---|---|---|---|---|---|
| Day 7-i.p. | | 68.5 | 0 | 4.0 | |
| Day 7-i.c. | 83.6 | 52.8 | 1.3 | 0.9 | Not protected |
| Day 7-i.c. | 67.2 | 16.0 | 1.2 | 0 | |
| Day 9-i.c. | 67.2 | 16.9 | 0 | 0.4 | |
| Day 11-i.c. | 67.2 | 12.8 | 1.8 | 0 | Protected |
| Day 14-i.c. | 67.2 | 3.1 | 0.9 | 0 | |
| H-2b Control | | - | - | 66.2 | |

Cytotoxic activity was measured in a $^{51}$Chromium release assay in splenocytes, collected from mice given MAb and infected i.c. $^{51}$Chromium labelled targets were: $H\text{-}2^d$ - Balb/c C17, $H\text{-}2^b$ - MC57/MEF.

H. Virus Binding Assay

It is of interest to identify the protective epitopes recognized by MAb to GP-1. Therefore we initiated studies of the mechanism of virus neutralization and attempted to identify these epitopes at the molecular level. Since we have already mapped the 2-11.10 epitope to amino acid 173 of GP-1, we focused on 1) the biological activity of these MAbs in binding inhibition, and 2) mapping the sequential (peptide) epitope 67.2 (section I below).

A binding assay was established to study the *in vitro* inhibitory effect of antibody on LCMV replication *in vitro* and *in vivo*. Binding of radiolabeled ($^{35}$S) LCMV showed saturation kinetics at both $4^o$ and $37^o$ with approximately 3-fold more virus bound at $37^o$, suggesting that internalization follows rapidly at $37^o$ (Fig. 4). In the presence of 0.1% sodium azide, an inhibitor of cellular energy production, an intermediate level between the $4^o$ and $37^o$ saturation curves was observed. The full library of MAbs against GP-1 of LCMV was tested for ability to inhibit this virus binding (Table 10), and it was noted that the most efficient inhibition of binding was achieved with MAbs 36.1, 67.2 and 67.5 (sister clones) and 2-11.10. The latter two MAbs were shown previously to be protective if administered passively to mice either prior to or after LCMV infection. Thus epitopes have been defined which elicit antibody which inhibits virus-cell interaction. These correspond in some cases (e.g. 2-11.10 and WE 67.2) to protective epitopes and as such are logical targets for vaccination.

Fig. 4. Binding curves of radiolabeled LCMV-Arm 4 to Vero cells.

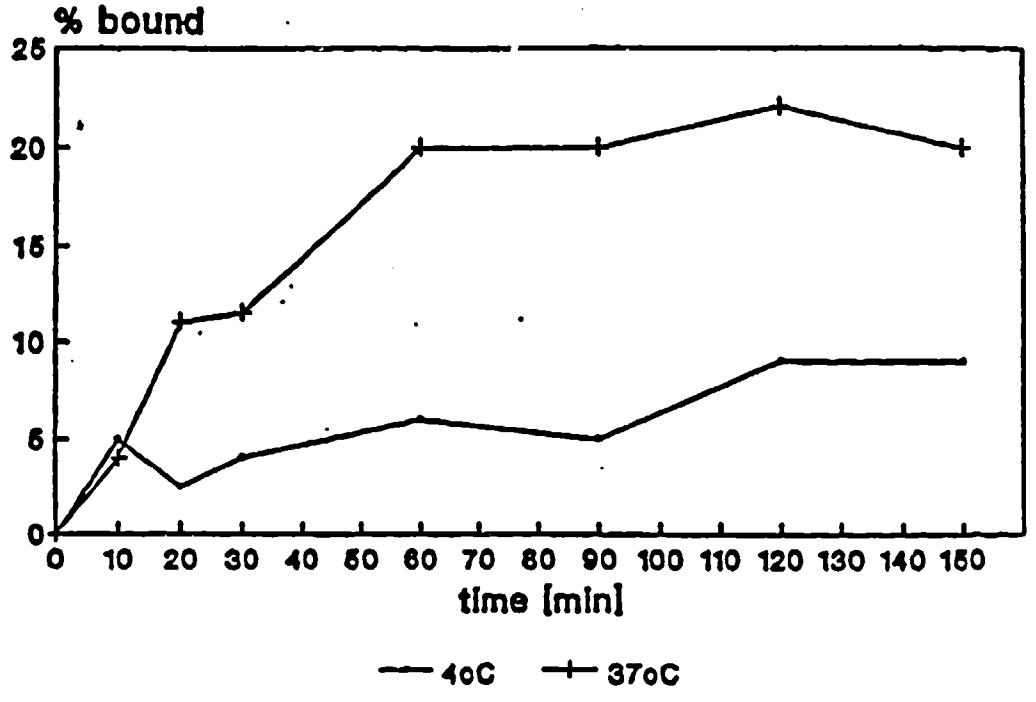



Table 10

**Effects of monoclonal antibody or polyclonal antisera on the binding of labeled LCMV-Arm 4 to Vero cells.**

| | | | Inhibition | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 37°C | | | 4°C | | |
| MAb | Epitope | Neutral. | 1:50 | 1:100 | 1:1000 | 1:50 | 1:100 | 1:1000 |
| WE-36.1 | GP-1A | + | ++ | ++ | + | +++ | ++ | + |
| WE-258.4 | GP-1A | + | - | - | - | - | - | - |
| WE-197.1 | GP-1A | + | - | - | - | - | - | - |
| WE-6.2 | GP-1A | + | - | - | - | - | - | - |
| WE-40 | GP-1A | + (WE) | + | + | - | + | - | - |
| WE-327.3 | GP-1B | - | - | - | - | - | - | - |
| WE-2.9 | GP-1B | - | - | - | - | - | - | - |
| WE-18.8 | GP-1C | - | - | - | - | - | - | - |
| WE-67.5 | GP-1C | - | ++ | + | - | ++ | + | - |
| WE-67.2 | GP-1C | - | ++ | + | - | ++ | + | + |
| 2.11.10 | GP-1D | + (Arm) | ++ | + | - | ++ | + | + |
| WE-33.6 | GP-2A | - | + | - | - | + | + | - |
| WE-83.6 | GP-2A | - | + | - | - | - | - | - |
| Arm 9.7.9 | GP-2B | - | + | - | - | + | - | - |
| WE 1-1.3 | NP | - | - | - | - | - | - | - |
| MHV 5B11.5 | - | - | - | - | - | - | - | - |
| Polyclonal | | | | | | | | |
| Guinea pig | LCMV | + | + | + | - | + | - | + |
| Guinea pig | normal | - | - | - | - | - | - | - |

Note: +++ inhibition between 100-75 %; ++ inhibition between 75-50%; + inhibition between 50-25% and - inhibition between 25-0%.

## I. Mapping of Peptide Epitopes on GP-C

In a continuation of our efforts to map and characterize the topography of GP-C and its cleavage products GP-1 and GP-2, synthetic peptides derived from the complete sequence of the GP-C precursor were made and characterized (Fig. 5). We screened the GP-1 peptide sequences against our panel of MAb to GP-1 by ELISA, and the binding patterns of these antibodies against the peptides are illustrated in Table 11. Note that one peptide in particular, 201-216, gave very high background binding of several monoclonals as indicated in matrix scores of 5-9 or 10 (asterisk). Furthermore, some monoclonals such as 9-7.9 gave high binding scores against several peptides in spite of the fact that this antibody recognizes a GP-2 sequence (GP-C 370-382) (Weber and Buchmeier, 1988). On the basis of these observations and other inconsistencies in the results, it was decided that this approach was not sufficiently reliable to map epitopic sites.

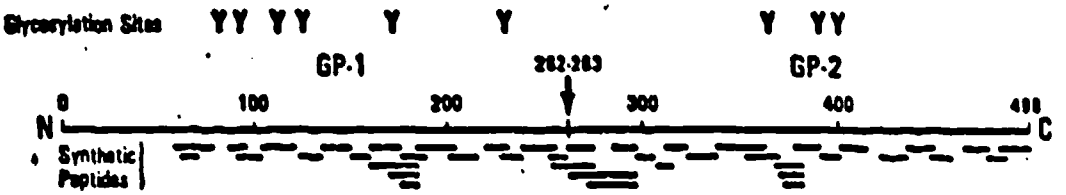
## J. Macromolecular Structure of the Glycoprotein Spike

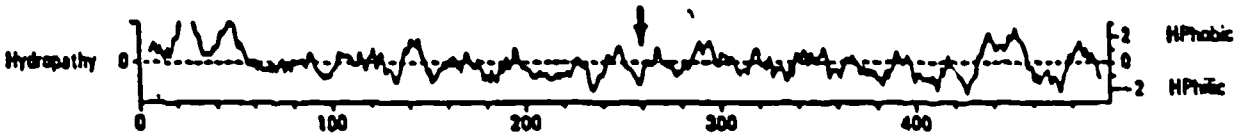
Dr. John Burns, a molecular virologist experienced in protein chemistry, joined my laboratory group to continue work on the structure of the arenavirus spike. Dr. Burns has performed experiments to look in more detail at the biosynthesis and folding of the LCMV glycoproteins as a model for Lassa. We feel that this comparison is justified in view of the overall high degree amino acid similarity (>77%) between the glycoproteins of these two viruses, and their similar biology (Fig. 6). Dr. Burns has performed two kinds of experiments. The first was precisely timed pulse chase studies to define the time required for synthesis and processing of GP-C to GP-1 and GP-2. By pulse labeling for 5 minutes and chasing for various intervals, it was shown that 75 minutes elapsed between GP-C biosynthesis and proteolytic cleavage. By inhibiting intracellular transport at low temperatures (16$^{o}$C, 25$^{o}$C), it was shown that cleavage occurred in the Golgi apparatus. These results, as well as the results of other transport studies, were reported in Virology (Wright et al., 1990).

## K. N-terminal Sequences of GP-1 and GP-2 of LCMV and Other Arenaviruses

In order to better define the GP-1 and GP-2 structural proteins more accurately, we utilized the algorithm of von Heijne to predict the most likely sites of signal peptide cleavage for the arenaviruses LCMV, Lassa, Pichinde and Tacaribe, for which sequence is available. Table 12 illustrates the most likely cleavage sites and their von Heijne scores. Note that in each case a long signal sequence of ca. 58 amino acids is predicted at the amino terminus of GP-1. We have utilized direct protein microsequencing to confirm this result and to identify the N-terminal residue of GP-2 for LCMV (Fig. 7). The sequence MYGLK at the N-terminus of GP-1 corresponds to residues 59-63 of the GP-C open reading frame, precisely where cleavage was predicted

Fig. 5. **SYNTHETIC PEPTIDES FROM LCMV GP-C SEQUENCE**




LCMV - Peptides

| | GP-C | Sequence |
|---|---|---|
| GP-1 | 59-79(83B) | MYGLKGPDIYKGVYQFKSVEF |
| | 62-71 | LKGPDIYKGV |
| | 87-96 | TMPNACSANN |
| | 92-104 | CSANNSHNYISMG |
| | 104-121 | (CGG)TSGLELTFTNDSIISHN |
| | 123-133 | CNLTSAFNKTFD |
| | 125-145(84B) | LTSAFNKTFDHTLMSIVSSV |
| | 135-150 | (CGG)DHTLMSIVSSLHLSIR |
| | 150-160 | RGNSNYKAVSC |
| | 160-176 | CDFNNGITIQYNLTFSD |
| | 160-185 | CDFNNGITIQYNLAFSDEQSAQSQC |
| | 170-184 | YNLAFSDEQSAQSQC |
| | 176-184 | DEQSAWSQC |
| | 176-189 | DAQSAQSQCRTFRG |
| | 184-205 | CRTFRGRVLDMFRTAFGGKYMR |
| | 206-216 | GGKYMRSGWGWTGSDGK |
| | 220-232 | CSQTSYQYLIIQN |
| | 228-239(H21B) | LIIQNGTWENHC |
| | 239-256 | CTYAGPFGMSRILLSQEK |
| | 253-262 | SQEKTKFFTR |
| | 254-275 | SQEKTKFTRRLAGTFTWTLSDS |
| GP-2 | 262-275 | RRLAGTFTWTLSDS |
| | 272-283(H22B) | LSDSSGVENPGGYC |
| | 272-285 | |
| | 285-297 | CLTKWMILAAELK |
| | 297-306 | CFGNTAVAK |
| | 307-315 | CNVNHDAEF |
| | 312-332(47B) | DAEFCDHLRLID |
| | 323-338 | (CGG)DYNKAALSKFKEDVES |
| | 338-363 | (CGG)SALHLFKTTVNSLISD |
| | 353-370 | DQLLMRNHLRDLMGVPYC |
| | 368-382 | PYCNYSKFWYLEHAK |
| | 370-382 | CNYSKFWYLEHAK |
| | 378-391(48B) | LEHAKTGETSVPKC |
| | 391-401 | CWLVTNGSYLN |
| | 401-415 | (CGG)NEIKFSDQIEQEADN |
| | 422-435 | (CGG)RKDYIKRQGSTPLA |
| | 436-450 | (CGG)ALMDLLMFSTSAYLIS |
| | 448-458 | LVSIFLHLVKIGGC |
| | 465-478 | (C)KGCSCPKPHRLTNK |
| | 477-487 | NKGICSCGAFK |
| | 483-498 | CGAFKVPGVKTIWKRR |

Table 11

Results of comparison of monoclonal antibodies versus synthetic peptides following ELISA

| GP-C | Blank - | WE 36.1 | WE 258.4 | WE 197.1 | WE 6.2 | WE 40 | WE 327.3 | WE 2.9 | WE 18.8 | WE 67.5 | WE 67.2 | 2.11.10 | WE 33.6 | WE 83.6 | Arm 9.7.9 | WE 2.9 | WE 258.4 (5 ul) | WE 327.3 (5 ul) | WE 1.1.3 | G.P. LCMV |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ANTIBODY | | | | | | | | | | | | | | | | | | | |
| 59-79 | 0 | 2 | 2 | 0 | 0 | 3 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | * |
| 62-71a | 0 | 0 | 2 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 62-71b | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| 87-96 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| 92-104 | 0 | 0 | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 0 |
| 104-121 | 0 | 0 | 9 | 1 | 1 | 1 | * | 1 | 1 | 1 | 2 | 3 | 1 | 0 | 4 | 0 | 2 | 3 | 0 | 5 |
| 123-135 | 0 | 0 | 4 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| 135-150 | 0 | 0 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 2 |
| 150-160 | 0 | 0 | 2 | 0 | 0 | 0 | 4 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| 160-185 | 0 | 0 | 5 | 1 | 0 | 0 | 6 | 0 | 1 | 0 | 1 | 2 | 1 | 1 | 0 | 0 | 2 | 3 | 0 | 3 |
| 170-184 | 0 | 0 | 3 | 1 | 0 | 0 | 4 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| 176-184 | 0 | 0 | 3 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 1 | 1 | 0 | 0 |
| 176-189 | 0 | 0 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 1 | 1 | 1 | * |
| 184-205 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 4 |
| 201-216 | 0 | 0 | * | 1 | 1 | 7 | * | * | 0 | 0 | 4 | 1 | 5 | 9 | * | * | * | 9 | 1 | * |
| 220-232 | 0 | 0 | 2 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 1 | 1 | 1 | 2 |
| 228-239 | 0 | 0 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 | 3 | 2 | 0 | 2 |
| 239-256 | 0 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 1 |
| 253-262 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| 254-275 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 3 |
| 262-275 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 2 | 1 | 0 | 3 |
| 272-285 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Blank | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 370-382 | - | - | - | - | - | - | - | - | - | - | 0 | 0 | 9 | 9 | 9 | - | 0 | 0 | 0 | - |

Note: The original optical density scale from 0 to 2.0 is transferred in to matrix scale from 0 to * (10), thus a score of 1 corresponds to an OD of 0.2, etc.

by the algorithm. The GP-2 sequence GTFTWT matched residues 266-271 of GP-C and was two residues to the carboxyl side of the predicted ARG-ARG cleavage recognition site. Building upon this result, we have performed direct amino acid sequencing of the N termini of GP-1 and GP-2 of LCMV and several other arenaviruses in order to identify the limits of these proteins and to understand their post-translational processing in the infected cell (Burns, Milligan and Buchmeier, J. Virol., submitted). Viruses were highly purified, and their polypeptides separated on a high resolution 5-15% gradient gel. Bands were transferred to nitrocellulose, stained with Ponceau and eluted for sequencing in a gas phase ABI microsequenator. Two runs each of NP, GP-1 and GP-2 of LCMV-Arm were made. NP yielded no sequence due to N terminal blocking of that polypeptide, a common problem due to acylation or amidation of the N-terminal amino acid, making it uncleavable by Sanger's reagent. GP-1 and GP-2 sequences were more informative, consistently yielding 5 and 6 residues respectively. Both sequences were quite clean and unequivocally matched segments of the predicted cDNA sequence of GP-C. GP-1 had an N-terminal sequence of MYGLK in both runs, matching exactly with residues 59-63 of GP-C. GP-2 had a sequence of GTFTWT, matching residues 266-271 of GP-C, just two residues toward the C terminus from the -RR sequence previously predicted by us as the recognition site for GP-C -> GP-1 + GP-2 proteolysis (Buchmeier et al., 1987). Similar results were obtained with the New World arenaviruses Pichinde and Tacaribe (Fig. 8), unequivocally locating the N termini of GP-2 in both viruses and of GP-1 in Pichinde.

Several observations can be made regarding these data. First, LCMV and Lassa show very similar sequences in amino acids 1-58 but begin to diverge substantially from 59-67, suggesting sequence differences in the free amino termini of each species GP-1. Secondly, both viruses have a long, substantially hydrophobic N terminal sequence covering residues 1-59. This is an extraordinarily long signal, and raised the possibility of a functional role for this N-terminal stretch of amino acids in anchoring and/or directing the transport of GP-C through the membrane secretory pathway. In order to address that question, we are preparing antisera to peptides in the signal sequence to track the intracellular processing and transport of this molecule through the secretory pathway. This data will be necessary to rationally approach <u>in vitro</u> expression of the arenavirus glycoproteins from cDNA and to rationally design GP-C expression vectors.

Fig. 6. Amino Acid Similarity Between LCM-Arm and Lassa-Jos GP-C Glycoprotein precursors.

```
BESTFIT of: Armgpc.Seq  check: 4604  from: 1  to: 498

REFORMAT of: armgpc.jou  check: 4604  from: 1  to: 498  22-AUG-1986 18:28
(No documentation)

to: Lagpc.Seq  check: 7584  from: 1  to: 491

REFORMAT of: lagpc.  check: 7584  from: 1  to: 491  2-SEP-1986 10:53
(No documentation)

Symbol comparison table: Gencorediskː[Gcgcore.Rundata]Swgappep.Cmp
CompCheck: 1254

       Gap Weight:  3.000      Average Match:  0.540
    Length Weight:  0.100   Average Mismatch: -0.396

          Quality:  508.2              Length:    502
            Ratio:  1.035                Gaps:      6
Percent Similarity: 77.160  Percent Identity: 61.523

Armgpc.Seq x Lagpc.Seq    November 12, 1986  12:54  ..

  1 MGQIVTMFEALPHIIDEVINIVIIVLIVITGIKAVYNFATCGIFALISFL 50
    ||||||:|:.:|||:|:||:|||:|.| |:..:|::||||||||.:|:.||
  1 MGQIVTFFQEVPHVIEEVMNIVLIALSVLAVLKGLYNFATCGLVGLVTFL 50

 51 LLAGRSCGMYGLKGPDIYKGVYQFKSVEFDMSHLNLTMPNACSANNSHHY 100
    ||.||||      ...:||||||:..:|::|. ||:||| .|. ||||||
 51 LLCGRSC......TTSLYKGVYELQTLELNMETLNMTMPLSCTKNNSHHY 94

101 ISMG.TSGLELTFTNDSIISHNFCNLTSAFNKKTFDHTLMSIVSSLHLSI 149
    | :| ..|||||:||.|||.|.||||..| .|. :||.||||:|.:|||||
 95 IMVGNETGLELTLTNTSIINHKFCNLSDAHKKNLYDHALMSIISTFHLSI 144

150 RGNSNYKAVSCDFNNG.ITIQYNLTFSDAQSAQSQCRTFRGRVLDMF.RT 197
    .. ..|.|:||||||.| |.:|||||. | |..|..:| |. . ||: | |
145 PNFNQYEAMSCDFNGGKISVQYNLSHSYAGDAANHCGTVANGVLQTFMRM 194

198 AFGGKYMRSGWGWTGSDGKTTWCSQTSYQYLIIQNRTWENHCTYAGPFGM 247
    |:||.|:  :.|:...|     | ||||||||||| |||:|| :. | .:
195 AWGGSYIALDSCRCNWD.....CIMTSYQYLIIQNTTWEDHCQFSRPSPI 239

248 SRILLSQEKTK..FFTRRLAGTFTWTLSDSSGVENPGGYCLTKWMILAAE 295
    : : |  ::|:  ::.||| ||||||||||.| :.|||||||:||:.||
240 GYLGLLSQRTRDIYISRRLLGTFTWTLSDSEGKDTPGGYCLTRWMLIEAE 289

296 LKCFGNTAVAKCNVNHDAEFCDMLRLIDYNKAALSKFKEDVESALHLFKT 345
    |||||||||||| .||.|||||||:|:||.|: ::|.:.: .::|:..
290 LKCFGNTAVAKCNEKHDEEFCDMLRLFDFNKQAIQRLKAEAQMSIQLINK 339

346 TVNSLISDQLLMRNHLRDLMGVPYCNYSKFWYLEHAKTGETSVPKCWLVT 395
    .||.||.|||:|:||||:|||:|||||:|||:|..|| ||:|||||.
340 AVNALINDQLIMKNHLRDIMGIPYCNYSKYWYLNHTTTGRTSLPKCWLVS 389

396 NGSYLNETHFSDQIEQEADNMITEMLRKDYIKRQGSTPLALMDLLMFSTS 445
    ||||||||||||:|||:|||||||||.|:|:.|||.|||:|:||::|||
390 NGSYLNETHFSDDIEQQADNMITEMLQKEYMERQGKTPLGLVDLFVFSTS 439

446 AYLVSIFLHLVKIPTHRHIKGGSCPKPHRLTNKGICSCGAFKVPGVKTVW 495
     ||:|||||||||||||| | ||||||||.:.|||||| :| |||.. |
440 FYLISIFLHLVKIPTHRHIVGKSCPKPHRLNHMGICSCGLYKQPGVPVKW 489

496 KR 497
    ||
490 KR 491
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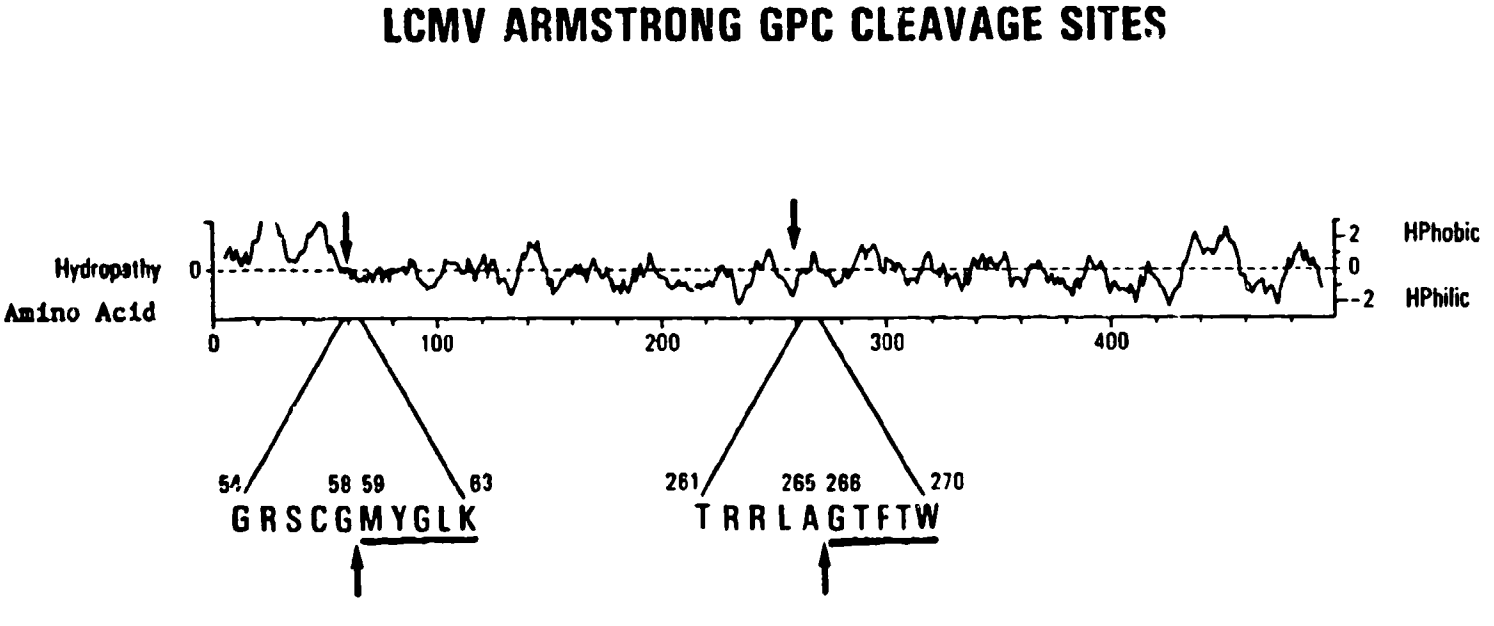
Table 12

Predicted signal peptidase cleavage site* on arenavirus GPC

| | LCMV (Arm) | | LCMV (WE) | | Lassa | | Tacaribe | | Pichinde | |
|---|---|---|---|---|---|---|---|---|---|---|
| Rank | Residue | Score | Residue | Score | Residue | Score | Residue | Score | Residue | Score |
| 1 | 58 | 8.305 | 58 | 7.320 | 58 | 6.332 | 58 | 8.106 | 16 | 5.576 |
| 2 | 56 | 5.954 | 56 | 7.017 | 56 | 5.433 | 56 | 4.758 | 56 | 3.489 |
| 3 | 34 | 4.816 | 54 | 4.594 | 34 | 5.006 | 53 | 4.561 | 59 | 3.367 |

*computer analysis of amino acid sequences based on algorithm of von Heijne (Fazakerley and Ross, 1988).

Fig. 7




Note: Amino terminal sequences MYGLK for GP-1 and GTFTWT were confirmed by microsequencing.

## ARENAVIRUS GPC CLEAVAGE SITES

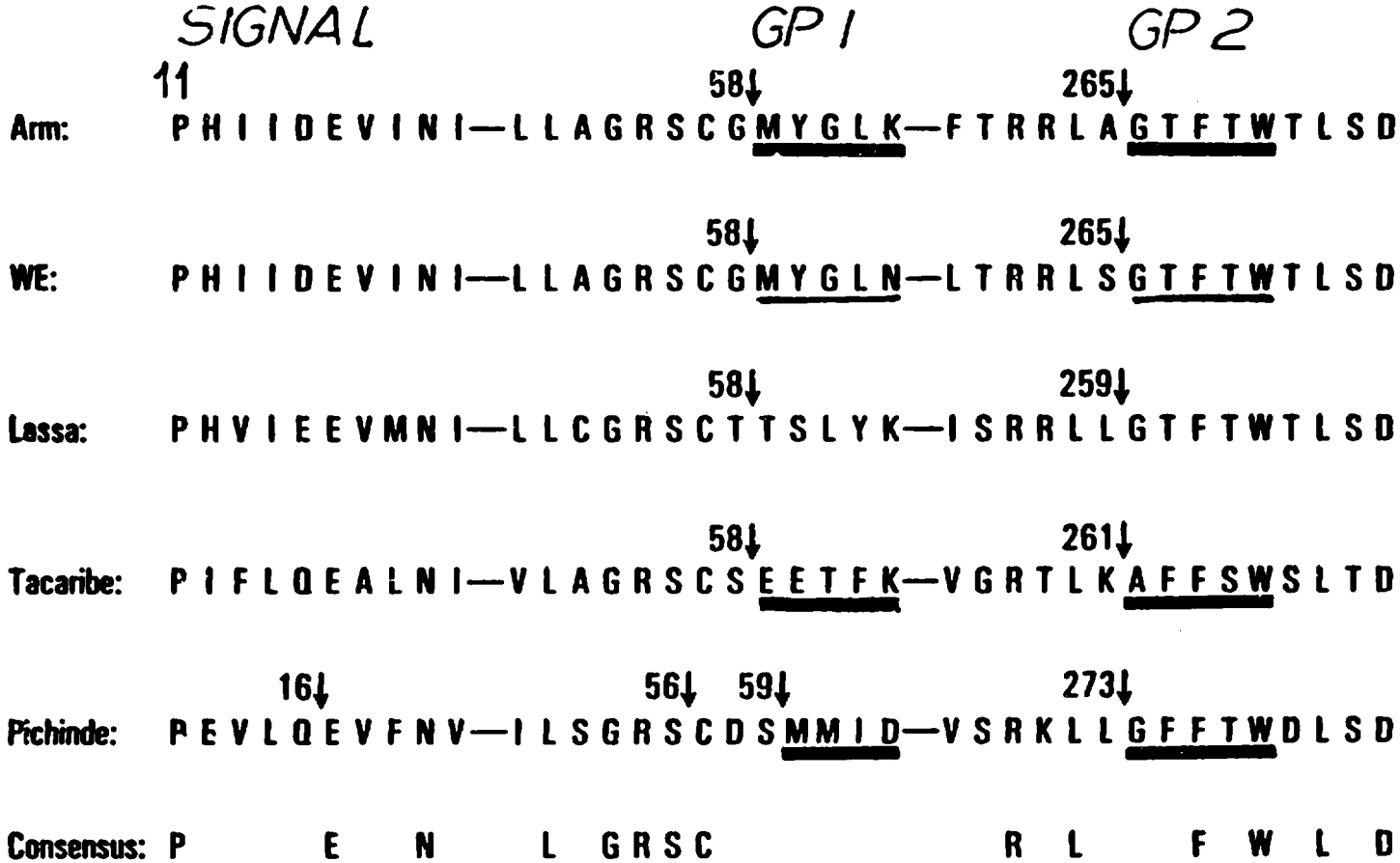



Fig. 8. Underlined sequences have been confirmed by N-terminal amino acid sequencing of virion structural proteins.

## L. Protein-protein Interactions in the Glycoprotein Spike

Continued studies of the macromolecular structure of the LCM virion spike have yielded a better picture of the association between GP-1 and GP2 and their association with the internal virion proteins (Burns and Buchmeier, 1991). Using the membrane permeable crosslinking agent dimethyl suberimidate (DMS), which cross links lysines within an 11A radius, we found that GP-1 formed homo-oligomeric complexes of the form $(GP\text{-}1)_n$ or where N = 1 to 4. Based on this observation we can make two conclusions: First, that GP-1 forms a tetramer of like molecules, and second, that GP-1 is not covalently linked to GP-2 nor does it have available lysines within 11A of similar reactive groups in GP-2. GP-2, in contrast to GP-1, showed complex patterns of interaction with other viral structural proteins. GP-2 was observed in monomeric, dimeric, trimeric and tetrameric homo-oligomers as well as in hetero-oligomers of the form (GP-2:NP), and $(GP\text{-}2\text{:}NP)_2$. Additional possible complexes of GP-2 with the 12kd Z protein were also occasionally observed (Salvato et al., 1992) (Fig. 9A).

Using a membrane impermeable cross linker, DTSSP, both GP-1 and GP-2 were found to exist as homo-oligomers of the form $(GP\text{-}1)_N$ and $(GP\text{-}2)_N$, where N = 1 to 4 (Fig. 9B). Use of this cross linker, which does not penetrate the virion envelope, prevented GP-2:NP complex formation. The cross linking data are summarized in Table 13. Although the GP-1 homotetramer appears to be stabilized by disulfide bonds, no evidence of covalent or disulfide bonding between GP-1 and GP-2 molecules was observed. Therefore it seems most likely that GP-1 and GP-2 interactions which form the spike are stabilized by either ionic or hydrophobic interactions.

Table 13

Theoretical and experimentally determined molecular weights of crosslinked complexes

| GP-1 | | | | GP-2 | | | | NP | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein Complex | Molecular Weight ($M_r$) Theoretical | DMS | DTSSP* | Protein Complex | Molecular Weight ($M_r$) Theoretical | DMS | DTSSP | Protein Complex | Molecular Weight ($M_r$) Theoretical | DMS | DTSSP |
| GP-1 | 44,000 | 46,000 | 40,000 | GP-2 | 35,000 | 34,000 | 35,000 | NP | 63,000 | 56,000 | 57,000 |
| $(GP\text{-}1)_2$ | 88,000 | 84,000 | 82,000 | $(GP\text{-}2)_2$ | 70,000 | 68,000 | 71,000 | (GP-2:NP) | 98,000 | 87,000 | - |
| $(GP\text{-}1)_3$ | 132,000 | 118,000 | 117,000 | (GP-2:NP) | 98,000 | 87,000 | - | $(NP)_2$ | 126,000 | 108,000 | 114,000 |
| $(GP\text{-}1)_4$ | 176,000 | 160,000 | 149,000 | $(GP\text{-}2)_3$ | 105,000 | 102,000 | 106,000 | $(GP\text{-}2:NP)_2$ | 196,000 | 173,000 | - |
| | | | | $(GP\text{-}2)_4$ | 140,000 | - | 129,000 | | | | |
| | | | | $(GP\text{-}2:NP)_2$ | 196,000 | 173,000 | - | | | | |

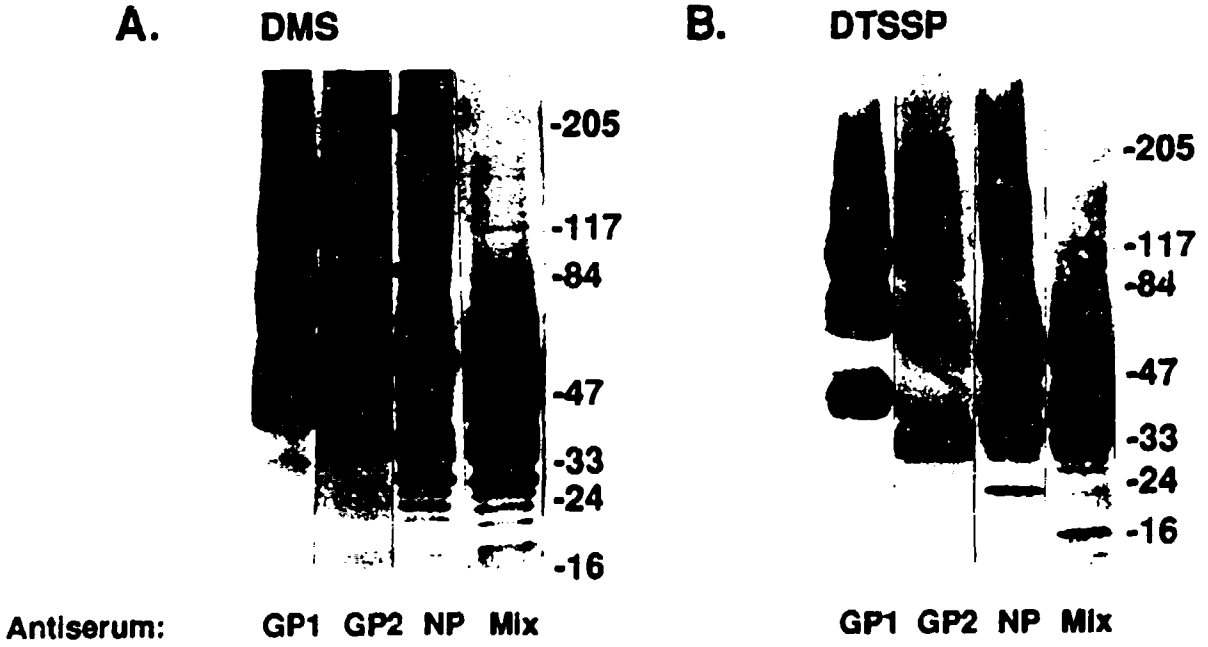
DTSSP gels were run under non-reducing conditions due to reversibility of crosslinker. Under non-reduced conditions GP-1 has a higher migration rate.

DMS crosslinking was performed using 1 mg/ml DMS for 90m at room temp.

DTSSP crosslinking was performed using 2 mg/ml DTSSP for 90m at room temp.

Experimentally determined molecular weights were obtained by comparing the observed relative migration for a polypeptide complex with a standard curve of relative migration rates vs. known molecular weights for prestained molecular weight markers (16,000-205,000).

Fig. 9




Immunoblot of crosslinked Arm-4 (A. DMS, B. DTSSP). Aliquots of purified LCMV were crosslinked using the membrane-permeable reagent, DMS (panel A), or the membrane-impermeable reagent, DTSSP (panel B), both in 100 mM triethanolamine-HCl, pH 8.2. Following incubation periods of 30 minutes (DTSSP) or 90 minutes (DMS), crosslinking was quenched by the addition of 1 M glycine until a final concentration of 20 mM glycine was obtained. Crosslinked virus preparations were disrupted using reducing (DMS) or non-reducing (DTSSP) electrophoresis sample buffer and heating at 95-100 C for four minutes. These samples were analyzed by immunoblotting following electrophoresis on 5-15% Laemmli gradient gels. Immobilon P membrane strips containing the transferred samples were probed using the appropriate rabbit anti-peptide sera specific for GP-1 (lane 1), GP-2 (lane 2), NP (lane 3) as described. Control virus (not crosslinked) was disrupted in the presence (lane 4 top panel) or absence (lane 4 bottom panel) of reducing agent and analyzed in parallel with the crosslinked preparations using a mixture of the three sera.

M. In Situ Hybridization Studies of LCMV RNA Distribution in Infection

By the application of in situ hybridization to thin sections of paraffin embedded tissues we have been able to determine with high resolution the cell-types containing lymphocytic choriomeningitis virus nucleic acid in the tissues of persistently infected mice (Fazakerley et al., 1991). We confirmed and extend previous observations of viral persistence in the brain, lung, liver, kidney, pancreas, thyroid, and reticuloendothelial system. In addition, we demonstrated for the first time persistence of viral nucleic acid in specific cell-types in the thymus, lymph nodes, testes, bladder, adrenal, parathyroid and salivary glands. The cell types infected were consistently observed among several animals. In lymphoid tissue, signal was predominantly located in the T-dependent areas of the spleen and lymph nodes. Viral nucleic acid was also present in cells of the thymic medulla. This has important implications for the deficiency in T-cell function observed in persistently infected mice. In the testes, viral nucleic acid was detected in spermatogonia but not differentiating spermatocytes. In this tissue at least, persistence is related to the differentiation state of the cell. Endocrine and exocrine dysfunctions have been described in persistently infected mice and we now report that the highest levels of viral nucleic acid were found in the adrenal gland. The infection of endocrine and exocrine tissue was not pantropic, specific cell-types expressed viral nucleic acid in each tissue. In the adrenal cortex, cells of the zona reticularis and zona fasciculata but not the zona glomerulosa were positive. In the adrenal medulla, signal was predominantly localized over adrenaline secreting cells. Infection of the renal tubules, transitional epithelium of the bladder, and the ducts of the salivary gland indicate the likely sites of virus production for the dissemination of arenavirus infections. These experiments provide the technical basis for in situ hybridization studies of human clinical material.

N. Refinement of a model of the structure of the arenavirus spike structure.

A priori computer predictions of the conformation of proteins such as the arenavirus GP-C polyprotein are of mixed value (Jennings, 1989). On one hand they provide a basis to predict with reasonable certainty gross topographical features such as N-terminal signal sequences and transmembrane domains. These methods are less reliable however when asked to predict more complex secondary and tertiary structures. We have used two such programs, the Chou-Fasman (1978) and Garnier-Oglethorpe-Robson (1978) algorithms, to attempt to predict the secondary and tertiary structure of GP-C. Figure 10 illustrates the results summarized in one figure by the plot structure (University of Wisconsin Genetics Computer Group software package) utility. Note in the Hopp and Woods (1981) hydrophilicity plot at the top that there are two extensive regions of hydrophobic peaks (peaks with negative overall values). These correspond to the 58 amino

acid N-terminal signal sequence (residues 1-58) which we described above, and the predicted transmembrane domain near the C-terminus of GP-C (boxed residues 432-458). Note as well a sequence near the amino terminal end of GP-2 (amino acids 266-290; dashed box) which is predicted by both the Chou-Fasman and Garnier algorithms to contain beta sheet-betaturn-beta sheet configuration. This sequence corresponds to the major GP-2 CTL epitope described by Whitton et al. (1988a) for GP-2 of LCMV. Between the CTL site and the transmembrane domain is a predicted extended alpha helix punctuated by a short beta sheet region at 370-390. In the GP-1 sequence there is little evidence of consistency between the two programs, although there are several predicted alpha helical and beta sheet regions predicted by both programs which are consistent with a globular structure.

Looking more closely at these structures we sought evidence of conservation among known GP-C sequences for various arenaviruses. Examining the hairpin loop, membrane spanning and carboxy terminal cytoplasmic domains (Fig. 11), it was evident that there was a high degree of sequence similarity between LCMV and Lassa and less between the Old World (LCM, ARM and WE; Lassa) viruses and the New World viruses (Tacaribe and Pichinde) in these regions. A similarity of considerable potential interest was observed in the region of predicted extended alpha helix in GP-2 (between GP-C residues 318 and 370). In this region which contains an extended alpha helical "heptad repeat" reported by Auperin et al. for Lassa virus (1986), we found that this predicted heptad repeat structure was conserved for all of the viruses for which sequence information is available. Although the amino acid sequences diverged (Fig. 12), the predicted heptad repeat periodicity indicative of alpha helix was conserved. This type of structure is found in the stalk region of Influenza HA (Wilson et al., 1981) and of the coronavirus spike glycoprotein S (deGroot et al., 1987), suggesting that this region of GP-2 serves as the stalk for the arenavirus spike. Also of interest is the fact that the highly conserved B cell antigenic site described by this laboratory (Weber and Buchmeier, 1987) lies immediately adjacent to the heptad repeat in amino acids 370-380.

To get a better look at the spike we performed cryoelectron microscopic (Milligan et al., 1984) examination on highly purified LCMV. This technique, which involves no fixation, images the virus directly in vitreous ice and offers minimal distortion of surface structures such as the spike. Purified LCMV-ARM (1 mg/ml; > $10^{10}$ pfu/ml) in TNE buffer was applied to carbon coated holey support films on grids. The grids were blotted and quick-frozen in liquid ethane slush in $LN_2$ and stored in $CN_2$. For examination the grids were mounted on a cold stage and visualized using a Phillips CM12T electron microscope at 100 kV. A number of images were taken at various levels of defocus to emphasize various aspects of the virion structure. As evident in Figure 13, the virions were spherical particles of variable diameter consisting of a dense (nucleoprotein) core enclosed by a lipid bilayer. The outer surface of the bilayer is studded with

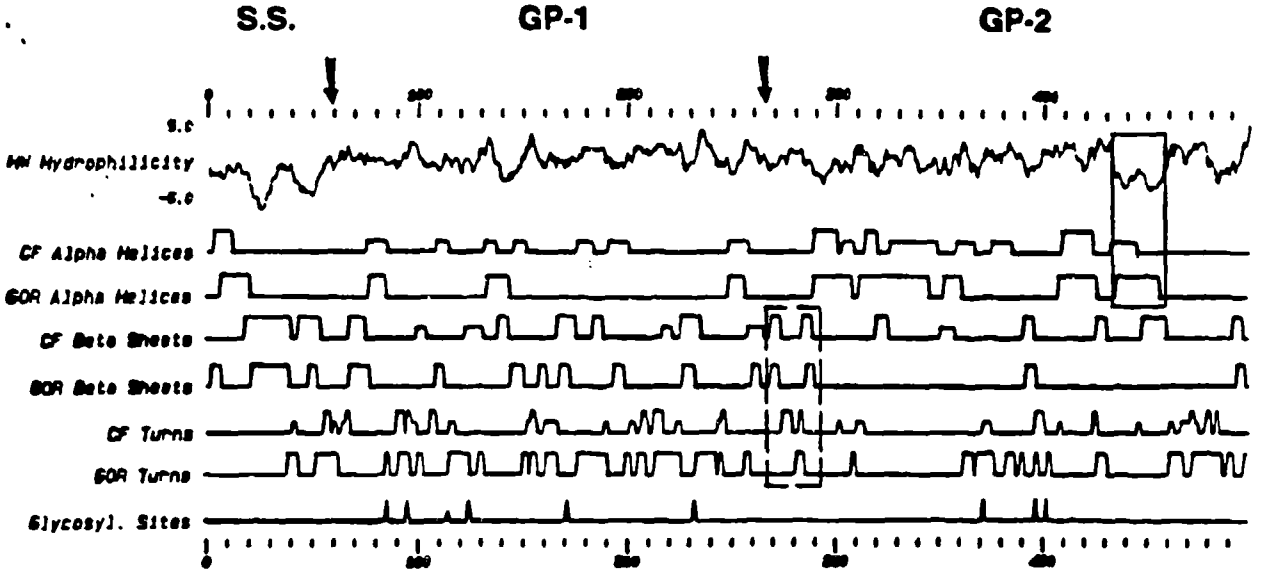



Figure 10. Predicted secondary structure of the LCMV glycoprotein precursor, GP-C. Predicted secondary structure plots of LCMV GP-C were generated on a VAX computer using the reported amino acid sequence of GP-C and the predictive algorithms of Hopp and Woods (1988), Chou and Fasman (1978) and Garnier, Osguthorpe and Robson (1978). The location of potential N-linked glycosylation sites are identified based on the presence of the amino acid consensus Asn-X-Ser/Thr. The proposed signal sequence (S.S.) and GP-1:GP-2 cleavage sites are indicated on the top line. The predicted beta sheet-reverse turn-beta sheet region of the proposed hairpin loop (dashed box) and the hydrophobic alpha-helical membrane spanning domain (solid box) of GP-2 are marked.

*1. Hairpin Loop Structure:*

| | |
|---|---|
| Arm: | G T F T W T L S D S S G V E N P G G Y C L T K W M I L |
| WE: | - - - - - - - - - - - - - - - - - - - - - - - - - - - |
| Lassa: | - - - - - - - - - - E - K D T - - - - - - - R - - L I |
| Pichinde: | - F - - - D - - - - - - - Q H V - - - - - - - E Q - A - I |
| Tacaribe: | A F - S - S - T - P L - M - A - - - - - - - S - - - L V |

*2. Membrane Spanning Domain:*

| | |
|---|---|
| Arm: | P L A L M D L L M F S T S A Y L V S I F L H L V K I P |
| WE: | - - - - - - - - - - - - - - - - - I - - - - - F - R - - |
| Lassa: | - - G - V - - F V - - - - F - - I - - - - - - - - - - |
| Pichinde: | - - - - T - I C F W - L V F - T I T V - - - I - G - - |
| Tacaribe: | - I T - V - I C F W - - V F F T S T L - - - - I G F - |

*3. Carboxy-Terminal Cytoplasmic Domain:*

| | |
|---|---|
| Arm: | C S C G A F K V P G V K T V W K R R |
| WE: | - - - - - - - - - - - - - - I - - - - |
| Lassa: | - - - - L Y - Q - - - P V K - - - |
| Pichinde: | - - - - Y Y - Y G R N L - N G |
| Tacaribe: | - R - - K Y L P L K K P - I - H - - H |

Figure 11. Comparison of the sequences of topographic landmarks of several arenavirus glycoproteins.

# CONSERVATION OF HEPTAD REPEAT REGIONS AMONG ARENAVIRUS GLYCOPROTEINS SUGGESTS A COMMON ALPHA-HELICAL AND COILED-COIL STRUCTURE

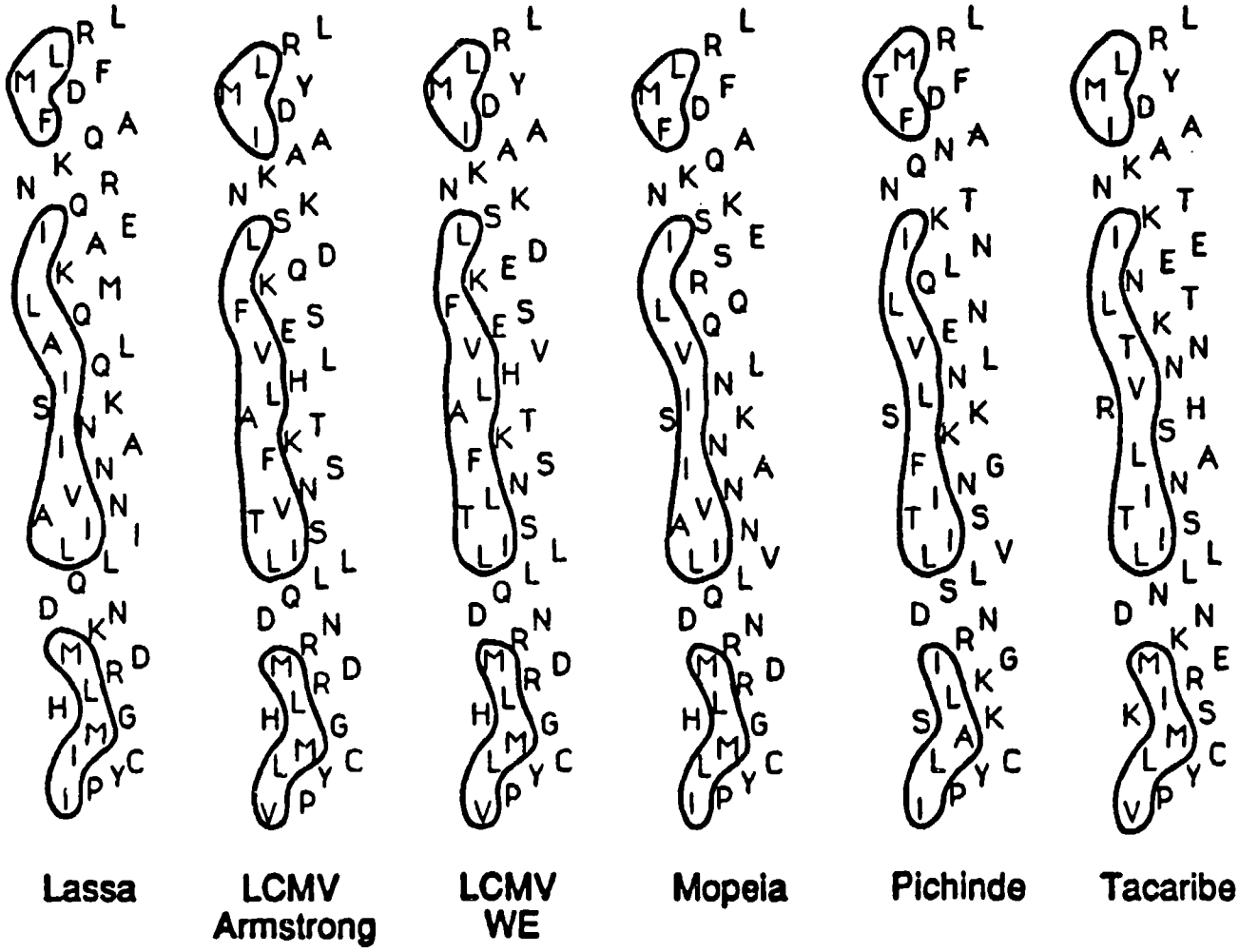



Figure 12. Helix-net array of the heptad repeat regions of the arenavirus GP-2 (equivalent) molecules. The published amino acid sequences of the arenavirus GP-2 heptad repeat regions are depicted in side-by-side helix net arrays aligned with the reported coiled coil domain of Lassa virus. Amino acids 309 (F) and 360 (I) of Lassa virus aare marked for reference. The hydrophobic face of each GP-2 molecule is outlined to illustrate the high degree of conservation among the viruses.

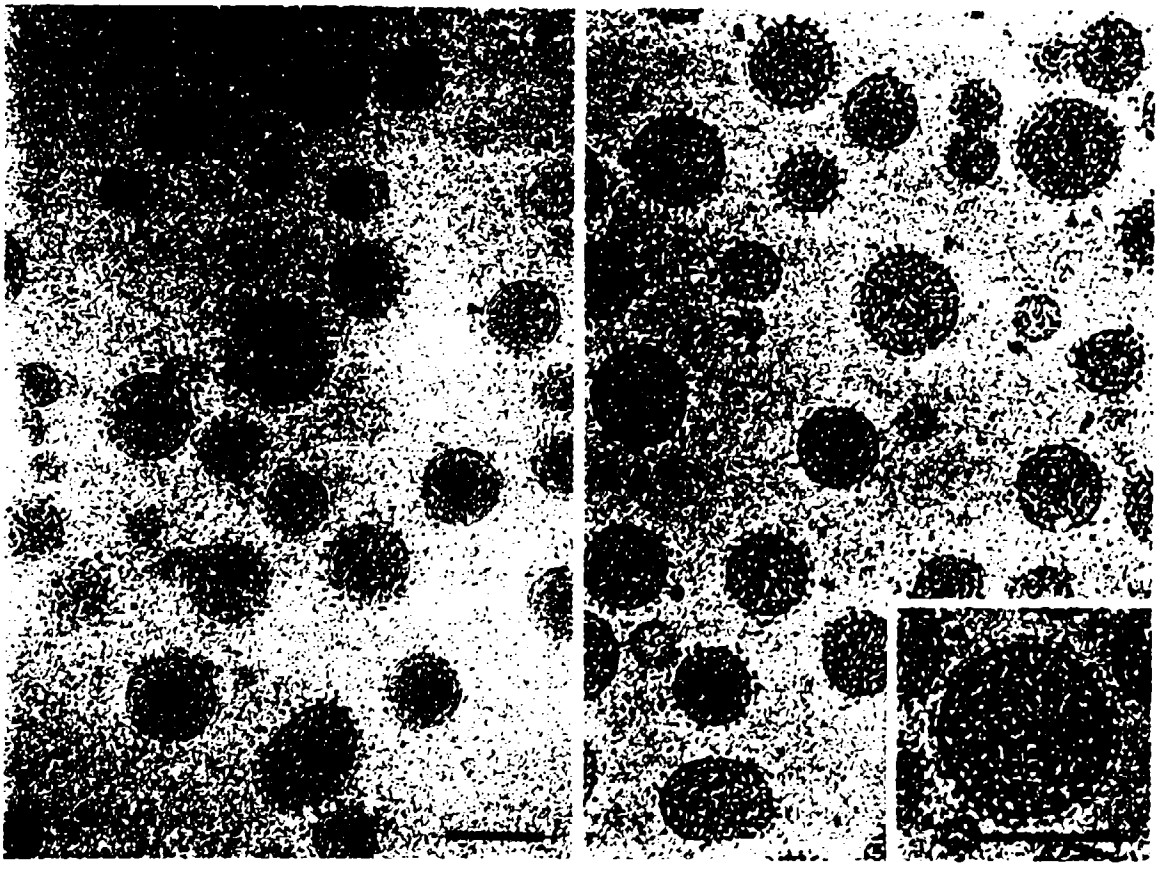


Figure 13. Cryo-electron micrographs of purified LCM virions. Purified LCMV preparations were analyzed by cryo-electron microscopy using defocus values of 1.5 u (left panel) or 3.0 u (right panel and inset). In the left panel the lipid bilayer of the virion envelope is emphasized (see arrow head). The right panel and inset emphasize the surface topography and t-shaped glycoprotein spikes (see arrow heads). Magnification of the left and right panels is 167,100x and the inset magnification is 232,750x. Bar scale equals 1000 A.

projections, the LCMV glycoprotein spikes. At a focus of -1.5 microns, where 50 A spacings are emphasized in the images, the bilayer is clearly visible (Fig. 13, left panel). The high electron-scattering density of the phosphate head groups of the lipids give rise to the characteristic trilamellar appearance of the bilayer. The spikes, while apparent in these images, are more clearly visualized in more strongly defocussed images (-3.0 microns, Fig. 13, right panel). Details in the images suggest that the spikes are T-shaped, with the rodlike stalk anchored in the lipid bilayer and the crosspieces lying parallel to the bilayer at a distance of approximately 80-100 A (8-10 nm) from the surface.

Having established these features of the spike structure, we sought to establish precisely the interaction of GP-2 with the envelope. Previous studies from this laboratory (Burns and Buchmeier, 1991) established that GP-2 was an integral membrane protein which could be crosslinked to the nucleocapsid protein, NP, using the membrane-permeable crosslinker dimethyl suberimidate (DMS). Briefly, we performed an exhaustive proteinase K digestion of highly purified LCMV followed by rebanding of the virus and separation and identification of the proteins remaining in the digested virions by SDS-PAGE and Western blotting. A schematic diagram of this protocol is represented in Figure 14. Peptide antisera used for this experiment corresponded to amino acids 130-144 of NP (antibody A), amino acids 59-79 of GP-C (1-20 of GP-1; antiserum B) and residues 483-498 of GP-C (exact C-terminus of GP-2; antiserum C). Figure 15 illustrates the results of this experiment. NP remained unaltered after proteinase K digestion due to its internal localization in the virion. GP-1 and GP-2 in contrast were largely digested by PK and a new polypeptide band of approximately 7800 da was evident. This band was detected only with antiserum C and not with A or B or with two additional peptide antisera representing amino acids 272-285 or 378-391 of GP-C. We sequenced this low molecular weight band by Edman microsequencing and identified an unambiguous N terminal sequence of gly-ser-thr-pro-leu, which corresponds to residues 430-434 of GP-C. Based on this data we conclude that the spike is anchored by a 68 amino acid (430-498) transmembrane and cytoplasmic domain at the C-terminus of GP-2. This domain includes the predicted transmembrane hydrophobic domain (Figure 10) and interestingly, in LCMV also contains five basic amino acids (Lys or Arg) in the last 12 residues at the C terminus of the cytoplasmic tail. These residues are likely to interact with the viral RNA and/or ribonucleoprotein complex within the virion. Considering all of the accumulated data we feel confident in proposing a working model (Figure 16) for the structure of GP-2 (Burns, Milligan and Buchmeier, J. Virol., submitted, 1992).

Forces that stabilize GP-1/GP-2 interaction have also been studied in detail. From Triton-X 114 extraction, as well as studies with nonionic detergents and urea, we can conclude that GP-1/GP-2 macromolecular spikes are not disulfide linked (Burns and Buchmeier, 1991). These molecules are however separated by

Fig. 14

# Sample Preparation and Immunoblotting of Control and Proteinase K-Digested Virions to Establish the Membrane Orientation of GP-2

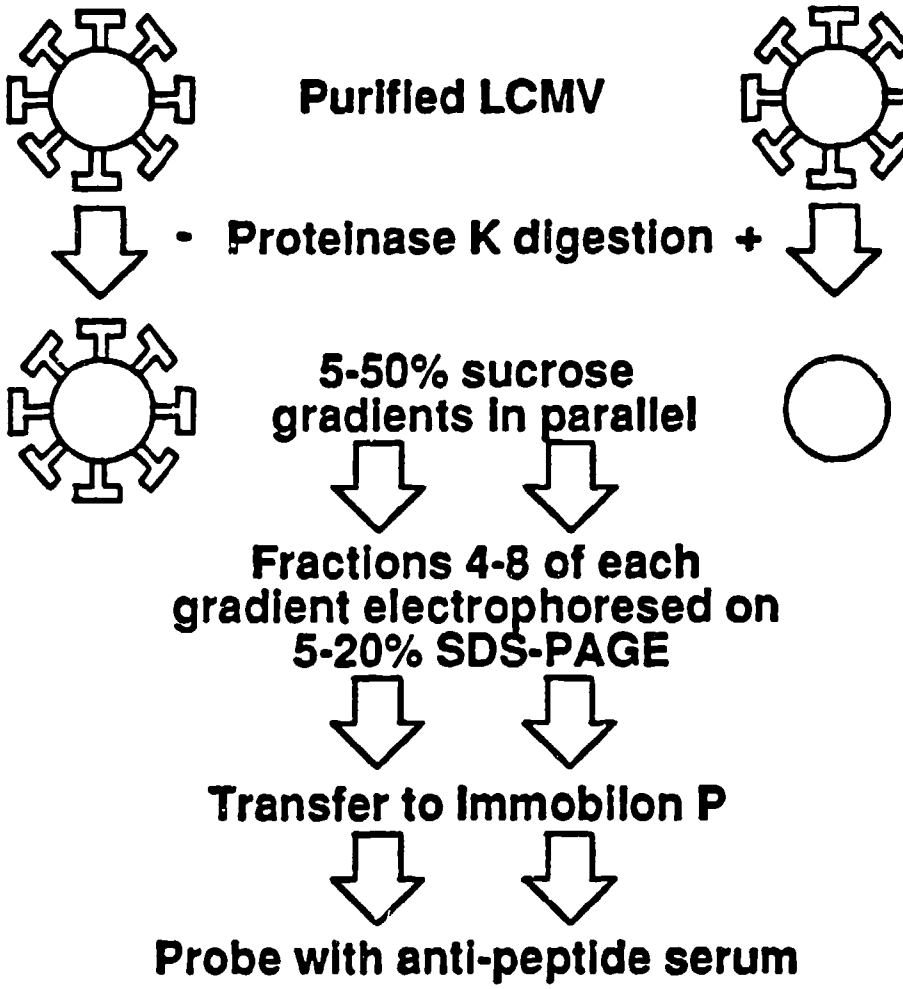
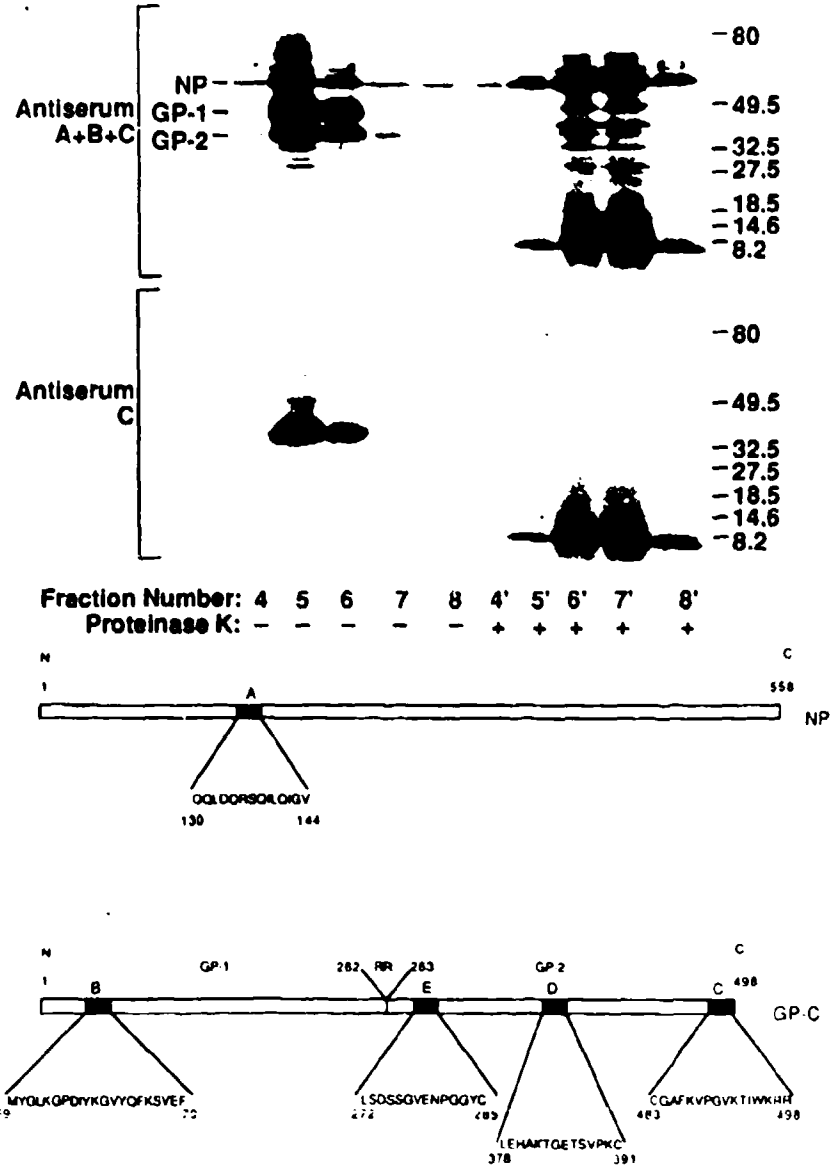


Figure 15. Isolation of a transmembrane fragment of GP-2. Purified LCM virions were digested with Proteinase K (500 ug/ml) for 30 min. at 37C. Digestion was terminated by the addition of PMSF to a final concentration of 25 mM. Control (non-digested) virions were incubated and PMSF treated in parallel. The protease-digested and control virus preparations were re-purified on 5-50% sucrose (w/w) gradients and fractionated by bottom puncture. Aliquots of fractions containing control (lanes 4, 5, 6, 7 and 8) or protease digested (lanes 4', 5', 6', 7' and 8') virions were analyzed on parallel immunoblots after disruption and electrophoresis on 5-20% SDS-polyacrylamide gradient gels. The blot shown in the upper panel was probed with a mixture of rabbit anti-peptide sera A, B and C (specific for NP, GP-1 and the carboxy-terminus of GP-2, respectively). The blot shown in the middle panel was probed only with anti-peptide sera C, specific for the carboxy-terminus of GP-2. Anti-peptide sera D and E reacted with GP-2 in the control preparation but were unable to detect any proteolytic cleavage fragments in the digested virus preparation (data not shown). The bottom panel shows the location and identity of the peptide sequences used to generate rabbit antisera.

# IMMUNOBLOT OF PROTEINASE K DIGESTED Arm 4 IDENTIFIES A CARBOXY-TERMINAL MEMBRANE ANCHOR ON GP-2



## Model of the LCMV GP-2 Monomer
(GP-2 exists as a tetramer)

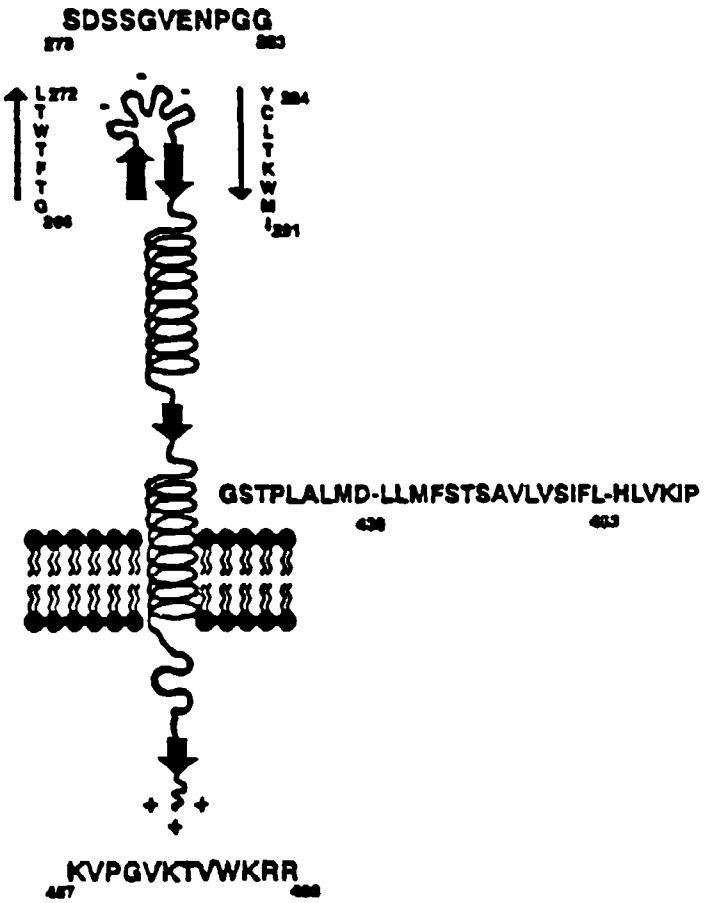



Figure 16. Schematic representation of the structure of an isolated GP-2 monomer. Working model of an isolated GP-2 monomer (the native structure of GP-2 is a homotetramer) illustrating the amino-terminal hairpin loop structure, the alpha-helical (coiled coil) domain, alpha-helical membrane spanning domain (sequence indicated adjacent to the lipid bilayer) and highly basic cytoplasmic tail domain. Single-letter amino acid sequences are included where significant. Alpha helical domains are shown as coils, beta turns are shown as heavy black arrows. Charged amino acid residues, believed to participate in intermolecular ionic bond formation, are indicated (+/-).

incubation with high concentrations of salts such as 1M NaCl or LiCl. Figure 17 illustrates this fact. Following incubation for 30-60 min in 1M LiCl, virions were banded on 10-50% sucrose gradients. In the salt-stripped virions a band of virions stripped of GP-1 but still containing GP-2 wask17H near the bottom of the gradient in fractions 4-6; the same fractions contained intact virions in the controls. Near the top of the gradient we found isolated GP-1 in fractions 11-13. These results have been extended to include LCMV, Pichinde and Tacaribe viruses, all of which contain GP-1 recoverable by this method (Fig. 18). We have established using conformation-dependent MAbs that the GP-1 recovered in this way is immunochemically native (Fig. 19). The observation of a GP-1 (G1) in Tacaribe virus was very satisfying since a number of groups have concluded that TAC had only one glycoprotein molecule, G (Gard et al., 1987). This erroneous conclusion was reached because of the comigration of TAC G1 and G2 glycoproteins in SDS-PAGE. Tacaribe clearly has a full length GP-C precursor as indicated by recently published sequence data (Franze-Fernandez et al., 1987). The MAb used to demonstrate TAC G1, 2.2.1, is a neutralizing MAb raised in this laboratory with collaboration of Dr. C. R. Howard (Howard et al., 1985).

O. Forces which stabilize GP-1 tetramers and GP-1/GP-2 spikes.

The influence of disulfide bonds on GP-1 tetramer stability has been explored in detail (Burns and Buchmeier, 1991). Briefly, GP-1 homotetramers can be stabilized and made resistant to sulfhydryl reagents by crosslinking with sulfo-DST. At a SDST concentration of 10 mg/ml, monomeric through tetrameric GP-1 species were seen. Conversely, in the absence of crosslinkers GP-1 homotetramers were exquisitely sensitive to reducing agents. The following sequential changes were noted. At 0 mM DDT (unreduced), monomeric through tetrameric GP-1 species were seen. At 1-3 mM DDT, only monomers and dimers remained. At higher concentrations of DDT ($\geq$ 200 mM) monomeric GP-1 predominated; moreover, between 3 and 30 mM the apparent mobility of GP-1 changed from ca. 38,000 to 44,000 kDa. Coincident with this mobility shift we observed a loss of GP-1 immunoreactivity with the disulfide-dependent conformation-sensitive MAb 2-11.10 (Wright, Salvato and Buchmeier, 1989) (Fig. 20). Based on these observations we propose the model for inter- and intramolecular disulfide bond interactions illustrated in Figure 21.

P. Purification of Native GP-1.

We have established that we can purify native GP-1 molecules from Old and New World arenaviruses by salt stripping and sucrose gradient centrifugation, however the low concentration of GP-1 (ca. 10% of total virion protein) in the virus requires that we explore alternative methods to prepare GP-1 for purification. Consistent with this aim we have obtained Vaccinia constructions expressing LCMV GP-C and Lassa GP-C. These will be used in a transient expression system to produce larger quantities of

# IONIC BONDS ARE ESSENTIAL FOR THE GP-1:GP-2 INTERACTION

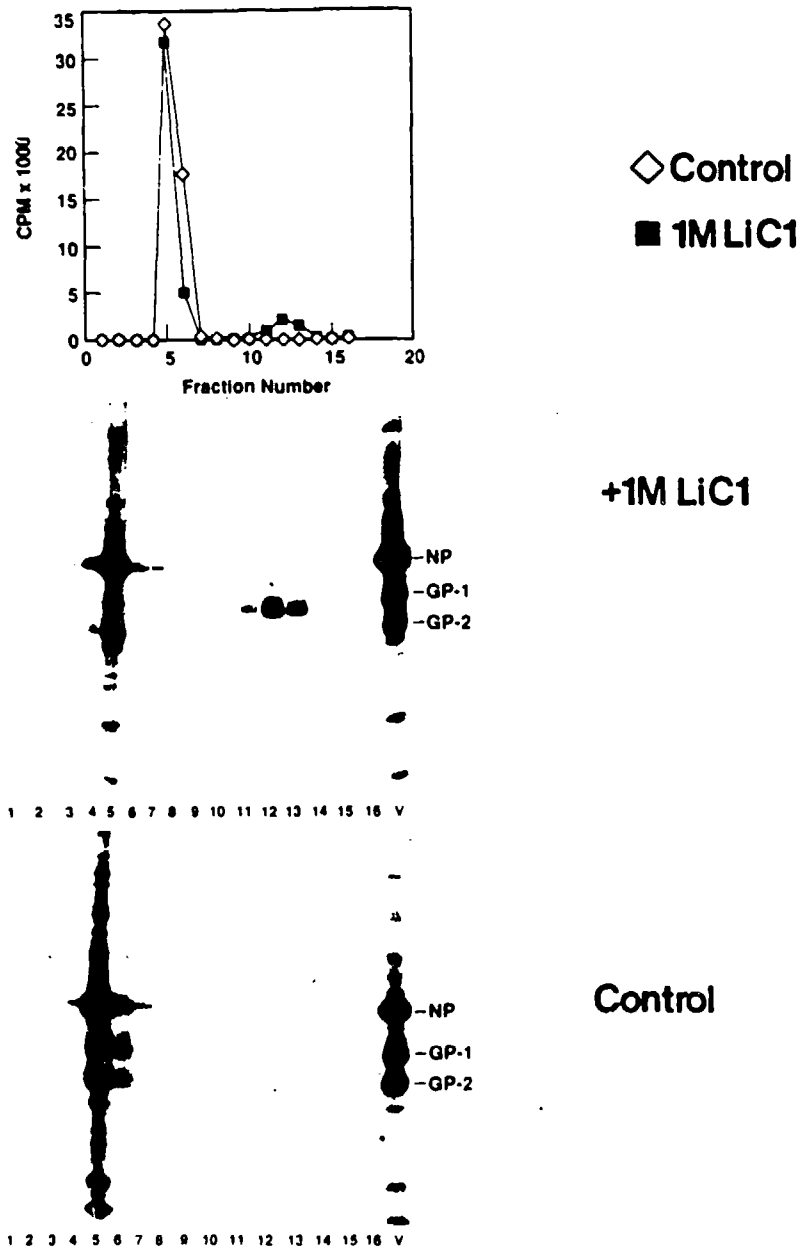



Figure 17. Typical radioactivity profiles from sucrose gradient fractions of salt treated and control Arm 4 preparations. Two samples of purified LCM virus (60 ug) were pelleted in an Airfuge rotor, resuspended in 1 M LiCl (salt-treated) or TNE (control) and incubated at 37C as described in Materials and Methods. The preparations were then centrifuged for 18 hours on parallel 5-50% continuous sucrose gradients in an SW 50.1 rotor. Each sucrose gradient was fractionated into 300 ul fractions and aliquots (50 ul) were counted in 3 ml Hydrofluor (panel A, black squares- LiCl treated virus, white diamonds-control virus). Fractions from each sucrose gradient were electrophoresed on 10% Laemmli gels following heating for 3 minutes in 2% SDS, 20mM DTT and 500mM Urea (panel B: 1 M LiCl treated virus, panel C: control virus) . Fractions are numbered 1 to 16 from the bottom to the top of each gradient. A sample of the starting virus preparation (V) was included as a marker.

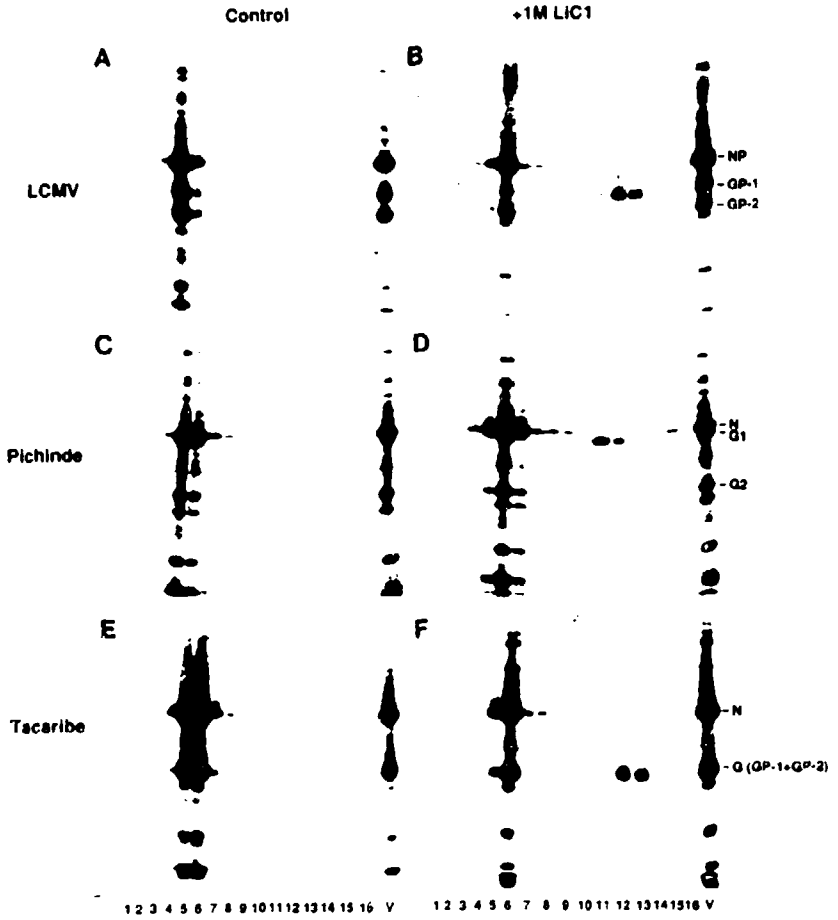
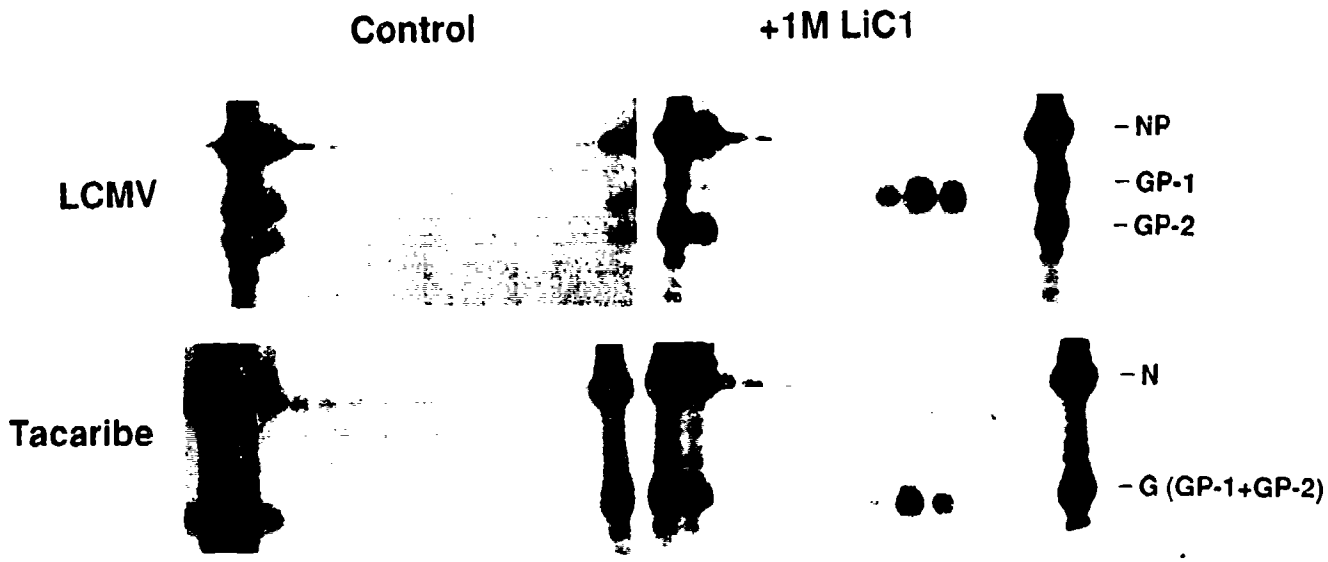
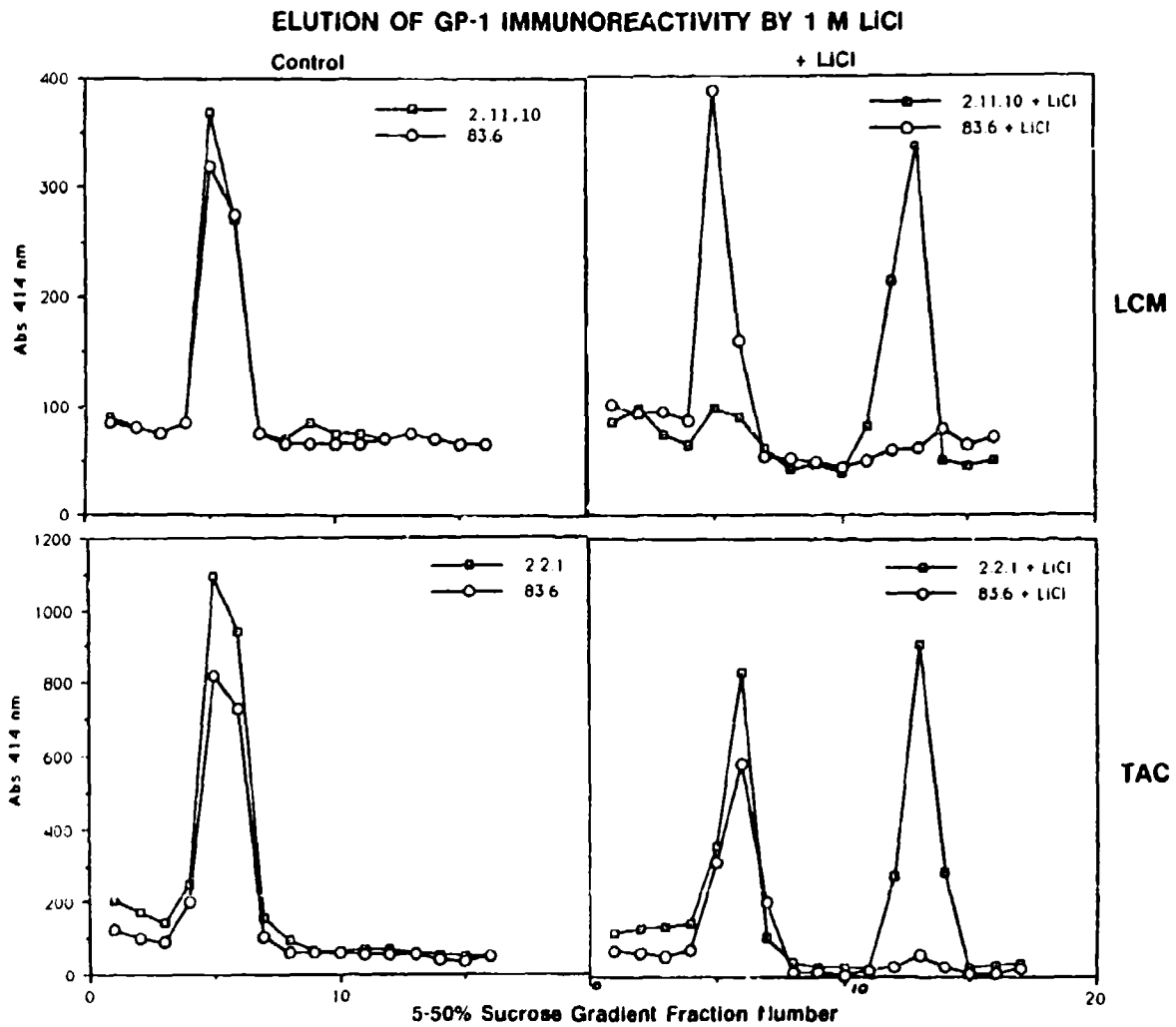



Figure 18. LiCl extraction and isolation of GP-1 glycoproteins from LCMV, Pichinde and Tacaribe viruses. See legend to Figure 7 for details.

Figure 19. (following page). Preservation of immunoreactivity in GP-1 species isolated by LiCl extraction of LCMV and TAC. Monoclonal antibodies used were 2-11.10 (anti LCMV GP-1), 83.6 (anti GP-2 panspecific) and 2-2.1 (anti TAC GP-1). Individual gradient fractions illustrated in top panel were assayed by ELISA in bottom panel.


Control
+1M LiC1
LCMV
Tacaribe
- NP
- GP-1
- GP-2
- N
- G (GP-1+GP-2)


ELUTION OF GP-1 IMMUNOREACTIVITY BY 1 M LiCl
Control
+ LiCl
2.11.10
83.6
2.11.10 + LiCl
83.6 + LiCl
LCM
2.2.1
83.6
2.2.1 + LiCl
83.6 + LiCl
TAC
Abs 414 nm
5-50% Sucrose Gradient Fraction Number

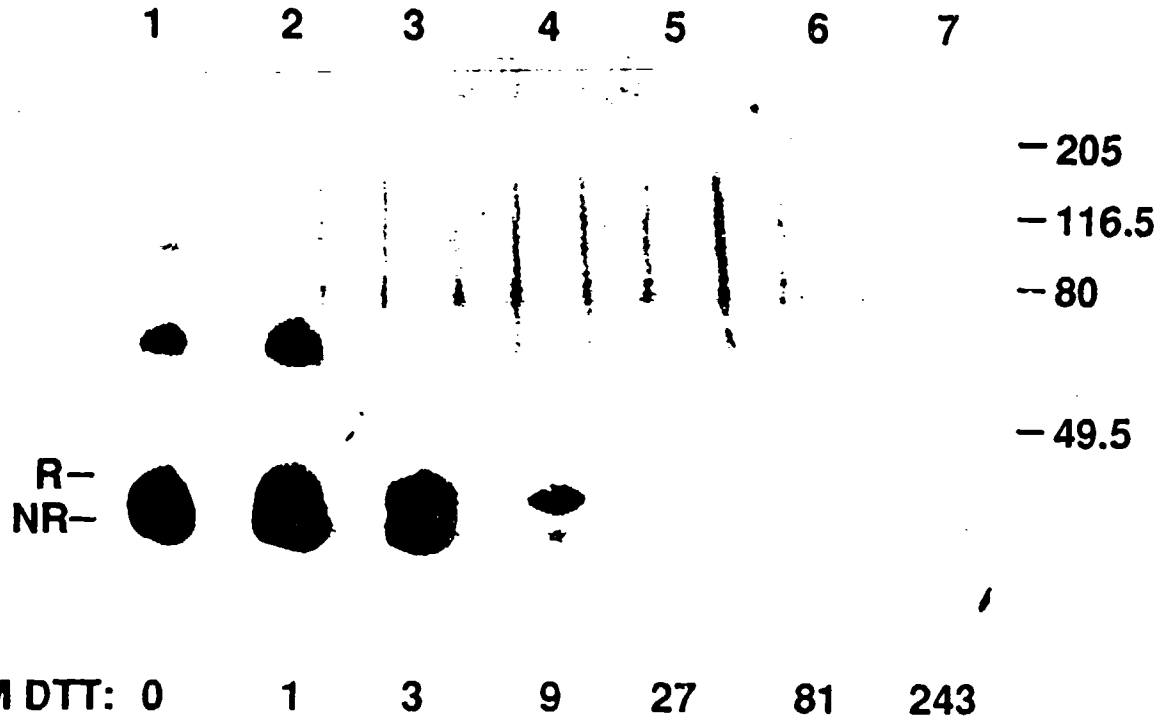



Figure 20. Immunoblot of non-reduced and reduced CHAPS disrupted Arm 4 probed with MAb 2-11.10. Purified LCMV (11 ug) was solubilized by incubation on ice for 5 minutes with 10 mM CHAPS under non-reducing conditions (lane 1) or in the presence of increasing concentrations of DTT, as indicated. These disrupted viral preparations were loaded onto 10% Laemmli gels, without heating or further reduction, electrophoresed at 4C and immunoblotted using the conformation dependent neutralizing anti-GP1 monoclonal antibody 2-11.10.

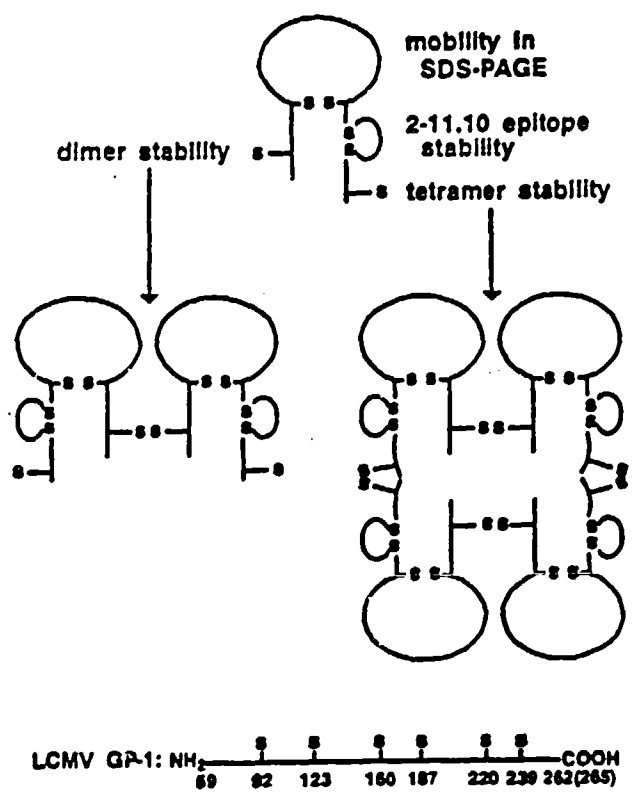



Figure 21. The six cysteine residues in GP-1 form two intramolecular and two intermolecular disulfide bonds.

protein than recoverable from virions. In our coronavirus work we have used the late cowpox promoter CAE I, which drives substantially higher levels of protein synthesis than does the 7.5 promoter more commonly used, and we will attempt to engineer the LCM and Lassa GP-C genes into that vector (p1246 and derivatives). One unexpected problem which we have encountered in using Vaccinia is the apparent failure of the cell to fully process GP-C. Figure 22 illustrates one such experiment, in which cells were infected with a vector expressing full length LCMV WE GP-C, then immunoprecipitated with MAb 33.6 which recognizes both GP-C and its cleavage product GP-2. While cleavage of GP-C to GP-2 was clearly evident in the virus control (lane 15), no cleavage was seen with the Vaccinia vector (lane 12). Instead an accumulation of uncleaved GP-C was observed. We will explore the basis of this to determine whether it represents a general phenomenon or is specific to this vector (VVB5). We will also attempt to engineer soluble glycoproteins by deletion of the transmembrane domain.

While we have demonstrated that we can isolate native antigenic GP-1, we are currently immunizing animals to confirm that this material is immunogenic. An extension of these experiments will be to determine whether GP-1 immunization elicits protective levels of humoral antibody using the model of antibody mediated protection we have recently described (Wright and Buchmeier, 1991).

Summing up the state of our understanding of the structures of the arenavirus glycoproteins, as it stands now we have prepared a schematic model of the glycoprotein spike (Fig. 23).

Q. Mechanism of antibody mediated protection against lethal arenavirus infection

Three potential outcomes of infection in mice with lymphocytic choriomeningitis virus are possible: (1) an acute asymptomatic infection when immunocompetent adults are inoculated extraneurally, (2) an acute fatal lymphocytic choriomeningitis which develops following intracranial inoculation of immunocompetent mice, or (3) a life-long persistent infection following inoculation of immunocompromised or neonatal mice. It is well established that CD8+ T-cells are required for viral clearance but the supporting role of antibody as well as the relative importance of antibody and T-cells in resistance to reinfection have not been examined fully.

Virus-specific antibodies of the IgG1 isotype are found in the serum of LCMV carrier mice; still the infection is not cleared. In contrast, anti-LCMV antibodies of the IgG2 isotype predominate in convalescent sera following acute infections. These antisera effectively neutralize virus in vitro and reduce viral titers in vivo in passive transfer experiments. Furthermore, the presence of anti-LCMV monoclonal antibodies can prevent the fatal T-cell mediated lymphocytic choriomeningitis (Wright and Buchmeier, 1991). These studies indicate that antibodies may play an

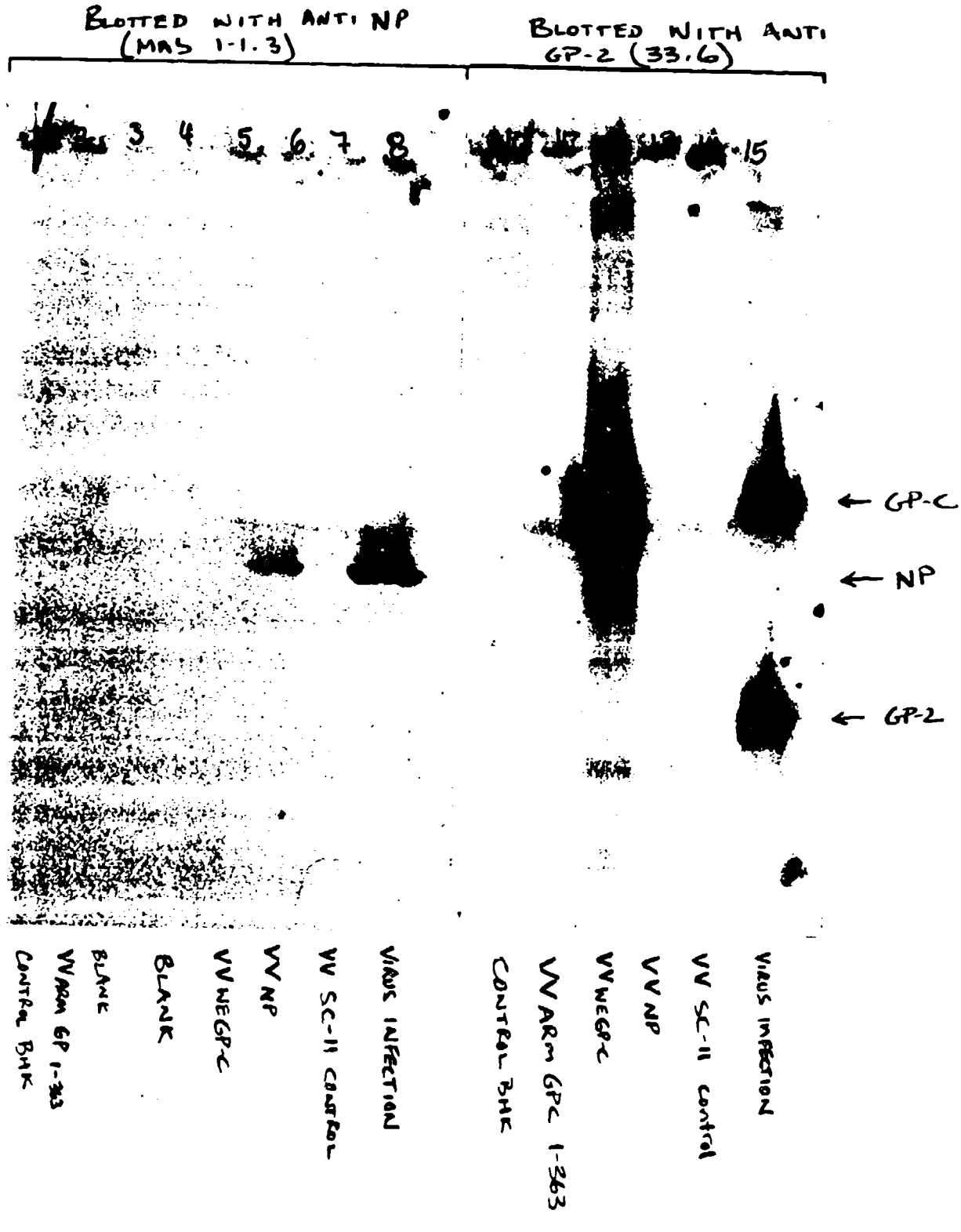



Figure 22. Immunoreactivity of LCMV glycoprotein produced by Vaccinia expression vector (see text for details).

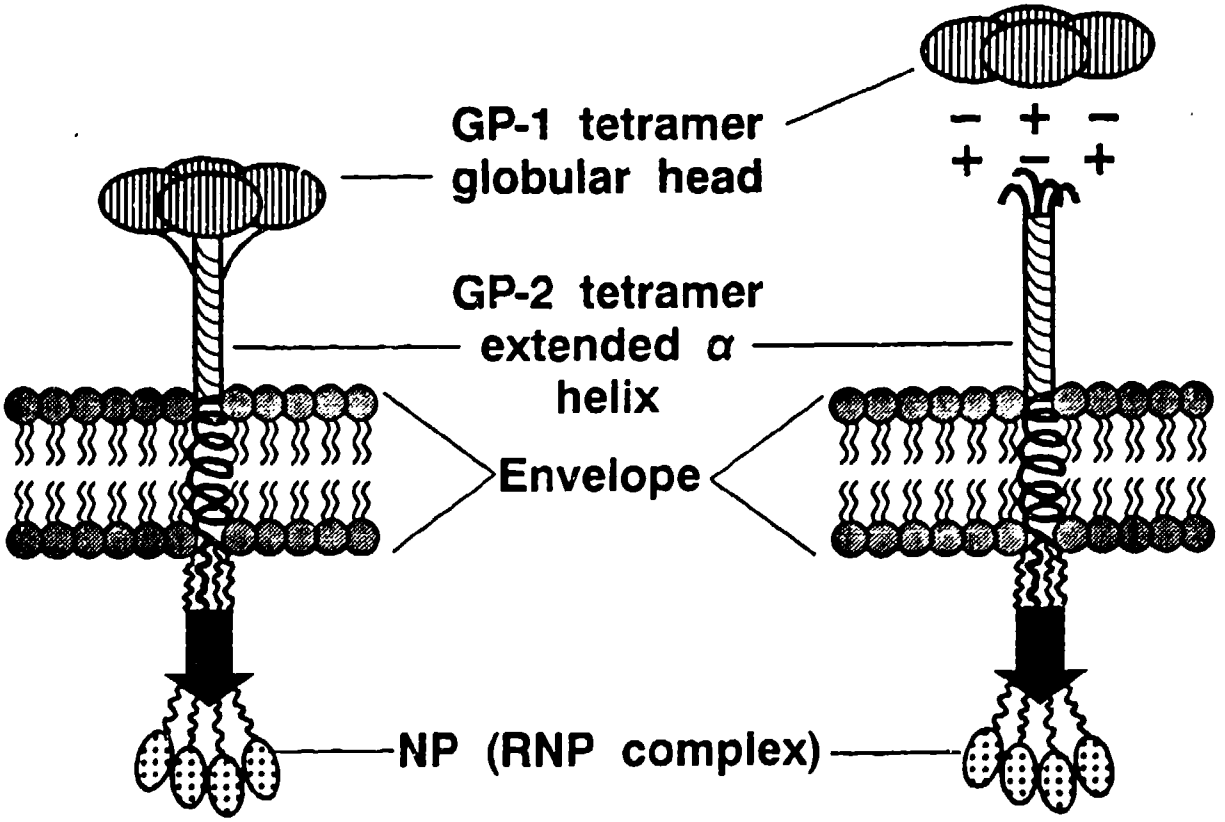



Figure 23. Proposed working model of the LCMV glycoprotein spike. The disulfide linked GP-1 homotetramer forms the crossmember component of the LCMV spike which associates with the amino-terminal hairpin loop of the GP-2 tetrameric stalk via ionic interactions. The alpha helical coiled-coil domain of the GP-2 homotetramer forms the linear region of the stalk. The glycoprotein spike is anchored in the virion envelope by a stretch of 15-25 amino acids and contains, within the virion, a highly charged carboxy-terminus allowing for ionic interaction with the ribonucleoprotein (RNP) complex.

important auxillary role in controlling LCMV infections. Therefore studies were initiated to further evaluate the requirements for and role of antibodies in resistance to LCMV infection.

Our success in demonstrating protection of adult mice against lethal LCM disease led us to perform a series of experiments to address the mechanism of humoral protection. The first series of

experiments explored the potential for passively acquired immunity among suckling pups born of immune mothers. Dams were immunized by infection with $10^5$ pfu of LCMV-ARM 30 days prior to mating, and the litters produced were nursed either on the immune birth mothers or on nonimmune foster mothers, then challenged at 10 or 14 days of age or at 5 weeks. It is evident from the data shown in Table 14 that pups nursed on immune mothers were solidly protected against viral challenge at 10 or 14 days postpartum, but that this protection substantially diminished by 5 weeks. Moreover, nursing of pups born of nonimmune mothers on immune foster mothers and the reciprocal combination established that protection was transmitted in milk. To eliminate the possibility of immune T-cells transferred either transplacentally or in milk, we passively transferred MAb 2-11.10 to nursing mothers postpartum and then challenged the pups at 14 days of age with either ARM-4, which is recognized by 2-11.10, or ARM-5, which is not (Wright and Buchmeier, 1991). Table 15 shows that only mice receiving 2-11.10 and challenged with ARM-4 were protected; ARM-5 challenged mice were not. Thus the specificity of transmammary protection in vivo exactly mirrors that of the 2-11.10 MAb in vitro.

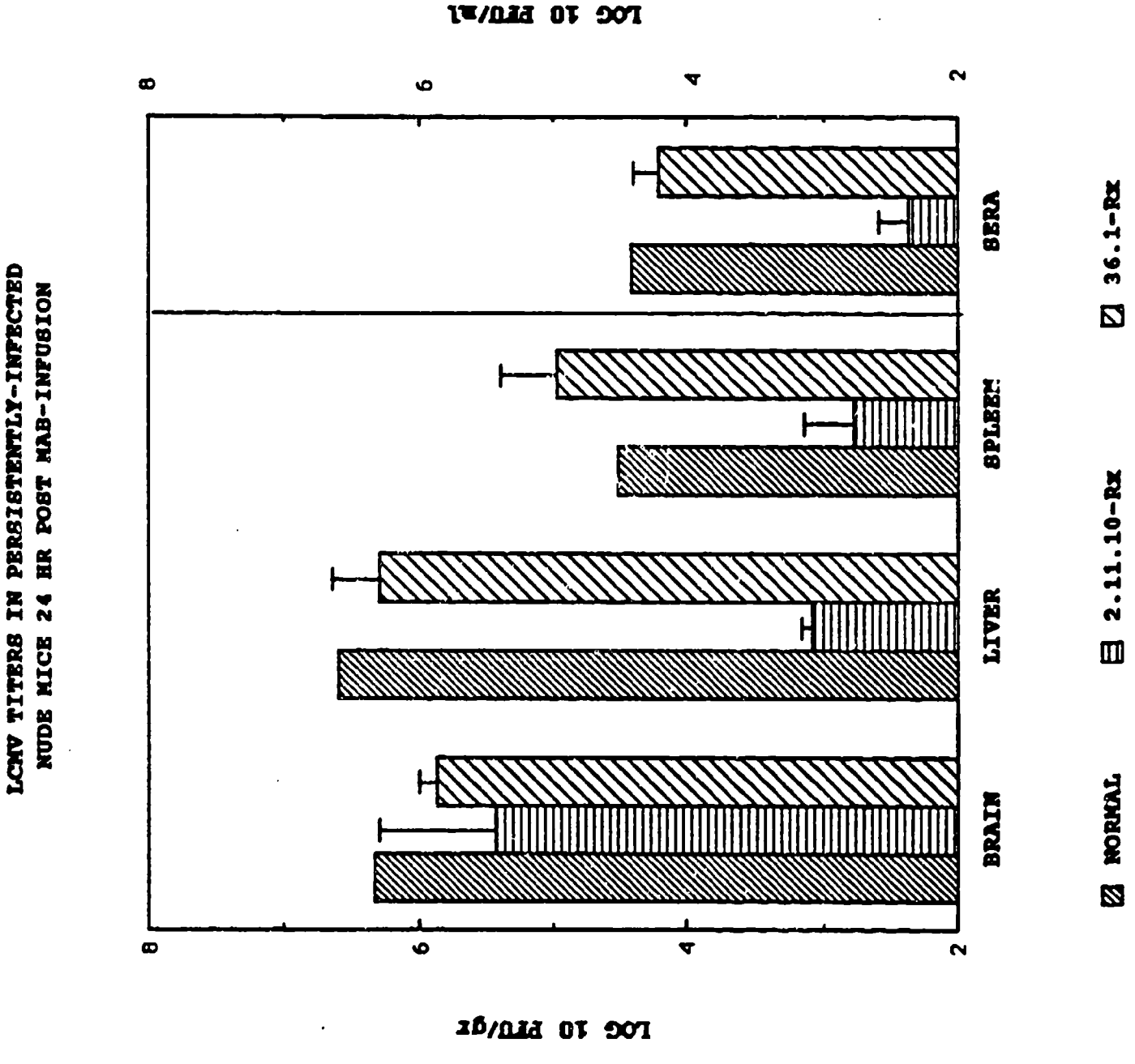
The ability of antibody to clear viral infection was tested by passively treating nude/nude LCMV carrier mice. Figure 24 shows that a single dose of MAb 2-11.10 or 36.1 reduced virus titers substantially within 24 hours. To determine whether MAb could block establishment of a persistent infection in nude mice we transferred antibody to a cohort of nude mice, then challenged with virus. As seen in Figure 25, MAb delayed the rise in virus titer which normally occurs during the first 7 days following infection, but eventually with clearance of the passive antibody titers rose to levels equivalent to those in untreated controls.

From these experiments it appears that even in the absence of a fully functional T-cell response, antibody provides a potentially useful means of reducing virus burden in an established infection in vivo.

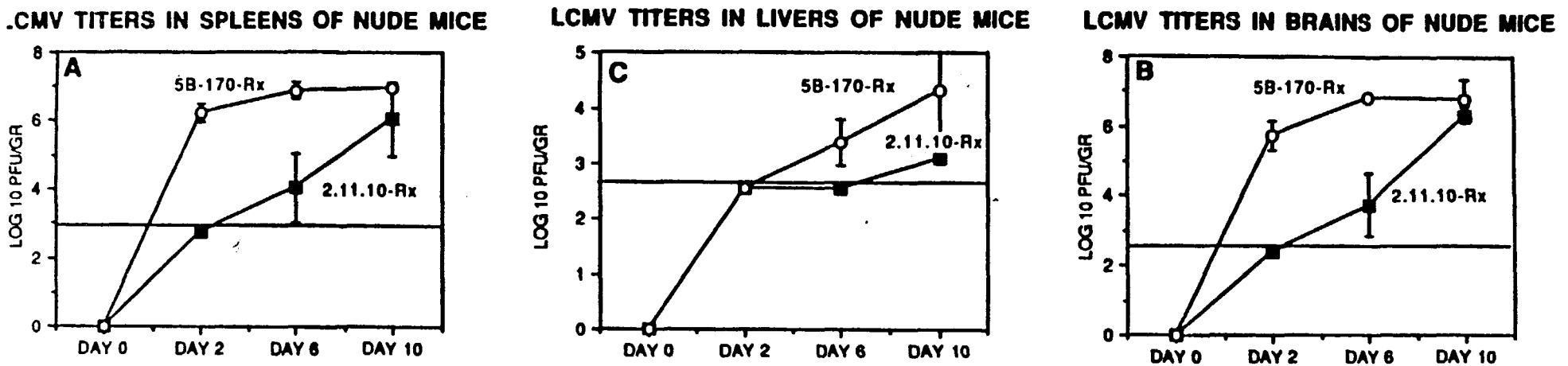
Humoral protection against challenge did not require the complement pathway. Table 16 illustrates that both B10.D2/o SnJ and SWR/J mice which are complement component C5 deficient were protected by MAb. Protection does however require a completeantibody molecule. $F(ab')_2$ fragments prepared from MAb 2-11.10 retained essentially full virus neutralizing activity measured in vitro but lost the ability to protect against challenge in vivo (Table 17). Taken with the complement data, these results suggest that Fc receptor- bearing cells may serve as important effectors in humoral protection (Baldridge and Buchmeier, 1992). We are continuing to investigate the role of antibody dependent cellular cytolysis (ADCC) in protection against arenavirus infection.

These results clearly indicate that preexisting antibody protects against lethal arenavirus infection and that the role of a humoral response has been underestimated. Therefore vaccination

FIGURE 24


LCMV TITERS IN PERSISTENTLY-INFECTED
NUDE MICE 24 HR POST MAB-INFUSION
LOG 10 PFU/gr
LOG 10 PFU/ml
8
6
4
2
BRAIN
LIVER
SPLEEN
SERA
NORMAL
2.11.10-Rx
36.1-Rx

# MAb 2.11.10 RESTRICTS THE SPREAD OF AN ACUTE VIRAL INFECTION IN NUDE MICE




Nude mice were infused with 0.2 ml of either MAb 2.11.10 (anti-LCMV) or MAb 5B-170 (anti-MHV) by ip injection on days -1 and 0. On day 0 the mice were challenged by ic inoculation with 1000 PFU ARM-4. The viral titers in the brain, spleen and liver were detemined on day 2, 6 and 10 following infection.

FIGURE 25

## SUCKLING PUPS OF IMMUNE DAMS ARE PROTECTED FROM LETHAL CHALLENGE

| BIRTH MOTHERS | FOSTER MOTHERS | AGE OF PUPS | PUPS WEANED | % SURVIVAL (TOTAL MICE) |
|---|---|---|---|---|
| IMMUNE | - | 10 DAYS | NO | 100% (6) |
| NONIMMUNE | - | 10 DAYS | NO | 0% (4) |
| IMMUNE | - | 14 DAYS | NO | 100% (6) |
| NONIMMUNE | - | 14 DAYS | NO | 0% (7) |
| IMMUNE | - | 5 WEEKS | YES | 29% (7) |
| NONIMMUNE | - | 5 WEEKS | YES | 0% (4) |
| NONIMMUNE | IMMUNE | 14 DAYS | NO | 73% (15) |
| IMMUNE | NONIMMUNE | 14 DAYS | NO | 0% (12) |

-- Pups challenged at 5 weeks of age were weaned when three weeks old.

-- Foster-nursed pups were switched to their foster mother within 24 hr of birth.

TABLE 14

## SUCKLING PUPS OF 2.11.10-TREATED DAMS ARE PASSIVELY PROTECTED.

| EXPT. | MATERNAL STATUS | AGE OF PUPS | VIRAL CHALLENGE | % SURVIVAL (TOTAL MICE) |
|---|---|---|---|---|
| 1 | NONIMMUNE | 14 DAYS | ARM-4 | 40% (5) |
| | NONIMMUNE | 14 DAYS | ARM-5 | 0% (5) |
| 2 | NONIMMUNE | 14 DAYS | ARM-4 | 83% (6) |
| | NONIMMUNE | 14 DAYS | ARM-5 | 0% (3) |

-- Nursing mothers were infused with 0.2 ml of 2.11.10 ascites every third day (EXPT 1) or 0.25 ml every other day (EXPT 2) post partum.

-- MAb 2.11.10 recognizes an epitope present on ARM-4 but not ARM-5.

TABLE 15

## ANTI-LCMV MAB PROTECT C5-DEFICIENT MICE FROM LETHAL LCM-DISEASE.

| MOUSE STRAIN | C5 DEFICIENT | VIRUS CHALLENGE | 2.11.10 TREATMENT | % SURVIVAL (TOTAL MICE) |
|---|---|---|---|---|
| B10.D2/oSnJ | YES | ARM-4 | - | 0% (4) |
| B10.D2/oSnJ | YES | ARM-4 | + | 100% (4) |
| B10.D2/nSnJ | NO | ARM-4 | - | 0% (4) |
| B10.D2/nSnJ | NO | ARM-4 | + | 100% (4) |
| SWR/J | YES | ARM-4 | - | 0% (12) |
| SWR/J | YES | ARM-4 | + | 83% (12) |
| SWR/J | YES | ARM-5 | - | 0% (12) |
| SWR/J | YES | ARM-5 | + | 0% (12) |

-- Mice treated with MAb 2.11.10 received 0.2 ml of ascites the day before and the day of viral challenge.

-- MAb 2.11.10 recognizes an epitope present on ARM-4 but not ARM-5.

TABLE 16

## $F(ab')_2$ FRAGMENTS FROM THE PROTECTIVE MAB 2.11.10 FAIL TO PROTECT MICE FROM LETHAL LCM DISEASE.

| GROUP | TREATMENT | SURVIVAL (TOTAL MICE) |
|---|---|---|
| RECIPIENTS OF: | | |
| 2.11.10 | 25ug | 25% (4) |
| $F(ab')_2$ | 25ug | 0% (4) |
| 2.11.10 | 50ug | 75% (4) |
| $F(ab')_2$ | 125ug | 0% (4) |

-- MAb 2.11.10 or the $F(ab')_2$ fragments of 2.11.10 were given 1x only concurrently with viral challenge.

-- $F(ab')_2$ fragments had equivalent neutralizing titers as MAb 2.11.10.

TABLE 17

strategies which stimulate such a protective antibody response are important goals for future research. Furthermore, the demonstration that passively administered antibody is able to reduce preexisting virus titers in vivo suggests that specific humoral immunotherapy with monoclonal antibodies of human origin or with recombinant human-mouse antibodies is a real possibility.

R. Effect of humoral immunotherapy on late neurological disease induced by LCMV in rats

LCMV inoculated intracerebrally into suckling rats results in development of a T-cell dependent neurologic disease characterized by cerebellar hypoplasia and necrosis and ataxia (Monjan et al., 1971, 1974; del Cerro et al., 1975). Ataxia is evident by 21 days after infection (at 4 days of age) and in our experience the rats survive for 3-4 months with residual neurologic deficits.

We utilized this model to determine whether passive immunotherapy could be of value in altering the course of this neurologic disease. This is particularly relevant since Junin virus infection in man is frequently complicated by late neurologic disease in patients receiving convalescent immune plasma. We utilized a panel of rat monoclonal antibodies described previously in this report for these studies. Table 18 describes the specificity and titers of these antibodies. In a manner similar to that observed in the mouse, we found that passively transferred rat MAb to GP-1 blocked the development of cerebellar

Table 18

Rat Monoclonal Antibodies to LCMV

| MAB | ANTIGEN[a] SPECIFICITY | % COMP.[b] WITH 2.11.10 | ISOTYPE | PRD50[c] | ELISA[d] TITER |
|---|---|---|---|---|---|
| 8-12 | GP-1(NP) | 80 | IgG2a | 5,623 | 12500 |
| 8-13 | GP-1 | 98 | IgG2b | >10,000 | 312500 |
| 8-24 | GP-1(NP) | 47 | IgG2a | 25 | 12500 |
| 8-40 | GP-1 | 90 | IgG2b | >10,000 | 62500 |
| 8-50 | GP-1 | 92 | IgG2b | >10,000 | 312500 |
| 8-14 | NP(GP-1) | 10 | IgG2a | 10 | 500 |
| 8-26 | NP | 15 | IgG2a | 10 | 62500 |
| 8-32 | NP | 15 | IgG2a | 16 | >312500 |

a) Antigen specificity was determined by western blot analysis.
b) A competitive RIA using radiolabeled 2.11.10 was used to further define the binding specificity of the rat antibodies. The mouse anti-LCMV monoclonal antibody 2.11.10 defines the GP-1d epitope found on the Armstrong-4 strain of LCMV.
c) The neutralizing capacity of the antibodies was determined by the plaque reduction method. The PRD50 represents the dilution required to eliminate 50% of the viral inoculum as measured by plaque assay on VERO cells.
d) The ELISA titers represent the reciprocals of the last dilution giving an absorbance reading of twice the negative control and greater than 0.1.

disease in Lewis rats (Fig. 26). As with the mouse model, protection was transferred from mother to nursing pup in the milk (Fig. 27). By immunocytochemistry no viral antigen was detectable in the brains of protected rats at 15-26 days.

These results establish that monoclonal antibody protects against the chronic neurologic disease induced by LCMV in the rat and suggest that a similarly designed strategy employing human or humanized monoclonals may be of value in treating acute arenavirus infections in man.

Although this contract has expired, this laboratory will continue to explore this model while also moving toward engineering human monoclonal antibodies to arenaviruses of clinical importance. We feel that the studies carried out under support of this contract provide a firm intellectual basis to justify this effort.

S. Function of the arenavirus spike: Early events in infection

Entry of RNA virions into the cell generally follows one of two pathways. Many viruses (e.g. Sendai, HIV) fuse directly with the plasma membrane of the cell and enter directly into the cytosol. Others such as myxo- and paramyxoviruses utilize an endocytic pathway in which virus is taken up into endosomes and in the acid environment a fusion activity (usually of the spike protein) is activated which mediates viral entry into the cytosol.

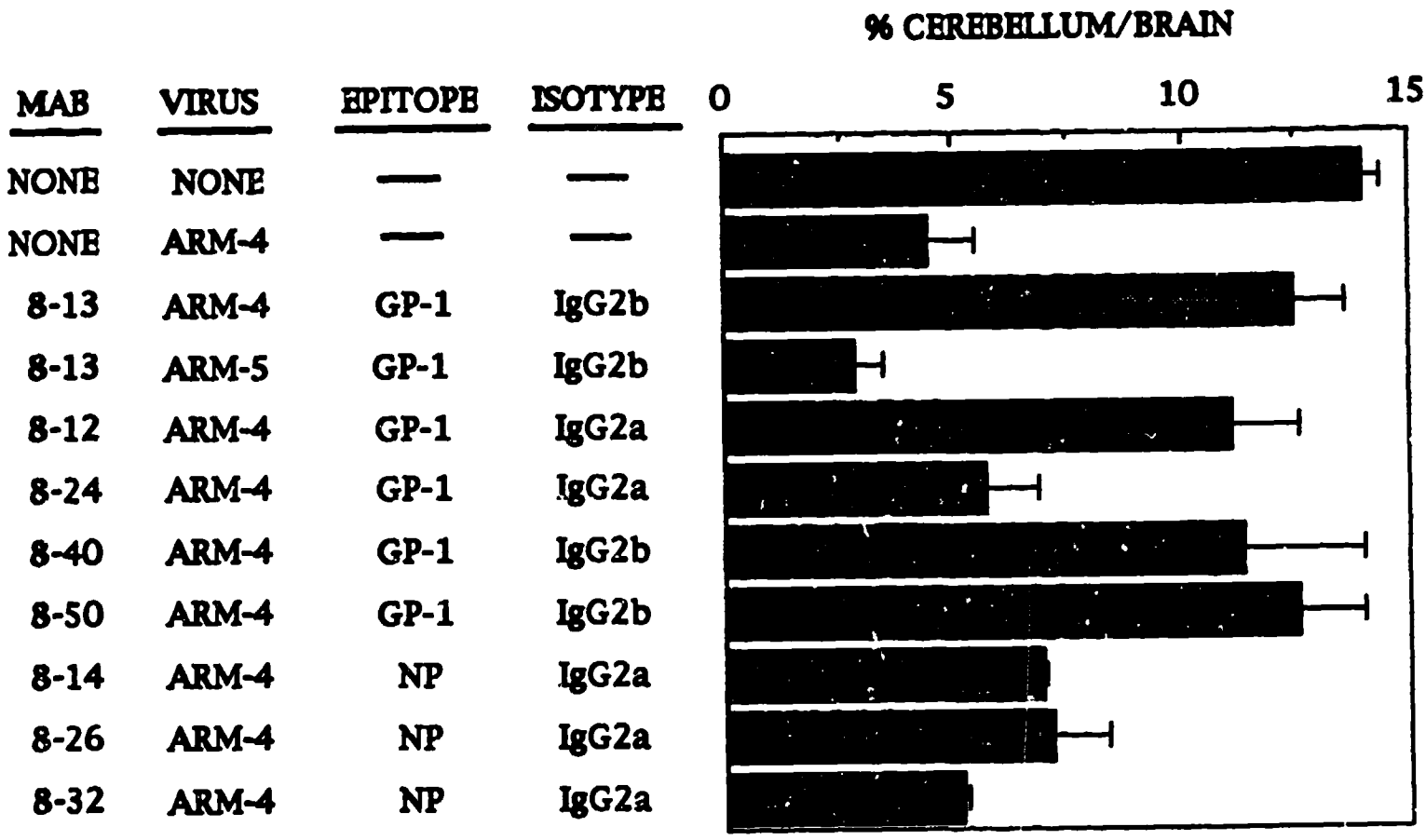
Knowledge of the early events in infection is important, as receptor binding and uncoating are two stages in replication which may be accessible to antiviral therapy. We have already shown that GP-1 binds to cellular receptors and can be inhibited by certain monoclonal antibodies. We sought as well to define the pathway of viral entry by inhibition of the endosomal pathway using chloroquine and by direct biophysical measurement of the pH dependent fusion activity of the glycoprotein spike.

Virus yields were quantitated in the presence and absence of 50 uM chloroquine added to cells either 1 hour before or at the time of infection. In both instances inhibition of virus yield was 92-92%, indicating that LCMV utilizes an endosomal route of entry.

The endosomal route predicts an acid-dependent fusion activity, therefore we performed fluorescence dequenching assays using R18 rhodamine labelled LCMV and multilaminar vesicles. Using this method we observed a pH dependent fusion of LCMV with both phosphatidyl serine and cardiolipin multilaminar vesicles (Figs. 28 and 29) with a pH optimum of 4.5-5, consistent with intralysosomal pH values.

Interestingly, we also observed that the GP-1 head of the viral spike protein eluted at low pH, suggesting that fusion activity is activated following dissociation of GP-1 and exposure of the N-terminal domain of GP-2. The mechanism and specificity of viral receptor binding, spike conformational change and fusion

Rat Monoclonal Antibodies to GP-1 Block Cerebellar Necrosis




| MAB | VIRUS | EPITOPE | ISOTYPE |
|---|---|---|---|
| NONE | NONE | — | — |
| NONE | ARM-4 | — | — |
| 8-13 | ARM-4 | GP-1 | IgG2b |
| 8-13 | ARM-5 | GP-1 | IgG2b |
| 8-12 | ARM-4 | GP-1 | IgG2a |
| 8-24 | ARM-4 | GP-1 | IgG2a |
| 8-40 | ARM-4 | GP-1 | IgG2b |
| 8-50 | ARM-4 | GP-1 | IgG2b |
| 8-14 | ARM-4 | NP | IgG2a |
| 8-26 | ARM-4 | NP | IgG2a |
| 8-32 | ARM-4 | NP | IgG2a |

Figure 26. The percentage of the cerebellum to total brain weight in 21 day old Lewis rats was assessed as a measurement of LCMV induced immunopathology. Suckling rats were challenged with either the Armstrong-4 or Armstrong-5 strain of LCMV at 4 days of age. Specific groups of rats were also treated with rat anti-LCMV monoclonal antibodies by ip injection of 0.1 ml ascites on the same day as the viral challenge. The monoclonal antibody 8-13 recognizes the Armstrong-4 but not the Armstrong-5 strain of LCMV.

Mother to Baby Transfer of Protection Against
LCMV Induced Cerebellar Hypoplasia

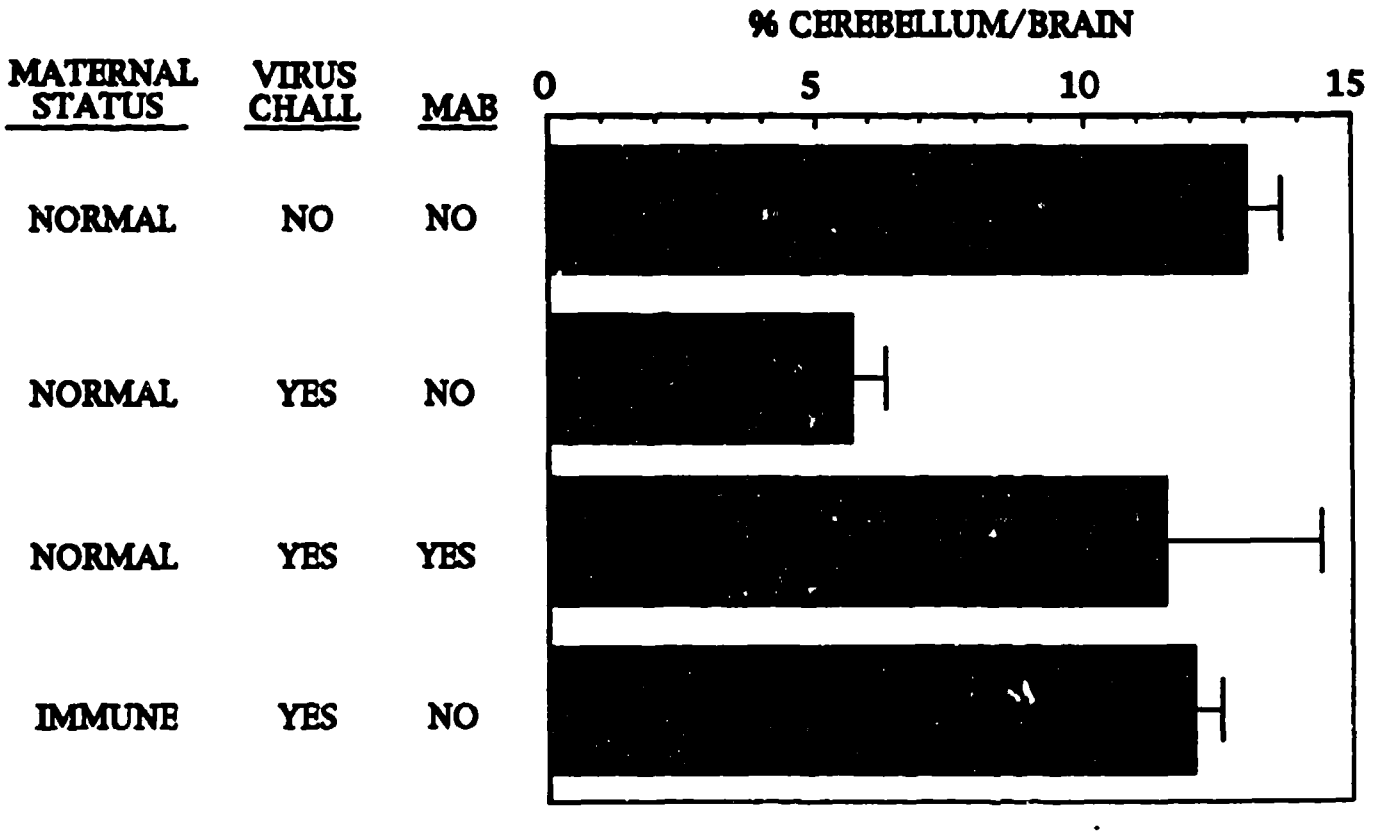



Figure 27. The assessment of maternally-transferred protection was determined by comparing the percentage of the cerebellum to total brain weight in 22 day old Lewis rats. Suckling rats born and nursed by normal or immune dams were challenged by ic inoculation of 1000 PFU of Armstrong-4 at 4 days of age. As a positive control for protection a group of pups born and nursed by a normal dam recieved the protective monoclonal antibody 8-13 by ip injection immediately following viral challenge.

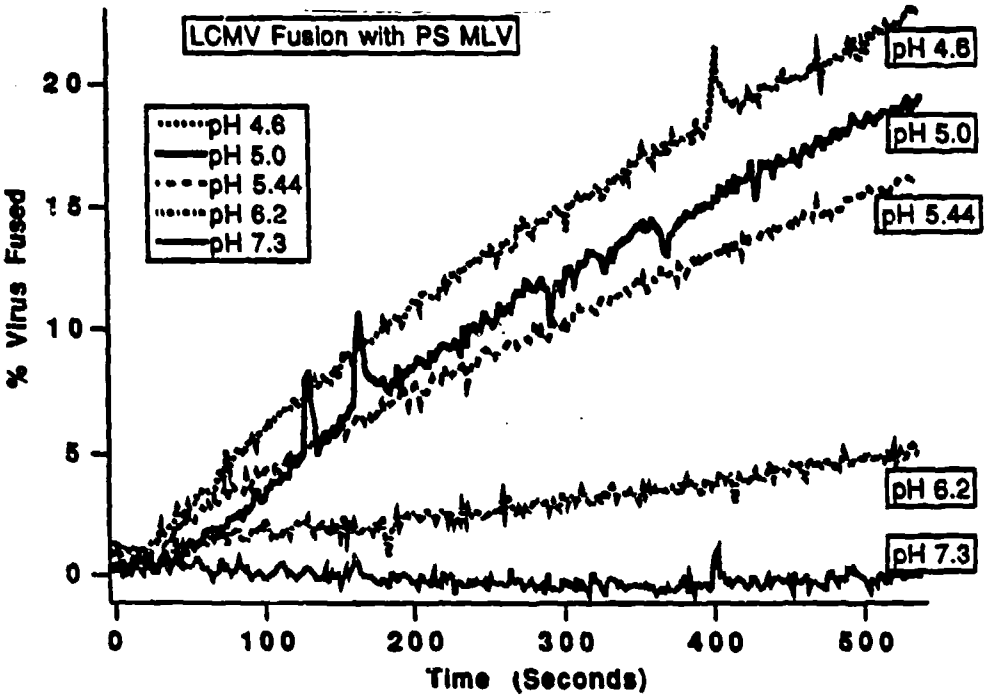



Figure 28.pH dependence of the kinetics of fusion of R18 (Octadecyl Rhodamine) labeled LCMV with phosphatidyl serine multilamellar vesicles (PS MLV): Ten μg of LCMV (viral protein) were added to a cuvette containing 10 μg of PS MLV in a PBS buffer at the appropriate pH. The cuvette and buffers were maintained at 37° C. An excitation wavelength of 560 was used and an emission wavelength of 590 was used to observe the R18 probe dequenching.

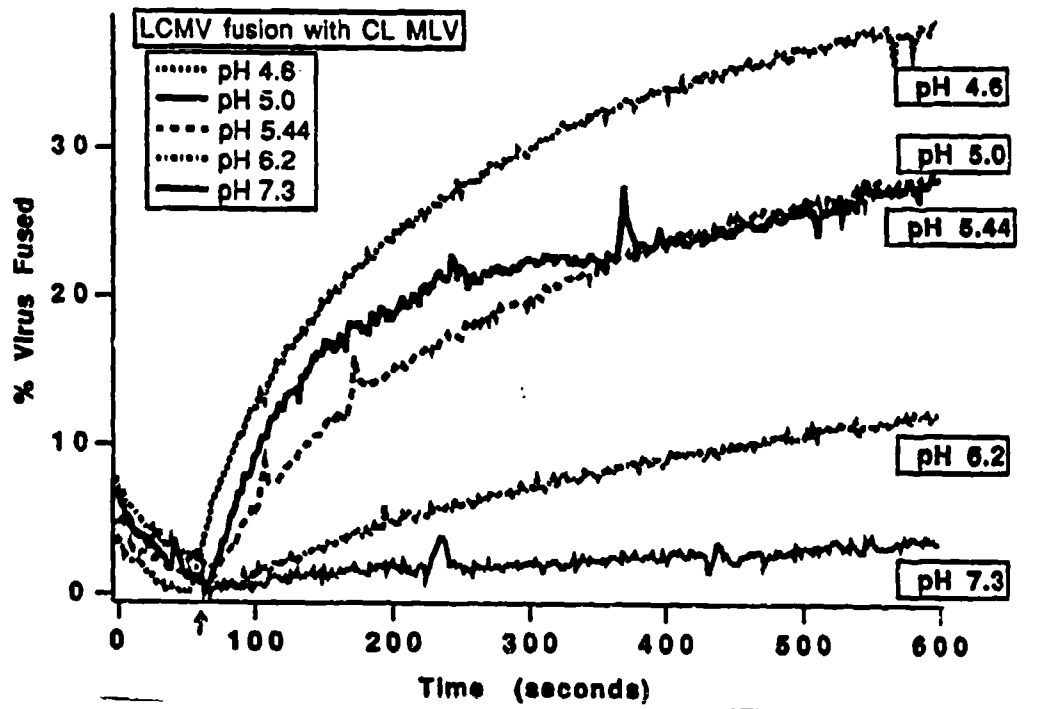



Figure 29.pH dependence of the kinetics of fusion of R18 (Octadecyl Rhodamine) labeled LCMV with cardiolipin multilamellar vesicles (CL MLV): Ten μg of LCMV (viral protein) were added to a cuvette at 60 seconds (arrow) containing 10 μg of CL MLV in a PBS buffer at the appropriate pH. The cuvette and buffers were maintained at 37° C. An excitation wavelength of 560 was used and an emission wavelength of 590 was used to observe the R18 probe dequenching.

are topics of continued study in this laboratory. It is crucial to understand these early events in arenavirus infection to design rational strategies for control of infection in man.

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Publications supported in part by this contract


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Chapter 9

# BIOLOGICAL AND GENOMIC VARIABILITY AMONG ARENAVIRUSES

**P. J. Southern and M. B. A. Oldstone**

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## I. INTRODUCTION

The arenavirus family contains several distinct viruses with markedly different biological properties.[1-5] Each virus is associated in the wild with a particular rodent host and is usually found in well-defined geographical regions, i.e., Lassa: West Africa, Junin: Argentina, Machupo: Bolivia. In contrast, lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, is widely distributed throughout the world (see Table 1). The viruses are maintained in natural rodent populations by both vertical and horizontal transmission. Despite a life-long viremia, the rodents normally show no overt signs of disease except under conditions of extreme crowding or stress. Primary human infection occurs from contact with infected animals or their excreta. Lassa fever transmission in man has occurred via contaminated blood or syringes.

LCMV infection of laboratory mice has provided an excellent model for virus-host interactions and virus persistence in vivo.[2] Different strains of LCMV have been studied in different laboratories, and substantial information has been accumulated relating to differences in pathogenic potential.[6] Recently, cDNA cloning and nucleotide sequencing experiments have generated information to examine differences between arenaviruses at the molecular level.[7-11] It is now possible to predict primary amino acid sequences for the major structural proteins (nucleocapsid protein [NP] and glycoprotein precursor [GP-C]) encoded by arenavirus genomic S RNA segments. Direct comparisons of these predicted protein sequences identify both conserved regions and divergent regions which may be involved with modifications to pathogenic potential.

In this chapter, we will review variations among arenaviruses and arenavirus-induced disease, especially in experimental laboratory infections. Correlations between disease states and viral gene products have been established using reassortant (mixed genotype) viruses,[12-14] and, with sequence information now available, it is possible to reconcile dramatic differences in biological properties of viruses with relatively small numbers of amino acid changes in viral structural proteins. There is a possibility that reassortant viruses may also arise in nature and be responsible for the appearance of new arenaviruses and a wider spectrum of diseases.

## II. HISTORICAL BACKGROUND

Perhaps the first record of an arenavirus infectious agent (subsequently to be named lymphocytic choriomeningitis virus) relates to a patient who had died during the 1933 epidemic of encephalitis in St. Louis. Using material obtained at autopsy, Armstrong and Lillie passed infection in monkeys and recovered a virus which, on the basis of pathological lesions in intracerebrally infected monkeys and mice, was designated the "virus of experimental lymphocytic choriomeningitis".[15] Shortly afterwards, Traub isolated a virus from an experimental mouse colony[16] and Rivers and Scott recovered viruses from two patients who had been treated for nonbacterial meningitis.[17] One of these patients was known to have worked with mice shown to be infected by Traub, but the source of infection for the second patient remained unknown. It was quickly realized that these independently isolated infectious agents were closely related, and the name lymphocytic choriomeningitis virus emerged.

There have been a number of documented cases of human disease caused by LCMV infection. For example, in the early 1970s an outbreak of LCMV occurred resulting in illness of children and adults. The source of virus was linked to persistently infected tumor cell lines and infected pet hamsters that had been obtained from a persistently infected breeding colony.[18] Most LCMV infections of adults result in subclinical, "influenza-like" illnesses and are usually resolved without further complication. The virus is, however, widely rep-

Table 1

| Virus | Disease | Locality | Rodent reservoir and vector | Person to person transmission | Laboratory model of human infection |
|---|---|---|---|---|---|
| Lymphocytic choriomeningitis | Grippe, aseptic meningitis, occasional more severe forms of meningoencephalomyelitis | Probably originated in Europe, now worldwide | *Mus musculus* natural host; colonized rodents, particularly mice and hamster, dog? | Never documented | Adult mouse inoculated intracerebrally |
| Junin | Argentinian hemorrhagic fever | Circumscribed area of Argentina: Buenos Aires to northwest | *Calomys musculinus*, possibly others | Occasional | Guinea pig |
| Machupo | Bolivian hemorrhagic fever | Beni region of Bolivia | *Calomys callosus* | Occasional, particularly spouses; recognized hospital outbreak | Rhesus monkey |
| Lassa | Lassa fever | Western Africa | *Mastomys natalensis* | Frequent; explosive intrahospital epidemics | Squirrel monkey |

resented in the U.S., as about 11 to 20% of the population show a positive serum antibody response against the virus.[4]

A number of distinct arenaviruses have been isolated and identified in the Americas and in Africa (reviewed in References 3, 4, and 19, see Table 1). In terms of human disease, Lassa virus in Africa and Junin and Machupo viruses in South America can cause severely debilitating and, on occasion, fatal infections. Individuals from outside areas face the greatest risk of serious infection, although infections are not uncommon among native populations. Clinical descriptions of human infections with Lassa, Junin, and Machupo viruses have recently been presented elsewhere[4] and will not be reviewed again here.

## III. PRINCIPLES LEARNED FROM LABORATORY MODELS OF LCMV INFECTIONS

Traub first described the phenomenon of persistent LCMV infection in laboratory mice and the fundamental pathogenic consequences of acute virus infection.[20] Intracerebral infection of an adult animal results in death 7 to 9 days postinfection, whereas equivalent infection of a newborn animal within the first 24 hr of life results in a persistent infection with life-long viremia. The acute lethal infection of adult animals was later shown by Rowe[21] to be immune mediated, as infection of adult, immunosuppressed animals resulted in a persistent infection. Persistently infected animals are not tolerant to the virus but produce antibodies directed against all known viral structural proteins that react with viral antigens in the circulation to form antigen-antibody complexes.[22,23] Accumulation of these complexes results in varying degrees of immune-complex disease. Indeed, LCMV persistent infection has been the model system for studying virus immune complexes, and many findings have subsequently been extended to diverse infections of man and animals (reviewed in References 24 and 25).

The prevention of lethal, intracerebral infection in adult mice by immunosuppression and the appearance of immune complex disease in persistently infected mice established an important involvement for the host immune response in the pathogenesis of virus-induced disease. For example, study of the immune response to LCMV during acute infection led to the first description of cytotoxic T lymphocytes (CTL)[26] and the finding that such cells recognized both a specific viral determinant and a syngeneic major histocompatibility protein.[27] These observations, first recorded with LCMV infection, have been extended throughout the realm of microbiology to other animals, man, and infectious agents. In both acute and persistent infections, disease frequently follows from the immune response to the virus rather than being caused directly by virus replication. More recent work has described the ability of this nonlytic virus to replicate in differentiated cells and alter their specific differentiation product, leading to altered homeostasis and disease.[28]

## IV. LCMV VARIANTS (STRAINS) AND DIFFERENT BIOLOGIC PROPERTIES

LCMV has been recognized as a manipulable and reproducible model for infection of laboratory mice, and several investigators have established independent virus isolates that have subsequently been passaged under different conditions. This has generated an extensive and sometimes conflicting literature for LCMV, however, it is now clear that discrepancies between published reports often reflect the generation of viral variants (strains) with fundamentally different properties.

The variability resulting from infections with many different combinations of animal and virus strains is summarized in Table 2. When mice are infected within the first 24 hr after birth (newborns), there is an initial period of active virus replication and release of progeny

**Table 2**
**LCMV STRAIN AND DISEASE ASSOCIATION**

| LCMV-induced disease or phenomena | Disease in LCMV strains | | | | |
|---|---|---|---|---|---|
| | ARM | E-350 | WE | PASTEUR | TRAUB |
| Growth Hormone (GH) deficiency in persistently infected C3H/St mice | | | | | |
| Death | >95% | >95% | <5% | 30% | <5% |
| Hypoglycemia | + + + + | + + + + | nil | + + | nil |
| Poor growth | + + + + | + + + + | nil | + + | nil |
| % GH cells containing viral antigen | >95% | >95% | <10% | 40% | <13% |
| Hyperglycemia abnormal glucose tolerance test in SWR/J, BALB mice, β cells in islets of Langerhans of pancreas containing virus | + + + | + + + | + + | + + | + + |
| Immune complexes in persistently infected SWR/J mice | + + + + | + + + + | + + + | + + + | + |
| Acute death of adult guinea pigs | nil with $< 10^5$ PFU | | + + + + with 1 PFU | | |

virus particles followed by an altered pattern of viral gene expression that marks the progression from acute to persistent infection. Molecular details of this regulatory change are still being described. There is a significant reduction in the release of infectious virus particles that correlates with reduced expression of the viral glycoproteins, but viral nucleic acid sequences and viral nucleoprotein continue to accumulate.[28,29] In most circumstances, LCMV infection of newborn animals results in maintenance of low levels of virus ($10^3$ to $10^5$ pfu per gram of tissue or per ml of serum) and substantial amounts of intracellular viral nucleic acid throughout the life-span. Circulating antibodies directed against viral proteins combine with viral antigens to form immune complexes that frequently complicate the infection. For example, certain mouse strains, i.e., SWR/J, are high level antibody responders to LCMV, but others, like BALB/WEHI, are low responders.[7] Responses are controlled by a number of host genes including immune response genes (Ir) located within the histocompatibility complex. Non-H2 genes also play a role. Some persistently infected animals manifest subtle alterations in specialized cell functions, e.g., hyperglycemia[30] (due to infection of beta cells of the islets of Langerhans) and decreased thyroid hormone[31] ($T_3$ and $T_4$) levels due to persistent infection of thyroid follicular cells.

A severe growth hormone deficiency disease occurs in 13- to 30-day-old C3H St mice that have been inoculated at birth with LCMV Arm or E-350 strains. This results in approximately 95% of the animals dying from low blood sugar. These infected animals fail to grow at the same rate as uninfected littermates and, at the time of death, show about a 50% weight reduction relative to controls. Such animals have lowered growth hormone levels in the pituitary, and reconstitution experiments involving the introduction of rat pituitary cells (the GH3 cell line) that secrete growth hormone allow the infected mice to develop normally and maintain normal blood glucose levels.[32,33] This reconstitution experiment suggests that a defect in growth hormone is responsible for abnormal growth and development. LCMV ARM and E-350 replicate extensively in the growth hormone-producing cells, while the other LCMV strains, Traub and WE, that fail to induce growth hormone disease, replicate poorly in growth hormone-synthesizing cells.[34] Interestingly, these virus strains (Traub, WE)

**Table 3**
**LCMV RNA SEGMENT AND DISEASE ASSOCIATION**

| LCMV-induced disease state or phenomena | LCMV strain | | LCMV RNA segment causing disease |
|---|---|---|---|
| | Virulent | Avirulent | |
| Growth hormone deficiency in persistently infected C3H/St mice (poor growth, hypoglycemia, death) | ARM | WE | S RNA ARM |
| Acute death in adult guinea pigs | WE | ARM | L RNA WE |
| Immune complexes in persistently infected SWR/J mice | ARM | TRALB | S RNA ARM |
| Interferon-induced liver necrosis and death of BALB/WEHI mice | WE<br>ARM | ARM<br>WE<br>ARM WE | L RNA of WE<br>S RNA of ARM |
| Induction and generation of virus specific H2 restricted cytotoxic T lymphocytes | ARM | PASTEUR | S RNA of ARM |

replicate in C3H mouse liver and spleen and show a typical, widespread distribution of viral nucleic acid sequences and infectious virus in most tissues. Hence, the growth hormone disease correlates with virus replication in selected cells of the anterior pituitary. However, it is not yet clear whether the differences in LCMV strains and disease potential are exerted at the level of virus adsorption and uncoating or at the level of virus replication within the growth hormone-producing cells of the anterior pituitary. Reassortant viruses, made between an LCMV strain that causes disease (Arm) and one that does not (WE), have been used to establish that the growth hormone disease is associated with the S RNA segment of the Arm strain[3] and, by implication, genes encoded by that segment. The S RNA encodes the nucleocapsid protein and glycoproteins.[2] This suggests that the growth hormone disease may reflect a tropism of infection rather than differential replication because the viral replicase functions are encoded by the L RNA segment.[4]

## V. REASSORTANT GENOTYPE LCM VIRUSES

The isolation and characterization of reassortant viruses from unique parental LCMV strains has produced considerable new information[3,14] (Table 3). Simultaneous infections with the two parental viruses allowed random interactions between input L and S genomic RNA segments, and reassortant viruses of mixed genotype were identified in the progeny virus population by screening with monoclonal antibodies and nucleic acid hybridization probes. Recovery of both pairs of potential reassortant viruses (for example, Arm L/WE S and WE L/Arm S) has allowed an unambiguous assignment of biological function to a genomic RNA segment. In this way, growth hormone disease in C3H mice has been mapped to the Arm genomic S RNA segment,[3] and a lethal infection in adult guinea pigs correlates with the presence of the WE genomic L segment.[4] Target specificity for H2-restricted CTL killing has also been mapped to the LCMV genomic S RNA segment.[14]

The frequency of recovery of reassortant viruses appears to have varied according to the pairing of parental LCMV strains. Reassortants between Arm and Pasteur were recovered at significantly greater frequency than Arm and WE reassortants.[3,14] This suggests that transcription and/or replication signals for Arm and Pasteur may be more closely related than for Arm and WE, and raises the possibility of mutational change at regulatory sites as a prerequisite for successful propagation of reassortant viruses. Experiments involving direct

RNA sequencing of virion RNA preparations are now in progress to examine the frequency of sequence change at the population level (Salvato et al., unpublished result(s)).

## VI. MOLECULAR BIOLOGY AND SEQUENCING

Recently, a number of laboratories have initiated molecular cloning experiments with arenavirus genomic RNA segments.[7-11] Different cloned cDNA sequences are now available for the following applications:

1. Evaluation of the complete genetic potential of the viruses.
2. Production of hybridization probes to monitor viral gene expression and gene regulation.
3. Comparisons of nucleotide and (predicted) protein sequences for the different arenaviruses.
4. Investigations of the molecular basis of arenavirus-induced disease.

The genomic organization of the viral S segment involves an unusual ambisense gene coding arrangement[7,10,12] (reviewed in Volume I, Chapter 9). Both the major viral structural proteins NP and GP-C are encoded by the S RNA segment — NP mRNA is complementary to the genome, whereas GP-C mRNA is in the sense of the genome. The NP and GP-C coding regions do not overlap and are separated by a short intergenic hairpin. The hairpin region and the ambisense gene organization are likely to be involved in regulation and discrimination between transcription and replication,[7,12] but detailed schemes are not presently available.

The genetic structure of the viral L RNA segment is not as well defined. There is a very long open reading frame which is apparently involved with synthesis of a 150- to 200-kdalton viral polymerase or replicase protein. This coding region is associated with a mRNA that is complementary to the L segment.[7,8]

## VII. CODING ASSIGNMENTS

The major viral structural proteins NP and GP-C have been mapped to the S RNA segment by both genetic and biochemical techniques.[13-15] Definitive experiments using antisera to synthetic peptides derived from regions of the predicted protein sequences have shown that the gene order for the S segment is 3′ NP, GP-2, GP-1, 5′.[14,15,16] Cleavage of the GP-C precursor, to release the mature GP-1 and GP-2 species, has been mapped to residues 262-263 in GP-C.[14] Antisera raised against synthetic peptides that converge from either side of this site recognize, respectively, GP-1 in the amino-terminal part of GP-C, and GP-2 in the carboxy-terminal part. The cleavage site, containing two adjacent basic amino acids, is conserved between LCMV Arm and WE, Pichinde, and Lassa viruses. A high-molecular-weight putative polymerase (L protein) originally assigned to the L segment by size considerations has now been detected using antibodies to L-derived synthetic peptides.[16] Similar experiments using antipeptide antibodies will be used to evaluate additional potential protein coding regions that may be detected in genomic L cDNA clones.

## VIII. TERMINAL SEQUENCE HOMOLOGIES

The 3′ terminal sequences of the genomic L and S RNAs are identical for 17 of the first 19 positions, and for the S segment, the 5′ terminal sequence is complementary to the 3′ sequence.[7,8] There is no sequence information currently available for the genomic 5′ L terminus, but, by analogy with S and other single-stranded RNA viruses, we can anticipate preservation of the complementary sequence character. These terminal sequences probably

**Table 4**
**SIGNIFICANT AMINO ACID CHANGES IN GP-C[a]**

| GP-C residue | LCMV arm | LCMV WE |
|---|---|---|
| 110 | L | P |
| 133 | T | S |
| 173 | T | S |
| 174 | F | S |
| 177 | A | P |
| 181 | Q | M |
| 216 | K | Y |
| 240 | T | R |
| 253 | S | A |
| 265 | A | S |
| 313 | A | E |

[a] GP-C residues 1 to 262 = GP-1; 263 to 498 = GP-2.

represent binding sites for the viral RNA-dependent RNA polymerase and/or a nucleation site for the binding of NP in the formation of ribonucleoprotein complexes. Auperin and Bishop[41] have noted the presence of an additional G residue at the exact 5′ terminus of Pichinde and Lassa S genomic segments and have suggested that this may function to discriminate between the 5′ ends of the genomic sense and genomic complementary sense RNAs. This additional G residue was not reported in the complete sequence of the WE genomic S segment,[9] so any suggested function for control of replication requires further experimental support.

## IX. NUCLEOTIDE AND PROTEIN SEQUENCE CONSERVATION

The LCMV strains Armstrong (Arm) and WE represent the most homologous pair of arenaviruses for which sequence information is presently available. In the S protein coding regions there is 80 to 85% conservation of nucleotide sequence with transitions occurring much more frequently than transversions, and the sequences can be aligned without any significant insertion or deletion. Conservation of amino acid sequences is somewhat higher (90 to 95%), indicating the silent character of many of the nucleotide changes. There are only a limited number of amino acid changes that might be expected to produce significant changes in the structures of the folded proteins, and well-characterized differences in biological properties or reactivities with neutralizing monoclonal antibodies[42] may reside in single amino acid changes[10,11] (Table 4).

On the basis of protein sequence relatedness, LCMV shows somewhat more homology to Lassa than Pichinde, and Lassa and Pichinde are are no more closely related to LCMV than they are to each other.[11] The viral structural proteins show highly conserved regions which are interspersed with divergent regions. This type of arrangement was previously indicated from cross-protection studies and conserved and unique epitopes that had been defined by monoclonal antibodies.[11] The alignment of amino acid sequences for GP-C molecules indicates that the greatest diversity occurs in the region between residues approximately 120 to 240 in GP-1 (Table 5). This alignment has been made with the minimum number of gaps being introduced into the amino acid sequences and emphasizes sequence conservation among the GP-2 molecules. The mechanism of sequence evolution among arenaviruses remains to be elucidated, but there is now substantial cumulative evidence for a common ancestral virus that has diverged while becoming fixed in distinct geographical locations within specific rodent hosts.

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**Table 5**

ARENAVIRUS GLYCOPROTEINS

```
                                                                  GP-1   |  GP-2
          ARN   MGQIVTMFEA LPHIIDEVIN IVIIVLIVIT GIKAVYNFAT CGIFALISFL
           WE   MGQIVTMFEA LPHIIDEVIN IVIIVLIIIT SIKAVYNFAT CGILALVSFL
           LA   MGQIVTFFQI VPHVIDEVMN IVLIALSVLA VLKGLYNFAT CGLVGLVTFL
           PV   MGQIVTLIQS IPEVLQEVFN VALIIVSVLC IVKGFVNLHR CGLFQLVTFL
    CONSERVED   MGQIVT      P    EV N    I          K   N    CG   L  FL

          ARN   LLAGRSCGMY GLKGPDIYKG VYQFKSVEFD MSHLNLTMPN ACSANNSHHY
           WE   FLAGRSCGHY GLNGPDIYKG VYQFKSVEFD MSHLNLTMPN ACSVNNSHHY
           LA   LLCGRSCT.. ....TSLYKG VYELGTLELN METLNMTMPL SCTKNNSHHY
           PV   ILSGRSCDSH MIDRRHNLTH VEFNLTRMFD NL......PQ SCSKNNTHHY
                 L GRSC                 M                P   C  NN HHY

          ARN   ISMGTS...G LELTFTNDSI CSNKFCNLTS AFNKKTFDHT LMSIVSSLHL
           WE   ISMGSS...G LEPTFTNDSI CSNKFCNLTS ALNKKSFDHT LMSIVSSLHL
           LA   IMVGNET..G LELTLTNTSI INHKFCNLSD AMNKNLYDHA LMSIISTFHL
           PV   YRGPSNTTWG IELTLTNTSI ANETSGNTSN SGGLGYGNIS NCDRTREAGH
                      G  E T TN SI        N

          ARN   SIRGNSNYKA VSCDFNNG.. .......... .ITIQYNLTF SDAQSAQSQC
           WE   SIRGNSNYKA VSCDFNNG.. .......... .ITIQYNLSS SDPQSAMSQC
           LA   SIFNFNQYEA MSCDFNGS.. ....K..... .ISVQYNLSH SYAGDAANHC
           PV   TLKWLLNELH FNVLHVTRHI CARCKTVEGA GVLIQYNLTV GDRGGFVGRH
                                                    QYNL

          ARN   RTFRGRVLDH F.RTAFGGKY MRSGWGWTGS DGKTTW.CSQ TSYQYLIIQN
           WE   RTFRGRVLDH F.RTAFGGKY MRSGWGWTGS DGYTTW.CSQ TSYQYLIIQN
           LA   GTVANGVLQT FNRMAWGGSY I......ALD SGRGNWDCIM TSYQYLIIQN
           PV   LIASLAQIIG DPKIAWVGKC FNNCSGGTCR LINCEGGTH. ..YNFLIIQN
                          A  G                                 Y  LIIQN

                                                    GP-1   |  GP-2
          ARN   RTWENHCTYA ..GPFGMSRI LLSQEKTKFF TPRLAGTFTW TLSDSGGVEN
           WE   RTWENHCRYA ..GPFGMSRI LFAQEKTKFL TPRLSGTFTW TLSDSGGVEN
           LA   TTWEDHCQFS RPSPIGYLGL LSQRTRDIYI SPRLLGTFTW TLSDSEGKDT
           PV   TTWENHCTYT ...PMATIRM ALQRTAYSSV SPKLLGFFTW DLSDSSGQHV
                 TWE HC       P                    R L G FTW  LSDS G

          ARN   PGGYCLTKWM ILAAELKCFG NTAVAKCNVN HDAEFCDMLR LIDYNKAALS
           WE   PGGYCLTKWM ILAAELKCFG NTAVAKCNVN HDEEFCDMLR LIDYNKAALS
           LA   PGGYCLTRWM LIEAELKCFG NTAVAKCNEK HDEEFCDMLR LFDFNKQAIQ
           PV   PGGYCLEQWA ITWAGIKCFD NTVMAKCNKD HNEEFCDTMR LFDFNQNAIK
                PGGYCL  W     A  KCF  NT  AKCN   H  EFCD  R L D N  A

          ARN   KFKEDVESAL MLFKTTVNSL ISDQLLMRNH LRDLMGVPYC NYSKFWYLEH
           WE   KFKQDVESAL MVFKTTLNSL ISDQLLMRNH LRDLMGVPYC NYSKFWYLEH
           LA   RLKAEAQMSI QLINKAVNAL INDQLIMKNH LRDIMGIPYC NYSKYWYLNH
           PV   TLQLNVENSL MLFKKTINQL ISDSLVIRNS LKQLAKIPYC NYTKFWYIND
                          M L I D L   N  L       PYC NY K WY

          ARN   AKTGETSVPK CWLVTNGSYL NETHFSDQIE QEADNMITEM LRKDYIKRQG
           WE   AKTGETSVPK CWLVTNGSYL NEIHFSDQIE QEADNMITEM LRKDYIKRQG
           LA   TTTGRTSLPK CWLVSNGSYL NETHFSDDIE QQADNMITEM LQKEYMERQG
           PV   TITGRHSLPQ CWLVHNGSYL NETHFKNCWL WESQNLYNEM LMKEYEERQG
                TG  S P  CWLV NGSYL NE HF          N   YN L K Y  RQG

          ARN   STPLALMDLL MFSTSAYLVS IFLHLVKIPT HRHIKGGSCP KPHRLTNKGI
           WE   STPLALMDLL MFSTSAYLIS IFLHFVRIPT HRHIKGGSCP KPHRLTNKGI
           LA   KTPLGLVDLF VFSTSFYLIS IFLHLVKIPT HRHIVGKSCP KPHRLNHNGI
           PV   KTPLALTDIC FWSLVFYTIT VFLHIVGIPT HRHIIGDSCP KPHRITRNSL
                 TPL L D     S   Y     FLH V IPT HRHI G  CP KPHR

          ARN   CSCGAFKVPG VKTVWKRR
           WE   CSCGAFKVPG VKTIWKRR
           LA   CSCCLYKQPG VPVKWKR
           PV   CSCGYYKYQR NLTNG
                CSCG  X
```

## X. CONSIDERATIONS FOR THE FUTURE

The availability of cloned arenavirus cDNA sequences should support many further advances in our understanding of virus gene regulation and expression and the mechanisms of virus pathogenesis. The isolation of a reassortant virus from a laboratory mixed infection that has a pathogenic potential possessed by neither of the parental virus strains (Table 3)[44,45]

is particularly significant. This may be indicative of a mechanism that has contributed to the diversity of known arenaviruses, and may account for the appearance of new arenaviruses with new disease associations. It is now possible to examine the expression of individual viral genes to assess their relative importance for recognition and interaction with the host immune system. Also, cDNA genes can be mutated or recombined in vitro and reintroduced into cells or animals either as double-stranded DNA or RNA to identify alterations in biological properties. These approaches should define epitopes within the viral proteins that relate directly to pathogenic potential, and this may provide the key to an effective vaccine strategy for arenaviruses.

## ACKNOWLEDGMENTS

This is Publication Number 4487-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA. This work was supported in part by USPHS grants NS-12428, AI-09484, and AG-04342 and by USAMRIID contract C6234. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents. We thank Kathy Nasif and Gay Schilling for secretarial assistance.

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VIROLOGY 183, 620–629 (1991)

# Protein–Protein Interactions in Lymphocytic Choriomeningitis Virus

JOHN W. BURNS AND MICHAEL J. BUCHMEIER[1]

*Division of Virology, Department of Neuropharmacology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037*

*Received January 16, 1991; accepted April 22, 1991*

**The structural organization of the lymphocytic choriomeningitis virus (LCMV) particle has been examined by Triton X-114 phase separation and nearest neighbor analyses in order to define protein–protein interactions in the virion. Extraction with Triton X-114 established that the 44-kDa membrane glycoprotein, GP-1, is a peripheral protein and that the 35-kDa glycoprotein, GP-2, is an integral membrane protein. Membrane permeable and membrane impermeable crosslinking reagents were used to establish the structural organization of the virion. Results obtained with both types of crosslinking reagents demonstrated that both GP-1 and GP-2 were assembled as native homotetramers. No covalent or disulfide linkages were found between GP-1 and GP-2, nor were these glycoproteins crosslinked. Protein complexes composed of GP-2 and NP were observed after treatment with a membrane permeable crosslinker (DMS) but not after treatment with the membrane impermeable crosslinker (DTSSP), localizing the site of the GP-2:nucleocapsid protein (NP) interaction to the interior of the virion. The interaction of GP-2 with NP may be important in directing the maturation and budding of LCM virions.** © 1991 Academic Press, Inc.

## INTRODUCTION

Biological studies of lymphocytic choriomeningitis virus (LCMV) have provided major insights in viral immunology and pathogenesis. Recent observations have identified new concepts of viral persistence and virus-induced diseases of the endocrine and central nervous systems. Cytolysis of virus-infected cells, mediated by T-lymphocytes, was first described using the LCMV model (Cole *et al.*, 1972). Likewise, MHC restriction of the cytotoxic T-lymphocyte (CTL) response to infected cells was first described using the LCMV model (Zinkernagel and Doherty, 1974). While early studies of viral persistence focused on the biology of LCMV infections *in vivo*, later reports attempted to provide molecular explanations for the phenomenon of persistence (Oldstone and Buchmeier, 1982; Ahmed *et al.*, 1984; Pircher *et al.*, 1990). In addition, immunologic studies have provided information on immune recognition of LCMV by B- and T-lymphocytes (Parekh and Buchmeier, 1986; Whitton *et al.*, 1988a,b; Wright *et al.*, 1989), virus-induced immune suppression (Ahmed *et al.*, 1984; Oldstone *et al.*, 1988), and delayed-type hypersensitivity (Lehmann-Grube, 1988; Moskophidis *et al.*, 1989, 1990).

LCMV, the prototype member of the Arenaviridae, contains a single-stranded, ambisense genome consisting of two RNA segments, L and S. Sequencing of both genomic segments has recently been completed (Romanowski *et al.*, 1985; Riviere *et al.*, 1985; Southern *et al.*, 1987; Salvato *et al.*, 1988; Salvato and Shimomaye, 1989). Open reading frame analysis revealed four primary translation products. These are encoded in a unique ambisense arrangement and include the 200-kDa putative RNA polymerase (L) and the 11- to 14-kDa Z protein on the L segment. The 63-kDa nucleocapsid protein (NP) and the 75-kDa glycoprotein precursor, GP-C, are encoded on the S segment. L and NP are encoded in a genomic complementary sense from the 3′ end of L and S, respectively, while Z and GP-C are encoded in message sense from the 5′ end of the genomic L and S RNA segments. Three additional small open reading frames (each capable of encoding a polypeptide of less than 100 amino acids) have been identified but there is no evidence to indicate that these are utilized (Salvato and Shimomaye, 1989).

Arenavirus particles are roughly spherical with an average diameter of 90–120 nm and are covered with distinct 5- to 10-nm club-shaped spikes projecting from the envelope (Vezza *et al.*, 1977; Pedersen, 1979). It has been proposed that the spikes, seen by electron microscopy, are composed of one or both of the glycoproteins (Gard *et al.*, 1977; Vezza *et al.*, 1977; Compans and Bishop, 1985). Protease digestion of virions, using either chymotrypsin or bromelain, resulted in the removal of the spikes with a concomitant loss of the viral glycoproteins from the virion (Gard *et al.*, 1977; Buchmeier *et al.*, 1978). By analogy with other enveloped viruses, the spike is presumably involved in receptor binding to the cell surface. This possibility is supported by the observation that several monoclonal antibodies to GP-1 block binding of LCMV to tissue

[1] To whom requests for reprints should be addressed.



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culture cells (G. van den Dobbelstein and M. Buchmeier, unpublished data).

To establish a structural basis for the observed biological activities and immunochemical properties of LCMV it is essential to derive a working model for the structural organization of the virion and its constituent proteins. We are interested in localizing the immunologically and biologically significant domains of the glycoprotein spike on its three-dimensional structure. In this communication we report the findings of our structural analyses of the LCM virion.

## MATERIALS AND METHODS

### Cells and virus

Baby hamster kidney cells (BHK) were maintained in DMEM supplemented with 8% fetal calf serum, glutamine, and antibiotics. The LCMV-Armstrong clone 4 (Arm-4) was used throughout these studies. This virus was originally isolated from LCMV CA 1371 and was triple plaque-purified (Wright *et al.*, 1989). Stocks of virus were grown in 850-cm$^2$ roller bottles containing semiconfluent BHK cells at a low multiplicity of infection. Virus was purified as previously described (Buchmeier and Oldstone, 1979) with minor modifications. First, NaCl was omitted from the polyethylene glycol precipitation step and two continuous gradients of 20–60% and 25–45% Renografin (v/v) in 10 m*M* Tris–HCl, 100 m*M* NaCl, 1 m*M* EDTA, pH 7.4 (TNE) were used consecutively to band the virus. Purified virions were pelleted and resuspended in a minimal volume of TNE. Protein concentrations were determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). Virus preparations routinely contained 1.0–1.5 mg protein/ml/liter of starting supernatant and contained typical LCM virions on examination by electron microscopy. For radiolabeled virus, $^{35}$S-Translabel (ICN, Costa Mesa, CA) was added to a concentration of 15 $\mu$Ci/ml 24 hr after infection. Labeled virus was harvested after an additional 24-hr incubation and purified as described above.

### Triton X-114 phase separation

Purified $^{35}$S-labeled virus was separated into aqueous and detergent phases using Triton X-114 for three cycles of: 5 min incubation at 4°, 3 min incubation at 30°, and centrifugation through 6% sucrose for 3 min at 325 *g* as described (Bordier, 1981). Commercially available purified membrane grade Triton X-114 was used (SurfactAmps X-114, Pierce, Rockford, IL). Upon completion of the phase separation, aqueous and detergent phase samples were analyzed on a 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel followed by autoradiography.

### Triton X-100 disruption and sucrose density gradient centrifugation of LCMV

Purified radiolabeled virus particles suspended in TNE were incubated in 1% Triton X-100 for 30 min at 37°. The sample was fractionated by centrifugation at 4° on a 5–50% sucrose density gradient (w/v in TNE) in an SW 50.1 rotor for 18 hr at 35,000 rpm. The gradients were fractionated by bottom puncture and 0.25- to 0.3-ml fractions were collected. Electrophoresis sample buffer (300 m*M* Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 10% SDS, 0.05% bromphenol blue) was added to aliquots of each fraction to obtain a final concentration of 2% 2-mercaptoethanol, 2% SDS. The samples were heated at 95–100° for 3–4 min and analyzed on a 10% SDS–polyacrylamide gel (Laemmli, 1970) followed by autoradiography. Sedimentation coefficients were estimated by comparison with IgM (19 S), IgG (7 S), and horseradish peroxidase (3.8 S) standards run in parallel.

### Immunoblotting of mildly disrupted or crosslinked Arm-4

Mildly disrupted virus preparations were obtained by incubating aliquots of purified virus on ice for 5 min in TNE containing 1% SDS, 10 m*M* 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma Chemical Co., St. Louis, MO; Hjelmeland, 1980), or 12 m*M* *n*-octyl $\beta$-D-thioglucopyranoside (OSG, Calbiochem, La Jolla, CA; Saito and Tsuchiya, 1984), in the presence or absence of 1 *M* urea. To determine the effect of disulfide reduction, increasing amounts of dithiothreitol (DTT) were added to specific aliquots as indicated. These mildly disrupted preparations were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) on 10% gels (Laemmli, 1970). Separated proteins were electrophoretically transferred to Immobilon P membranes (Millipore, Bedford, MA) for 2 amp-hr in 25 m*M* Tris–HCl, 192 m*M* glycine, 20% methanol, pH 8.3. All SDS–PAGE and electrophoretic transfer procedures were performed at 4°. Membranes were blocked with BLOTTO (Johnson *et al.*, 1984) for 1 hr and incubated overnight in the appropriate rabbit antiserum in dilute BLOTTO (one part BLOTTO in four parts PBS). The remainder of the immunoblot procedure was completed as previously described (Burns *et al.*, 1989).

Crosslinking was done by incubating purified virus suspended in 100 m*M* triethanolamine–HCl, pH 8.2 (TE), with freshly prepared solutions of homobifunctional crosslinking reagents (all from Pierce, Rockford,

IL) in TE. Crosslinking reagents and reaction conditions used were: dimethyl suberimidate–2HCl (DMS) 1 mg/ml, 90 min at room temperature; 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), 3.6 mg/ml, 30 min on ice; or disulfosuccinimidyl tartarate (S-DST), varying concentrations, 30 min on ice (Davies and Stark, 1970; Garoff and Simons, 1974; Smith *et al.*, 1978; Lee and Conrad, 1985). Following incubation, the reactions were quenched by the addition of 1 *M* glycine to a final concentrations of 20 m*M* for DMS and DTSSP and 50 m*M* for S-DST. Electrophoresis sample buffer was added to a final concentration of 2% 2-mercaptoethanol (2ME), 2% SDS, 1 *M* urea. Due to the reversibility of DTSSP crosslinking under reducing conditions, nonreducing electrophoresis sample buffer (without 2ME) was used with DTSSP-crosslinked virus. Crosslinked preparations were heated at 95–100° for 4 min, loaded onto 5-15% SDS–polyacrylamide gradient gels, electrophoresed at 30 mA, transferred to Immobilon P membranes, and immunoblotted as described.

### Antisera

Rabbit antipeptide antisera were raised by inoculating New Zealand white rabbits with synthetic peptide conjugated to keyhole limpet hemocyanin. Peptides corresponding to amino acids 59–79 (GP-1) or 378–391 (GP-2) of GP-C or 130–144 of NP were used as immunogens. The generation and specificity of the peptide antisera have been described elsewhere (Buchmeier *et al.*, 1987).

## RESULTS

### Detergent extraction of LCMV structural proteins

To establish the nature of the membrane association of GP-1 and GP-2, we performed a Triton X-114 phase separation. This procedure differentiates peripheral from integral membrane proteins on the basis of partitioning into aqueous and detergent fractions in the presence of Triton X-114 (Bordier, 1981). As seen in Fig. 1, GP-1 was quantitatively extracted into the supernatant fraction (S), identifying it as a peripheral protein. In contrast, GP-2 was fractionated into the detergent phase pellet (P), consistent with the behavior of an integral membrane protein. These findings were further supported by the results of separation of the virion proteins in sucrose gradients (Fig. 2). Following velocity sedimentation of Triton X-100 disrupted virions, a significant amount of GP-1 was found in a highly enriched peak near the top of the gradient (fractions 13–15) and had a sedimentation coefficient of slightly less than 3.8 S. Based on an empirically determined sedimentation coefficient for a 40- to 44-kDa protein of

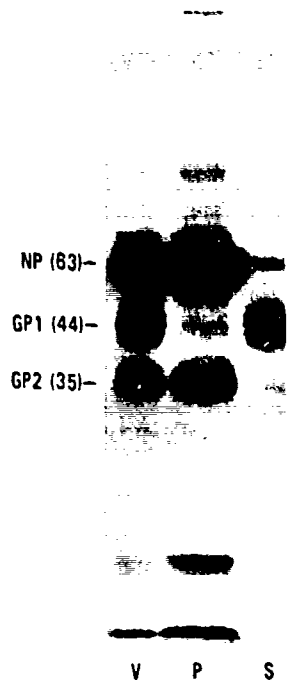



FIG. 1. Purified $^{35}$S-LCMV was subjected to Triton X-114 phase separation as described (Bordier, 1981). The final detergent pellet (P) was resuspended in TNE to a volume equal to the supernatant fraction (S). An aliquot of the original virus preparation (V) was included for comparison. Electrophoresis sample buffer was added until all samples contained 2% 2-mercaptoethanol, 2% SDS and heated for 4 min at 95°. Samples were electrophoresed on a 10% polyacrylamide gel containing SDS. The gel was then impregnated with a fluorigraphic enhancer, dried, and put on X-ray film (Kodak X-Omat AR) at −70°.

2.93–3.13 S (Young, 1987), we believe the GP-1 found in these upper fractions is most probably a monomer. It should be noted that some GP-1 is also present in more dense fractions, indicating that oligomeric forms or possibly aggregates were present. GP-2, in contrast, was found primarily associated with the pellet and in the more dense fractions of the gradient where NP was also concentrated.

### Tetrameric structure of native GP-1

A previous report demonstrated that a homopolymer of GP-1 was released from virions by incubation in 1% SDS at 4° (Wright *et al.*, 1989). To further investigate the native structure of GP-1, we used ionic, nonionic, and zwitterionic detergents to solubilize the GP-1 polymer in the presence or absence of 1 *M* urea. The immunoblot results shown in Fig. 3 demonstrate that under all solubilization conditions utilized, a homotetrameric GP-1 complex was detected.

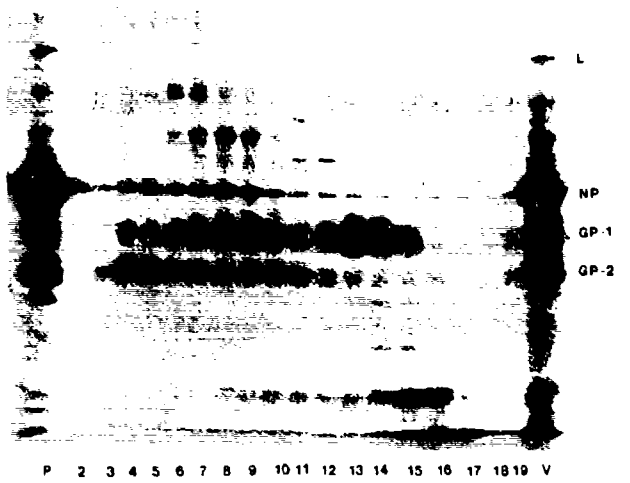



FIG. 2. Purified $^{35}$S-LCMV (200 μg) was incubated in 1% Triton X-100 for 30 min at 37°, layered onto a 5-ml 5–50% preformed sucrose gradient (w/v in TNE), and centrifuged for 18 hr at 35,000 rpm in a Beckman SW 50.1 rotor. The gradient was fractionated by bottom puncture and 250- to 300-μl fractions were collected. The pelleted material (P) at the bottom of the tube was suspended in 300 μl TNE. For comparison, an aliquot of the starting virus preparation was included (V). Samples were prepared and electrophoresed as described in Fig. 1. Sedimentation standards, run in parallel, were immunoglobulin M (19 S, fraction 5), immunoglobulin G (7 S, fraction 10), and horseradish peroxidase (3.8 S, fraction 12).




FIG. 3. Immunoblot of ionic, nonionic, and zwitterionic detergent solubilized GP-1 polymer. LCM virions (1.1 μg) were gently solubilized by incubation on ice for 5 min with 1% SDS (lanes 1 and 2), 12 m*M* OSG (lanes 3 and 4), or 10 m*M* CHAPS (lanes 5 and 6) in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 1 *M* urea. These mildly disrupted viral preparations were loaded onto 10% Laemmli gels without heating or reduction, electrophoresed at 4°, and analyzed by immunoblotting using rabbit anti-GP-1 peptide serum as described. The numbers 1, 2, 3, and 4 (right-hand margin) identify monomeric, dimeric, trimeric, and tetrameric forms for GP-1, respectively.

Next, we wanted to evaluate the role of disulfide bridges in the stabilization of the GP-1 homotetramer. It has been reported that reduction significantly affected the conformation of GP-1 as determined by electrophoretic mobility shifts in polyacrylamide gels (Bruns *et al.*, 1983a) and reactivity with neutralizing monoclonal antibodies specific for GP-1 (Wright *et al.*, 1989). We disrupted purified virus with 10 m*M* CHAPS under nonreducing conditions and in the presence of increasing concentrations of DTT. These disrupted virus preparations were examined by immunoblotting using rabbit antipeptide serum specific for GP-1. A ladder of bands representing oligomeric structures of the form $(GP\text{-}1)_1$, $(GP\text{-}1)_2$, $(GP\text{-}1)_3$, and $(GP\text{-}1)_4$ was seen under nonreducing conditions (Fig. 4, lane 1). With the addition of DTT at concentrations as low as 1 m*M*, the homotetramers began to dissociate (Fig. 4, lane 2). A resistant homodimer was observed under all reducing conditions examined. The intensity of the dimeric band varied inversely with the concentration of DTT (Fig. 4, lanes 2–7). Similar results were obtained using 2ME (data not shown) and demonstrate that disulfide bridges are essential to the stability of the native GP-1 tetramer.

Closer analysis of the monomeric form of GP-1 seen after reduction with low levels of DTT (Fig. 4, lanes 2–4) indicated that mild reduction resulted in the appearance of two monomeric bands containing GP-1. This suggested there were two populations of GP-1 monomer when mildly reducing conditions were employed, the reduced 44-kDa form and the slightly faster migrating nonreduced form. We believe these results suggest that an intramolecular disulfide bond maintains the monomeric conformation of GP-1 as determined by

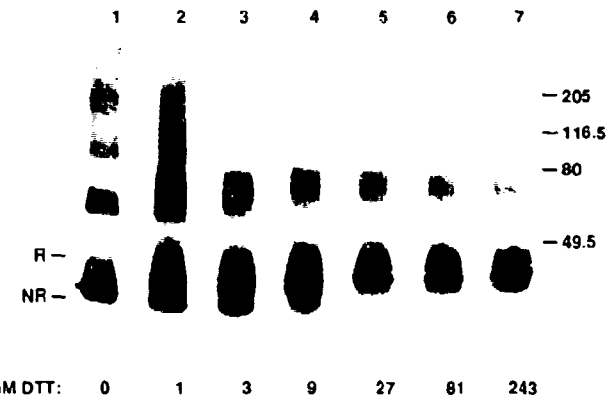



FIG. 4. Immunoblot of nonreduced and reduced CHAPS disrupted Arm-4. Purified LCMV was gently solubilized with 10 m*M* CHAPS under nonreducing conditions (lane 1) or in the presence of 1 m*M* (lane 2), 3 m*M* (lane 3), 9 m*M* (lane 4), 27 m*M* (lane 5), 81 m*M* (lane 6), or 243 m*M* (lane 7) DTT. Solubilization and immunoblotting conditions were as described in Fig. 3.

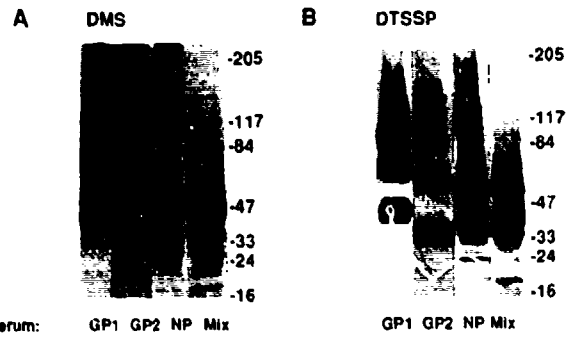



FIG. 5. Immunoblot of crosslinked Arm-4 ((A) DMS, (B) DTSSP) Aliquots of purified LCMV were crosslinked using the membrane permeable reagent, DMS (A) or the membrane impermeable reagent, DTSSP (B), both in 100 m*M* triethanolamine-HCl, pH 8.2. Following incubation periods of 30 min (DTSSP) or 90 min (DMS), crosslinking was quenched by the addition of 1 *M* glycine until a final concentration of 20 m*M* glycine was obtained. Crosslinked virus preparations were disrupted using reducing (DMS) or nonreducing (DTSSP) electrophoresis sample buffer and heating at 95–100° for 4 min. These samples were analyzed by immunoblotting following electrophoresis on 5–15% Laemmli gradient gels. Immobilon P membrane strips containing the transferred samples were probed using the appropriate rabbit antipeptide sera specific for GP-1 (lane 1), GP-2 (lane 2), or NP (lane 3) as described. Control virus (not crosslinked) was disrupted in the presence or absence of reducing agent and analyzed in parallel with the crosslinked preparations using a mixture (Mix) of the three sera.

its migration rate in polyacrylamide gels. When this disulfide bond was intact, the monomeric form of GP-1 migrated with an apparent molecular weight of approximately 40,000, representing a single population of "native" GP-1 monomers. Reduction of this disulfide bond resulted in a conformational change, presumably an unfolding of the molecule such that the electrophoretic migration of GP-1 decreases to ca. 44,000 and represents a second population of GP-1 monomers.

## Analysis of crosslinked virus

To investigate the topography of the virion, nearest neighbor analysis was performed by crosslinking intact virions using either DTSSP, a membrane impermeable reagent, or DMS, a membrane permeable reagent. These reagents crosslink lysine residues and other primary amines within 11 (DMS) or 12 Å (DTSSP). All proteins containing reactive amines within a proximity of 11–12 Å will theoretically be crosslinked using DMS, while only those located external to the membrane will be crosslinked by DTSSP.

The results of crosslinking virions prior to disruption are shown in Fig. 5. When the membrane permeable reagent DMS (Fig. 5A) was used, the GP-1 antiserum detected three protein complexes in addition to the reduced 44K monomer of GP-1 (lane 1). None of the complexes which reacted with the GP-1 antiserum were detected by either the GP-2 or the NP antisera (Fig. 5A, lanes 2 and 3), identifying them as homooligomers of GP-1. The apparent molecular weights of these crosslinked GP-1 complexes are listed in Table 1.

In addition to the monomeric 35K form of GP-2, at least five protein complexes were detected by the GP-2 antiserum in the DMS-crosslinked preparations (Fig. 5A, lane 2). Three of these complexes were not detected by the antisera specific for GP-1 or NP (Fig. 5A, lanes 1 and 3). We believe these represent the homodimeric, -trimeric, and -tetrameric forms of GP-2. Again this conclusion is supported by their apparent molecular weights (Table 1). The two remaining complexes detected by the GP-2 antiserum comigrated with two bands which were reactive with NP antiserum (Fig. 5A, lanes 2 and 3), suggesting that they are GP-2:NP heterooligomers. The molecular weights of these comigrating bands, 87,000 and 173,000, are close to that predicted for heterodimeric (GP-2:NP) and heterotetrameric $(GP\text{-}2:NP)_2$ complexes. The slowest migrating band seen in the panel probed with NP antiserum (Fig. 5A lane 3) may be either a higher ordered complex of NP or an NP:L dimer.

The pattern of protein complexes detected using the membrane impermeable crosslinking reagent DTSSP is shown in Fig. 5B. Again, the GP-1 antiserum reacted with four bands, consistent with monomers, homodimers, -trimers, and -tetramers of GP-1 (Fig. 5B lane 1). The pattern of protein complexes detected by the GP-2 antiserum following DTSSP crosslinking differs from that observed with DMS-crosslinked preparations. The GP-2:NP complexes observed in the DMS-crosslinked preparations using either GP-2 or NP antisera were not detected (Fig. 5B lanes 2 and 3). This result supports the previous observation that the two comigrating bands seen after DMS crosslinking contained complexes of GP-2 and NP. The four bands detected using the GP-2 antiserum are consistent with monomeric through homotetrameric forms of GP-2 (Fig. 5B lane 2). Table 1 summarizes the predicted and observed molecular weights for the complexes identified in these experiments.

We occasionally detected additional higher ordered structures after crosslinking and were concerned about their origin. It is important to bear in mind that crosslinking agents react not only with preexisting protein complexes but also with uncomplexed proteins located within the "reach" of the linker arm. Therefore, not every oligomeric band may represent a native macromolecular complex. To address this issue we performed additional crosslinking experiments under conditions designed to optimize the crosslinking of native

TABLE 1

THEORETICAL AND EXPERIMENTALLY DETERMINED MOLECULAR WEIGHTS OF CROSSLINKED COMPLEXES

| GP-1 | | | | GP-2 | | | | NP | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Protein complex | Molecular weight ($M_r$) | | | Protein complex | Molecular weight ($M_r$) | | | Protein complex | Molecular weight ($M_r$) | | |
| | Theoretical | DMS | DTSSP* | | Theoretical | DMS | DTSSP | | Theoretical | DMS | DTSSP |
| GP-1 | 44,000 | 46,000 | 40,000 | GP-2 | 35,000 | 34,000 | 35,000 | NP | 63,000 | 55,000 | 57,000 |
| $(GP\text{-}1)_2$ | 88,000 | 84,000 | 82,000 | $(GP\text{-}2)_2$ | 70,000 | 68,000 | 71,000 | (GP-2:NP) | 98,000 | 87,000 | — |
| $(GP\text{-}1)_3$ | 132,000 | 118,000 | 117,000 | (GP-2:NP) | 98,000 | 87,000 | — | $(NP)_2$ | 126,000 | 108,000 | 114,000 |
| $(GP\text{-}1)_4$ | 176,000 | 160,000 | 149,000 | $(GP\text{-}2)_3$ | 105,000 | 102,000 | 106,000 | $(GP\text{-}2:NP)_2$ | 196,000 | 173,000 | — |
| | | | | $(GP\text{-}2)_4$ | 140,000 | — | 129,000 | | | | |
| | | | | $(GP\text{-}2:NP)_2$ | 196,000 | 173,000 | — | | | | |

*Note.* The theoretical molecular weight for a given complex is the sum of the molecular weights of each individual component. Experimentally determined molecular weights were obtained from the relative electrophoretic mobility plot generated using prestained high and low-molecular-weight standards (Bio-Rad). Due to the sensitivity of DTSSP to reduction, all DTSSP crosslinked preparations were analyzed under nonreducing conditions (*)

structures and minimize the chance that independent molecules or structures would become crosslinked. We chose to employ the membrane impermeable homobifunctional crosslinking reagent S-DST. This reagent has a linker arm of 6.4 Å, about half that of either DMS or DTSSP. Crosslinking procedures were performed at 4° for 30 min using prechilled reagents in an effort to restrict protein mobility in the membrane. To further enhance crosslinking of native oligomeric structures, we crosslinked virions over a 125-fold S-DST concentration range (0.08–10 mg/ml). As seen in Fig. 6, even at the highest crosslinker concentration used (lanes 5 and 6), complexes larger than the previously described homotetramers of GP-1 or GP-2 were not detected. These findings are totally consistent with our previous crosslinking experiments and further substantiate the tetrameric structure of both GP-1 and GP-2.

## DISCUSSION

There has been great expectation for the potential use of synthetic peptides and recombinant expression vectors as vaccines against infectious agents (Lasky and Obijeski, 1984; Moss, 1986; Vadja *et al.*, 1990). For this approach to be effectively utilized, information concerning the identity of appropriate B- and T-cell epitopes must be available. An increasing number of potentially protective B-cell epitopes has been shown to be conformational in nature, complicating the effective employment of this type of vaccine. To date the structures of very few infectious agents or their subcomponents have been analyzed rigorously enough to address conformations of biological or immunological significance. The knowledge gained from the few structures that have been carefully analyzed has provided insight concerning receptor binding (Weis *et al.*, 1988; Rossmann and Palmenberg, 1988), viral uncoating (Smith *et al.*, 1986; Kim *et al.*, 1990), and other inherent biological activities (Colman *et al.*, 1983). A great deal has also been learned about the requirements for epitope recognition (Wiley *et al.*, 1981; Hogle *et al.*, 1985; Minor *et al.*, 1986), escape from immune surveillance (Hogle *et al.*, 1986; Rossmann, 1989), and virus neutralization (Prasad *et al.*, 1990).

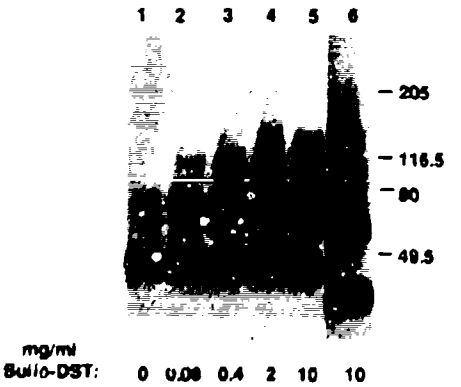



FIG. 6. Crosslinking of LCMV with S-DST verifies the homotetrameric structure of both GP-1 and GP-2. Purified LCMV (22 μg) suspended in 1/10 M triethanolamine HCl, pH 8.2, was crosslinked with the 6.4 Å membrane impermeable crosslinking reagent S-DST at various concentrations (lane 1, control; lane 2, 0.08 mg/ml; lane 3, 0.4 mg/ml; lane 4, 2 mg/ml; and lanes 5 and 6, 10 mg/ml). Crosslinked and control preparations were disrupted with reducing electrophoresis sample buffer, divided into two aliquots and electrophoresed in parallel on 5–15% Laemmli gradient gels, transferred to immobilon P membranes, and probed with rabbit antipeptide sera specific for GP-1 (lanes 1–5) or GP-2 (lane 6). Molecular weights were obtained using prestained high molecular weight standards (Bio-Rad).

We have been interested in the post-translational processing, transport, and immunology of the LCMV glycoproteins. Recent efforts in this laboratory have focused on (1) generating a B-cell epitope map (Parekh and Buchmeier, 1986; Weber and Buchmeier, 1988), (2) the conformational requirements of the neutralizing epitopes (Wright *et al.*, 1989), and (3) the processing and modification of the glycoprotein precursor, GP-C (Buchmeier *et al.*, 1987; Wright *et al.*, 1990). The results reported here describe the protein–protein interactions and topography of the LCM virion and spike. This information allows us to draw several conclusions concerning the structure of the LCM virion and its glycoprotein spikes.

To determine the degree of GP-1 and GP-2 membrane association, we performed Triton X-114 detergent extraction. This technique has been used to examine membrane interactions of many cellular and viral proteins (Bordier, 1981; Simons and Warren, 1984; Gething *et al.*, 1986; Mason, 1989). Our results clearly demonstrate fractionation of LCMV GP-1 into the aqueous phase using Triton X-114, indicating that GP-1 is a peripheral membrane protein. In contrast, GP-2 behaved as an integral protein. Additional support for this conclusion comes from our findings with sucrose gradient fractionation of virions disrupted with Triton X-100. In such gradients the upper, more slowly sedimenting fractions were found to contain large amounts of GP-1 in a soluble form, while the more rapidly sedimenting and pelleted fractions contained much of the GP-2 and NP. This may suggest, indirectly, a possible association between these two molecules. These findings are consistent with previous reports which showed that GP-1 was more accessible to iodination and antibody binding at the virion surface than was GP-2 (Buchmeier and Oldstone, 1979; Bruns *et al.*, 1983a).

To further investigate LCMV protein–protein interactions, we employed an immunoblot procedure for analyzing the protein complexes obtained after either mild disruption or crosslinking of virus particles. The availability of sequence-specific antisera to GP-1, GP-2, and NP allowed us to unequivocally determine the components of each complex, obviating the need for two-dimensional gel analysis. Chemical crosslinking was performed using the homobifunctional crosslinking reagents DMS, DTSSP, or S-DST. Comparison of the results obtained with DMS to those obtained using DTSSP allowed us to distinguish associations occurring external to the membrane from those occurring internally.

It has previously been reported that dissociation of virus particles under mild, nonreducing conditions followed by immunoblotting revealed homopolymeric forms of GP-1 (Wright *et al.*, 1989). We confirmed and extended this earlier observation by identifying the homopolymer as a tetramer of GP-1. The existence of a native GP-1 tetramer was further substantiated by results obtained following chemical crosslinking of the virion. Observation of the tetramer was independent of the crosslinking reagent employed. We also investigated the effects of urea and reducing agent on the stability of this structure. The results demonstrated that the GP-1 homotetramer was stable in the presence of 1 *M* urea but was dissociated in the presence of reducing agents, as detected by our immunoblotting procedure. This finding suggests that intermolecular disulfide bonds are important in maintaining the tetrameric structure of GP-1. The presence of a partially reduction-resistant disulfide-linked dimer suggests that the GP-1 tetramer may be a dimer of homodimers as reported for the Sendai HN and F membrane glycoproteins (Markwell and Fox, 1980; Sechoy *et al.*, 1987).

The second viral glycoprotein, GP-2, can be crosslinked to itself using any of the crosslinking reagents and to NP using DMS. The externally exposed portion of GP-2 appears to be a homotetramer, but when virions were crosslinked with DMS, heterodimeric and heterotetrameric oligomers with NP were also observed. When DTSSP was used these heterooligomeric complexes were not observed. Thus it can be concluded that the GP-2:NP interaction occurs within the viral envelope, sequestered from the crosslinking effects of DTSSP but not DMS. In this regard, it is of interest that there are three lysine residues, the substrate for these crosslinking reagents, within the last 12 amino acid residues at the carboxy-terminus of GP-2, the intracellular domain we believe to be interacting with NP ($_{487}$-KVPGVKTVWKRR$_{498}$).

Interactions between viral spike glycoproteins and internal nucleocapsid proteins have been reported for several other RNA viruses including Semliki Forest virus (Helenius and Kartenbach, 1980), mouse hepatitis virus (Sturman *et al.*, 1980), and Sindbis virus (Burke and Keegstra, 1976). Previous reports on arenavirus morphogenesis described patches on the surface of infected cells that contained viral glycoprotein spikes with dense granules, possibly nucleocapsids, underlying them (Murphy *et al.*, 1970; Murphy and Whitfield, 1975). It has been proposed that the interaction between the LCMV ribonucleoprotein complex, of which NP is a part, and GP-2 anchored in the infected cell membrane may be essential in allowing the virion to undergo the final steps of assembly and budding (Dubois-Dalcq *et al.*, 1984).

The lack of demonstrable association of GP-1 with GP-2 was unexpected. We believe GP-1 does interact with GP-2 to form the morphological spike, but the

complex was not observed due to the limitations of the crosslinking technique. Alternatively, GP-1 may exist as a readily dissociable peripheral protein that does not interact with other viral proteins but is anchored to the membrane by other means. The failure of crosslinking reagents to effectively complex all components of glycoprotein spikes has been reported with other viruses. For example, the E3 glycoprotein of Semliki Forest virus has been clearly established as a component of the virus glycoprotein spike (a trimer containing one molecule each of E1, E2, and E3) but is not crosslinked to E1 or E2 using similar techniques (Ziemiecki and Garoff, 1978; Ziemiecki *et al.*, 1980). Similarly, the HIV spike glycoproteins, gp41 and gp120, are not complexed using crosslinking reagents (Schawaller *et al.*, 1989; Weiss *et al.*, 1990).

To summarize our data: (1) GP-1 is a peripheral protein whose native structure is a disulfide-linked homotetramer, (2) GP-2 is an integral membrane protein, also assembled as a tetramer, that we believe spans the lipid bilayer and may interact with NP via a carboxy-terminal cytoplasmic tail. In other experiments we have shown that the C-terminal tail of GP-2 is protected from proteolysis in the intact virion (data not shown).

On the basis of previous reports and the data presented here, we propose the following model for the LCMV glycoprotein spike. After initial folding, processing, and oligomerization in the endoplasmic reticulum, GP-C is transported to the Golgi where it is cleaved in either the trans-Golgi compartment or the trans-Golgi network to yield GP-1 and GP-2 (Wright *et al.*, 1990; Buchmeier and Oldstone, 1979). We believe that following cleavage, GP-1 and GP-2 remain associated and form a single glycoprotein spike. This spike is composed of a GP-1 tetramer noncovalently associated with a membrane spanning GP-2 tetramer. We believe the GP-1:GP-2 interaction occurs through a minimal contact surface. Such an interaction would explain our crosslinking results in which we could easily detect homotetramers of either GP-1 or GP-2 but not GP-1:GP-2 heterooligomers.

Although a structural model of the LCMV has been previously reported (Bruns *et al.*, 1983b), many features of that model can no longer be reconciled with the present knowledge of the virion polypeptides and coding capacity of the virus. The entire LCMV genome has been sequenced and the potential open reading frames have been identified (Southern *et al.*, 1987, Smith *et al.*, 1987; Salvato and Shimomaye, 1989). The virion proteins identified by nucleotide sequencing and open reading frame analysis are: L, the putative RNA polymerase; NP, the nucleocapsid protein; GP-C, the 70–75K precursor glycoprotein which is cleaved to yield GP-1 and GP-2, and Z, an 11–14K protein, possibly involved in transcription regulation through a zinc finger domain (Salvato and Shimomaye, 1989). The utilization of all of these open reading frames has been confirmed using synthetic peptide antibodies derived from the predicted amino acid sequences. There has been no evidence for RNA splicing events in the arenaviruses, so it is unlikely that the virion encodes additional polypeptides. Therefore we favor the model proposed here which is completely consistent with these data and allows for an equimolar ratio of GP-1 and GP-2 molecules in the virion as predicted by the cotranslational coding and post-translational processing of GP-C (Buchmeier *et al.*, 1987) and reported for both Pichinde virus (Vezza *et al.*, 1977) and LCMV (Bruns *et al.*, 1983a).

Studies are currently underway which should allow us to more precisely define the structure of the LCMV glycoproteins and identify their biologically and immunologically significant domains.

## ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Michelle Zandonatti, Cynthia K. Davis, and Wendy Ewing. The assistance of Drs. Thomas Gallagher and Jory Baldridge in their critical reading of the manuscript is also gratefully acknowledged. This is Publication Number 6682-NP from the Department of Neuropharmacology, Research Institute of Scripps Clinic, La Jolla, CA. This work was supported by NIH Grant AI 16102 and U.S. Army Contract C-6234. J.W.B. was supported by NIH Training Grant GM 07437. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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JOURNAL OF VIROLOGY, Aug. 1991, p. 3995–4000
0022-538X/91/083995-06$02.00/0
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Vol. 65, No. 8

# Isolation of an Arenavirus from a Marmoset with Callitrichid Hepatitis and Its Serologic Association with Disease

CHARLES B. STEPHENSEN,[1]* JAMES R. JACOB,[2] RICHARD J. MONTALI,[3] KATHRYN V. HOLMES,[4] ELIZABETH MUCHMORE,[5] RICHARD W. COMPANS,[6,7] EUGENE D. ARMS,[7] MICHAEL J. BUCHMEIER,[8] AND ROBERT E. LANFORD[2]

*Department of Public Health Sciences, School of Public Health,[1] Department of Microbiology, School of Medicine,[6] and Electron Microscopy Core Facility, Comprehensive Cancer Center,[7] University of Alabama at Birmingham, Birmingham, Alabama 35294; Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78284[2]; Department of Pathology, National Zoological Park, Smithsonian Institution, Washington, D.C. 20008[3]; Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814[4]; Laboratory for Experimental Medicine and Surgery in Primates, New York University Medical Center, New York, New York 10016[5]; and Department of Neuropharmacology, Division of Virology, Research Institute of Scripps Clinic, La Jolla, California 92037[8]*

Received 7 January 1991/Accepted 14 April 1991

**Callitrichid hepatitis (CH) is an acute, often fatal viral infection of New World primates from the family Callitrichidae. The etiologic agent of CH is unknown. We report here the isolation of an arenavirus from a common marmoset (*Callithrix jacchus*) with CH by using in vitro cultures of marmoset hepatocytes and Vero-E6 cells. Enveloped virions 67 to 133 nm in diameter with ribosomelike internal structures were seen in infected cultures. Immunofluorescence and Western immunoblot analysis using CH-specific antisera (principally from animals exposed to CH during zoo outbreaks) revealed three antigens in cells infected with this CH-associated virus (CHV). These antigens had the same electrophoretic mobilities on sodium dodecyl sulfate-polyacrylamide gels as did the nucleocapsid, GP2, and GPC proteins of lymphocytic choriomeningitis virus (LCMV). Monoclonal antibodies specific for these arenavirus proteins also reacted with the three CHV antigens. Conversely, the CH-specific antisera reacted with the nucleocapsid, GP2, and GPC proteins of LCMV. CHV thus appears to be a close antigenic relative of LCMV. The serologic association of CHV with several CH outbreaks implicate it as the etiologic agent of this disease.**

Callitrichid hepatitis (CH) occurs in discrete epizootics in zoo collections of tamarins and marmosets (14). Twelve outbreaks have occurred in the United States since 1980, killing 57 animals. Included among the deaths were callitrichid species that are considered endangered in the wild, such as the golden lion tamarin (*Leontopithecus rosalia*), which is being bred in U.S. zoos for release into its native habitat in Brazil. CH epizootics are thus a threat to this breeding program. Premortem signs of CH are nonspecific, including dyspnea, anorexia, weakness, and lethargy, and are frequently followed by prostration and death. Postmortem findings include jaundice, pleural and pericardial effusions (occasionally sanguinous), subcutaneous and intramuscular hemorrhage, and hepatosplenomegaly. Diffuse hepatocellular necrosis with the formation of acidophilic bodies and mild inflammatory infiltrate is a consistent finding (12, 14). The typical CH syndrome can be experimentally produced in marmosets by parenteral inoculation with a bacteria-free liver filtrate from an animal with CH (12). Sera from callitrichids with CH and from some asymptomatic animals exposed to the disease contain antibodies that react with three antigens found in the livers of animals that died of CH (17). These antigens appear to be viral proteins belonging to the suspected etiologic agent of CH, termed CH-associated virus (CHV).

In the studies described here, we set out to cultivate CHV in primary cultures of marmoset hepatocytes (10) because hepatocytes are one of the targets of CHV infection in vivo and in vitro cultures of these cells are known to be permissive for the replication of other hepatotropic viruses (7, 8). Our initial goal was to demonstrate replication of CHV in cell culture by using CH-specific antisera from convalescent animals (17). Electron microscopy (EM) was then used to identify key morphologic characteristics of the CHV virion. Finally, since CHV appeared to be an ultrastructurally typical arenavirus, we tested (i) arenavirus-specific monoclonal antibodies (MAbs) for their ability to react with CH-specific antigens and (ii) CH-specific antisera for their ability to react with lymphocytic choriomeningitis virus (LCMV), the prototypic Old World arenavirus.

## MATERIALS AND METHODS

**Hepatocyte cultures.** A liver wedge biopsy was obtained from a 2.5-year-old male common marmoset (*Callithrix jacchus*). Hepatocytes were isolated by perfusion with collagenase as previously described (7, 8, 10) and were plated at a density of $10^6$ cells per 60-mm dish (Primaria, Falcon). Dishes were pretreated with rat tail collagen. Hepatocytes were allowed to attach for 3 h in Williams medium E supplemented with 5% fetal bovine serum, at which time the cultures were washed and changed to a serum-free medium supplemented with growth factors and hormones (10). The cultivation and characterization of baboon and chimpanzee hepatocytes in serum-free medium have been described (7, 8, 10). Secretion of apolipoproteins A1 and E by the hepatocyte cultures was monitored by Western immunoblot as stringent markers of differentiation. Stable levels of secre-

* Corresponding author

tion of these markers were observed over a 66-day period in culture (data not shown).

**Vero cell cultures.** Vero-E6 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, N.Y.) with 5% fetal bovine serum.

**Virus inoculation.** Liver (0.3 g) from experimentally inoculated common marmoset EXP2 (12) was thawed, placed in 6 ml of Dulbecco's modified Eagle's medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) buffer, homogenized, and spun at 1,500 × *g* for 10 min at 4°C, and the supernatant was frozen at −85°C until used. EXP2 was inoculated with liver filtrate from an emperor tamarin (*Saguinus imperator*) that died with CH at the Oklahoma City Zoo (14). EXP2 subsequently developed CH and died 7 days after inoculation (12). The homogenate was diluted five- or sixfold with cell culture medium and filtered through a sterile 0.22- or 0.45-μm-pore-size filter, and 0.5 ml was used to inoculate cells cultured in 60-mm dishes. Vero cells were also inoculated with LCMV-Armstrong obtained from the American Type Culture Collection (Rockville, Md.) essentially as described for CHV.

**Immunofluorescence.** Hepatocytes were fixed in cold acetone, and Vero-E6 cells were fixed in a cold 1:1 mixture of acetone-methanol. Binding of callitrichid antisera (diluted in phosphate-buffered saline [PBS] containing 0.2% bovine serum albumin) and murine MAbs (diluted in PBS containing 0.5% normal goat serum) was detected by using fluorescein isothiocyanate-(FITC)-labeled protein A and fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), respectively.

**Western immunoblots.** Cell monolayers were extracted by using Laemmli sample treatment buffer (9) with the following composition: 4% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 50 mM Tris, 100 mM NaCl, 10% glycerol, and 0.05 mg of bromophenol blue per ml. SDS-polyacrylamide gel electrophoresis (PAGE) and Western immunoblot analysis were performed as described previously (17), using 10% acrylamide gels and a Mini-Protean II gel apparatus (Bio-Rad, Rockville Centre, N.Y.). $^{125}$I-protein A was used as the reporter molecule. Two types of molecular mass markers were used: (i) prestained standards (rainbow markers; Amersham, Arlington Heights, Ill.) and (ii) unstained standards (high-range standards; Bethesda Research Laboratories, Gaithersburg, Md.) visualized by staining with India ink (6). Marmoset liver samples were prepared for Western blot analysis as described previously (17).

**EM.** Cell monolayers on 60-mm dishes were fixed in 1% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide in PBS, and dehydrated in graded ethanol solutions. The fixed monolayers were then released from the plastic surface by using propylene oxide, embedded in Polybed 812, and sectioned with a diamond knife. Sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope.

**Antisera.** CH-specific sera available for use in this study were drawn from callitrichids exposed to CH at the Oklahoma City Zoo, Oklahoma City, Okla. (OKCZ1; emperor tamarin), the Brookfield Zoo, Chicago, Ill. (BZ1; Goeldi's monkey; *Callimico goeldii*), the Lincoln Park Zoo, Chicago, Ill. (LPZ; emperor tamarin), and Marineworld, Vallejo, Calif. (MW2; saddleback tamarin; *Saguinus fusciolis*). While BZ1 was asymptomatic, the other three tamarins had clinical and pathological evidence of CH (14). CH-specific serum R306 was raised in a rabbit against the 54-kDa putative viral antigen identified in the livers of marmosets with CH (17).

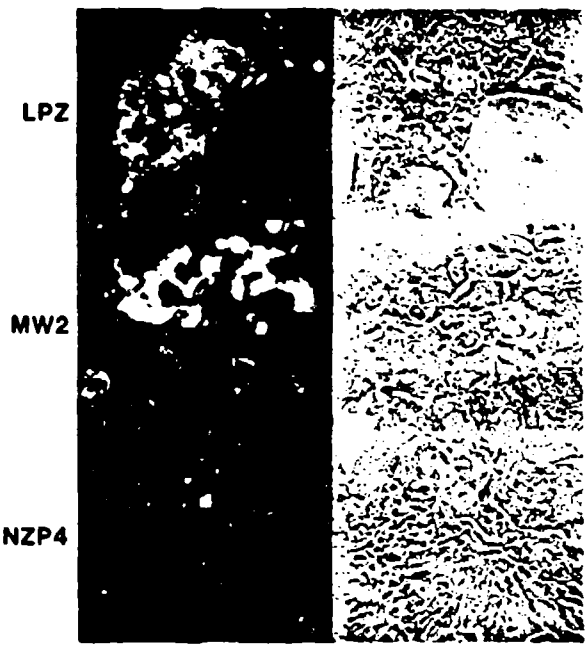



FIG. 1. Immunofluorescence analysis. CHV-inoculated marmoset hepatocyte cultures with CH-specific tamarin antisera LPZ and MW2 show punctate cytoplasmic fluorescence, while negative control serum NZP4 did not react (1:100 dilutions). Cultures were fixed in acetone 9 (LPZ) and 11 (MW2 and NZP4) days after inoculation with an infectious liver filtrate from a common marmoset (EXP2) with CH. Results were similar on days 3, 5, 7, and 28 after infection. The fluorescent (left) and phase-contrast (right) photographs are of the same field. CHV-specific sera BZ1 and OKCZ1 reacted similarly to LPZ and MW2 (data not shown).

Negative control sera were from two golden lion tamarins never exposed to CH (NZP3 and NZP4). The reaction of these sera with CH-specific antigens has been described in detail (17). A polyclonal anti-LCMV mouse ascitic fluid was purchased from Microbiological Associates (Rockville, Md.).

**MAbs.** MAb 3B-3.1, raised against Pichinde virus, recognizes a highly conserved epitope on the nucleocapsid protein of both New World and Old World arenaviruses (4), while MAb 1-1.3, raised against LCMV, recognizes a different epitope present only on the nucleocapsid protein of Old World arenaviruses (3). MAbs 33.6 and 9-7.9, both raised against LCMV, recognize two different epitopes within one antigenic site on the virion surface glycoprotein GP2. The epitope recognized by MAb 33.6 is found on both New World and Old World arenaviruses, while the epitope recognized by MAb 9-7.9 is restricted to Old World arenaviruses (20).

## RESULTS

**Evidence of CHV replication in cell culture.** Hepatocytes inoculated with liver filtrate from a marmoset with CH were examined by immunofluorescence using CH-specific sera from animals exposed to CH during four separate CH outbreaks (17). All four sera reacted with inoculated but not uninoculated cultures on all postinoculation days (Fig. 1). Typically, approximately 50% of cells in the inoculated cultures showed punctate cytoplasmic fluorescence, with

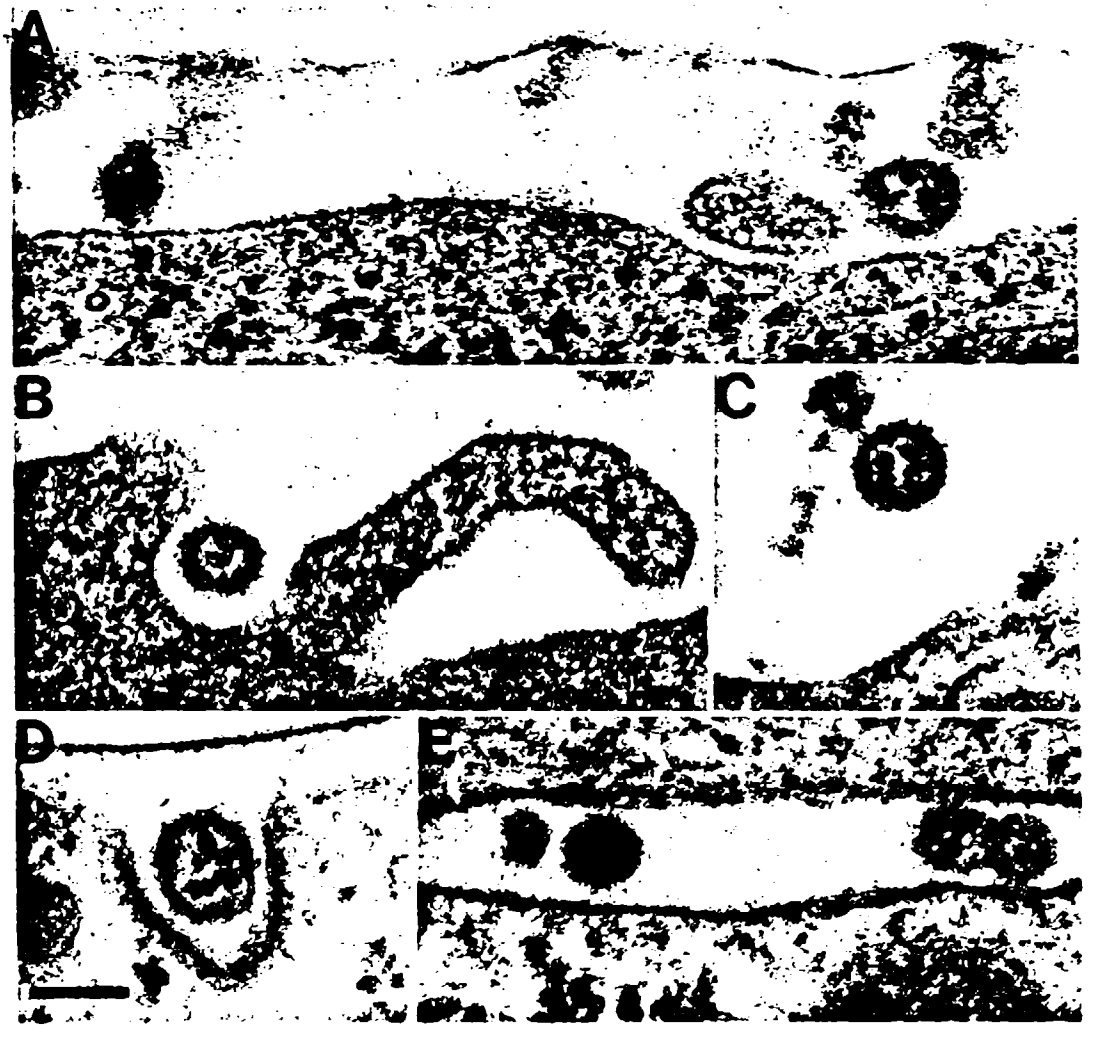



FIG. 2. Presence of typical arenavirus particles in CHV-inoculated marmoset hepatocyte and Vero-E6 cultures. Transmission EM analysis of marmoset hepatocytes (A and B) and Vero- E6 cells (C to E) 3 and 4 (E) days after inoculation with infectious liver filtrate from a common marmoset (EXP2) with CH revealed virions with morphologic characteristics of the family *Arenaviridae*. Final magnifications are ×135,000. Bar represents 100 nm.

some cells containing large inclusions. Control sera from seronegative animals showed no specific fluorescence (Fig. 1). Hepatocytes inoculated with cell-free supernatants removed from liver filtrate-inoculated cells 11 to 13 days after infection showed the same pattern of fluorescence as the liver filtrate-inoculated cells, while sham-inoculated cultures showed no specific fluorescence (data not shown). No obvious cytopathic effects were seen in the inoculated hepatocyte cultures. These data indicate that CHV productively infects in vitro cultures of marmoset hepatocytes.

EM examination of hepatocyte cultures was performed to identify the ultrastructural characteristics of the CHV virion. Sections from parallel inoculated and uninoculated cultures fixed 3, 7, and 11 days after inoculation were examined under code to prevent bias. Enveloped virus particles 67 to 133 nm in diameter with an apparent glycoprotein fringe of approximately 10 to 15 nm and electron-dense internal structures which appeared to be ribosomes were seen extracellularly in the day 3 inoculated culture (Fig. 2). These virions resemble members of the family *Arenaviridae*. These virus particles were not seen during extensive examination of the uninoculated cultures. Since arenaviruses can often be cultivated in Vero-E6 cells (11), cultures of these cells were inoculated with the infectious liver filtrate. No cytopathic effects were seen, but EM examination revealed typical arenaviruslike virions (Fig. 2). Inoculated Vero-E6 cells also showed a pattern of punctate cytoplasmic fluorescence when examined with CH-specific sera (data not shown). Uninoculated cultures showed no specific fluorescence. CHV thus appears to replicate in Vero-E6 cells as well as in hepatocytes.

**Antigenic relationship of CHV to arenaviruses.** To determine whether CHV was antigenically related to members of the family *Arenaviridae*, CHV-inoculated hepatocytes and Vero-E6 cells were examined by immunofluorescence using four arenavirus-specific MAbs. MAbs 3B-3.1 and 1-1.3, which are specific for the nucleocapsid protein, reacted only with inoculated cells, showing a pattern of punctate cytoplasmic fluorescence in both hepatocytes (Fig. 3) and Vero-E6 cells (data not shown), similar to the pattern seen

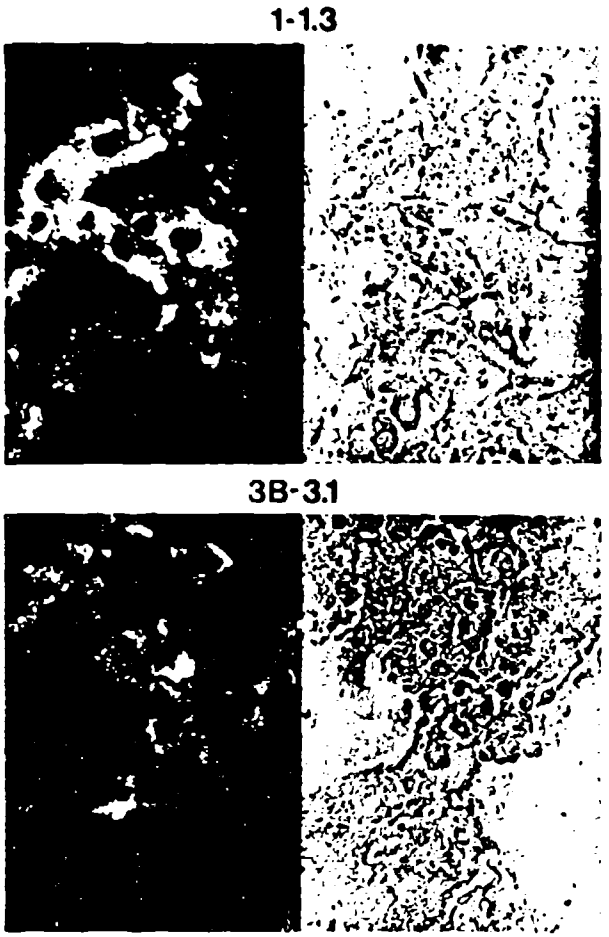



FIG. 3. Immunofluorescence analysis of inoculated marmoset hepatocyte cultures with arenavirus nucleocapsid protein-specific MAbs 1-1.3 and 3B-3.1, showing punctate cytoplasmic fluorescence. Cultures were fixed in acetone 3 (1-1.3) and 5 (3B-3.1) days after inoculation with infectious liver filtrate from a common marmoset (EXP2) with CH. The MAbs were diluted 1:20 (1:100 dilutions gave identical results). The fluorescent (left) and phase-contrast (right) photographs are of the same microscopic field.

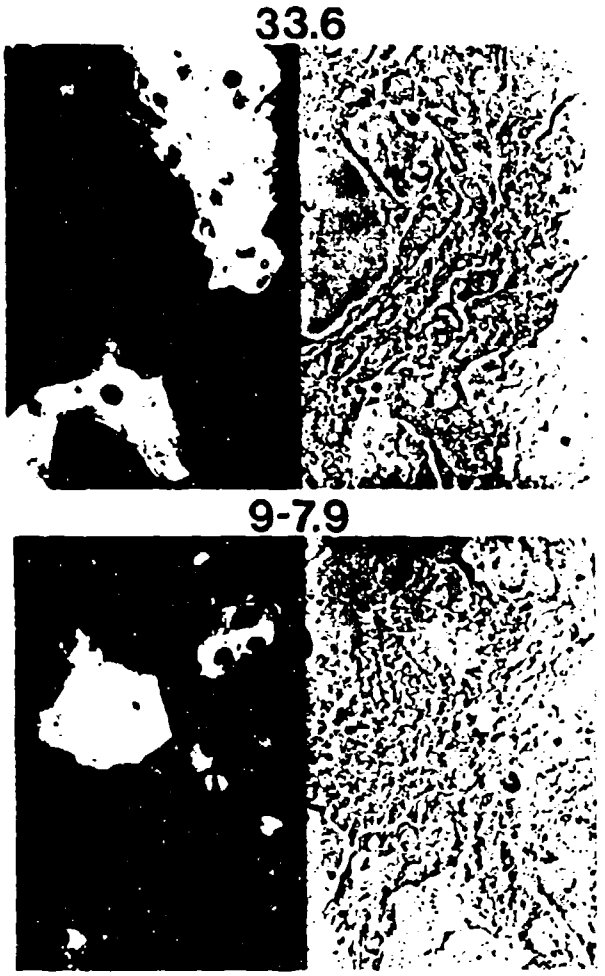



FIG. 4. Immunofluorescence analysis of inoculated marmoset hepatocyte cultures with arenavirus GP2-specific MAbs 33.6 and 9-7.9, showing smooth cytoplasmic fluorescence. Cultures were fixed in acetone 3 (33.6) and 5 (9-7.9) days after inoculation with infectious liver filtrate from a common marmoset with CH (EXP2). The MAbs were diluted 1:20 (1:100 dilutions gave identical results). The fluorescent (left) and phase-contrast (right) photographs are of the same microscopic field.

with the CH-specific sera. MAbs 33.6 and 9-7.9, which are specific for the surface glycoprotein GP2, gave a pattern of diffuse cytoplasmic fluorescence when reacted with CHV-inoculated hepatocytes (Fig. 4) and Vero-E6 cells (data not shown). No specific fluorescence was seen when MAb 33.6 was incubated with uninoculated hepatocytes or Vero-E6 cells or when normal mouse ascitic fluid was incubated with CHV-infected hepatocytes (data not shown). Whereas MAbs 3B-3.1 and 33.6 recognize highly conserved epitopes found in both New and Old World arenaviruses, MAbs 1-1.3 and 9-7.9 recognize epitopes restricted to Old World arenaviruses. These data suggest that CHV belongs to the Old World family of arenaviruses. Polyclonal anti-LCMV ascitic fluid also reacted specifically with CHV-inoculated cells (data not shown), reinforcing this conclusion.

To determine the apparent molecular masses of the CHV antigens identified by the arenavirus-specific MAbs, extracts of CHV- and LCMV-infected cells were analyzed by Western blot. MAb 1-1.3 recognized the nucleocapsid protein of LCMV and an apparently homologous CHV protein of the same electrophoretic mobility (Fig. 5). The molecular mass of the LCMV nucleocapsid protein is reported to be 63 kDa (2), although the mass relative to the standards used here is closer to 54 kDa, as we previously reported for CHV (17). This inconsistency is apparently due to the use of different molecular mass standards in these two studies. MAb 33.6 identifies the GP2 and GPC proteins of LCMV and also identifies two CHV antigens of the same electrophoretic mobilities (Fig. 5). These are presumably the homologous CHV glycoproteins. (MAb 33.6 also recognized an antigen of 47 to 51 kDa in LCMV-infected Vero cells [Fig. 5]. The identity of this antigen is uncertain.) When extracts of CHV-infected cells were run in adjacent lanes on the same gel and were subsequently probed with the arenavirus MAbs and the CH-specific sera from infected animals, the three CH-specific antigens identified by these sera were shown to comigrate with the nucleocapsid, GP2, and GPC proteins identified by the MAbs, suggesting that both the CH-specific

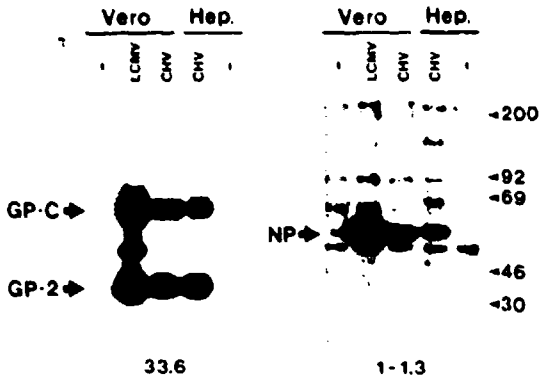



FIG. 5. Evidence that the GPC, GP2, and nucleocapsid proteins of LCMV have the same electrophoretic mobilities as the homologous CHV proteins. Cultures of marmoset hepatocytes (Hep.) and Vero-E6 cells (Vero) were inoculated with infectious liver filtrate from a common marmoset with CH (EXP2) and with LCMV-Armstrong (Vero-E6 cells only). Extracts (20 μl per lane) made 3 days postinfection from parallel inoculated (LCMV and CHV) and uninoculated (−) cultures were analyzed by Western blot using MAbs specific for LCMV nucleocapsid (1-1.3) and GP2 and GPC proteins (33.6). The LCMV nucleocapsid protein (NP) and GPC and GP2 proteins are indicated. The MAbs were diluted 1:100, and a secondary rabbit anti-mouse immunoglobulin G (Sigma, St. Louis, Mo.) was used for amplification. Sizes on the right are indicated in kilodaltons.

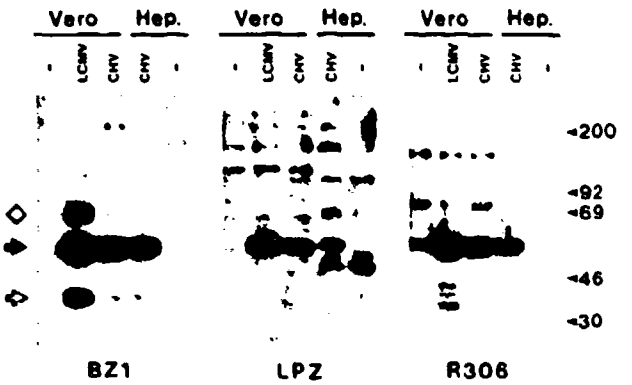



FIG. 6. Evidence that CH-specific antisera recognize the GPC (diamond), GP2 (open arrow), and nucleocapsid (closed arrow) proteins of LCMV. Cultures of marmoset hepatocytes (Hep.) and Vero-E6 cells (Vero) were inoculated with infectious liver filtrate from a common marmoset with CH (EXP2) and with LCMV-Armstrong (Vero-E6 cells only). Extracts (20 μl per lane) made 3 days postinfection from parallel inoculated (LCMV and CHV) and uninoculated (−) cultures were analyzed by Western blot using CH-specific sera from callitrichids BZ1 (1:100 dilution) and LPZ (1:50 dilution) and rabbit serum R306 (1:25 dilution). Serum R306 was raised against the putative CHV nucleocapsid protein partially purified from the liver of an infected animal by preparative SDS-PAGE. This preparation also contained normal cellular proteins, some of which are recognized by this serum. Thus, a cellular antigen of similar molecular mass to the CHV nucleocapsid protein is seen faintly in the uninoculated Vero-E6 cell extract, even though R306 was preabsorbed against normal marmoset liver and acetone-methanol-fixed Vero-E6 cells. Sizes on the right are indicated in kilodaltons.

sera and the MAbs identify the same viral proteins (data not shown).

Because arenavirus-specific antibodies were shown to react with CHV antigens (Fig. 3 to 5), CH-specific sera were tested by Western blot to determine whether reciprocal cross-reactions occurred against LCMV proteins. Sera from tamarins BZ1 and LPZ, and serum R306 raised against the 54-kDa CHV antigen, reacted with the nucleocapsid protein of LCMV in infected Vero-E6 cells and with the apparent nucleocapsid protein of CHV in infected hepatocytes and Vero-E6 cells (Fig. 6, closed arrow). Serum BZ1 also clearly identified the LCMV proteins GP2 (open arrow) and GPC (diamond) as well as antigens of similar mobility in the CHV-infected cells (Fig. 6; the CHV glycoproteins are less abundant in these extracts, making these bands much lighter than the corresponding LCMV bands). None of the CH-specific sera reacted with the arenavirus glycoprotein GP1. The significance of this is uncertain, since the presence of GP1 in these cultures was not confirmed by using anti-GP1 antibodies. The reaction of CH-specific sera with LCMV proteins further strengthens the close relationship of CHV to members of the Old World group of arenaviruses.

## DISCUSSION

CHV is an ultrastructurally typical arenavirus. It is enveloped, apparently buds from the cytoplasmic membrane (no intact virions were seen intracellularly), has a glycoprotein fringe, contains ribosomelike internal structures, and has a diameter ranging from 67 nm (condensed spherical particles) to 130 nm (pleiomorphic particles with electron-lucent areas within the virion cross section), as has been described for LCMV (5, 15).

CHV is antigenically related to the Old World arenaviruses. This was demonstrated clearly by the reaction of the arenavirus-specific MAbs with CHV and the reciprocal reaction of CH-specific sera with LCMV-Armstrong. The Old World arenaviruses are geographically limited to Africa, with the single known exception of LCMV, which can be found in Europe, Asia, Africa, and the Americas (11). Considering the geographic origin of this CHV isolate, Oklahoma City, CHV may be more closely related to LCMV than to other Old World arenaviruses. A close relationship between CHV and LCMV is also supported by the cross-reaction of several CH-specific sera with LCMV-Armstrong.

CHV is antigenically linked to several outbreaks of CH. Sera from animals exposed at the Oklahoma City Zoo, the Brookfield Zoo, the Lincoln Park Zoo and Marineworld (Vallejo, Calif.) reacted with CHV in infected hepatocytes and Vero-E6 cells by immunofluorescence and Western blot analysis. Similarly, the rabbit immune serum raised against the 54-kDa CHV antigen purified from an experimentally infected marmoset also reacted with CHV. These data strongly implicate CHV as the etiologic agent of CH.

The pathologic changes caused by CHV infection are strikingly similar to those seen in typical arenavirus infections. For example, Lassa fever and CH have several similarities, including an acute course, involvement of multiple organs (including liver and spleen), petechial hemorrhages (although not prominent in either CH or Lassa fever), nonsanguinous pleural and pericardial effusions, and, most strikingly, a pattern of microscopic liver changes that includes multifocal hepatocellular necrosis with formation of acidophilic bodies and minor inflammation. This type of liver lesion is characteristic of CH and is the most consistent microscopic finding of Lassa fever (12, 19). Experimental infection of cynomolgus monkeys with LCMV-WE also

produced an acute, often fatal disease with some similarities to CH, although hemorrhagic signs are much more pronounced than in CH (13). These similarities support the argument that CHV is the etiologic agent of CH.

Arenaviruses are typically maintained in rodent reservoirs by causing persistent infections during which infectious virus is excreted in the urine (11). This suggests that CHV may also persist in such a reservoir species. Rodents (including *Mus musculus*, a known host of LCMV) are common inhabitants of zoos and may be the principal reservoir of CHV. Another possible reservoir could be African rodent species (*Mastomys* and *Paromys*) that serve as reservoirs for the Lassa and Mopeia arenaviruses, respectively, in Africa. Such species are kept on exhibit in some zoos.

Zoo workers exposed to CHV-infected primates or rodents may be at risk of infection. Two zoo veterinarians who cared for callitrichids with CH were found to be seropositive for CHV, although neither recalled being ill at the time of exposure (unpublished observations). Human CHV infection could thus be asymptomatic, as is sometimes the case with LCMV (1). However, LCMV can also cause symptomatic disease in humans ranging from a flulike illness to meningitis to a fatal, hemorrhagic syndrome (16, 18), suggesting that CHV be treated as a serious health hazard for zoo personnel.

The data reported here strongly implicate CHV as the etiologic agent of CH, although marmoset inoculation studies with a clonally derived inoculum grown in cell culture are needed to definitively establish this etiologic relationship. The exact relationship of CHV to other arenaviruses should also be further explored. Sequencing of relevant portions of the genome and preparation of MAbs are now possible, since we have shown that the virus can be grown in cell culture. Preparation of CHV-specific reagents will also allow the examination of histologic specimens to determine whether affected animals from other CH outbreaks show antigenic evidence of infection with CHV. Finally, serologic studies will be facilitated by the availability of purified CHV antigen and will allow for surveillance of callitrichid populations (and reservoir species) for evidence of infection. Such information should enable zoos to prevent future CH outbreaks and avoid the losses that have plagued many U.S. zoos over the past decade.

## ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of L. R. Melsen and Sharon Blount. We thank Kenneth Burk for numerous helpful conversations during the course of these studies. We also thank J. B. McCormick and Bertha Farrar of the Centers for Disease Control, Atlanta, Ga., for providing the Vero-E6 cells.

This work was supported in part by a research grant from Biotech Resources Inc., San Antonio, Tex., and by Public Health Service grants AI27203, RR00463, and CA13148 from the National Institutes of Health.

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JOURNAL OF VIROLOGY, July 1992, p. 4252–4257
0022-538X/92/074252-06$02.00/0
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Vol. 66, No. 7

# Mechanisms of Antibody-Mediated Protection against Lymphocytic Choriomeningitis Virus Infection: Mother-to-Baby Transfer of Humoral Protection†

JORY R. BALDRIDGE AND MICHAEL J. BUCHMEIER*

*Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037*

Received 26 November 1991/Accepted 30 March 1992

**The role of antiviral antibodies in resistance to lymphocytic choriomeningitis virus (LCMV) infection was explored. Immune serum and monoclonal antibodies prevented fatal T-cell-mediated immunopathology following acute LCMV infections. In addition, 10- and 14-day-old mice that received maternally derived anti-LCMV antibodies through nursing were protected from an otherwise lethal LCMV challenge. Detailed investigation of the mechanism(s) by which these antiviral antibodies provided protection was carried out by using anti-LCMV monoclonal antibodies. Protection correlated directly with the ability of the antibodies to reduce viral titers in the tissues of conventional (K. E. Wright and M. J. Buchmeier, J. Virol. 65:3001–3006, 1991) and nude mice. However, this reduction was not simply a reflection of virus neutralizing activity, since not all antibodies which neutralized in vitro were protective. A correlation was also found between immunoglobulin isotype and protection: all of the protective antibodies were immunoglobulin G2a (IgG2a), while IgG1 antibodies mapping to the same epitopes were not. Protection appeared to be associated with events controlled by the Fc region. Functional $F(ab')_2$ fragments which retained in vitro neutralizing activity were not protective in vivo. Furthermore, this Fc-associated function was not related to complement-mediated cell lysis, since C5-deficient mouse strains were also protected. These results suggest a role for antibody in protection from arenavirus infections and indicate that a distinct immunoglobulin subclass, IgG2a, may be essential for this protection.**

Passive humoral immunotherapy is currently the treatment of choice for the arenavirus infection in humans caused by Junin virus (9) and has been used successfully to treat Lassa fever (24). In nonhuman primates, the combined use of immune plasma and ribavirin is more effective than either therapy alone and thus looks promising for clinical use (19). Despite the demonstrated effectiveness of passive antibody therapy, the mode of action remains poorly understood. Because of the risks inherent in using human convalescent-phase sera, monoclonal antibody (MAb) therapy has become an attractive alternative. Defining the protective mechanisms by which antibodies mediate protection is critical so that therapeutic MAbs and vaccine regimens can be appropriately targeted to protective epitopes while avoiding potentially enhancing reactions. In this study, we investigate the requirements for and mechanism of action of humoral protection against lymphocytic choriomeningitis virus (LCMV) infection.

Although the protective role of antibody in viral infections is appreciated, it remains poorly understood. Antiviral antibodies are predominantly of the immunoglobulin G2a (IgG2a) isotype (8), while antibodies produced against soluble proteins are predominantly of the IgG1 subclass (8, 33). This distinct difference in isotype production most likely relates to differences in cytokines induced in response to the antigen (13). Production of IgG2a antibodies requires T-cell help in the form of gamma interferon, which is generated in large quantities during viral infections (13), while IgG1 production is more dependent on interleukin-4 (38). Although no clear protective advantage has yet been ascribed to the IgG2a immunoglobulins elicited during viral infections, this immunoglobulin subclass efficiently activates the complement system, which may be advantageous to the host (12, 18).

The arenavirus LCMV can trigger three distinct outcomes during infections of mice: (i) an acute asymptomatic infection followed by viral clearance and life-long immunity when immunocompetent adults are inoculated extraneurally, (ii) an acute fatal, T-cell-mediated lymphocytic choriomeningitis (LCM) which develops following intracerebral (i.c.) inoculation of immunocompetent adults, and (iii) a life-long persistent infection following inoculation of neonatal or immunocompromised adult mice. Studies of the humoral response to LCMV infections have demonstrated that antiviral antibodies are produced during both the acute and the persistent infections (4, 26). During acute infections, complement-fixing antibodies can be detected as early as 6 days postinfection and reach peak titers in 2 to 3 weeks (4). Neutralizing antibodies appear later and have specificity for the major viral glycoprotein, GP-1 (2, 4). Also following an acute infection, the majority of virus-specific antibodies are of the IgG2a isotype, but mice enduring persistent infections produce predominantly low-affinity IgG1 antibodies (36, 37). The complexing of these antiviral antibodies with viral antigens during persistent infections leads to chronic glomerulonephritis (29).

The protective capacity of anti-LCMV antibodies was first demonstrated by Rowe (34), but with limited success. More recently, Thomsen and Marker (35) demonstrated that LCMV infections are restricted by passively transferred antibodies, suggesting that preformed antibodies may play a dominant role in resistance to reinfection. Finally, Wright and Buchmeier (39) showed that passively acquired MAbs

* Corresponding author.

† Publication 7132-NP from the Department of Neuropharmacology, The Scripps Research Institute.

directed against the GP-1 glycoprotein can attenuate fatal T-cell-mediated LCM disease. In this report, we extend those observations, using the model of antibody-mediated attenuation of LCM disease to investigate the mechanisms by which antiviral antibodies provide protection.

## MATERIALS AND METHODS

**Virus.** The Armstrong strain 4 (ARM-4) and Armstrong strain 5 (ARM-5) variants of LCMV were used for these studies. These strains differ in their sensitivity to neutralization by MAb 2.11.10 (40) but show no difference in the ability to induce lethal acute LCM disease following inoculation of immunocompetent mice (39). Both viral variants were originally plaque purified from a stock of Armstrong CA-1371. Working stocks of virus were obtained from infected BHK-21 cells (multiplicity of infection of 0.1) 48 h postinfection and stored in 1-ml aliquots at −70°C.

**Mice.** Female BALB/cByJ, SWR/J, and A/J mice of matched age (4 to 6 weeks) were obtained from the rodent breeding colony at The Scripps Research Institute. Athymic BALB/Wehi-nude male and female mice of matched age (4 to 6 weeks) were also obtained from the rodent breeding colony at The Scripps Research Institute. Seven-week-old B10.D2/nSnJ and B10.D2/oSnJ female mice were purchased from Jackson Laboratory (Bar Harbor, Maine).

**MAb treatment.** Mice were passively immunized with anti-LCMV MAbs by intraperitoneal (i.p.) injection of 0.2 ml of crude ascitic fluid on the day before and the day of viral challenge, unless otherwise noted. Generation and characterization of the murine anti-LCMV MAbs have been described in detail previously (3, 30).

**Viral challenge and immunizations.** Mice were challenged by i.c. inoculation of 1,000 PFU (unless otherwise indicated) of virus. The virus was diluted in Dulbecco modified Eagle medium for delivery of 50 μl for adult mice and 10 μl for neonates. Immune mothers were immunized with $10^5$ PFU by i.p. inoculation 30 days or more prior to mating.

**$F(ab')_2$ fragment preparation.** MAb 2.11.10 (IgG2a) was purified from ascitic fluid on protein A columns (Pierce, Rockford, Ill.) (11). The antibody was dialyzed and concentrated in an Amicon filtration cell (Amicon, Beverly, Mass.). MAb at a concentration of 10 mg/ml in 20 mM sodium acetate buffer (pH 4.0) was mixed 2:1 with immobilized pepsin in 20 mM sodium acetate buffer (pH 3). The mixture was incubated overnight at 37°C on a tube rotater. The digestion was stopped by removal of the pepsin, and the undigested MAb was removed by protein A column chromatography. The unbound fraction was collected as the $F(ab')_2$ and purity checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Activity of $F(ab')_2$ was confirmed by an in vitro plaque reduction neutralization assay.

## RESULTS

**Passively transferred immune serum and maternal antibodies protected mice from T-cell-mediated LCM.** Previous experiments from this laboratory have demonstrated that LCMV-specific MAbs attenuate immunopathologic effects of lethal LCM disease (39). In our initial experiments, we sought to determine whether similar attenuation could be achieved by using a passively transferred anti-LCMV polyclonal antiserum. In the first set of experiments, recipient BALB/c mice were given serum from immune BALB/c donors and then challenged by i.c. inoculation with ≥1,000 50% lethal doses ($LD_{50}$) (500 PFU) of the ARM-4 strain of LCMV. Fifty percent of the mice that received only one infusion of serum (0.2 ml) on the day of challenge survived (Table 1, experiment 1). When a second 0.2-ml dose of immune serum was given to recipients, all of the mice survived the challenge (Table 1, experiment 2).

TABLE 1. Attenuation of lethal LCM by passively transferred immune serum

| Expt | Group[a] | % Survival[b] |
|---|---|---|
| 1 | Normal control | 0 |
| | Immune control | 100 |
| | Serum recipients | 50 |
| 2 | Normal control | 0 |
| | Immune control | 100 |
| | Serum recipients | 100 |

[a] Donor BALB/c mice were immunized with $10^5$ PFU of ARM-4 30 days prior to bleeding. Recipient BALB/c mice were injected i.v. with 0.2 ml of serum from immune donors immediately prior to viral challenge for experiment 1 or i.p. infusions of serum (0.2 ml per mouse) the day before and the day of viral challenge for experiment 2. The immune serum donors were from a group of mice treated identically to the immune control mice.

[b] Mice (four per group) were challenged with 500 PFU (≥1,000 $LD_{50}$) of LCMV ARM-4 by i.c. inoculation.

Infection of neonatal mice with LCMV normally results in a life-long persistent infection; however, by 10 days of age, mice challenged with virus die of acute LCM. We took advantage of this age-dependent change in susceptibility to disease to study acquired resistance to LCMV infection in a mother-baby model. Passage of immunoglobulin from mother to baby across the placenta and in the colostrum is well known (16). Therefore, we sought to determine whether protective levels of anti-LCMV antibodies could be obtained in this manner. Ten- and 14-day-old suckling pups were challenged i.c. with 1,000 PFU of LCMV (≥2,000 $LD_{50}$). The pups born to and nursed by immune dams had a 100% survival rate, while all pups born to and nursed by normal mothers died (Table 2). Pups nursed by immune mothers had

TABLE 2. Protection from lethal challenge of pups nursing on immune dams

| Birth mothers[a] | Foster mothers[b] | Age of pups upon challenge[c] | Pups weaned[d] | % Survival[e] (total no. of mice) |
|---|---|---|---|---|
| Immune | | 10 days | No | 100 (6) |
| Nonimmune | | 10 days | No | 0 (4) |
| Immune | | 14 days | No | 100 (6) |
| Nonimmune | | 14 days | No | 0 (7) |
| Immune | | 5 wk | Yes | 29 (7) |
| Nonimmune | | 5 wk | Yes | 0 (4) |
| Nonimmune | Immune | 14 days | No | 73 (15) |
| Immune | Nonimmune | 14 days | No | 0 (12) |

[a] The pups were born to either nonimmune or immune dams as indicated. Immune dams were given $10^5$ PFU of LCMV by injection 30 or more days prior to mating.

[b] Within 24 h of birth, foster-nursed pups were switched to either nonimmune or immune foster mothers as indicated.

[c] Pups were challenged at the indicated age with 1,000 PFU of LCMV via i.c. inoculation.

[d] Weaning was done at 3 weeks of age.

[e] The surviving mice exhibited no evidence of LCMV in the brain, liver, or spleen 30 days postchallenge.

TABLE 3. Protection from LCMV challenge of suckling pups of 2.11.10-treated dams

| Expt[a] | Viral challenge[b] | % Survival (total no. of mice) |
|---|---|---|
| 1 | ARM-4 | 40 (5) |
| | ARM-5 | 0 (5) |
| 2 | ARM-4 | 83 (6) |
| | ARM-5 | 0 (3) |

[a] The mothers were nonimmune when their pups were born but were subsequently infused with 0.2 ml of MAb 2.11.10 ascites every third day (experiment 1) or every second day (experiment 2) from the time of birth until the pups were challenged.

[b] Pups were challenged at 14 days of age with 500 PFU (≥1,000 $LD_{50}$) of LCMV ARM-4 or ARM-5 by i.c. inoculation. The ARM-4 strain of LCMV has the GP-1D epitope as defined by MAb 2.11.10 (40), while the ARM-5 strain lacks this epitope.

anti-LCMV antibody titers equivalent to those of their mothers. Titers for mothers and pups were both 1/655,360, as determined by enzyme-linked immunosorbent assay (ELISA). Maternally derived protection was transient; pups born of and nursed by immune mothers lacked resistance (71% mortality) when challenged at 5 weeks of age after weaning at 3 weeks (Table 2).

To determine whether protection was being transferred to the pups through the milk or across the placenta, foster-nursing experiments were perform. . In these experiments, pups born to immune and nonimmune mothers were switched within 24 h of birth and nursed by foster mothers. Those pups born to nonimmune mothers and nursed by immune dams had a 73% survival rate following i.c. challenge. However, none of the pups born to immune mothers and foster nursed by nonimmune dams survived the i.c. challenge (Table 2). In addition, dams passively immunized (postpartum) by infusion of the protective MAb 2.11.10 provided their suckling pups with antigen-specific protection against lethal LCM disease (Table 3). Pups challenged by i.c. inoculation with the ARM-4 strain of LCMV had a high survival rate (40 to 83%), while pups challenged with a 2.11.10-resistant strain of LCMV, ARM-5 had 100% mortality. The survival rate was proportional to the amount of MAb 2.11.10 given to the mothers.

**Protection is related to reduced viral titers.** Having established that antiviral antibody can effectively protect against LCM disease, we sought to investigate the mechanism(s) of protection. Previous studies had shown that viral titers in the brains of BALB/c mice challenged i.c. with LCMV were reduced if protective MAbs were administered near the time of challenge (39). To ascertain that the reduction in viral burden was the result of antibody-mediated events and was not T-cell dependent, we assayed viral titers in the tissues of nude mice. Athymic nude mice were infused with MAb on days −1 and 0 and then challenged with 1,000 PFU of ARM-4 by i.c. inoculation. On days 2, 6, and 10 after virus challenge, tissues samples were removed and LCMV titers were determined. At 2 days postchallenge, virus could not be detected in mice treated with MAb 2.11.10 (Fig. 1), while control mice treated with an unrelated control MAb (anti-mouse hepatitis virus; 5B-170) had titers exceeding $10^6$ PFU/g of tissue. The threshold for detection of infectious virus in tissues was approximately 600 PFU/g. By 6 days after challenge, viral titers in 2.11.10-treated mice had risen to detectable levels in the spleens and the brains, although the virus concentrations in these tissues remained approximately 100-fold less than those of the control mice. By day 10 after challenge, the LCMV titers in 2.11.10-treated mice were similar to those in the control group. This same general pattern was also seen in the livers, although the overall titers were lower in both 2.11.10-treated and control mice (Fig. 1C).

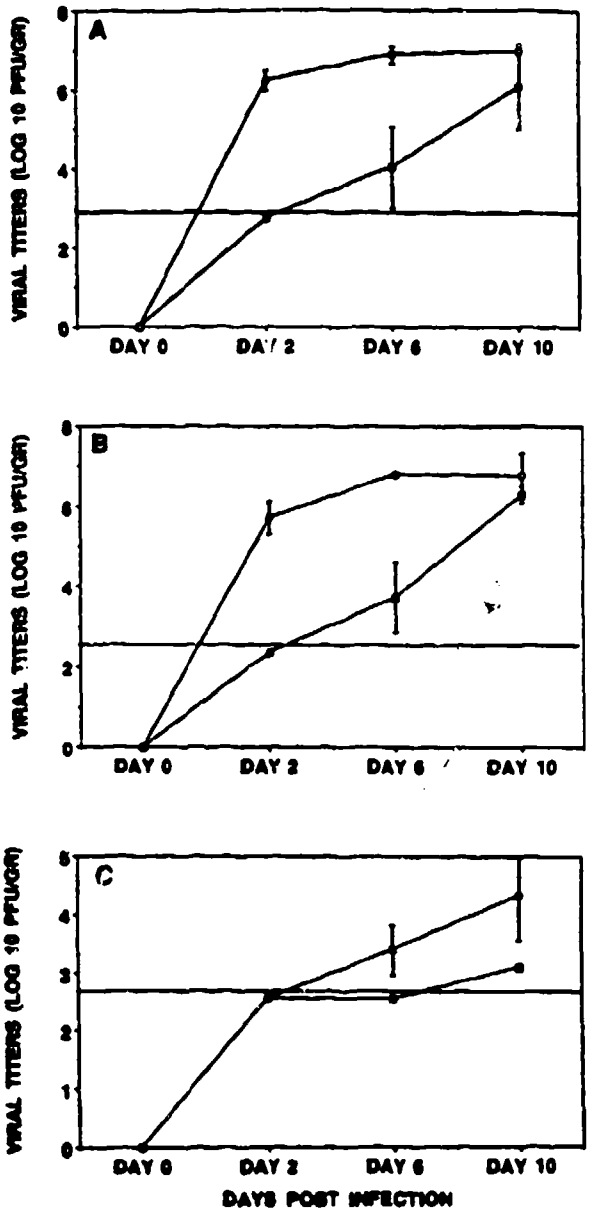



FIG. 1. LCMV titers during acute infection. Nude mice were infused with 0.2 ml of ascitic fluid containing either MAb 2.11.10 (anti-LCMV; ■) or MAb 5B-170 (anti-mouse hepatitis virus; ○) by i.p. injection on days −1 and 0. On day 0, the mice were challenged by i.c. inoculation with 1,000 PFU of ARM-4. The spleens (A), brains (B), and livers (C) of infected mice were removed for assessment of viral titers on days 2, 6, and 10 following challenge. Each point on the graph represents the mean of four samples. The limit of sensitivity (about 2.8 $\log_{10}$ PFU/g) is indicated by the horizontal lines in each graph.

We also evaluated the capacity of MAbs to reduce viral

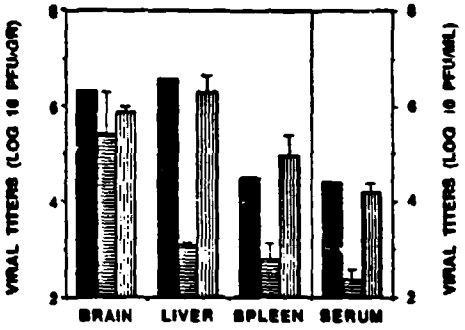



FIG. 2. Viral titers during persistent LCMV infection. To establish persistent infections, nude mice were inoculated i.c. with 500 PFU of ARM-4 35 days prior to MAb treatment. Mice received no MAb (■) or ascitic fluid containing MAb 2.11.10 (■) or MAb 36.1 (■) by i.p. injection. Twenty-four hours after MAb infusion, viral titers were determined in each tissue. Each bar represents the mean of three samples except that for the normal control, which represents titers from one mouse.

titers of persistently infected nude mice. We found that 24 h after MAb infusion, viral titers in mice treated with protective MAb 2.11.10 were decreased by 100- to 1,000-fold (Fig. 2) compared with the titers of mice treated with the MAb 36.1 (nonprotective, anti-LCMV; Table 4). This reduction in viral titers occurred in the spleen, sera, and livers of mice infused with MAb 2.11.10. Titers in the brains of either group of mice were not reduced, likely reflecting the inability of the MAbs to cross the blood-brain barrier (15).

**Isotype and epitope specificity of the in vivo protection was restricted to MAb of the IgG2a subclass.** MAbs specific for the peripheral glycoprotein GP-1 protected mice from lethal LCM, but MAbs against the transmembrane glycoprotein GP-2 or the nucleocapsid protein did not (39). We set up experiments to further investigate the mechanisms by which the anti-GP-1 MAbs mediated protection. Specifically, MAbs of the IgG1 and IgG2a isotypes were compared by in vitro neutralization assays, ELISA, and in vivo protection assays. All of the MAbs which protected mice were of the IgG2a isotype, while neither of the IgG1 MAbs (36.1 and 18.8) was protective even though MAb 36.1 neutralizes virus in vitro (Table 4). The protective capacity of the anti-LCMV

TABLE 4. Comparison of anti-GP-1 MAb by neutralization assay, ELISA, and passive protection assay

| MAb[a] | Epitope[b] | Isotype | Neutralization titer[c] | ELISA titer[d] | % Survival (total no. of mice) |
|---|---|---|---|---|---|
| 197.2.1 | GP-1A | IgG2a | 3,548 | 218,700 | 75 (8) |
| 258.2.11 | GP-1A | IgG2a | 2,512 | 218,700 | 88 (8) |
| 36.1 | GP-1A | IgG1 | >25,000 | 656,100 | 0 (12) |
| 67.2 | GP-1C | IgG2a | 4,266 | 218,700 | 88 (8) |
| 18.8 | GP-1C | IgG1 | <10 | 218,700 | 0 (8) |
| 2.11.10 | GP-1D | IgG2a | 12,023 | 656,100 | 100 (40) |

[a] Mice were infused with 0.2 ml of ascitic fluid the day before and the day of viral challenge.
[b] Mapped by Parekh and Buchmeier (30).
[c] Reciprocal of the dilutions of ascitic fluid yielding 50% reduction in PFU of LCMV on Vero cells.
[d] Reciprocal of the last dilutions of ascitic fluid giving absorbance values of ≥0.1 and at least twice that of the negative control.

TABLE 5. Ability of anti-LCMV MAb to protect C5-deficient mice from lethal LCM disease

| Mouse strain | C5 deficient | Virus challenge[a] | 2.11.10 treatment[b] | % Survival (total no. of mice) |
|---|---|---|---|---|
| A/J | Yes | ARM-4 | − | 0 (4) |
| | | ARM-4 | + | 100 (4) |
| | | ARM-5 | − | 0 (4) |
| | | ARM-5 | + | 0 (4) |
| SWR/J | Yes | ARM-4 | − | 0 (12) |
| | | ARM-4 | + | 83 (12) |
| | | ARM-5 | − | 0 (12) |
| | | ARM-5 | + | 0 (12) |
| B10.D2/oSnJ | Yes | ARM-4 | − | 0 (4) |
| | | ARM-4 | + | 100 (4) |
| B10.D2/nSnJ | No | ARM-4 | − | 0 (4) |
| | | ARM-4 | + | 100 (4) |

[a] Mice were challenged i.c. with 1,000 PFU of either ARM-4 or ARM-5 on day 0. The ARM-5 strain lacks the GP-1D epitope that is recognized by 2.11.10 (40).
[b] Mice were infused with 0.2 ml of 2.11.10 MAb ascitic fluid on days −1 and 0 by i.p. injection.

antibodies did not depend upon a specific epitope within the GP-1 glycoprotein. MAbs to three of the four known antibody epitopes on GP-1 were protective (Table 4). MAbs to the fourth epitope were not tested.

**Anti-LCMV MAbs mediate protection independent of the complement cascade.** Since IgG2 antibodies are known to be strong activators of the complement cascade (12), we sought to investigate whether complement was required for protection. The A/J, SWR/J, and B10.D2/oSnJ strains of mice lack complement component C5, precluding the completion of the either the classical or the alternate complement cascade (6, 10). When these mice were administered the protective MAb 2.11.10, they resisted the lethal challenge of ARM-4 virus in a manner similar to that of normal mice (Table 5).

**$F(ab')_2$ fragments of MAb 2.11.10 fail to protect mice from lethal LCM.** $F(ab')_2$ fragments of the protective MAb 2.11.10 were prepared by pepsin digestion and infused into mice to assess the requirement of the Fc region for protection. Even though the $F(ab')_2$ preparations neutralized virus with efficiency equivalent to that of intact antibody, the $F(ab')_2$ fractions were far less efficient or not protective at all against lethal LCM disease in recipient mice (Table 6). As little as 63 μg of MAb 2.11.10 given one time only protected 100% of the recipients. In contrast, recipients infused intravenously (i.v.) with a total of 1,100 μg of $F(ab')_2$ fragments (220 μg/day for 5 consecutive days) all succumbed to LCM disease, although one of four mice receiving 440 μg of $F(ab')_2$ fragments i.v. (110 μg/day for 4 days) survived. Additionally, all four mice receiving 125 μg of $F(ab')_2$ by i.p. injection failed to survive viral challenge (data not shown). By ELISA, we demonstrated that $F(ab')_2$ fragments were present in the serum of mice for greater than 24 h following i.p. or i.v. infusion (data not shown), indicating that the lack of protection was not related to an inability of the fragments to gain access to or rapid removal from the vasculature.

## DISCUSSION

Previous studies from this laboratory demonstrated that MAbs specific for the GP-1 glycoprotein of LCMV could be used therapeutically to prevent lethal LCM disease (39). In

TABLE 6. Evidence that the Fc region of the protective MAb 2.11.10 is critical for protection against lethal LCM disease

| Recipient of[a]: | Neutralization titer[b] | Treatment[c] (μg) | % Survival |
|---|---|---|---|
| 2.11.10 | 1:1,584 | 41 | 25 |
| | | 63 | 100 |
| $F(ab')_2$ | 1:1,318 | 440 | 25 |
| | | 1,100 | 0 |

[a] Normal recipient BALB/cByJ mice (four per group) were infused with purified MAb 2.11.10 or purified $F(ab')_2$ fragments of 2.11.10 by i.v. injection. All mice, including naive controls, were challenged with 100 PFU (>200 $LD_{50}$) of LCMV ARM-4 by i.c. inoculation.

[b] Reciprocal of the dilutions of 100 μg of purified 2.11.10 or $F(ab')_2$ yielding 50% reduction in PFU of LCMV on Vero cells.

[c] Recipients were infused with MAb 2.11.10 immediately prior to viral challenge one time only. Recipients of $F(ab')_2$ fragments were infused once a day for 4 consecutive days with 110 μg (total of 440 μg) or once a day for 5 consecutive days with 220 μg (total 1,100 μg) beginning the day of viral challenge. MAb 2.11.10 and the $F(ab')_2$ fragments were suspended in phosphate-buffered saline (pH 7.2).

the work presented here, we extend these findings to show that passively derived immune serum and maternal antibodies can also attenuate fatal LCMV-induced immunopathology. In addition, the use of MAbs to investigate the mechanism by which protection is mediated showed (i) that protection correlated with the ability of the antibody to specifically impede the rate of viral infection and (ii) that Fc-associated functions other than activation of the complement cascade were required for protection.

While there is little doubt that cytotoxic T lymphocytes (CTL) are essential for the clearance of primary LCMV infections (5, 7, 14, 20, 28), the role that antibodies play during infections is largely unexplored. Previous attempts to demonstrate passive protection with immune sera have been unsuccessful (27), but our data clearly indicate that anti-LCMV antibodies effectively attenuate T-cell-dependent LCM disease. It appears that antibodies mediate this protection by containing viral replication below levels at which the CTL response becomes destructive. The ability of antibodies to restrict viral spread and replication has previously been demonstrated by Thomsen and Marker (35) and Wright and Buchmeier (39) for conventional mice and was confirmed here for athymic nude mice (Fig. 1 and 2). The mechanisms by which antibodies retard viral spread remains unclear, although as with other models, there does not appear to be a strict correlation between protection and in vitro neutralizing activity (1, 23, 25, 31). However, the anti-LCMV MAbs do seem to require a functional Fc region, since $F(ab')_2$ fragments equivalent to whole antibody in the capacity to neutralize virus in vitro lost most if not all of their capacity to protect in vivo (Table 6). Mathews et al. (25) obtained similar results for MAbs against Venezuelan equine encephalitis virus. It does not appear that the complement-binding capacity of the Fc region is required, since no difference was observed between normal and C5-deficient mice. Similar results were found with MAbs specific for herpes simplex virus (1) and Venezuelan equine encephalitis virus (25). This observed lack of complement dependence coupled with the requirement for an intact Fc raises the possibility that protection involves antibody-dependent cellular cytotoxicity. In this respect, it is interesting that all of the protective MAbs were of the IgG2a isotype and the two nonprotective MAbs were both of the IgG1 subclass. These results are similar to those reported by Kaminski et al. (21), who found that MAbs of the IgG2a isotype were more effective than IgG1 antibodies at reducing tumor growth in vivo and at mediating antibody-dependent cellular cytotoxicity reactions to the lymphoma in vitro. Whether or not the correlation that we found between antibody subclass and anti-LCMV protection will be supported by continued analysis remains to be determined. However, the putative relationship is intriguing given that mice cleared of LCMV infections produce predominantly anti-LCMV antibodies of the IgG2a isotype, while mice with persistent infections produce antiviral antibodies predominantly of the IgG1 isotype (36, 37).

Maternal antibodies are passively transferred to offspring across the placenta and/or through milk (16). While this form of protection has been demonstrated in several viral models (17, 22, 32), we now extend the observation to include passive protection against LCM disease with maternal antibodies. This finding supports the work showing that such passively acquired antibodies can provide an effective restraint against LCMV infections (35, 39). In this regard, the antibodies may function most efficiently as an initial barrier impeding viral spread in the circulation and preventing the initial explosion of viral replication seen in nonimmune hosts until infection-terminating CTLs can develop. This may be best illustrated by comparing the results of mother-baby passive transfer experiments presented here and those demonstrated by Oldstone and Dixon (29). In the present experiments, 10- or 14-day-old pups which passively acquired antibody prior to viral infection became resistant to lethal LCM disease and cleared the virus (Table 1). In contrast, Oldstone and Dixon showed that neonatal mice with a preexisting persistent infection were unable to clear the infection when foster nursed by immune mothers (29). In fact, the antibodies were deleterious to these pups, causing accelerated immune complex disease and death.

While the exact mechanism by which antibodies mediate viral clearance remains to be determined, it is clear that they can provide an efficient protective buffer until sterilizing CTLs are elicited. It seems likely that antibodies function most efficiently in a prophylactic mode by containing viral titers below a threshold at which the T-cell response becomes destructive. This situation is most likely to occur during a secondary infection, as suggested by Thomsen and Marker (35), but could also be generated artificially by prior vaccination to induce an humoral response. These studies serve to reinforce the notion that elicitation of a strong humoral response against GP-1 epitopes should be considered a goal in attempts to vaccinate against pathogenic arenavirus infections of humans.

## ACKNOWLEDGMENTS

We thank Michelle Zandonatti and Tanya McGraw for excellent technical assistance and Jim Johnston for manuscript preparation.

This work was supported by Army contract C-6324 and PHS grants AI 16102. J.R.B. was supported by NIH training grant AI 07354.

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Edit. M.H.V. van Regenmortel and A.R. Neurath
Immunochemistry of viruses, II. The basis for serodiagnosis and vaccines.
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# Arenaviridae and bunyaviridae 12

K.E. WRIGHT[1], C. SCHMALJOHN[2], A. SCHMALJOHN[2] and M.J. BUCHMEIER[3]

[1]Department of Microbiology, University of Ottawa, Ottawa, Canada, [2]USAMRIID, Ft. Detrick, Frederick, MD, U.S.A. and [3]Research Institute of Scripps Clinic, Department of Neuropharmacology, 10666 North Torrey Pines Road, La Jolla, CA 92037, La Jolla, CA, U.S.A.

## 1. Arenaviridae

The arenaviridae are a group of 13 enveloped RNA animal viruses sharing common group-specific antigens as well as the unique morphological feature consisting of electron-dense 20-nm bodies within the virion. The presence of these dense bodies, which have subsequently been shown to consist of host cell derived ribosomes (Carter et al., 1973; Pederson, 1973; Pederson and Konigshofer, 1976; Farber and Rawls, 1975), suggested the group name from *arenosus* (L. Sandy) (Rowe et al., 1970a). The prototype virus of the family is lymphocytic choriomeningitis virus (LCMV). Serological analysis and geographic distribution of the viruses and their natural rodent hosts has led to a functional division of the arenaviridae into two groups. These are the Old World arenaviruses which include the African arenaviruses Lassa (LAS), Mopeia (MOP), Mobala (MOB) and LCMV, and the new world arenaviruses, Amapari (AMA), Tacaribe (TAC), Junin (JUN), Machupo (MAC), Tamiami (TAM), Parana (PAR), Pichinde (PIC), and Latino (LAT). All of these, with the possible exception of TAC, establish lifelong persistent infections in a single or limited species of rodent and are transmitted to man by direct contract with, or aerosolization of rodent excreta. No substantial evidence of an arthropod vector exists for the arenaviruses. Several members of the group are etiologic agents of severe human disease (Table 12.1).

## 2. Properties of Arenaviridae

### 2.1. Morphology, morphogenesis and virion composition

The arenaviridae are pleomorphic, enveloped viruses with a mean diameter of 110–

130 nm (Fig. 12.1). The envelope is formed from the host cell plasma membrane by budding, and contains sparse, 10-nm club-shaped projections composed of viral glycoproteins (Murphy et al., 1970; Gard et al., 1977; Vezza et al., 1977; Buchmeier et al., 1978).

TABLE 12.1

Arenaviruses

| Virus | Natural host | Geographic distribution | Naturally occurring human disease |
|---|---|---|---|
| **Old World** | | | |
| Lymphocytic choriomeningitis (LCM) | *Mus musculis* | Americas, Europe | Undifferentiated febrile illness, aseptic meningitis rarely serious |
| Lassa | *Mastomys natalensis* | Africa | Lassa fever, mild to severe and fatal disease |
| Lassa-like viruses from Africa | *Mastomys, Praomys* | Mozambique, Zimbabwe, Cent Afr Rep | Unknown |
| **New World** | | | |
| Junin | *Calomys musculinus* | Argentina | Argentine hemorrhagic fever (AHF) rarely serious |
| Tacaribe | *Artibeus* bats | Trinidad, West Indies | None |
| Machupo | *Calomys musculinus* | Bolivia | Bolivian hemorrhagic fever (BHF) |
| Amapari | *Oryzomys gaeldi*<br>*Neacomys guianae* | Brazil | None |
| Parana | *Oryzomys bucinnatus* | Paraguay | None |
| Tamiami | *Sigmodon hispidus* | USA (Florida) | Antibodies detected |
| Pichinde | *Oryzomys albigularis* | Colombia | None |
| Latino | *Calomys callosus* | Bolivia | Unknown |
| BeAn 293022 (proposed flexal virus) | *Oryzomys* spp. | Brazil | Unknown |

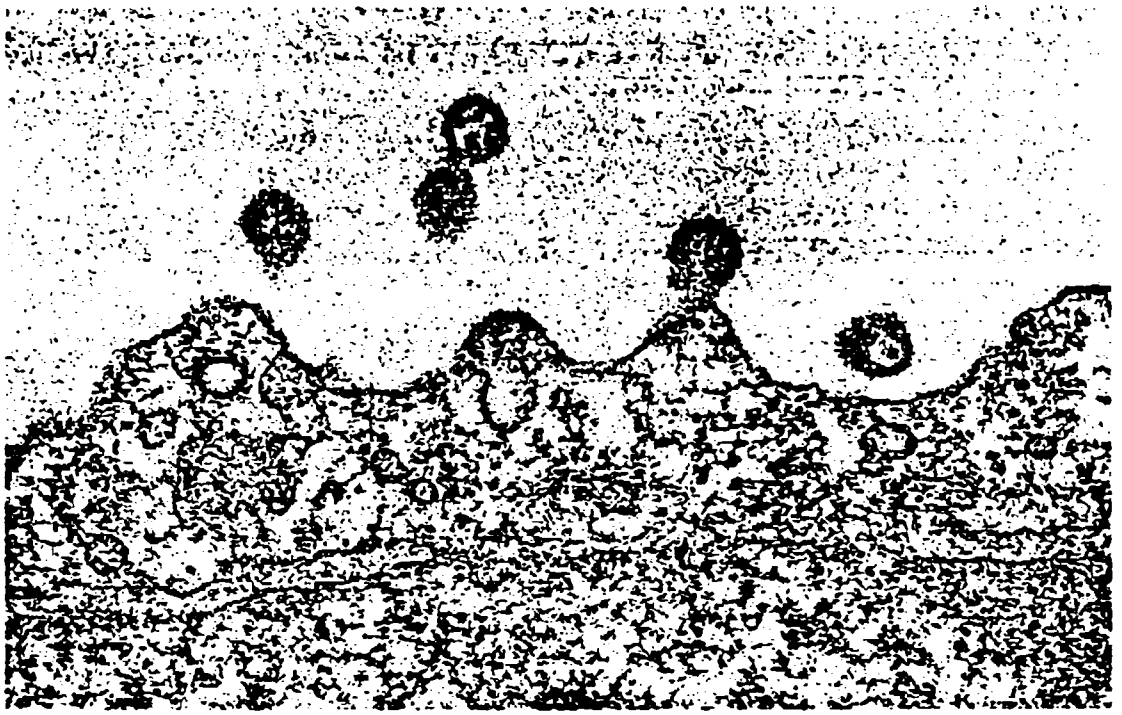


Fig. 12.1. LCM virus budding from the plasma membrane of infected BHK-21 cells. Typical 110-nm virions containing numerous electron-dense 20 nm particles are evident. (x 26 000).

Internal nucleocapsid structures have been difficult to visualize, but the presence of 20-nm electron-dense granules thought to be host cell ribosomes is characteristic (Murphy and Whitfield, 1975). Electron microscopic studies of PIC virions disrupted under controlled conditions have demonstrated a filamentous structure consisting of viral RNA and the nucleocapsid protein (N or NP) which resembled a string of beads 4 nm in diameter (Young and Howard, 1986).

Two strands of genomic RNA are present. These are a large (L) RNA (7.2 kb) and a smaller 3.4 kb RNA (S) (Vezza et al., 1977). The arenaviruses have been considered to be negative strand viruses (Carter et al., 1974), but the coding strategy of both the strands of RNA is ambisense, with both L and S containing positive and negative sense genes (Auperin et al., 1984a; Bishop and Auperin, 1987; Salvato et al., 1989). The L and S RNAs are unique except for a shared 3′ terminal sequence that is complementary to sequences at the 5′ termini (Auperin et al., 1984b; Romanowski et al., 1985; Southern and Bishop, 1987). The S RNA encodes the viral nucleocapsid protein in the negative sense at the 3′ end and the viral glycoprotein precursor, GP-C, in the positive sense at the 5′ end. Similarly, L encodes a 200-kDa polypeptide thought to be the virion RNA polymerase in the negative sense at the 3′ end and a newly described putative zinc binding protein, Z, in the positive sense at the 5′ end (Fig. 12.2).

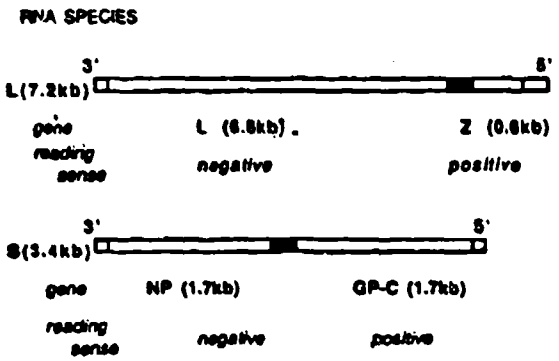



Fig. 12.2. Genetic organization of the LCMV, L and S RNAs. Four known genes, L, Z, NP and GP-C encode their respective proteins as described in the text. Black bars indicate non-coding intergenic hairpin regions and 3' and 5' non-coding regions of each RNA segment.

## 2.2. Serological relationships

Antigenic cross-reactions among the arenaviruses have been demonstrated in vitro using indirect immunofluorescence (IF), complement fixation (CF), ELISA and neutralization (Nt) assays. Antigens present on both Old and New World arenaviruses can be identified readily by immunofluorescence (Rowe et al., 1970b; Wulff et al., 1978; Buchmeier et al., 1981; Howard et al., 1985; Sanchez et al., 1989). Generally speaking, antisera directed against the New World viruses react with LCMV antigens, but anti-LCMV antiserum has a more restricted reactivity against New World viruses, staining AMA, minimally TAC, and not MAC virus (Rowe et al., 1970b). Monoclonal antibodies (mAb) raised against various arenaviruses have revealed epitopes on the viral proteins responsible for these cross reactivities. A monoclonal antibody which reacts with an epitope contained within amino acids 370–382 of the GP-C glycoprotein precursor of LCMV also reacts with LAS, MOP, MOB, PIC, TAC, JUN, AMA and PAR viruses (Buchmeier et al., 1980; Weber and Buchmeier, 1988). mAbs directed to either GP-1 or NP of LCMV do not cross-react with the New World viruses, but one mAb epitope detected on NP of PIC is shared by LAS and LCMV (Buchmeier et al., 1981). These data indicate that group-specific epitopes present on both new and Old World arenaviruses are located on internal proteins (NP) and surface glycoproteins.

Monoclonal antibodies also define subgroup and type-specific antigens. Subgroup-specific antigens appear to be restricted to NP, as mAb directed to NP but not GP of TAC and JUN react with viral antigen in cells infected with other New World viruses (Howard et al., 1985; Allison et al., 1984). Most of the anti-PIC monoclonals directed to NP are virus-specific, but three anti-NP mAb detect an epitope(s) on other New World viruses, namely TAM and PAR (Buchmeier et al., 1981). Within the Old World group, LCMV antiserum reacts with LAS and other African isolates, but with reduced titer compared to the reaction with homologous

antigen. Antiserum to LAS reacts only weakly with LCMV (Peters et al., 1987). Similarily, a mAb specific for NP of LCMV reacts with LAS and MOP, both African arenaviruses, but does not react with any New World arenaviruses (Buchmeier et al., 1980, 1981). mAbs directed to LAS virus GP-2 and NP cross-react with other African viruses, demonstrating that group-specific epitopes are present on both proteins (Gonzalez et al., 1984). Reactivity of these mAb against New World viruses has not been reported. Group specific antigens are also detected by complement fixation (Wiebenga et al., 1964; McKenzie et al., 1965; Calisher et al., 1970, Casals et al., 1975), and this response is likely directed against epitopes on NP (Buchmeier et al., 1980).

Virus or strain-specific antigens probably exist on all three structural proteins. Neutralization epitopes on GP-1 are largely virus specific, but cross-reactivity has been reported between closely related viruses, such as TAC and JUN (Henderson and Downs, 1965; Weissenbacher et al., 1975, 1976; Howard et al., 1985).

All strains of LCMV, with the exception of certain clonal isolates of LCMV Armstrong, share a single neutralization epitope defined by both mAb and polyclonal antisera on GP-1 (Parekh and Buchmeier, 1986; Wright et al., 1989a). One isolate of LCMV Armstrong containing a mutation at position 173 of GP-C bears a second neutralization epitope that distinguishes it from other Armstrong isolates, and other strains of LCMV (Buchmeier and Parekh, 1987; Wright et al., 1989a). Non-neutralization, type-specific epitopes have also been described on the glycoproteins of LCMV (Buchmeier, 1984; Parekh and Buchmeier, 1986), TAC (Allison et al., 1984; Howard et al., 1985), and the African arenaviruses (Gonzalez et al., 1984).

Cross-reactions described by in vitro assays may have in vivo correlates. Guinea pigs and marmosets immunized with TAC virus developed low levels of neutralizing antibody to JUN that appeared late after immunization (Weissenbacher et al., 1975, 1976; Coto et al., 1980; Weissenbacher et al., 1982). When subsequently challenged with JUN, a secondary neutralizing anti-JUN response occurred, indicating that the animals were primed by cross-reacting antigen on TAC (Coto et al., 1980; Weissenbacher et al., 1982).

The significance of neutralizing antibody to LAS in vivo is unclear since infection of guinea pigs with MOP, MOB or LCMV does not induce cross-reactive neutralizing antibody to LAS, but these animals are protected against LAS challenge (Peters et al., 1987). Clearly the relative importance of B and T cell-mediated immune responses in LAS infection remains to be elucidated.

### 2.3. Natural hosts and diseases

Arenaviruses persist in nature by virtue of their ability to establish lifelong persistent infection in rodent hosts. These naturally infected hosts show few if any symptoms of infection, reproduce normally, and transmit infection to their offspring in utero or neonatally. Each virus is restricted in nature to specific species of rodents, al-

though in the laboratory, other rodents can be infected with many of the arenaviruses (Peters et al., 1987). Horizontal transmission of virus also occurs but does not result in virus persistence. In both acute and persistent infections virus is excreted in the urine (Peters et al., 1987; McCormick, 1987).

*2.3.1. Old World arenaviruses*

Among the Old World arenaviruses, LAS and LCMV are known to cause human disease. Viruses antigenically related to LAS have been isolated in Central African Republic, Zimbabwe, Mozambique and South Africa (Wulff et al., 1977; Johnson et al., 1981; Gonzalez et al., 1983; McCormick, 1987). Some of these viruses persist in the same rodent host (*Mastomys natalensis*) as LAS, and are transmitted to humans as indicated by significant incidence of seropositivity, but no clinical disease has been associated with these infections (McCormick, 1987). LCMV infection produces a spectrum of illness ranging from subclinical, or mild influenza-like symptoms, to aseptic meningitis (Casals, 1984).

Lassa virus also produces a spectrum of illness in humans, but is largely restricted to West Africa, where in Sierra Leone infection rates have been estimated to range from 5% to 20% of the population. Of these, 10% to 25% will present with classical symptoms, 5% to 8% will be hospitalized, and 1% to 2% will die (McCormick, 1987).

The natural host of LAS is the rodent *Mastomys natalensis* (Monath et al., 1974). As with LCMV in the mouse, neonatal infection of the rodent results in lifelong persistence of the virus in liver, kidney, lymph nodes, lung and brain, with shedding in the urine, throat secretions and blood (Walker et al., 1975). Transmission to humans occurs from contact with excreta in the home, either by aerosol, ingestion or through cuts and abrasions in the skin (Monath, 1975; Casals, 1984). Person-to-person transmission is also possible and presents a substantial risk in the hospital setting (McCormick, 1987).

*2.3.2. New World viruses*

Two New World arenaviruses, JUN and MAC, cause disease in man. JUN virus is transmitted to humans from field rodents of the genus *Calomys*, causing Argentine hemorrhagic fever (AHF) (Weissenbacher et al., 1987). Persistently infected rodents excrete JUN in saliva and urine and human infection occurs through skin abrasions, conjunctiva or through respiratory mucosa from contaminated dust (Weissenbacher et al., 1987). About 30% of infections are subclinical. Of the remaining infections, the majority recover spontaneously, but total mortality can reach 16% in untreated patients (Peters, 1984; Casals, 1984). Like Lassa fever, all organ systems are affected, but hemorrhagic manifestations are more frequent. Treatment of acute phase patients with immune plasma has proven effective in reducing mortality to below 1%. Convalescence is lengthy but usually without sequelae, although a small number of patients experience transient late neurological symptoms (Peters, 1984). Pathologi-

cal findings in fatal cases of AHV are not severe enough to indicate the cause of death. Most consistent is the observation of depletion of the bone marrow and lymphoid necrosis. Death appears to be the result of hypovolemic shock due to plasma leakage, suggesting a lesion in the vascular endothelium similar to that described in LAS (Casals, 1984, Peters, 1984).

Bolivian hemorrhagic fever (BHF) results from infection with Machupo virus. This infection is 'house-associated' and is contracted from persistently infected rodents of the species *Calomys callosus* in much the same way that Lassa fever virus is passaged to humans in Africa (Casals, 1984). Human to human transmission is rare. Clinically BHF is very similar to AHF, although inapparent infections with Machupo virus are rare. Mortality ranges from 5% to 30% (Casals, 1984). Machupo virus has largely been controlled through an aggressively applied program of rodent control (Johnson et al., 1966).

Other New World viruses persist in specific rodents in nature with the exception of TAC which was isolated from fructivorous bats of the genus *Artibeus* (Table 12.1). None of these viruses cause disease in humans, although inapparent infections with PIC virus have been documented in a laboratory setting (Buchmeier et al., 1974).

## 3. Potential targets of immune responses

### 3.1. Structure and morphology

#### *3.1.1. Structural proteins*

Quantitatively, the major structural protein of the arenaviruses is NP (60–68 kDa), which has been estimated to constitute 58% of total protein in virions (Vezza et al., 1977). In the virion, NP is complexed with genomic RNA in a ribonuclear protein (RNP) (Gard et al., 1977; Buchmeier et al., 1978; Young and Howard, 1983, 1986; Bruns et al., 1983a, 1986). It has been difficult to consistently observe a nucleocapsid structure in the arenaviruses by electron microscopy, however disruption of virus particles releases an RNP complex which has been examined (Pedersen and Konigsfoter 1976; Buchmeier et al., 1978). The RNP of PIC is a linear array of globular nucleosomes each about 4–5 nm in diameter which are supercoiled into helical structures which form circles (Young and Howard, 1983, 1986). Several minor protein species have been observed in purified virions of various arenaviruses. These include a minor protein of 77 or 79 kDa associated with the RNPs of TAM and TAC (Gard et al., 1977; Saleh et al., 1979). A nonglycosylated protein of approximately 15 kDa is consistently observed in preparations of LCMV and may represent the gene product of the Z gene, a putative zinc finger protein found in LCMV (Salvato et al., 1989, Salvato and Shimomaye, 1989) and TAC (Iapalucci et al., 1988, 1989). Other nonglycosylated proteins have been described for LCMV (Bruns et al., 1983), JUN (Martinez Segovia and deMitri, 1977), LAS virus (Clegg and Lloyd,

1983) and PIC virus (Harnish et al., 1981). Some of these have been shown to be degradation products of NP while others are of unknown origin.

A 200-kDa L protein has been visualized for PIC (Harnish et al., 1981), LCMV (Bruns et al., 1983b; Buchmeier and Parekh, 1987), and other African arenaviruses (Gonzalez et al., 1984), and presumably is present in all the arenaviruses. This L protein is associated with the viral RNP complex, and has been shown by using antibodies generated to synthetic peptides to correspond to the product of the 6.6 kb L gene located on the large RNA (Singh et al., 1987; Salvato et al., 1989).

The arenaviruses contain a single glycoprotein gene (GP-C) which encodes a polypeptide of 75–76 kDa for LCMV (Buchmeier and Oldstone, 1979; Harnish et al., 1981; Saleh et al., 1979; Clegg and Lloyd, 1983, 1984; Lukasevich and Lemeshko, 1985). GP-C is cleaved post-translationally in LCMV, LAS, MOP, MOB and PIC viruses to yield two structural glycoproteins, GP-1 and GP-2 (G1 and G2) of approximately 44–55 and 35–41 kDa. In contrast, JUN, TAC and TAM viruses have only a single structural glycoprotein (Gard et al., 1977; Boersma et al., 1982). The basis for this difference is unclear, but the sequence of TAC virus GP-C (Franze-Fernandez et al., 1987) lacks the paired basic amino acid sequence, ARG-ARG or ARG-LYS, which provides the cleavage recognition sequence for LCMV, LAS and PIC viruses (Buchmeier et al., 1987).

### 3.2. Morphogenesis

Both the major glycoproteins, GP-1 and GP-2, of PIC (Vezza et al., 1977), LCMV (Buchmeier et al., 1978), and the single GP of TAC and TAM (Gard et al., 1977) are sensitive to proteolytic cleavage on the surface of virions. For viruses with two glycoproteins, they are present in the virion in equimolar amounts (Vezza et al., 1977), but their arrangement in the envelope is not known. It appears that for LCMV, GP-1 is more accessible on the surface of infected cells than GP-2, as the latter is difficult to detect either by surface iodination of infected cells or virions (Buchmeier and Oldstone, 1979; Bruns et al., 1983b) or by immunofluorescence using mAb specific for GP-2. Bruns and Lehmann-Grube (1983) have proposed that in the viral envelope, a single molecule of GP-2 is complexed with three molecules of GP-1, or that one GP-1 is linked to another larger glycoprotein (gp85). Such an arrangement seems unlikely since GP-1 and GP-2 are produced by GP-C cleavage and are represented in the virion in equimolar amounts. Furthermore, the origin of the gp85 glycoprotein described by these authors is unclear, and cannot be explained on the basis of the known genetic map of LCMV. Other studies have suggested that LCMV GP-1 and GP-2 are present in the virion in homo-oligomers with up to tetrameric complexes of GP-1 observable in SDS denatured, non-reduced virions (Wright et al., 1989a).

The GP-1 and GP-2 glycoproteins are produced by post-translational cleavage of the cell associated precursor GP-C. Cleavage is mediated by a cellular protease at

a site defined by the sequence ARG-ARG at positions 262–263 of GP-C (Buchmeier et al., 1987). Full-length GP-Cs of LCMV (Buchmeier and Parekh, 1987) and TAC (Saleh et al., 1979) contain mannose-rich oligosaccharides, but the cleaved glycoproteins contain only complex oligosaccharides, indicating that they undergo further processing either prior to or just after cleavage. Recent studies with LCMV have shown that cleavage of GP-C occurs immediately after trimming of the oligosaccharide side chains, but before the molecule is transported to the plasma membrane. This data is consistent with cleavage occurring in the trans Golgi or immediate post-Golgi compartment (Wright and Buchmeier, unpublished data, 1989) (Wright et al., 1989b). A small fraction of GP-C becomes fully processed in the absence of cleavage, therefore transport is not dependent on cleavage. Others have reported full-length GP-C on the surface of radioiodinated LCMV infected cells (van der Zeijst et al., 1983a), and we observe on occasion full-length GP-C in purified virions (Parekh and Buchmeier, unpublished observations), hence cleavage may not be highly efficient. Experiments with PIC have shown that GP-C does not appear on the surface of cells infected with ts mutants in which cleavage fails to occur (Shivaprakash et al., 1988).

### 3.3. Protein functions

Although the arenaviruses are ambisense, an RNA-directed RNA polymerase is required for copying genomic RNA to functional messenger RNAs. Such an activity has been associated with RNP complexes, of PIC (Leung et al., 1979), and LCMV (Fuller-Pace and Southern, 1989). Although it has yet to be directly established, the L protein is likely to be RNA polymerase. L is associated with the RNP, its size is consistent with other RNA polymerases, and sequence comparisons of the predicted protein indicate segments conserved with other viral polymerases (Singh et al., 1987, Salvato et al., 1989).

A protein kinase activity has also been described associated with the RNP of LCMV (Howard and Buchmeier, 1983). This enzyme preferentially phosphorylates serine and threonine residues on NP in vitro. Attempts to find phosphorylated proteins in mature virions have been largely unsuccessful, but one report describes a soluble phosphorylated form of NP, termed p63E in LCMV (Bruns et al., 1986).

No hemagglutinating, neuraminidase or fusion activities have been associated with arenavirus glycoproteins. GP-1, the major surface glycoprotein, is responsible for binding to host cells. Monoclonal antibodies to two closely linked GP-1 epitopes, only one of which neutralized virus in vitro, inhibited virus binding to susceptible cells by up to 95% (Buchmeier, 1989, unpublished observations).

## 4. Mechanisms of immunity

### 4.1. Antibody-defined epitopes

#### *4.1.1. Immune responses to virus infection*

As for most viruses, the immune responses elicited depend on the route of infection, as well as on the age, genetic background and immune status of the host. Comprehensive studies have been reported for LCMV, and data exist for other arenaviruses as well. In the natural hosts, arenaviral infection occurs congenitally or at birth. Early attempts to find circulating antibody in mice chronically infected with LCMV were negative, and it was surmised that persistent infection was accompanied by immunologic tolerance to viral antigens (Buchmeier et al., 1980). However, it was subsequently demonstrated that hosts persistently infected with LCMV had both complexed (Oldstone and Dixon 1967, Buchmeier and Oldstone, 1979) and free antibody (Oldstone et al., 1980) to the virus. In LCMV carrier mice, anti-viral antibody was detected in the serum by indirect immunofluorescence and by immune precipitation (Buchmeier et al., 1980). Serum from *C. musculinus* persistently infected with JUN has high-titered levels of Nt activity that is ineffective in clearing virus (Weissenbacher et al., 1987). In contrast, *C. callosus* infected with MAC have no demonstrable Nt antibody (Johnson et al., 1973). These animals are not tolerant since antibody can be detected by indirect immunofluorescence (IF) (Johnson et al., 1973; Webb et al., 1973). LAS virus also may persist in the presence of antibody, but the majority of animals do not mount an antibody response (Walker et al., 1975).

Infection of adult animals with LCMV yields an acute disease which may be symptomatic or asymptomatic depending upon dose and route of infection and genetic background of the mouse. Infection by the peripheral route results in the development of antibody detectable by CF or ELISA by day 5 coincident with decline in virus titers (Buchmeier et al., 1980). Antibody titers remain high for several months (Buchmeier et al., 1980). Neutralizing antibody appears from 7 days to 3 weeks after infection, depending on the virus dose and the mouse strain (Kimmig and Lehmann-Grube, 1979). Both CF and Nt antibody persist for at least a year, and probably longer (Kimmig and Lehmann-Grube, 1979).

Other arenaviruses have not been studied in their natural hosts to the same extent as LCMV. Machupo induces antibody detectable by CF, Nt and IF in a proportion of infected adult *Calomys*, and the presence of Nt antibody was found to correlate with virus elimination (Webb et al., 1973; Johnson et al., 1973). Animals not producing Nt antibody became persistently infected (Johnson et al., 1973). Lassa virus also induced persistent infection in adult *Mastomys*, and CF but not Nt antibody was detected (Walker et al., 1975).

The arenaviruses differ in their ability to elicit, and their sensitivity to Nt anti-

body. LCMV induces Nt antibody in adult mice, but at low frequencies (Buchmeier and Parekh, 1987). Tacaribe, JUN and MAC all induce Nt ab in rodents and humans, but Nt antibody develops rather late in infection (Casals, 1984: Peters, 1984). Nt antibody to LAS virus appears so late in convalescence that it can have little effect in virus clearance (Casals, 1984) while PIC fails to induce a Nt response in any host (Howard, 1987).

*4.1.2. Identification and expression of B cell epitopes*

The availability of monoclonal antibodies to arenaviruses has made detailed mapping of B cell epitopes possible (Fig. 12.3) (Howard et al., 1985: Parekh and Buchmeier, 1986). Using competitive binding assays with a panel of mAbs directed against LCMV, it was demonstrated that Nt mAb recognized a single antigenic site on GP-1 (Parekh and Buchmeier, 1986). One isolate of LCMV Armstrong, ARM-4, expressed a second Nt site (Parekh and Buchmeier, 1986: Wright et al., 1989a). Competitive binding and neutralization kinetic studies of TAC indicated the existence of two neutralization sites on the glycoprotein of this virus (Howard et al., 1985).

In addition to the major neutralization site on GP-1, two topographically linked non-neutralization sites have been identified. Three closely linked epitopes have been described on GP-2 of LCMV. Two of these have been mapped to a single stretch of amino acid sequence, GP-C 370–382. These epitopes can be distinguished on the basis of critical internal contact residues and virus specificity (Weber and Buchmeier, 1988).

Extensive epitope mapping of NP has not been carried out, but virus cross-reactivity patterns observed with NP specific mAb suggest the existence of at least three

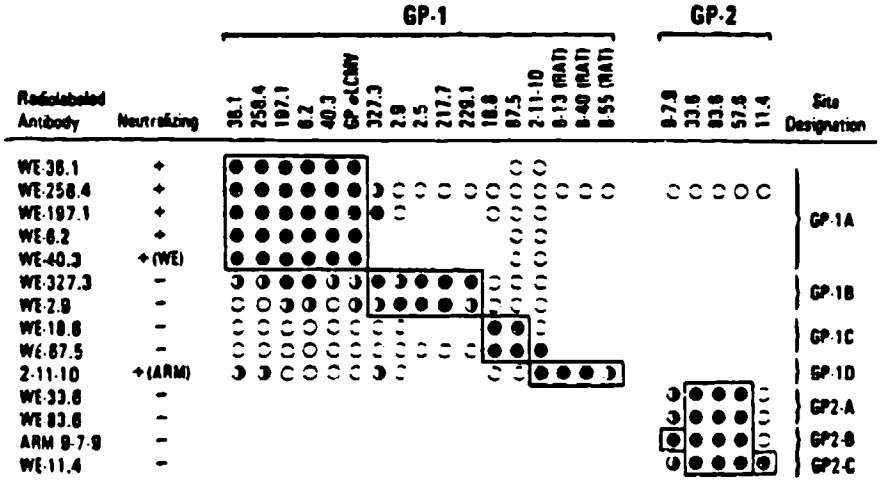



Fig. 12.3. Summary of mAb epitope mapping data for LCMV. Inhibition of binding of radiolabeled antibody by unlabeled competing antibody is indicated as follows, Filled circles, > 80% inhibition by unlabeled antibody; half-filled circles, 40–80% inhibition; open circles < 40% inhibition. Virus neutralizing antibodies are indicated. Reproduced from Parekh and Buchmeier, 1986, with permission.

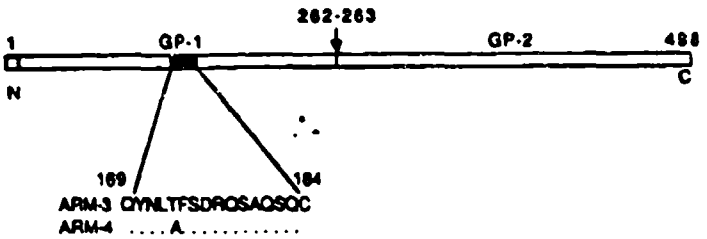



Fig. 12.4. Location of two antigenic sites on LCMV GP-C. A polymorphic neutralizing determinant has been shown to be expressed on mutant strain ARM-4 but is lost on ARM-3 correlating with an Ala → Thr substitution at position 173 of GP-C of these viruses (Wright, Salvato and Buchmeier, 1989).

antigenic sites on NP of PIC, one virus-specific, one group-specific and one that is shared with LCMV and LAS virus (Buchmeier et al., 1981). Complement-fixing antibody is directed to NP (Geschwender, 1976; Buchmeier et al., 1977), but it is unclear whether all sites on NP induce CF antibody.

Efforts to map Nt epitopes on GP-1 have been hindered by their conformational nature. Three of the four sites described on GP-1, including the neutralization site, are conformation-dependent, that is, destroyed by denaturation. Consequently epitope mapping using synthetic peptides has not been feasible. Despite this limitation, one epitope, GP-1D, recognized by mAb 2-11.10, has been partially localized. This epitope is disulfide-dependent, hence was destroyed by reducing agents. Using mutant strains of LCMV-Arm which differed in their binding of 2-11.10 we found that substitution of threonine for alanine or lysine at position 173 of GP-C abrogated binding activity (Fig. 12.4). Insertion of threonine completed a concensus *N*-linked glycosylation site ASN X THR at positions 171–173 and resulted in addition of an extra oligosaccharide side chain on GP-1 (Wright et al., 1989a). Thus, it is likely that antibody binding was blocked by the presence of this sugar chain.

### *4.1.3. Identification of protective epitopes*

A role for antibody in prevention or clearance of arenavirus infections may be underestimated. Generally, it is thought that neutralizing antibody does not play a significant role in clearing primary virus infections, but under certain conditions such a role can be demonstrated in arenavirus infections. In JUN virus infections the treatment of choice for acutely ill patients has been passive transfer of immune plasma (Maitzegui et al., 1979). This procedure results in rapid reduction of virus burden and reduction in morbidity and mortality. Animal studies also suggest that passive administration of serum containing Nt antibody can protect animals against primary infection with JUN. Studies in animals also demonstrate a similar phenomenon with LAS virus (Fisher-Hoch and McCormick, 1987; Peters et al., 1987), but in human patients, transfer of Nt antibody has little therapeutic effect (McCormick et al., 1987).

Studies with LCMV have defined B cell epitopes that attenuate T-cell-mediated disease in adult LCMV infected mice (Wright and Buchmeier, 1989). Passively transferred mAb against both Nt and non-Nt epitopes on GP-1 protected mice from fatal immune-mediated disease after intracranial infection and did so by limiting growth of virus in the brain. mAbs directed against either GP-2 or NP were not protective. These results indicate that although T cells may be responsible for clearing virus during primary infection, pre-existence of antibody or rapid induction of appropriate B cell responses protect against secondary infections. These data indicate that the protective effect of antibody is not restricted to those antibodies that neutralize virus in vitro.

### 4.2. Cellular responses

#### *4.2.1. Immune responses to virus infection*

T-cell responses to viral products are a central feature of arenavirus pathobiology. In LCMV infection, for example, cytotoxic T-cells are clearly important in virus clearance, and when their generation is blocked, persistent infection ensues. On the other hand, these same T-cells, when focused in an inflammatory infiltrate in the brain, cause lethal choriomeningitis. Clearly it is desirable to harness the beneficial functions of CTL while minimizing tissue destruction.

T-cell responses to the arenaviruses have usually been most extensively studied in the mouse where a natural host virus relationship exists with LCMV. Adult mice infected with LCMV develop both delayed-type hypersensitivity (DTH), as demonstrated by footpad swelling, (Tosolini and Mims, 1971) and cytotoxic T cell responses (Zinkernagel and Doherty, 1974). Cytotoxic T cells are pivotal in mediating virus clearance after a peripheral infection of adult mice with LCMV, and absence of this response leads to lifelong virus persistence (Marker and Volkert, 1973; Byrne and Oldstone, 1984; Moskophidis et al., 1987). Induction of, and target cell recognition by, CTL map to the S RNA segment of LCMV, the segment encoding NP and GP-C (Riviere et al., 1986). Studies examining the cross-reactivity of CTLs elicited by various strains of LCMV indicate that all five strains of the virus share common CTL epitopes, but some also have unique determinants (Ahmed et al., 1984a). The pattern of cross-reactivity also varies with the MHC background of the mouse, indicating determinants are recognized selectively in the context of different MHC class I molecules (Ahmed et al., 1984). The characteristic pattern of cross-reactivity observed in H-2b and H-2d mice is also observed when examining cross-reactivity of cloned CTLs from each strain of mouse (Byrne et al., 1984). These data suggest that there are multiple CTL determinants, and that recognition of these varies with the genetics of the host.

These observations were confirmed using LCMV proteins expressed in vaccinia vectors. H-$2^b$ mice recognize both NP and GP-C, but the majority of CTL clones

from these mice were directed against the latter. On the other hand, H-$2^d$ and H-$2^q$ mice appear to generate CTL activity mainly to NP, with a small proportion of CTL clones recognizing GP-C (Oldstone and Whitton, 1989; Whitton et al., 1988a). Analysis using a series of vaccinia-GP recombinants truncated at the C-terminus has mapped more precisely the epitopes recognized by H-$2^b$ mice (Whitton et al., 1988b). In C57B/6 (H-$2^b$) mice epitopes were recognized on NP, on GP-1 between residues 1 and 218, and on GP-2 between residues 272–293 (Whitton et al., 1988b). Using a series of synthetic peptides spanning this region as targets, the GP-2 epitope has been further mapped to amino acids 278–286. Responses to this determinant are restricted by $D^b$. The few available H-$2^b$ CTL clones directed against NP recognize a determinant at the carboxy end of the molecule, between residues 301 and 558 (Oldstone and Whitton, 1989). In H-$2^d$ mice, the major epitope lies on NP and has been mapped to residues 121–127 (Oldstone and Whitton, 1989). Thus H-$2^d$ mice recognize structures different from those recognized by H-$2^b$ mice. The NP epitope is specifically restricted by $L^d$.

Pichinde virus does not cause disease in mice, but does elicit a DTH reaction after footpad inoculation (Wright, 1989, unpublished data) and a strong CTL response after primary and secondary infection by other routes (Walker et al., 1984). The cytotoxic response is thought to be directed to GP-C, as infection of syngeneic cells with a vaccinia recombinant expressing PIC NP does not render the cells susceptible to lysis (Ozols et al., 1988). In certain strains of hamster, PIC induces DTH after inoculation in the footpad; in other strains of hamster such a response is missing and thought to be actively suppressed (Chan et al., 1983). Unlike the other viruses examined, JUN induces a good CTL response in mice, but does not elicit footpad swelling (Barrios et al., 1982). Cytotoxic cell responses to LAS virus, LCMV and MOP have been studied in guinea pigs (Jahrling and Peters, 1986; Peters et al., 1987). Such a response could be measured in splenocytes from guinea pigs inoculated 15 days previously with any of the 3 viruses.

*4.2.2. Protective epitopes and epitopes inducing T cell-mediated disease*

Primary infection with LAS virus, like LCMV, is thought to be cleared mainly by T-cell-mediated responses. Guinea pigs can be protected from challenge with LAS by transfer of immune splenocytes if the splenocytes are harvested early in infection (Peters et al., 1987), but not late. Recombinant vaccinia viruses expressing LAS GP-C or NP have been constructed and used in protection experiments. Immunity to both proteins protects guinea pigs from death although not from infection (Auperin et al., 1988; Auperin et al., 1987; Clegg and Lloyd, 1987). When non-human primates were vaccinated with the same constructs, only the virus expressing GP-C was protective. As observed with guinea pigs, monkeys became infected, but infection caused few if any symptoms (Auperin et al., 1988). Guinea pigs were protected by immunization with LAS GP-C but did not produce anti-LAS antibody (Auperin et al., 1987). These results suggest that protective T cell epitopes for guinea pigs lie

on both GP-C and NP, whereas those for monkeys reside on GP-C.

Pichinde may also contain a protective epitope on NP. A vaccinia construct containing NP from PIC was able to modulate disease and delay death in MHA hamsters after challenge with PIC (Ozols et al., 1988). These results parallel earlier results showing that the transfer of IL-2 into susceptible hamsters prior to infection slowed death (Wright et al., 1987) and suggest that immunization with NP may activate helper T-cells that synthesize IL-2.

The observation that a vaccinia-LCMV-NP recombinant virus elicited a CTL response when inoculated into mice suggested that such a construct might confer protective immunity. Both H-$2^d$ and H-$2^b$ mice were protected from lethal intracerebral challenge with LCMV after vaccination with a construct containing full-length NP (Klavinskis et al, 1989a, 1989b). Immunization with a construct expressing truncated NP (residues 1–201) only protected H-$2^d$ mice, demonstrating that the presence of a CTL epitope for H-$2^d$ mice on the vaccine correlated with protection (Klavinskis et al., 1989a, b). No anti-viral antibody was detected in these mice.

T-cell epitopes of LCMV inducing disease appear to be identical to those which are protective. Cytotoxic clones specific for either GP (restricted by H-$2D^b$) or NP (restricted by H-$2L^d$) can induce central nervous system disease in immunosuppressed syngeneic mice infected 5 days earlier with LCMV (Klavinskis et al., 1989c). As few as $10^{2.3}$ cloned CTL induced disease when injected intracerebrally into infected mice. When cells were transferred to immunosuppressed mice at the time of infection, virus titers remained low in the brain, and the animals survived (Klavinskis et al., 1989c).

*4.2.3. Modulation of T cell responses by virus*

Infection of neonatal mice with LCMV results in lifelong persistent infection without demonstrable CTL response. T-cells in neonatal mice are susceptible to infection with the virus, whereas T-cells in adult mice are relatively resistant (Lehmann-Grube et al., 1983; Doyle and Oldstone, 1978). Helper T-cells are preferentially infected in young mice (Ahmed et al., 1987; Tishon et al., 1988; Oldstone et al., 1988). It has been suggested that immature T-cells required to provide help for generation of LCMV-specific CTL are infected and destroyed or functionally inactivated early in life (Lehmann-Grube et al., 1983; Ahmed et al., 1987; Oldstone et al., 1988). During the course of infection, variant viruses with the ability to induce persistent infection in immunocompetent adult mice are selected in lymphoid tissues. As in neonates, persistence is associated with failure to mount a CTL response (Ahmed et al., 1984b). Although induction of and recognition by virus-specific CTL maps to the S segment of the genome, and hence relies on expression of NP and/or GP-C (Riviere et al., 1986), failure to mount a CTL responses against variant viruses maps to the L genomic segment (Salvato et al., 1988). Thus differences between the variant and wild-type viruses may not be due to alterations of specific T cell epitopes, but rather to alterations in polymerase or other heretofore uncharacterized genes en-

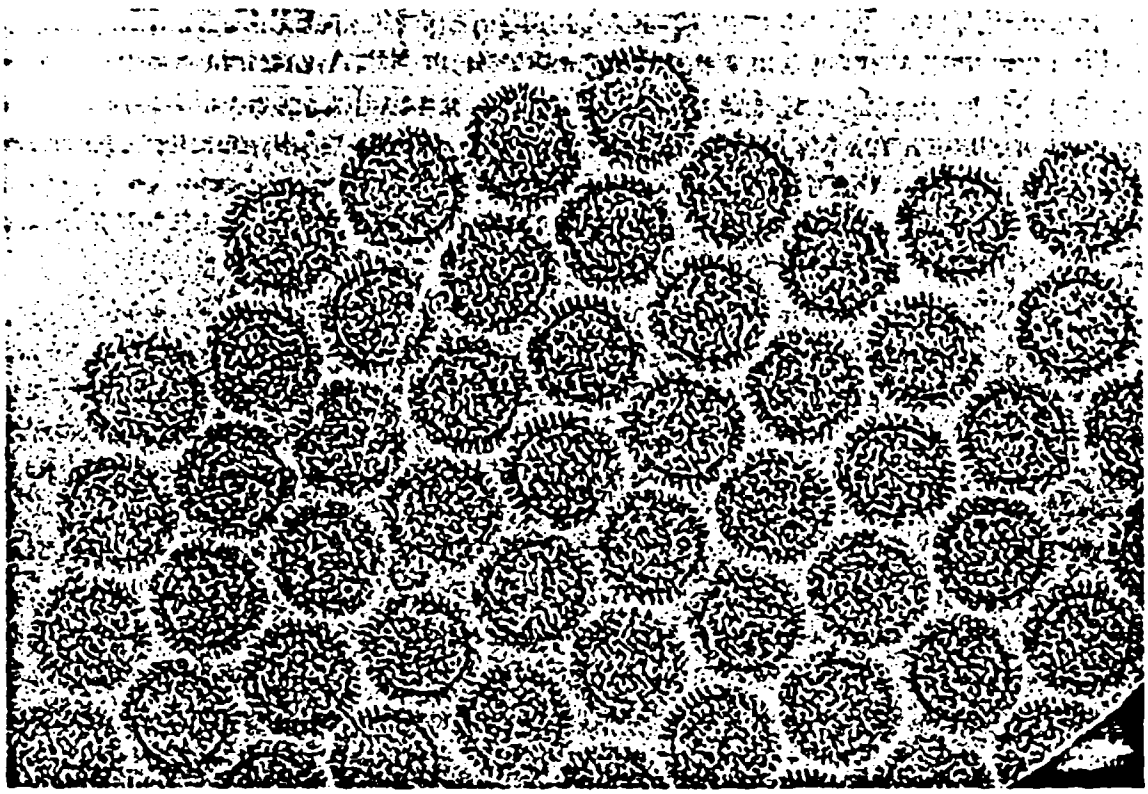


Fig. 12.5. Cryo-electron microscopy of Uukuniemi virus particles displaying surface glycoprotein projections. Particles are approximately 100 nm in diameter. Photograph courtesy M. Cyrklaff and C-H. von Bonsdorff.

coded on L. T-cells of the helper phenotype are preferentially infected in adult mice inoculated with an immunosuppressive variant of LCMV termed Clone 13, but not with wild-type, and total T cells numbers are decreased, so Clone 13 may express a different tropism than wild-type virus in its ability to infect mature T cells (Oldstone et al., 1988). Depletion of T-cells specific for LCMV is not lifelong. Carrier mice that have been cured of their infection by transfer of immune splenocytes are protected from a second challenge with the virus, and can generate LCMV-specific CTL (Jamieson and Ahmed, 1988).

Infection of macrophages with PIC is known to interfere with proliferative responses to macrophage growth factor (Friedlander et al., 1984), but it is not known whether macrophage antigen processing functions necessary for generation of immune responses are altered in any way. There have been suggestions that LCMV may depress some macrophage functions, such as phagocytosis (Gledhill et al., 1965) and lysozymal functions, but other studies indicate that infection with LCMV has no effect on macrophages (Mims and Wainwright, 1968; Oldstone et al., 1973, Schwartz et al., 1978).

*4.2.4. Vaccines*

At the present time, no safe vaccines exist for any of the pathogenic arenaviruses. Attempts have been made to develop live attenuated vaccines for MAC (Peters et al., 1987) and JUN (Guerrero et al., 1969). The latter was tested in human volunteers and appeared to be efficient in inducing neutralizing antibody (Ruggiero et al., 1974, 1981), but its use was discontinued because the virus had been passaged through heterodiploid cells and mouse brain.

A second generation live attenuated JUN vaccine designated candidate 1 is currently in field trials in Argentina. This strain, which was developed from the JUN strain XJ-44 by passage into FRhL-1 cells, is stably attenuated and tested without incident in over 100 human volunteers before initiating double blind placebo field trials in approximately 6000 individuals at risk in the endemic area of Argentina.

Recombinant approaches have been applied to efforts to vaccinate against Lassa. Vaccinia recombinants containing LAS proteins have been tested in guinea pigs and primates for efficacy against LAS challenge (Auperin et al., 1987; Clegg and Lloyd, 1987; Auperin et al., 1988). Only constructs containing GP-C were protective in monkeys, and although immunization reduced mortality, infection and viremia were not prevented (Auperin et al., 1988).

## 5. Bunyaviridae

Bunyaviridae is the largest known family of RNA animal viruses, with more than 250 serologically distinct members (Bishop et al., 1980; Karabatsos, 1978). The family has been divided into five genera based upon molecular and antigenic properties. Prototype viruses for the Bunyavirus, Hantavirus, Nairovirus, Phlebovirus and Uukuvirus genera, respectively are: Bunyamwera, Hantaan, Crimean-Congo hemorrhagic fever, sandfly fever Naples and Uukuniemi viruses (Bishop, 1985; Bishop et al., 1980; Schmaljohn and Dalrymple, 1983). Most viruses in the Bunyaviridae family are arthropod-borne (primarily by mosquitoes or ticks), with the exception of hantaviruses, which appear instead to be transmitted via aerosolized rodent excreta (Bishop et al., 1980; Lee et al., 1981) or by biting among rodents (Glass et al., 1988). Serious and fatal infections have been associated with certain bunyaviruses (e.g., LaCrosse encephalitis), hantaviruses (e.g., Korean hemorrhagic fever), nairoviruses (e.g., Crimean-Congo hemorrhagic fever), and phleboviruses (e.g., Rift Valley fever), but many are not known to infect humans. Although all viruses in the family share morphological and morphogenic properties, and are distinguished from other RNA viruses by a three-segmented, single-stranded genome, numerous differences in replication strategies and antigenic characteristics have been described for viruses in each genus.

## 6. Properties of Bunyaviridae

### 6.1. Morphology, morphogenesis and virion composition

Unique morphological features characteristic of viruses in each genus have been described (Martin et al., 1985), however, virions generally are 80–120 nm in diameter and appear as spherical particles. Penton-hexon clusters arranged in an icosahedral lattice were found on the surface of Uukuniemi virus (von Bonsdorff and Pettersson, 1975). The outer envelope of virion particles consists of a bilaminar membrane with integral surface projections of approximately 5–10 nm (Fig. 12.5). The internal composition of virions has been inferred from biochemical studies and is schematically illustrated in Fig. 12.6.

The three genome segments, designated as large (L), medium (M) and small (S), individually complex with nucleocapsid proteins to yield three separate ribonucleoprotein structures. Although at least one each of the L, M, and S ribonucleocapsids must be present in infectious virions, the ratio of those present varies (Bishop and Shope, 1979). Two virus-specified glycoproteins constitute the spike-like surface projections, one or both of which have transmembrane regions. No matrix (M) protein is found in virion particles, suggesting a direct interaction of the nucleocapsid and envelope proteins. Relative RNA and structural protein sizes of a representative member of each genus of the Bunyaviridae are illustrated in Fig. 12.7.

Morphogenesis occurs primarily in association with smooth membranes, particularly in the Golgi, and involves budding of morphologically complete virions into cytoplasmic vacuoles, transport of the vacuoles to the cell surface, and release of mature virions by exocytosis (Smith and Pifat, 1982).

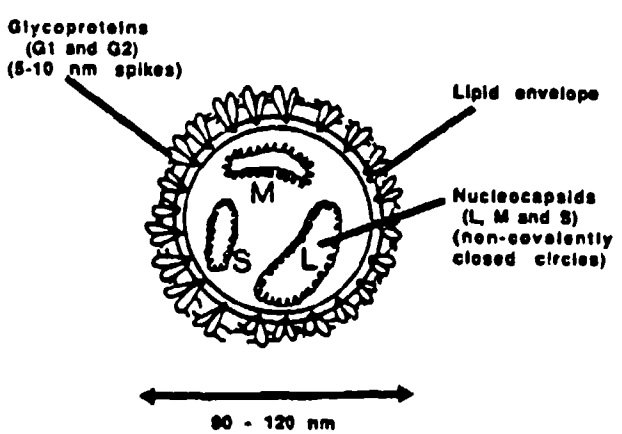



Fig. 12.6. Schematic representation of a Bunyaviridae virion. Particles contain three separate, non-covalently closed nucleocapsid structures consisting of the large (L), medium (M) or small (S) RNA segment complexed with nucleocapsid protein (N). A host-derived, bilaminar lipid envelope with integral virus-specified glycoprotein projections surrounds the nucleocapsids.

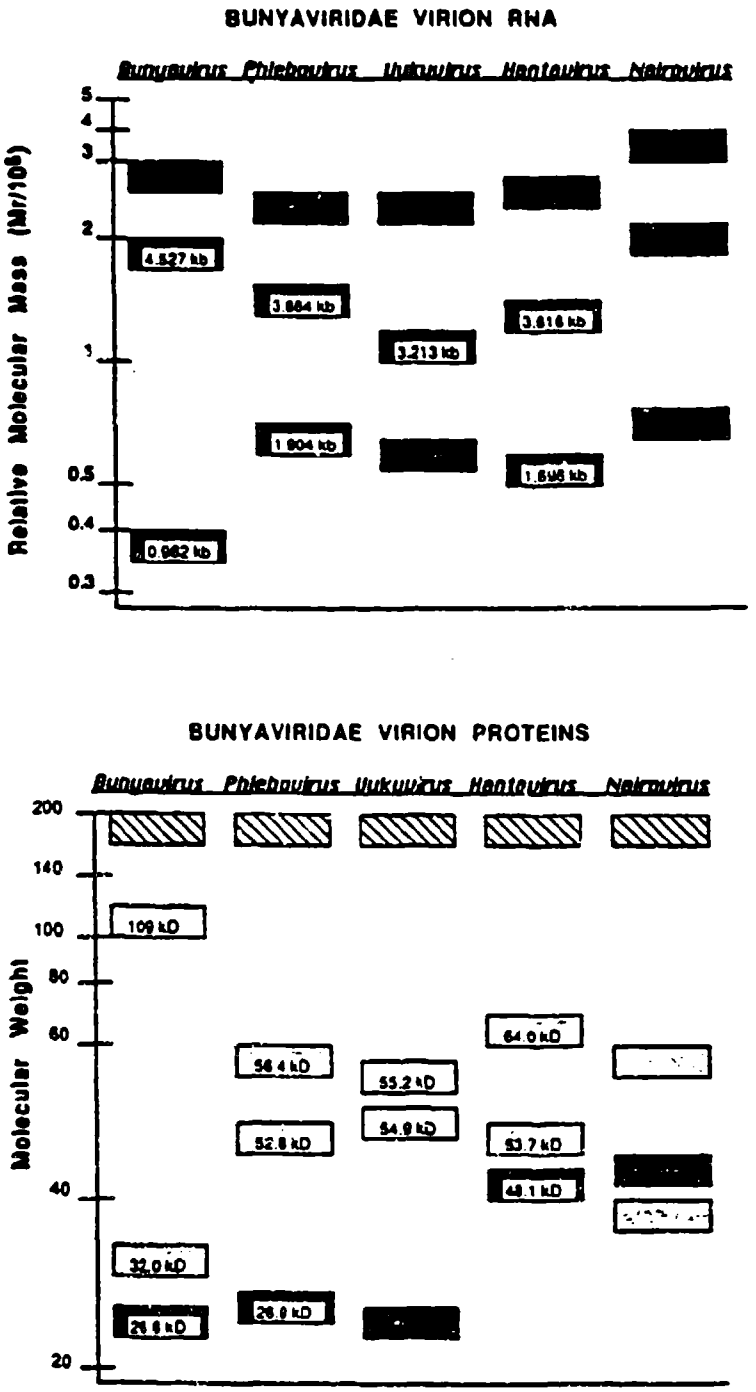



Fig. 12.7. Electrophoretic migration patterns of the three RNA genome segments and the structural proteins of a virus representative of each genus of the Bunyaviridae. Where sequence analysis is available, the number of nucleotides and the predicted molecular weights of proteins (non-glycosylated) are listed. Data were obtained from the following references: (1) Bunyavirus (Snowshoe hare virus) Akashi and Bishop, 1983; Fazakerley et al., 1988; (2) Phlebovirus (Rift valley fever virus) Collett et al., 1985; (Punta Toro virus) Emery and Bishop, 1987); (3) Uukuvirus (Uukuniemi virus) Ronnholm and Petterson, 1987; (4) Hantavirus (Hantaan) Schmaljohn et al., 1986a, 1987b); Nairovirus (Qalyub) Clerx and Bishop, 1981.

## 6.2. Coding assignments of viral genes

All viruses in the Bunyaviridae examined to date encode their nucleocapsid protein (N) in the S genome segment and their envelope proteins (G1 and G2) in the M genome segment. The coding strategies used to generate these proteins, however, differ dramatically among viruses in each genus (Fig. 12.8).

The simplest of the strategies described thus far is that of the hantaviruses for which known coding regions are strictly negative-sense. A single continuous open reading frame (ORF) located in the viral-complementary-sense S or M RNA, is used to encode the nucleocapsid protein (N) and the envelope proteins (G1 and G2), respectively (Schmaljohn et al., 1986a, 1987b). These structural proteins are apparently the only gene products of the hantavirus S and M segments.

Like hantaviruses, bunyaviruses encode their envelope glycoproteins in a continuous open reading frame in the viral complementary-sense RNA; however, at least

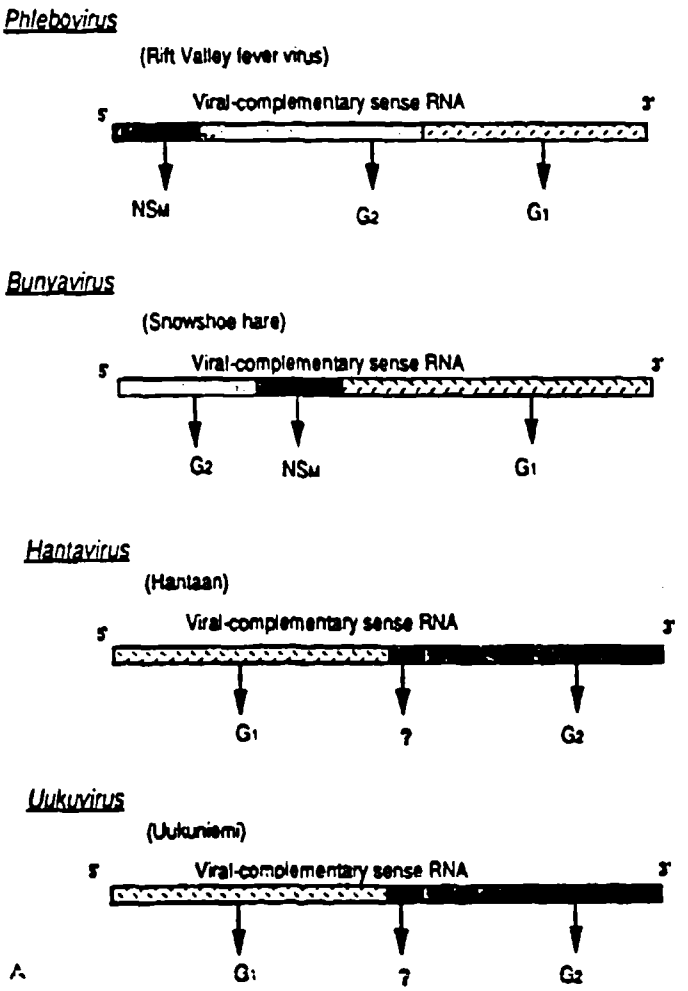
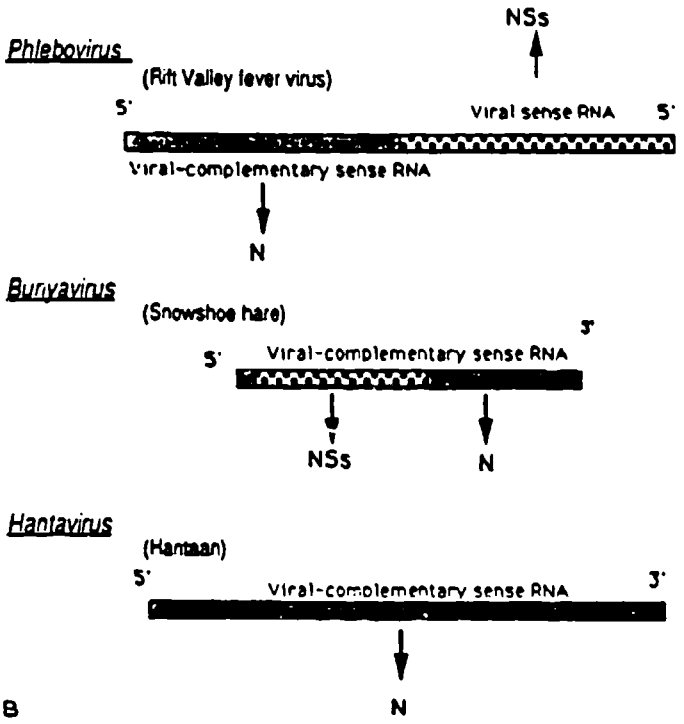



Fig. 12.8. Known coding assignments of the M and S genome segments of representative viruses in the Bunyaviridae. (A) The envelope glycoproteins (G1 and G2) of viruses in each genus examined to date

one non-structural protein ($NS_M$) is also encoded, and has been localized to the gene region between G1 and G2 coding sequences (Fazakerley et al., 1988). The bunyavirus S segment codes for N and a non-structural protein (NSS) using the same sequences but in two different overlapping reading frames of the viral-complementary sense RNA. (Akashi and Bishop, 1983; Akashi et al., 1984; Bishop et al., 1982; Cabradilla, et al., 1983, Gerbaud et al., 1987).

The continuous ORF of the viral-complementary-sense M segment of phleboviruses contains coding sequences for $NS_M$ which precede those of G1 and G2. Coding sequences for the phlebovirus N protein are found in the viral-complementary sense RNA of the S segment and sequences for $NS_s$ in a non-overlapping region of the viral-sense RNA. This strategy, which has thus far been reported for RNA viruses only in the S segments of phleboviruses, and viruses in the Arenaviridae family, has been termed 'ambisense' (Bishop, 1986). The S segment of uukuviruses also encodes N and $NS_s$, probably with an ambisense strategy, however, uukuvirus M segments,




are encoded in a single continuous open reading frame in the viral-complementary sense RNA. Nonstructural protein coding regions ($NS_M$) have also been identified within the open reading frame for viruses in the phlebovirus and bunyavirus genera. The carboxy-terminal sequences of the G1 proteins of viruses in the hantavirus and Uukuvirus genera have not been defined, thus small amounts of $NS_M$ coding information may exist between sequences encoding G1 and G2 as indicated by ? (B) Viruses in the phlebovirus, bunyavirus and hantavirus genera each encode their nucleocapsid protein (N) in the viral complementary senser RNA. Additionally, phleboviruses encode a nonstructural protein ($NS_s$) with nonoverlapping sequences of the viral sense RNA. Bunyaviruses encode $NS_s$ in an overlapping reading frame of the viral-complementary sense RNA. $NS_s$ protein(s) have not been reported for hantaviruses.

differ from those of phleboviruses in that G1 and G2 are the only gene products (Ronnholm and Pettersson, 1987). The function(s) of neither $NS_S$ nor $NS_M$ proteins have yet been determined for any virus in the family.

Little information is available for the L genome segments of viruses in the Bunyaviridae. The L segments have long been presumed to encode the virion-associated viral transcriptase (L protein) which is required to copy the negative-sense RNAs to messenger-sense RNAs. Direct proof of this was obtained by preparing reassortant bunyaviruses with mixed infections of Tahyna and LaCrosse. The L genome segments of these two viruses could be differentiated by nucleic acid analysis and the L proteins by electrophoretic migration. By examination of the nucleic acids and proteins of reassortant viruses, it was clearly established that the L protein sorts with the L genome segment (Endres et al., 1989).

Coding strategy information has not yet been reported for any of the genome segments of viruses in the Nairovirus genus.

### 6.3. Antigenic variation

Serological relationships among viruses in different genera of the Bunyaviridae have not been reported, and with the exception of phleboviruses and uukuviruses which display a low degree of amino acid homology in their envelope proteins (Ronnholm and Pettersson, 1987), no inter-generic relationships can be discerned by comparison of available nucleic acid or predicted amino acid sequences. These data suggest different origins or an early evolutionary divergence of the members of this family.

The viruses within each genus have been subdivided into serogroups based upon cross-reactivities in tests such as hemagglutination inhibition, neutralization and complement fixation. The largest genus, the Bunyavirus genus, is the best characterized serologically, and includes 16 serogroups with more than 100 distinct viruses. Even within a particular serogroup viruses display antigenic differences detectable by monoclonal antibody (mAb) reactivities. An example of this diversity can be observed with viruses in the Bunyamwera serogroup, in that people infected with one virus in this group are not protected from infection with another virus in the same group (Gonzalez and Georges, 1988). At the genetic level, even individual isolates of the same virus are distinguishable. For example, numerous isolates of LaCrosse virus, obtained in the same or different geographic locales, either at the same or different times, all were found to be genetically distinct by oligonucleotide fingerprint analyses, suggesting that a high and frequent rate of mutation occurs (Beaty, et al., 1988).

To examine antigenic variation and to localize point mutations which may be important in immunity, Battles and Dalrymple (1988) sequenced gene regions of 22 isolates of RVFV which were known to encode epitopes involved in neutralization and protection (see below). The isolates had been collected over a 34 year time period from six African countries. They were able to identify specific base variations

which resulted in amino acid coding differences and rendered isolates resistant to neutralization by mAbs defining the epitopes. Amino acid differences which correlated with loss or reduction of reactivity with the mAb in question involved changes of amino acid charge. In some cases, there was reduced mAb reactivity despite conversation of the peptide against which the mAb was known to react; presumably, this is explicable on the basis of amino acid changes in other juxtaposed regions of the viral glycoproteins. (reference crystallography of Ag-Ab interaction with influenza; Colman et al., 1987)

In addition to the resultant 'antigenic drift' such point mutations would incur, the segmented genomes of viruses in the family also allow for more dramatic 'antigenic shifts' attributable to segment reassortment. Reassortment among viruses in the California serogroup of bunyaviruses has been demonstrated in cell culture and in mosquitoes both in the laboratory and in nature. Reassortment was also obtained by interrupted feeding of mosquitoes on animals viremic with different viruses (Beaty et al., 1985). The ability to reassort, however, appears to be limited only to very closely related viruses. It has not been possible to obtain reassortment between viruses in different serogroups of the Bunyavirus genus, nor between viruses in different genera.

Host influences on viruses have also been suggested to play a role in the antigenic properties of bunyaviruses. Repeated passage of Bunyamwera and snowshoe hare viruses in cultured mosquito cells resulted in the loss of reactivity of certain monoclonal antibodies with the parent, mammalian cell culture passaged virus (James and Millican, 1986). Bunyaviruses, phlebovirus, uukuviruses and nairoviruses all replicate alternately in invertebrate and vertebrate hosts, and are generally noncytolytic in arthropod host cells, but cytolytic in vertebrate cells. Consequently, host factors may greatly influence the antigenic evolution of these viruses. In contrast, hantaviruses replicate only in vertebrate (usually rodent) hosts, often persistently, with little or no detectable cytopathology. Although numerous hantavirus strains can be differentiated by serological means, the extent and frequency of antigenic drift in hantaviruses has not been reported. Evidence exists that a particular strain of virus may be antigenically stable as suggested by studies in which antigenic differences between Hantaan virus (a rodent isolate) and two isolates from Korean hemorrhagic fever patients could not be detected with monoclonal or polyclonal antibodies. Furthermore, although the isolates came from different vertebrate hosts, and were obtained over an eight year time-frame, the predicted amino acid sequences of the envelope proteins of these viruses differed by less than 3% (Schmaljohn et al., 1988).

## 7. Antibody-mediated immunity

In general, neither structural nor nonstructural viral proteins are translocated to infected-cell surfaces in quantities sufficient to be demonstrated with antibodies. Thus,

the principal targets of protective antibodies appear to be virion surface glycoproteins, with little or no contribution from cytolytic mechanisms requiring complement or accessory cells. Two exceptions were noted. The first was observed by immuno-electron microscopy in cells infected with a hantavirus isolated from rats (R22 virus). The infected cells displayed a thick layer of antigen, which was thought to be virus-specific, located on top of, but distinct from the plasma membrane. It is unclear if or how this material is involved in viral morphogenesis as mature virions could be observed budding intracellularly into Golgi vesicles as is usual for viruses in the Bunyaviridae (Hung et al., 1985). It was not determined if the antigen layer had any significance for immunity. The second potentially important exception was noted with the phlebovirus, Rift Valley fever virus (RVFV), which expressed envelope glycoproteins and could be seen budding not only in Golgi but at plasma membranes in primary hepatocytes; in other cell types RVFV exhibited the more typical absence of cell-surface proteins (Anderson and Smith, 1987).

### 7.1. Neutralization

Neutralization of virion particles, i.e., reaction of antibody and virions in a manner which significantly reduces the number of observable infectious particles, presumably requires accessibility of virion proteins to the neutralizing antibody. The envelope glycoproteins of viruses in the Bunyaviridae are at least partially exposed on the virion surface as indicated by reactivity of antibodies with both G1 and G2 in studies such as immunoelectron microscopy, radioimmune precipitation, or enzyme treatment of intact virions. In contrast, antibodies to N do not react with virions. No information is available concerning the precise location of the L protein, however, it is assumed not to be exposed on the virion surface.

Because of obvious structural constraints, not all portions of G1 and G2 can be accessible to antibodies that might mediate neutralization. The G1 protein of LAC was found to be more susceptible to digestion by proteolytic enzymes than was G2, suggesting that more of G2 may be sequestered within the viral envelope. After enzyme treatment, the modified LAC virions demonstrated reduced neutralization by polyclonal sera, indicating that G1 plays an important role in neutralization of virion particles (Kingsford et al., 1983).

The involvement of G1 and G2 epitopes in virion neutralization has been more accurately defined by use of mAbs, which have been described for representative viruses in each genus except the Uukuvirus genus. Not surprisingly, mAbs reactive with nucleocapsid proteins have been found devoid of neutralizing (NT), hemagglutination-inhibiting (HI), or protective activities, though they provide useful serological reagents by virtue of the fact that nucleocapsid proteins may carry either virus-specific or conserved, genus-specific epitopes. In contrast, some mAbs reactive with G1 and G2 will demonstrate NT and/or HI activities and may protect animals from infection or disease. Where examined, both G1 and G2 are antigenically complex,

each possessing multiple epitopes that can be discriminated by mAb reaction patterns and mAb competition grouping. With phleboviruses (Keegan and Collett, 1986; Pifat et al. 1988,), hantaviruses (Dantas et al., 1986; Arikawa et al., 1989), and nairoviruses (J. Smith, personal communication), both G1 and G2 possess one or more epitopes defined by neutralizing or hemagglutination-inhibiting mAbs. With bunyaviruses, NT and HI activities have been attributed only to G1-specific mAbs, but G2-specific mAbs have not been obtained and the possibility that G2 bears immunologically relevant epitopes has not been formally excluded (Gonzalez-Scarano et al., 1982).

### 7.2. Humoral immunity

Humoral immunity is experimentally defined by the ability of transferred antibodies (as serum or monoclonal antibodies) to confer specific antiviral resistance to non-immune animals. It is not always synonymous with viral neutralization as it is commonly viewed, in that the protective mechanism(s) may be inadequately or even erroneously reflected by in vitro neutralization assays. This discrepancy was clearly illustrated by analyses of antibody-mediated protection with mAbs. For example, with the phlebovirus, Punta Toro virus (PTV), it was demonstrated that a mAb with very low neutralization activity (1:10) in vitro, nevertheless protected all animals from a lethal PTV challenge (Pifat et al., 1988). The ability of antibodies having poor neutralizing antibody in vitro to confer protection in vivo was previously noted with alphaviruses as well (Schmaljohn et al., 1982); however, the protective mechanism for viruses in the Bunyaviridae seems unlikely to correspond to the antibody-directed cytolysis observed with alphaviruses, but more likely involves virion opsonization (and clearance by cells of the reticuloendothelial system) or complement-mediated virolysis.

More confounding was the observation that certain mAbs or polyclonal monospecific antibodies exhibited good neutralizing activity in vitro but were ineffectual in confering protection in vivo. These were G2-specific in the case of PTV and specific for the homologous protein, G1, in RVFV (Smith et al., 1987; Pifat et al., 1988; Dalrymple et al., 1989). These observations could not be easily explained on the basis of immunoglobulin isotype, avidity, or other trivial possibilities. The same principle; i.e., that G2 of RVFV confers a greater level of antibody-mediated immunity than G1 despite equivalent or greater levels of neutralizing antibodies induced by the latter, was upheld by data involving mice immunized with vaccinia recombinants that expressed only either G2 or G1 (Dalrymple et al., 1989). These results demonstrate an inadequacy of conventional serology in predicting antibody activities in vivo and indicate the need for caution in extrapolation of in vitro principles to immunity.

### 7.3. Molecular definition of B cell epitopes

Competitive inhibition with panels of mAbs have provided rough estimates of the number and complexity of antigenic sites important in neutralization and protection of viruses in the Bunyaviridae. This methodology, however, does not permit localization of epitopes within the gene. Molecular techniques were employed with the phlebovirus RVFV which allowed determination of sequences encoding epitopes recognized by three neutralizing (and protective) and one non-neutralizing G2-specific mAbs (Keegan and Collett, 1986). Progressively smaller cDNAs, representing specific regions of the RVFV G2 gene, were expressed as β-galactosidase fusion proteins in *E. coli* and the expressed proteins were tested against individual mAbs. With this method it was possible to limit the three epitopes reactive with neutralizing mAbs to 11, 20, or 34 amino acids, and the epitope reactive with a non-neutralizing mAb to 14 amino acids within the G2 gene. The 20 and 34 amino acid epitopes overlapped, but the 20 amino acid epitope appeared to be structurally constrained (as indicated by the inability of the mAb to recognize denatured antigen) while the 34-amino-acid epitope was not (as indicated by mAb reactivity with both native and denatured protein). Synthetic peptides representing 9–14 amino acids within neutralizing sites were found to elicit antibody responses in animals, and in one case the resultant antibody displayed high titered neutralizing activity (Smith et al., 1987).

## 8. T cell responses

The roles that T cells play in immunity to viruses in the Bunyaviridae remain relatively poorly defined, perhaps because antibodies alone have proven sufficient to confer resistance in all cases examined. However, the prominent role of antibody-mediated resistance does not preclude a critical role for T cells in recovery from primary infection, in cross-reactive immunity, and possibly in immunopathology. Nor should the influence of T cells on quantitative and qualitative aspects of antibody responses be underestimated.

Through their work with hantaviruses, Asada et al.(1987, 1989) have provided the clearest evidence for the potential importance of T cells in resistance to a member of the Bunyaviridae. They showed that Hantaan-specific cytotoxic T lymphocytes (CTL) can be demonstrated if immune mouse lymphocytes are restimulated in vitro with Hantaan antigen. Similarly, they were able to demonstrate cross-reactive CTL by restimulating, with Hantaan, spleen cells from mice immunized with hantaviruses representing different serotypes from Hantaan (Prospect Hill and Puumala viruses). The bulk of the cytotoxic cells, assayed on Hantaan-infected peritoneal macrophages, bore a surface phenotype ($Thy1^+$, $L3T^-$, $Lyt2^+$) generally associated with class I-restricted CTL. Adoptive transfer of immune T cells, subsets of immune T cells, or serum to nonimmune mice were used to demonstrate that the abilities of mice to control Hantaan viremia were attributable not only to antibodies

but also, in part, to T cells bearing either helper (Thy 1+, L3T4+, Lyt2−) or cytolytic (Thyl+, L3T4−, Lyt2+) phenotypes. In one instance of cross-protection among related but dissimilar viruses (protection against Hantaan by immunization with Puumala) their evidence suggested that T cells may be particularly relevant (Asada et al., 1989).

Except for one preliminary report (Balady, 1987), in which vaccinia virus recombinants expressing RVFV envelope glycoproteins were shown to elicit CTL, the proteins that elicit T cell responses to viruses in the Bunyaviridae have not been defined. In contrast with humoral immune mechanisms, it has been demonstrated in many viral systems that T lymphocytes are somewhat indifferent to the presence or absence of native viral proteins on cell surfaces, and might well react with unfolded or fragmented proteins representing any of the viral gene products. Consequently, any and perhaps all of the virus-encoded proteins of Bunyaviridae may eventually be found to elicit some degree of T cell responsiveness. The challenge will be to distinguish which of the responses are genuinely important in immunity. With recent advances that include not only gene sequencing and thereby the potential exploitation of synthetic peptides, but also the synthesis of individual proteins or their fragments in a variety of expression systems, a more thorough understanding of the cellular immunology of Bunyaviridae can be anticipated.

## 9. Approaches to new vaccines

Vaccine development for viruses in this family has been guided by the observation that traditional killed vaccines, where it has been possible to make and test them, have generally been effective in experimental systems, underscoring the dominant role of antibodies in protection. In addition to killed vaccines, subunit preparations, live-attenuated viruses, and more recently, recombinant-expressed proteins or live recombinant viruses have been investigated as potential vaccines for viruses in the Bunyaviridae. Studies of RVFV vaccine candidates best illustrate the continuing evolution of thought and experimentation in vaccines against members of the Bunyaviridae.

The first RVFV vaccine developed was a formalin-inactivated virion preparation. This vaccine elicits virus-neutralizing antibodies, is exceptionally effective in preventing disease and death in laboratory animals, and has been used with apparent efficacy in people at risk from natural or laboratory exposure (Randall et al., 1964; Eddy et al., 1981); however, it is expensive to produce, is not particularly immunogenic (three inoculations are recommended), and it carries the inherent risk of incomplete inactivation. A candidate live-attenuated RVFV vaccine was derived from its more virulent progenitor by chemical mutagenesis and subsequent selection of a virus that appears to be highly attenuated in its virulent properties, yet replicates sufficiently to evoke antibodies and solid immunity in experimental animals includ-

ing domestic livestock, the main target of RVFV epidemics (Caplen et al., 1985; Morrill et al., 1987). Despite apparent multiple mutations resulting from successive passes in the presence of the chemical mutagen, (Caplen et al., 1985) such a vaccine carries the theoretical risk of reversion or reassortment and, even in the event that it proves a highly safe and successful vaccine, such a strategy for vaccine production may be difficult or impossible to reproduce with other viruses in the family.

Recombinant DNA based vaccine strategies are still under development, but offer exciting possibilities for disease prevention. Moreover, these strategies involve the significant advantage of avoiding propagation of large amounts of pathogenic viruses in the laboratory or vaccine production facility. As described above, *E. coli*-expressed, RVFV-β-galactosidase fusion proteins were used to definitively map three neutralization epitopes on G2 (Keegan and Collett, 1987). Collett et al. (1987), further tested the abilities of such proteins to serve as immunogens. Although results verified the protective potential of G2 immunization, the fusion proteins were otherwise disappointingly poor immunogens and protected only 70% of animals from a lethal challenge of RVFV even when high dosages of antigen were administered.

In contrast to the bacterial expression products, vaccinia recombinants which expressed both G1 and G2 were readily able to elicit antibodies and immunity in experimental animals and more importantly prevented abortion in pregnant sheep, a major consequence of RVFV infection of livestock (Collett et al., 1987; Dalrymple et al., 1989). The strain of vaccinia used to construct recombinants was found to play a role in successful induction of immunity in the sheep (but not in mice), suggesting that caution must be taken in projecting the success of a vaccine tested in laboratory animals to that obtained in naturally infected hosts. In agreement with results obtained by passive protection of animals with mAbs, when tested individually, recombinants expressing G2 were found to elicit a greater level of immunity than those expressing only G1 (Dalrymple et al. 1989). A potential advantage to the vaccinia expression system is the concomitant induction of CTL (Balady, 1987). However, it has the drawback of being intimately linked to the biology and immunology of the vaccinia virus vector, so that expression levels, vaccinia virus strain, and vaccinia-immune status may all be critical to vaccine efficacy.

Expression of the same RVFV genes by baculovirus recombinants (Autographa californica nuclear polyhedrosis virus), resulted in production of abundant amounts of G1 and G2 in lepidopteran insect cell cultures. The glycoproteins were biochemically and antigenically indistinguishable from authentic RVFV G1 and G2, and the expressed proteins were immunogenic in mice (Schmaljohn et al., 1989a). Recombinant-infected cells that contained both G1 and G2 elicited neutralizing antibodies in mice and conferred immunity to lethal challenge. G2 alone was apparently less immunogenic, but was also effective. Potential impediments with this vaccine strategy include the purification of viral antigens from insect cell contaminants, the possible influences of glycosylation differences between invertebrate and vertebrate

cells, and the theoretical concern that some important immunological component would be absent in the response elicited by a subunit vaccine.

Because they contained only glycoprotein genes, (unlike killed or live-attenuated vaccines) both the vaccinia and baculovirus recombinant immunogens elicited no antibodies to the nucleocapsid protein and therefore provided a convenient serological marker to discriminate vaccinated from infected individuals.

Similar but less extensive approaches have been used experimentally to evaluate experimental vaccines against other viruses in the Bunyaviridae. Recombinant vaccinia viruses have been shown to express apparently authentic glycoproteins of another phlebovirus, PTV, however protective properties of the expressed proteins have not yet been reported (Matsuoka, et al., 1988). Hantaan glycoprotein and nucleocapsid genes have been expressed in both vaccinia and baculovirus recombinants (Schmaljohn et al. 1987a, 1989b); antigens produced in both systems were able to elicit antibodies in animals. Vaccinia recombinants containing both G1 and G2 were particularly effective at inducing neutralizing antibody responses, but it is otherwise difficult to assess Hantaan vaccine efficacy because of the current lack of a suitable animal model for human disease.

## 10. Conclusion

The arenaviruses, and particularly LCMV, have long provided investigators with biological models of exceptional interest and merit. Over the last decade the molecular biology of this group has advanced to the extent that meaningful questions addressing molecular pathogenesis can be approached. The next generation of studies will combine biological and molecular approaches to elucidate mechanisms of viral persistence, immunosuppression, inhibition of luxury function, and to provide a safe and effective vaccine for diseases like Lassa fever and Argentine hemorrhagic fever.

With Bunyaviridae, molecular biology is providing a particularly important set of tools for unifying traditional serology, cellular and molecular immunology, and vaccine development. With the Bunyaviridae tested to date, there appear to be no unusual barriers to the expression of viral antigens by either vaccinia or baculovirus recombinants. Rapid advances in immunochemistry, previously thwarted by poor growth characteristics or high biohazard potential of important members of the Bunyaviridae, can be also expected for this diverse family.

## 11. Acknowledgments

This work was supported by PHS grant AI 16102 and DOD contract C6234.

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*Virus Research*, 22 (1992) 185–198

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VIRUS 00733

# Biochemical and immunological evidence that the 11 kDa zinc-binding protein of lymphocytic choriomeningitis virus is a structural component of the virus *

Maria S. Salvato [2], Karl J. Schweighofer [1], John Burns [1,a] and Elaine M. Shimomaye [1]

[1] *Department of Neuropharmacology, Scripps Clinic and Research Foundation, La Jolla, CA 92037 (U.S.A.) and* [2] *Department of Pathology, University of Wisconsin Medical School, Madison, WI 53706 (U.S.A.)*

(Received 15 October 1991; revision received 1 November 1991; accepted 5 November 1991)

**Summary**

The completed sequence of the arenavirus, lymphocytic choriomeningitis virus, revealed a new gene encoding a small protein with a single zinc-binding domain. The cDNA for this gene has been expressed in *E. coli* to produce fusion protein that has been used to raise antisera. The antisera facilitated the positive identification of the p11 'Z' gene product as a structural component of the virion. A related arenavirus, Tacaribe, has a comparable p11 gene product. The abundance of the p11 Z protein relative to other virion components has been determined by metabolic labeling. Triton X-114 extraction and dimethyl suberimidate-HCl crosslinking indicate that the p11 Z protein is a hydrophobic protein associated with the nucleocapsid of the virion core.

Arenavirus; Lymphocytic choriomeningitis virus; Zinc-binding protein; Antiserum; Triton X-114; Protein crosslinking

*Correspondence to:* M.S. Salvato, Department of Pathology, University of Wisconsin Medical School, 505 SMI, 1300 University Avenue, Madison, WI 53706, U.S.A.

[a] Present address: Department of Veterans Affairs, Medical Center, Palo Alto, CA 94304, U.S.A.

* Presented in part at the Eighth International Conference on Negative Strand Viruses, Charleston, SC, U.S.A., 15–20 September 1991.

## Introduction

Arenaviruses, like most other negative-strand RNA viruses, have a small single-stranded genome of 10–15 kb. Unlike other negative-strand viruses, arenaviruses contain two genome segments, a large (L) RNA segment of 7–8 kb and a smaller (S) segment of 3–4 kb, both of which bear ambisense coding arrangements. Three types of gene products have been described for the arenaviridae: the nucleocapsid protein (NP), the envelope glycoproteins (GP-1 and GP-2), and the RNA polymerase (L). Completion of the genomic sequence revealed an additional open reading frame in lymphocytic choriomeningitis virus (LCMV) (Salvato et al., 1988, 1989; Salvato and Shimomaye, 1989) and in the related arenavirus, Tacaribe (Iapalucci et al., 1989). Because of its single zinc-binding domain, the new LCMV gene product was designated p11 'Z'.

The function of the Z protein remains a matter of speculation. Other negative-strand RNA viruses have, in addition to polymerase, nucleocapsid, and envelope glycoproteins, two other functional categories of protein often designated non-structural (NS) or phosphoprotein (P), and matrix protein (M). The NS/P proteins are generally thought to act as mediators of transcription and are often found in a functional complex with the nucleocapsid and polymerase proteins. The M proteins are thought to mediate virion assembly and are found to be integral membrane proteins of the virion. The Z gene product is found both in association with virion cores and in association with the hydrophobic phase of a Triton X-114 extraction. These associations may be fortuitous or may be indicative of more than one function for the Z gene product. The possibility of alternate structures or modifications of the Z protein is still under investigation.

## Materials and Methods

### *Production of the Z gene cDNA*

The LCMV strain Armstrong isolate 53b was used for most of these studies, and the Armstrong isolate 4 was used for the crosslinking studies. Maintenance of virus stocks and purification of viral RNA have been described (Salvato et al., 1988). DNA copies of the LCMV Z gene were made by reverse transcription of viral RNA followed by amplification using the polymerase chain reaction (Saiki et al., 1988) with two flanking oligonucleotide primers (CGCACCGGGGATCCTAG·GCG$_{20}$ and TGTGTGTGTGCGTGTTCTG$_{445}$; subscript numbers refer to the base number from the 5′ end of the viral L RNA (Salvato and Shimomaye, 1989)). This cDNA was cloned into the *Sma*I site of pUC18 (Yanische-Perron et al., 1985) to make pZ-ORF.1.

### *High level expression of the LCMV Z gene in bacteria*

*Escherichia coli* strain BL21-DE3 (F$^-$ *hsdS gal* rB$^-$mB$^-$) is a lambda lysogen containing the T7 RNA polymerase gene downstream of the isopropyl-β-D-

thiogalactopyranoside (IPTG)-inducible *lac*UV5 promoter (Studier and Moffat, 1986). The plasmid pRK172 is a pBR322-derivative containing the T7 polymerase promoter Ø10, the translation start site for the T7 gene 10 protein, and a deletion for the pBR copy control region (described by McLeod et al., 1987). Plasmid pRK172/Z-ORF.1 was constructed as described in Fig. 1a. The AvaII site could only be cleaved in DNA prepared from *dcm*$^-$ strains such as BL21-DE3. An NdeI/AvaII adapter retained the bacterial ribosome binding site upstream of the Z gene reading frame and fused an additional 8 amino acids to the *N*-terminus of the Z gene product.

*Purification of Z fusion protein*

One liter of bacteria was grown to mid-log phase, incubated with 0.4 mM in IPTG for 1 h, pelleted, and suspended in 10 ml of GTC buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate pH 7, 30 mM dithiothreitol (DTT), 0.5% sarcosyl). Five milliliters of this lysate was loaded on a 300 ml column of Sephadex G-200 in GTC buffer. Three-milliliter samples were collected beginning with a void volume marked with blue dextran. Peak fractions containing the Z protein, 22–25, were pooled and dialysed overnight against a liter of buffer (10 mM $NaPO_4$, 30 mM DTT, pH 7.0). Precipitates that occasionally formed during dialysis contained contaminating proteins and were discarded. Half of the soluble Z protein was loaded in dialysis buffer onto a 4 ml Heparin agarose column, and washed in steps of increasing salt concentration (0.01 to 1.0 M NaCl): 90% of the Z protein eluted upon the addition of buffer containing 0.3 M NaCl. The yield of Z protein by this procedure is approximately 10–20 mg Z protein per liter of starting bacterial culture. This purification scheme provided sufficient fusion protein for antibody production. Z protein was occasionally purified by gel elution as described by Hager and Burgess (1980).

*Production of Z-specific antisera in rabbits*

Column purified Z protein was concentrated by precipitation with 4 vols. of acetone, resuspended to 1 mg/ml in 10 mM $NaPO_4$, 4 M urea and diluted 1 : 1 with adjuvant. Rabbits were injected at 2-week intervals: the first injection was 0.5 mg Z protein with complete Freunds adjuvant given intramuscularly (i.m.), the second, third, and fourth were 0.1 mg Z protein with incomplete Freunds (i.m.), and subsequent injections were 0.1 mg Z protein with 10% aluminum hydroxide hydrate (Aldrich) in phosphate-buffered solution (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$) given intraperitoneally.

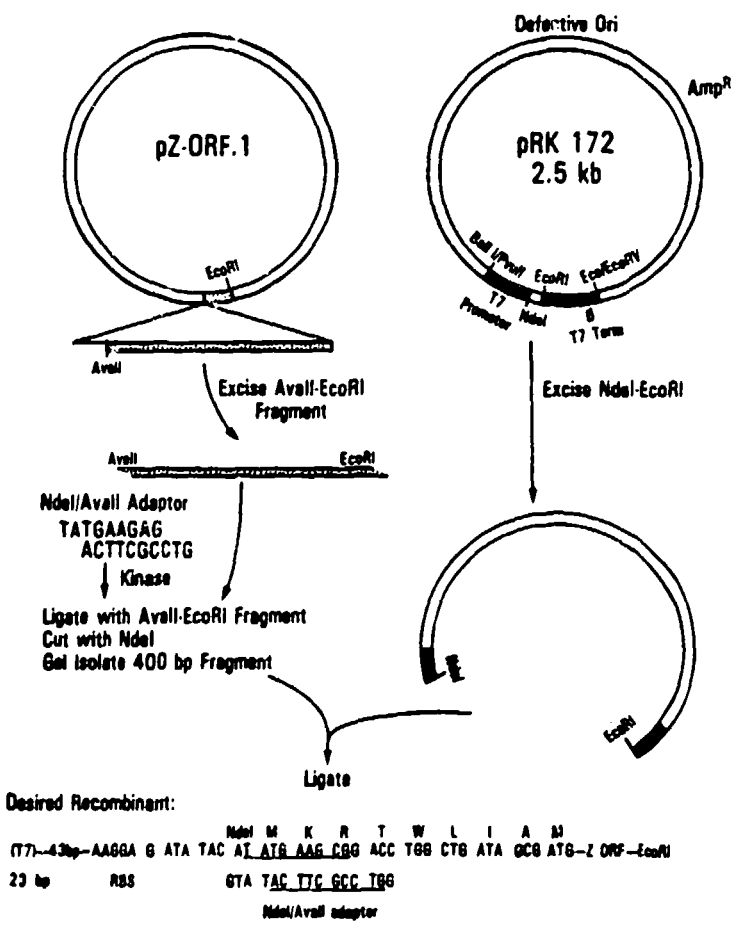
*Production of [$^{35}$S]cysteine-labeled virus*

Ten $T_{175}$ flasks of BHK-21 cells were grown to 80% confluence and infected with LCMV Armstrong at a multiplicity of 5 pfu/cell. At 32 h post-infection, media was replaced with media lacking cysteine (Select-Amine Kit, Gibco). At 33 h

post-infection, 0.5 mCi cysteine (NEN) was added per flask and harvested at 48 h post-infection. Virus was precipitated with 7% PEG, and banded twice on renografin gradients, then disrupted in SDS gel buffer and electrophoretically analysed by 15% SDS PAGE (Laemmli, 1970). Viral proteins were detected by autoradiography, excised, and counted in scintillation cocktail (Poly-Fluor, Packard).

*Immunoprecipitation of Z fusion protein and of p11 from metabolically labeled virions using the rabbit polyclonal antisera*

Bacteria BL21-DE3 containing pRK172/Z-ORF.1 was radiolabeled by incubation of 1 ml log-phase culture with 5 $\mu$Ci [$^{35}$S]cysteine for 30 min. Bacteria were



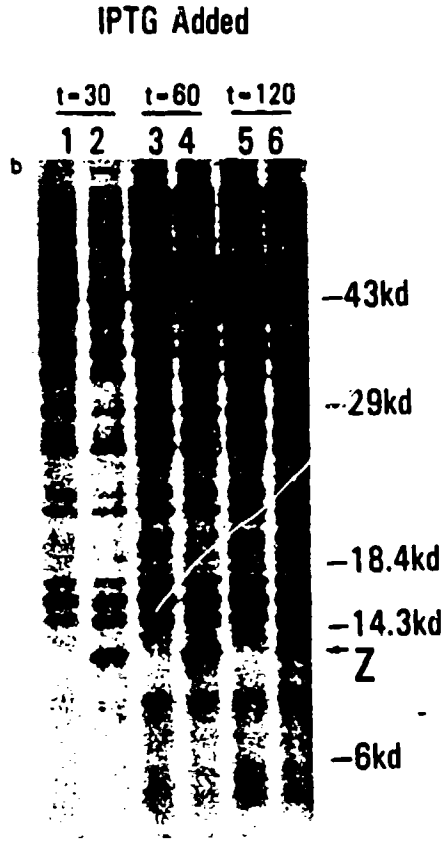



Fig. 1. High level expression of the LCMV Z gene in bacteria. (a) Cloning the Z gene cDNA into pRK172 and expression into *E. coli*. (b) IPTG induction of the T7 RNA polymerase gene in BL21-DE3 leads to higher Z protein expression. Bacteria were labeled with [$^{35}$S]cysteine and proteins were extracted at 30, 60 and 120 min after addition of IPTG and isotope (see Materials and Methods). Extracts were electrophoretically separated on 15% SDS-PAGE and autoradiographed. Lanes 1, 3 and 5 contain proteins from bacteria BL21-DE3/pRK172 (not expressing Z) and lanes 2, 4 and 6 contain proteins from bacteria BL21-DE3/pRK172/pZ-ORF.1 expressing Z protein.

pelleted, resuspended in 0.5 ml SDS-PAGE sample buffer (Laemmli, 1970), boiled 2 min, and diluted into 2 ml PBS. 100 $\mu$l of this extract was immunoprecipitated with rabbit polyclonal serum (diluted 1:100) as described (Wright et al., 1989).

To precipitate p11 Z protein from preparations of LCM virions, 200 $\mu$g of metabolically labeled virus ([$^{35}$S]cysteine as described above) is treated with 1% Triton X-100 for 30 min at 37°C, separated by equilibrium sedimentation from the other viral proteins (Burns and Buchmeier, 1991), and immunoprecipitated with rabbit polyclonal antiserum as described (Wright et al., 1989). In such disrupted virus preparations, the p11 Z protein is in the top protein fraction of the gradient (see fraction 16, Fig. 2 of Burns and Buchmeier, 1991). Immunoprecipitated

fractions from both bacterial extracts and from gradient fractions of Triton-disrupted virus, were separated for analysis on 15% SDS-PAGE.

*Triton X-114 extraction of virus*

[$^{35}$S]Cysteine-labeled virus was subjected to 3 cycles of Triton X-114 extraction to separate aqueous and detergent-associated components. One cycle involves a 5-min incubation at 4°C, a 3-min incubation at 30°C, and centrifugation through 6% sucrose for 3 min at 325 *g* as described (Bordier, 1981). Subsequent cycles used the aqueous phase of the previous cycle. Extracts were analyzed by 15% SDS-PAGE and autoradiography (Fig. 3).

*DMS crosslinking of virus*

LCMV Armstrong 4 was subjected to crosslinking with the bifunctional crosslinking agent dimethyl suberimidate-2HCl (DMS) as described by Burns and Buchmeier (1991). Crosslinked virus and non-crosslinked controls were denatured in gel sample buffer, run on 5–15% SDS-PAGE (Laemmli, 1970), transferred to immobilon P membranes (Millipore), and immunoblotted as described (Burns and Buchmeier, 1991). Rabbit polyclonal serum to Z protein (described above) and to NP amino acids 130–144 (described by Buchmeier et al., 1987) were used in immunoblotting.

## Results

*High level expression of the LCMV Z gene product in bacteria*

The p11 Z gene product was abundantly expressed as a fusion protein downstream of a T7 RNA polymerase promotor (Fig. 1). Expression of the fusion protein could be induced by the addition of IPTG to the culture (Fig. 1b). Bacterial extracts were column fractionated and the purified Z protein was used to raise antisera in rabbits as described in Materials and Methods.

*Use of polyclonal antisera to identify p11 Z as a structural component of the virion*

[$^{35}$S]Cysteine-labeled Z protein could be immunoprecipitated from bacterial extracts expressing fusion protein and from LCMV that had been treated with 1% Triton X-100 and fractionated on a 5–50% sucrose gradient. Immunoprecipitated samples were fractionated on 15% SDS-PAGE and subjected to autoradiography (Fig. 2). Lane A depicts immunoprecipited proteins of BL21-DE3/pRK172/Z-ORF.1 with control 'pre-bleed' antiserum, lane B employs the rabbit anti-Z serum (bleed 3 as described in Materials and Methods), whereas lane C depicts immunoprecipitated proteins from control bacteria (BL21-DE3/pRK172) not expressing Z fusion protein. The fusion protein migrates at approximately 14 kDa. Proteins in

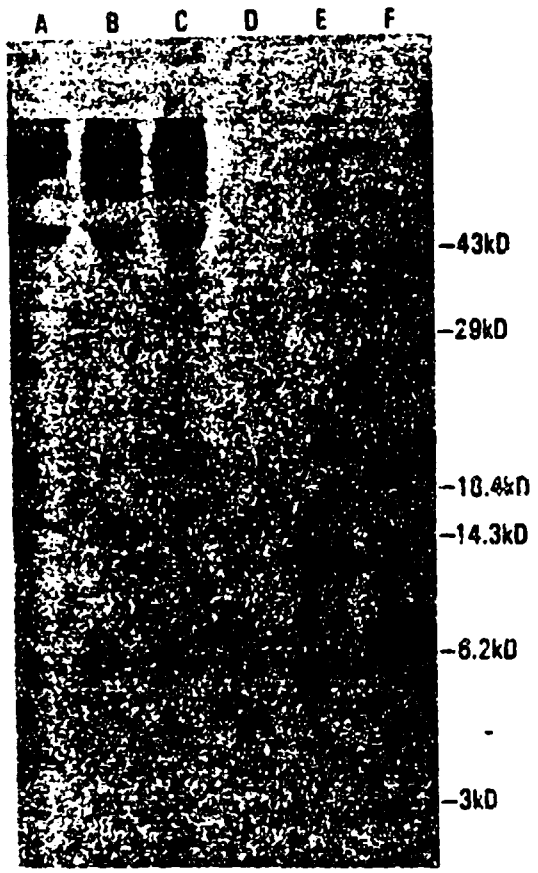



Fig. 2. Autoradiogram of immunoprecipitated [$^{35}$S]cysteine-labeled proteins. Lanes A–C are precipitates of bacterial extracts. A and B contained extracts expressing Z fusion protein; lane A employed control 'pre-bleed' serum and lane B employed anti-Z serum. Lane C contained an extract not expressing the Z fusion protein. Lanes D–F are precipitates of LCMV proteins that had been fractionated on a 5–50% Triton X-100/sucrose gradient. Lanes D and E contain the top fraction of the p11 protein of such a gradient; lane D employed control 'pre-bleed' serum and lane E employed the anti-Z serum. Lane F contained the middle fraction of the sucrose gradient devoid of any p11 protein and employed the anti-Z serum.

lanes D and E are from the top fraction of a 5–50% sucrose gradient sedimentation of Triton-disrupted virus; lane D has been immunoprecipitated with pre-bleed control serum, lane E has been immunoprecipitated with anti-Z serum. Lane F is immunoprecipitated proteins from the middle fraction of the 5–50% sucrose gradient with anti-Z serum. As is evident in Fig. 2 from Burns and Buchmeier (1991), envelope proteins and nucleocapsid proteins migrate near the middle of the 5–50% sucrose gradients; the L protein pellets with viral cores, and the p11 gene product (later identified by immunoprecipitation) migrates in the top protein fraction. Here we show the p11 gene product migrating at 11 kDa after immunoprecipitation with rabbit antiserum and gel electrophoresis.

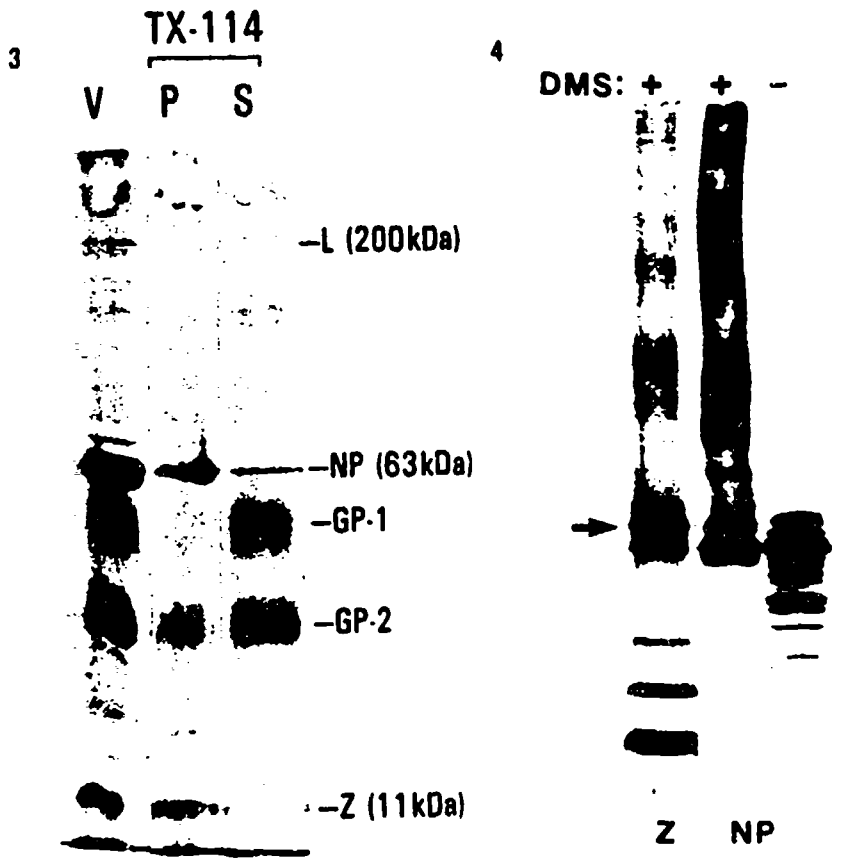



Fig. 3. Triton X-114 extraction of metabolically labeled virus. [$^{35}$S]Cysteine-labeled viral proteins were electrophoretically separated on 15% SDS-PAGE and autoradiographed. Lane V contains whole virus, lanes P and S contain the Triton X-114 extracted pellets and supernatants, respectively (see Materials and Methods).

Fig. 4. DMS crosslinking of LCMV. Virus was mildly crosslinked with DMS, denatured in SDS/mercaptoethanol sample buffer and subjected to electrophoresis (Laemmli, 1970). Proteins were transferred to membranes, incubated with antibody to Z protein (first lane) or to NP (second and third lanes), developed and photographed as described (Burns and Buchmeier, 1991). The arrow is pointing to a 75 kDa protein which appears in immunoblots with either anti-Z serum or anti-NP serum.

*Relative quantities of viral structural proteins determined by metabolic labeling with [$^{35}$S]cysteine*

[$^{35}$S]Cysteine-labeled viral proteins were separated by electrophoresis on 15% SDS-PAGE (as depicted in Fig. 3) excised from the gel, and quantitated (Table 1). The most abundant protein, NP, was fixed at 1500 copies per virion, as estimated for Pichinde, another arenavirus (Vezza et al., 1977).

TABLE 1

Incorporation of radiolabeled [$^{35}$S]cysteine into LCMV structural proteins

| LCMV protein | cpm/protein [a] | Residues/protein | Proteins/virion [b] |
|---|---|---|---|
| Labeling Expt. 1 | | | |
| L protein | 1720 | 1 cysteines | 22 |
| NP | 17888 | | 1500 |
| GP-1 | 5050 | 3–2 = 6 [c] | 657 |
| GP-2 | 7818 | 9 | 678 |
| Z | 3726 | 7 | 416 |
| Labeling Expt. 2 | | | |
| L protein | 8235 | 61 cysteines | 30 |
| NP | 63290 | 7 | 1500 |
| GP-1 | 17625 | 8–2 = 6 [c] | 650 |
| GP-2 | 27200 | 9 | 663 |
| Z | 15300 | 7 | 486 |

[a] Virion proteins were separated by SDS–PAGE, stained with Coomassie blue, excised and counted in scintillation cocktail (Poly-Fluor, Packard).

[b] Proteins/virion was calculated by assuming that each virion has 1500 copies of NP protein (Bruns and Lehmann Grube, 1983; Vezza et al., 1977). This number was used to calculate a specific activity for the radiolabeled precursors in the virion. A sample calculation for specific activity of the cysteine labeling is as follows: (63290 cpm per NP gel band)÷(7 cysteines per NP protein)×(1500 NP proteins per virion) = 4.5 cpm/cysteine. A sample calculation for the number of Z proteins per virion is: (15300 cpm/protein in the gel slice)÷(7 cysteines/protein)×(4.5 cpm/cysteine) = 486 copies of Z protein per virion. These calculations are within ±12% accuracy as determined by the quantitation of 3 different gel runs from each labeling experiment.

[c] After processing of 59 N-terminal residues from GP-1 only 6 of the 8 cysteines remain (Burns and Buchmeier, in preparation).

*The Z protein separates with the detergent-rich phase after Triton X-114 extraction*

[$^{35}$S]Cysteine-labeled virus was subjected to 3 cycles of extraction with Triton X-114 such that viral components partitioned into either the detergent-rich pellet or the aqueous supernatant. Fig. 3 is an autoradiogram of electrophoretically separated whole virus (lane V), detergent-rich pellet (lane P), and aqueous supernatant (lane S). The Z protein remains with NP and GP-2 in the detergent-rich pellet, whereas GP-1 and GP-2 are in the supernatant. This result has also been obtained at pH 6, pH 7, pH 8 and pH 9 and in the presence or absence of zinc, with little variation (data not shown).

*The Z protein crosslinks to NP in LCM virions*

Intact virions were treated with the membrane-permeable reagent DMS which crosslinks primary amines within 11 Å proximity to one another. Fig. 4 is an autoradiogram of two Western blots from one 5–15% SDS-PAGE of LCMV proteins. In the first and second lanes (DMS + ), virus was DMS crosslinked prior to denaturation in gel sample buffer and electrophoresis. In the third lane (DMS − ), virus was not crosslinked prior to denaturation and electrophoresis. The

lanes were excised, transferred to membranes, and the first lane was incubated with anti-Z serum, whereas the last two lanes were incubated with anti-NP serum. A prominent crosslinked product is indicated with an arrow, and it corresponds in molecular weight to approximately 75 kDa. The 75-kDa crosslinked product appears after incubation with either anti-Z serum or anti-NP serum (Fig. 4).

## Discussion

The polymerase (L protein), the nucleocapsid protein (NP), and two envelope glycoproteins (GP-1 and GP-2) are known to be components of the arenaviridae (Bruns and Lehmann-Grube, 1983; Vezza et al., 1977; Buchmeier and Parekh, 1987). The completed sequence of LCMV revealed the presence of an additional gene and binding studies with $^{65}ZnCl_2$ identified an 11 kDa zinc-binding virion component (Salvato and Shimomaye, 1989). Immunological and biochemical evidence that the p11 Z protein is a structural component of the LCM virion closely associated with the nucleocapsid core is presented here. The p11 Z gene product may have been overlooked previously because of its small size, causing it to comigrate electrophoretically with degradation products of NP, and because of its lack of methionines for metabolic labeling.

### *Immunological evidence that p11 Z is a structural component of LCMV*

The Z gene product was expressed as a fusion protein in bacteria (Fig. 1), and used to raise specific polyclonal antisera. These antisera immunoprecipitated the fusion protein from bacterial extracts and immunoprecipitated an 11-kDa virion protein from purified virus that had been metabolically labeled with [$^{35}$S]cysteine (Fig. 2). The polyclonal sera have also been used to visualize the Z gene product in the cytoplasm of infected cells (Salvato, unpublished) and for Western-blot analysis of viral proteins, such as is depicted in Fig. 4.

### *Biochemical characterization of p11 Z protein*

The abundance of p11 Z protein in relation to other virion proteins was determined by analysis of metabolically labeled virus. [$^{35}$S]Cysteine-labeled LCMV proteins were electrophoretically separated (Fig. 3, lane V) and quantitated (Table 1). Accordingly, the average virion should contain L : NP : GP-1 : GP-2 : Z in a ratio of approximately 30 : 1500 : 650 : 650 : 450 copies. This indicates that the p11 Z gene product is nearly as abundant in the virion as the envelope glycoproteins. The equimolar proportions of GP-1 and GP-2 agree with the findings of Vezza et al. (1977) and Bruns and Lehmann-Grube (1983).

Detergent treatment of LCM virions with Triton X-114 indicates that p11 Z partitions entirely into the hydrophobic, detergent-rich phase (Fig. 3). This result is corroborated by Fig. 1 in the paper by Burns and Buchmeier (1991), although the band for p11 Z is barely detectable due to the use of $^{35}$S-hydrolysate (mostly

methionine) for metabolic labeling. In contrast with Burns and Buchmeier, who show GP-2 only in the hydrophobic detergent-rich phase, we repeatedly find that GP-2 is distributed between both hydrophobic and aqueous phases. This may indicate differences in the virus isolates used (LCM Arm 53b lacks a GP-1 glycosylation found in the LCM Arm 4 isolate used by Burns and Buchmeier (Wright et al., 1989)), or a difference in the Triton X-114. Although hydrophobic partitioning is one criterion for integral membrane proteins, we cannot conclude that the Z protein is an integral membrane protein from this data because the p11 sequence contains only one hydrophobic region of 11 residues adjacent to its carboxyl terminus, and 15 hydrophobic residues are usually needed to span a lipid bilayer. On the other hand, several proteins with charged transmembrane regions have been described (Popot and Engelman, 1990) in which the charged residues are neutralized by interaction with other regions of the protein or with other proteins. Thus, additional information is needed before the relationship between the p11 Z protein and the virion envelope is determined.

The interaction of the LCMV Z protein with other virion components was investigated by mild crosslinking of virus particles with the bifunctional agent DMS. A 75-kDa product appeared upon crosslinking that is consistent with a combined NP (63 kDa) and Z (11 kDa) linkage. The fact that it appears in Western blots (Fig. 4) after incubation with both anti-Z serum and anti-NP serum further indicates that the 75 kDa product contains both NP and Z antigens. Crosslinking was not evident between Z and the envelope glycoproteins (J. Burns, unpublished); so the Z gene product is most closely associated with the nucleocapsid protein of the virion core. Transcription complexes of LCMV-infected BHK cells also contain the p11 Z gene product (M. Salvato, unpublished), but this association has neither been quantitated nor linked to a necessary function of p11 Z.

*Implications for the function of the p11 Z gene product*

Five functional categories have been identified for the proteins of negative-strand RNA viruses (NP, NS/P, M, GP, and L; see Introduction). NP, GP and L proteins have been identified for LCMV; so, by default, the p11 Z protein is likely to be an NS/P protein, an M protein, or both. NS/P proteins are transcription factors, which in the case of the vesicular stomatis virus (VSV) NS protein involves the ability to bind both the nucleocapsid protein and the RNA polymerase (Emerson and Schubert, 1987). The NS protein of VSV requires phosphorylation for function in transcription (Banerjee et al., 1987). Although the p11 Z protein binds a component of the transcription machinery (NP), no role in transcription has yet been demonstrated for p11. LCMV purified from $^{32}$P-labeled cells contains phosphorylated p11 Z protein (Salvato, unpublished), but the relevance of this to function is unknown.

M proteins in other negative strand RNA viruses mediate assembly, are hydrophobic in nature, and inhibit viral transcription (Ye et al., 1985; Weiner, et al., 1985; Ogden et al., 1986; Hull et al., 1988; Faaberg and Peeples, 1988; Li et al., 1989; Ye et al., 1989). The p11 Z protein is hydrophobic in nature, but the

relevance of this feature has not yet been corroborated by functional assays. Before the discovery of p11 Z it was believed that LCMV had no matrix protein (Bishop, 1990). When the LCMV Z gene sequence was first discovered, we speculated on the possible connection between its zinc-binding domain and a transcription factor function (Salvato and Shimomaye, 1989), and failed to acknowledge examples of proteins with zinc-binding motifs involved in virus assembly: the RNA-packaging nucleocapsid protein of retroviruses (Gorelick et al., 1988; Green and Berg, 1989), the influenza M1 protein (Wakefield and Brownlee, 1989), and the tobacco streak virus nucleocapsid protein (Sehnke et al., 1989). Recent evidence for crosslinking directly between the GP-2 envelope glycoprotein and NP (Burns and Buchmeier, 1991) supports the view that LCMV may not need an M protein to mediate the association of cores with envelopes.

Both the p11 Z gene product of LCMV, and p11 of the related arenavirus Tacaribe, have a conserved zinc-finger motif of two cysteines and two histidines ($C_2$–$H_2$) similar to transcription factor IIIA (Miller et al., 1985). In addition, an overlapping metal-binding motif, $C_2$–$C_2$, is also conserved in both the LCMV and Tacaribe p11 gene products. Spectroscopic and NMR studies of synthetic peptides indicate that the $C_2$–$H_2$ structure coordinates a zinc atom between an antiparallel $\beta$-sheet and a short $\alpha$-helix (Lee et al., 1989), whereas the $C_2$–$C_2$ structure has no $\beta$-sheet and an $\alpha$-helix that extends beyond the C-terminus of the zinc coordinating residues (Schwabe et al., 1990). A protein with overlapping zinc-coordinating motifs could alternate conformation depending on which residues coordinate the zinc atom. The possibility of two alternate structures of the p11 protein provides a basis for alternate functions of this protein. The function(s) of the Z protein will eventually be defined by its activity in transcription assays and by the nature of its involvement in the transcription of a synthetic recombinant LCMV template.

## Acknowledgements

This is Publication 6025-NP from the Department of Neuropharmacology, Scripps Clinic and Research Foundation, La Jolla, CA. This work was supported in part by USPHS Grants AI-09484 (M.S.S. and M.B.A. Oldstone) and AI-25522 (M.S.S.). J.B. was supported by NIH Training Grant GM 07437 and by research funding to M.J. Buchmeier (NIH Grant AI-16102 and US Army ~~Grant GM-07437~~). Contract C 6234 The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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*Virus Research*, 22 (1992) 199–206

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VIRUS 00737

# Rapid treatment of whole cells and RNA viruses for analysis of RNA by slot blot hybridization *

Jennifer L. McKimm-Breschkin

*Biomolecular Research Institute, Parkville (Australia)*

(Received 20 September 1991; revision received 21 November 1991; accepted 22 November 1991)

**Summary**

To avoid extensive manipulation for the purification of RNA from cells, several methods were evaluated for the direct release of RNA from influenza virus infected cells and supernatants using slot blot hybridization and non-radioactive probes. Treatment with an equal volume of 10 M aqueous guanidine hydrochloride produced the best hybridization signal. Less, but significant amounts of RNA were also released using the following treatments: dilute alkali (final concentration of 0.16 M NaOH) or 100°C/5 min or RNA sample buffer containing formamide/formaldehyde, then heating at 65°C/10 min. Despite the presence of large amounts of cell debris, RNA from guanidine hydrochloride treated whole cell extracts bound quantitatively to the positively charged nylon membranes. The sensitivity of RNA detection when whole cell extracts treated with guanidine hydrochloride were probed with a digoxigenin labelled cDNA probe was similar to the detection of RNA in highly purified, protein free samples. Three positively charged membranes were tested (from Amersham, ICN and Boehringer Mannheim) using two alkaline phosphatase substrates, NBT-X phos, and a chemiluminescent substrate, 3-(2'-spiroadamantane)-4-methoxy-4-(3'-phosphoyloxy)-phenyl-1-1,2-dioextane (AMPPD) and a peroxidase substrate, tetramethylbenzidine (TMB). The Boehringer Mannheim membrane had the highest sensitivity for the alkaline phosphatase substrates, but the peroxidase reaction with the TMB substrate was the most consistently sensitive, irrespective of which membrane was used. The ability to quantitatively detect RNA from whole cells without any purification will

*Correspondence to:* J.L. McKimm-Breschkin, Biomolecular Research Institute, 343 Royal Parade, Parkville 3052, Australia.

* Presented in part at the Eighth International Conference on Negative Strand Viruses, Charleston, SC, U.S.A., 15–20 September 1991.

In press, 1992
The Viruses,
M. Salvato, ed.,
Plenum Pub., NY

# GLYCOPROTEINS OF THE ARENAVIRUSES

John W. Burns[1] and M.J. Buchmeier*

Division of Virology
Department of Neuropharmacology
The Scripps Research Institute
10666 N. Torrey Pines Road, La Jolla, CA 92037

Phone (619) 554-7056
FAX (619) 554-6470

present address: Palo Alto V.A. Medical Center,
3801 Miranda Avenue (154C), Palo Alto, CA 94304

*to whom corresence should be addressed.

# I. INTRODUCTION

The members of the Arenaviridae are characterized by spherical to pleomorphic particles, 50-300 nm in diameter, with club-shaped projections extending outward 5-10 nm from the virion surface. The viral genome contains two segments of single stranded RNA transcribed in an ambisense manner. Each of the genome segments, designated L and S, encode two primary translation products which in most cases give rise to a total of five structural proteins. The prototype arenavirus, lymphocytic choriomeningitis virus (LCMV) contains a 200 kDa polymerase, L, a nucleocapsid protein, NP (63 kDa), two structural glycoproteins, GP-1 (44 kDa) and GP-2 (35 kDa) which are derived by cleavage of the glycoprotein precursor, GP-C (70-75 kDa) and an 11-14 kDa nonglycosylated protein, Z, of unknown function. Equivalent proteins have been reported for most of the other arenaviruses.

The arenaviruses are divided for taxonomic purposes into two categories, the Old World complex (e.g., LCM, Lassa, Mozambique) and the New World, Tacaribe complex (e.g., Tacaribe, Junin, Machupo) based on the geographic location in which they were isolated. Serologic cross-reactivity is greatest among members of a complex, and to a lesser extent between complexes(Howard and Simpson, 1930; Buchmeier et al, 1982; Weber and Buchmeier, 1988).

Proteolytic digestion of purified PIC, TAC or LCM virions resulted in "bald" or spikeless particles which had lost their structural glycoproteins, thus identifying the glycoproteins as the structural components of the spikes (Vezza et al., 1977; Gard et al., 1977; Buchmeier et al., 1978). Further characterization of the arenavirus glycoproteins has established them as major

targets of host immune responses. Serologic analyses have demonstrated that neutralizing antibodies are elicited only against the glycoproteins (Buchmeier et al., 1982; Bruns et al., 1983b; Howard et al., 1985; Parekh and Buchmeier, 1986; Ruo et al., 1991). Evaluation of the cellular immune response has identified epitopes for murine cytotoxic T-lymphocytes (CTL) on both of the structural glycoproteins (Whitton et al., 1988a, 1988b). Further, in conjunction with the various components of the immune response, the expression of the arenavirus glycoproteins (or the lack thereof) may play a major role in viral pathogenesis and persistence. These and other aspects of the glycoproteins of the arenaviruses will be the subject of this review.

## II. GLYCOPROTEIN EXPRESSION AND PROCESSING

The arenavirus genome encodes only a single translation product which becomes glycosylated, the precursor glycoprotein, GP-C. GP-C is encoded in the message sense on the S RNA segment, while the nucleocapsid protein, NP, is encoded in the anti message sense on S (Harnish et al., 1983; Riviere et al., 1985). Despite the message polarity of GP-C, because of the replication strategy of the ambisense genome NP mRNA is transcribed and translated prior to GPC gene expression. This regulatory process may play a major role in the establishment of arenavirus persistent infections (discussed below).

The LCMV GP-C open reading frame encodes 498 amino acids, including a 58-amino acid leader sequence. The GP-C precursor

has an apparent molecular weight of 70-80,000 kDa in its glycosylated form (see Table 1). Analyses of the LAS, LCM, PIC and TAC GP-C genes indicate they contain 8 to 16 potential N-linked glycosylation sites (Auperin et al., 1984, 1986; Franze-Fernandez et al., 1987, Southern et al., 1987; Wright et al., 1989). Wright et al. (1989) demonstrated that 8 of 9 potential N-linked glycosylation sites are utilized for LCMV. Harnish et al. (1981) estimated that up to 47% of the apparent molecular weight of PIC GP-C may be due to carbohydrate. Using radiolabelled sugars, it was demonstrated that both LCM and TAC GP-C initially contained only high mannose residues which were subsequently converted to complex carbohydrates (Buchmeier and Oldstone, 1979; Boersma et al., 1982). Further, it was determined that GP-1 contained glucosamine, fucose and galactose while GP-2 contained predominantly glucosamine and fucose. These findings were substantiated using a series of drugs that inhibit sequential steps in the processing of N-linked sugars, conclusively establishing that GP-C utilizes the cellular secretory pathway for its processing (Wright et al., 1990). In these experiments inhibition of glycosylation by tunicamycin resulted in blockage of glycoprotein processing and transport and failure to produce virions. Other inhibitors such as castantospermine, which allowed en bloc addition of the mannose-rich precursor chain, permitted processing transport and virion maturation. The Tacaribe glycoproteins reportedly also contain glucosamine, galactose, mannose, as well as terminal sialic acid residues (Boersma et al., 1982). Clearly the carbohydrate content reflects that of the host cell. We are not aware of

TABLE 1. Arenavirus Glycoproteins

| Virus | Non-structural | Structural | Reference |
|---|---|---|---|
| LCM | 74-75 kDa | 44, 35 kDa | Buchmeier & Oldstone, 1979 |
| LCM | ND | 130,85,60, 44,35 kDa | Bruns et al., 1983b |
| PIC | ND | 64,38 kDa | Vezza et al., 1977 |
| PIC | 79 kDa | 52,36 kDa | Harnish et al., 1981 |
| JUN | ND | 91,72,52, 38 kDa | M. Segovia & DeMitri, 1977 |
| JUN | ND | 44, 34-39 kDa | Grau et al., 1981 |
| LAS | 84 or 115 kDa | 52,39 kDa | Kiley et al., 1981 |
| LAS | 72 kDA | 45,38 kDaa | Clegg & Lloyd, 1983 |
| MAC | ND | 50,41 kDa | Gangemi et al., 1978 |
| MOB | ND | 48,37 kDa | Gonzalez et al., 1984 |
| MOP | ND | 48,35 kDa | Gonzalez et al., 1984 |
| MOZ | ND | 52, 39 kDa | Kiley et al., 1981 |
| TAC | 70 kDa | 42 kDa | Gard et al., 1977<br>Saleh et al., 1979 |
| TAM | ND | 44 kDa | Gard et al., 1977 |

ND - not described; kDa - kilodalton

reports describing the sulfation or phosphorylation of arenavirus glycoproteins. A single report describes the palmitylation of the LCM structural glycoproteins gp60 and gp130, reportedly a dimer of gp60 (Bruns and Lehmann-Grube, 1983), however subsequently the entire LCM genome has been sequenced and there does not appear to be an open reading frame corresponding to gp60.

The kinetics of viral glycoprotein synthesis has been examined, at various multiplicities of infection (MOI), with LCM, PIC and TAC. In cells acutely infected with LCM, NP expression was first observed at approximately 6 hours post-infection (p.i.) while GP-C expression was readily detectable at 24-48 hours p.i. at an M.O.I. of 1.0 (Buchmeier et al., 1978). Similar findings were reported for PIC with the exception that GP-C was detectable at 12 hours p.i. at high MOI infection (MOI 50), but not at lower MOIs (MOI 0.1; Harnish et al., 1981). In TAC infected cells NP was observed at 24-34 hours, increasing until 48 hours p.i. TAC GP-C was detected by 48 hours and increased until 60 hours p.i. (Saleh et al., 1979).

Several studies have examined the cleavage of the arenavirus glycoprotein precursor. Pulse-chase studies with LCM and PIC have demonstrated that GP-C is cleaved approximately 75-90 minutes after synthesis, resulting in the appearance of the two structural glycoproteins, GP-1/G1 and GP-2/G2 (Harnish et al., 1981; Wright et al., 1990). It appears that cleavage of LCM GP-C is a two-step process (Figure 1). Recent experiments in this laboratory have shown that GP-C of LCMV, TAC and PIC all contain a long (58 amino acid) signal sequence, which is likely conserved

Figure 1.

among all of the arenaviruses. This signal peptide is cleaved at a conserved signal peptidase site and removed prior to glycoprotein transport from the endoplasmic reticulum. We find no evidence that the long signal is incorporated into virions (Burns et al, manuscript in preparation). This finding is especially interesting since a CTL epitope has been mapped to the cleaved signal sequence (amino acids 34-43; Klavinskis et al., 1990) and thus constitutes the first reported CTL epitope contained within a signal sequence.

Cleavage of GP-C to form GP-1 and GP-2 occurs later in the secretory pathway, between the medial and the trans-Golgi network (Wright et al., 1990). Using synthetic peptides the second GP-C cleavage site has been localized to a stretch of nine amino acids which span the dibasic residues -Arg-Arg- at amino acids 262-263 (Buchmeier et al., 1987). Immune precipitation using antisera raised to synthetic peptides corresponding to LCM GP-C amino acids 59-79 and 378-391 allowed the mapping of GP-1 and GP-2 to the amino- and carboxy-terminal regions of GP-C (Buchmeier et al., 1987) These findings have been substantiated by amino-terminal sequence analysis of GP-1 and GP-2 which established that the amino terminus of GP-1 was met-59 and of GP-2 was gly-266 (Burns et al., unpublished data).

It is probable that the GP-C cleavage event is mediated by a golgi-associated furin-like protease acting at or following the dibasic residues, which are conserved among most arenaviruses (Figure 2). Amino-terminal sequencing of the structural glycoproteins of PIC and TAC indicates that equivalent cleavage events occur in both viruses (Burns et al., manuscript in

Figure 2.

preparation). Similar cleavage events are therefore likely in other members of the arenavirus family.

Cleavage of GP-C to yield GP-1 and GP-2 has been shown to require prior glycosylation (Wright et al., 1989). In the presence of tunicamycin, an inhibitor of N-linked glycosylation, both LCM and PIC produced an unglycosylated form of GP-C but cleavage was not observed (Harnish et al., 1981; Wright et al., 1990). Further, GP-C cloned into a baculovirus vector and expressed in Spodoptera cells was not cleaved, presumably due to the lack of proper glycosylation and processing in the insect cells (Matsuura et al., 1986).

## III. STRUCTURE AND ORGANIZATION OF THE GLYCOPROTEIN SPIKE

The arenavirus particle, visualized by electron microscopy, contains surface projections (spikes) that are reportedly 5-10 nm in length with a "club-shaped appearance (Murphy et al., 1970; Murphy and Whitfield, 1975; Young et al., 1981) (Figure 3). These spikes are closely spaced, but appear to be mobile in the viral membrane since osmotic swelling of the virion causes the spikes to become more widely spaced. When viewed "end on" at higher magnification, the spikes appear to have a hollow central axis, suggesting a macromolecular organization of multiple polypeptide chains (Murphy and Whitfield, 1975; Young et al., 1981). The arenavirus spike has been studied in detail and models have been proposed to explain its structure (Bruns and Lehmann-Grube, 1983; Young, 1987; Burns and Buchmeier, 1991; Burns et al., manuscript in preparation).

The composition of the arenavirus spike has been established

Figure 3.

by treating purified PIC, TAC and LCM virions with proteases. The resulting spike-less particles had lost their glycoproteins while the other structural proteins were unaffected by proteolysis (Vezza et al., 1977; Gard et al., 1977; Buchmeier et al., 1978). Quantitation of the molar ratios of the structural proteins of PIC demonstrated that equal numbers of G1 and G2 molecules were present in the virion (Vezza et al., 1977). Equal amounts of GP-1 and GP-2 were also found to be present in LCM particles (Bruns et al., 1983a).

Surface iodination of LCM-infected cells or virions resulted in incorporation of the label into GP-1, while GP-2 was labelled poorly or not at all (Buchmeier and Oldstone, 1979; Bruns et al., 1983b). These results suggested that GP-1 was more externally exposed than was GP-2, but must be qualified since only exposed tyrosine residues would be expected to label under the conditions used. Direct support for this conclusion was provided by experiment in which LCM virions were extracted using Triton X-114, which has been used to distinguish peripheral from integral membrane proteins (Bordier et al., 1981). The results of these experiments demonstrated that GP-1 and GP-2 were peripheral and integral membrane glycoproteins respectively (Burns and Buchmeier, 1991). The membrane spanning domain of GP-2 has been established by amino-terminal sequencing of a polypeptide fragment protected by the membrane from proteolysis. The GP-2 membrane spanning hydrophobic domain begins with amino acid 439 and extends for eighteen residues, however a longer fragment beginning with $Gly_{430}$ is protected from proteolysis in the virion envelope (Burns et al., 1991, manuscript in preparation). The C-

terminal end of GP 2 is highly charged; five of the 12 C terminal residues are Lys or Arg. It is likely that the cross linking interactions which we have observed between NP and GP-2 involve these basic residues (Burns and Buchmeier, 1991). These findings have firmly established the integral membrane nature of GP-2 and its orientation in the viral envelope.

The macromolecular organization of the structural glycoprotein complexes of LCM has been examined using detergents and cross-linking reagents. Solubilization of LCM virions at 4C with SDS, CHAPS or Triton X-100 in the absence of reducing agents caused the released of oligomeric structures consisting of GP-1 homo-polymers in complexes as large as homotetramers (Wright et al., 1989; Burns and Buchmeier, 1991). Further characterization using membrane impermeable cross-linking reagents (DTSSP and Sulfo-DST) demonstrated that both GP-1 and GP-2 were assembled in separate homotetrameric complexes. The results varied when a membrane permeable crosslinker was used. Hetero-oligomeric complexes of GP-2 crosslinked with NP were observed following crosslinking with the membrane permeable crosslinking reagent DMS (Burns and Buchmeier, 1991). Taken together these results demonstrated that GP-1 and GP-2 form separate homotetrameric complexes which are located outside the envelope. Within the viral envelope, GP-2 was crosslinked with NP, indicating that the cytoplasmic tail of GP-2 may interact with NP, a part of the ribonucleoprotein complex. The possibility that GP-2 and NP were associated within the virion had been proposed and and it was suggested this association may play a critical role in budding of

the virion (Dubois-Dalcq et al., 1984; Compans and Bishop, 1985).

The native conformation of both GP-1 and GP-2 has been investigated in the course of studies to establish the structure of the virion spike. Disulfide bonding is essential in maintaining the native conformation of GP-1 (Wright et al., 1989; Burns and Buchmeier, 1991). Neutralizing anti-LCM monoclonal antibodies (MAbs) failed to react with reduced virions (Wright et al., 1989). Likewise, polyclonal guinea pig and human anti-Lassa antisera failed to react with the reduced form of G1 in immunoblots (Clegg and Lloyd, 1983).

Computer prediction of the likely secondary of LAS GP-2 identified a domain likely to assume a coiled-coil conformation (Chambers et al., 1990). Comparison of this region of LAS GP-2 with other arenaviruses for which sequence is available indicates they all share a similar heptad repeat region approximately 50 amino acids long (Figure 4), suggesting that a coiled-coil "stalk" domain is common to the GP-2 molecules of all arenaviruses. Such a structure would be consistent with the club- or T-shaped spike seen in electron micrographs. Cumulatively, these data suggest that the 233-amino acid GP-2: 1) forms a homotetrameric complex, 2) contains a coiled-coil domain which assumes an elongated conformation, 3) has a 164-amino acid ectodomain and a 69-amino acid endodomain, 4) is anchored in the membrane by a hydrophobic stretch of 20-25 hydrophobic amino acids near the amino terminus of the carboxy-terminal endodomain, and 5) has a charged cytoplasmic tail through which it can interact with NP, a component of the ribonucleoprotein complex.

Figure 4.

Recently we began to address the nature of the interaction between GP-1 and GP-2. We have observed that GP-1 (G1) of LCM, Pichinde and Tacaribe viruses are eluted from the virion by incubation in 1M NaCl or LiCl (Burns et al., manuscript in preparation). Preliminary cryo-electron microscopic analyses of these self-stripped virions suggest that the club-shaped component of the spike has been lost. This observation suggests that GP-1 forms the club-chaped head of the glycoprotein spike and is associated via ionic interactions with the tetrameric GP-2 stalk.

Of further interest is the finding that a portion of the TAC "G" protein band is recovered in the same location in sucrose gradients that PIC G1 and LCM GP-1 are found. N-terminal sequencing of TAC "G" protein resulted in a mixture of two amino acids in each of five cycles of Edman degradation which exactly correlated with the predicted amino termini of TAC GP-1 and GP-2. Thus it appears that the two structural glycoproteins of TAC co-migrate in SDS polyacrylamide gel electrophoresis.

## IV. HOST IMMUNE RESPONSES

Pathogenesis associated with acute LCM virus infection is characterized by a vigorous T-cell mediated immune response against the virus. Indeed, it is this T-cell response directed against viral antigens which characterizes lethal acute LCM disease; if the response is suppressed by cyclophosphamide or gamma irradiation, little acute pathology is evident (Buchmeier et al., 1980). The evidence for immunopathologic disease with other members of the arenavirus family is not as well documented,

but the host immune response is likely to play an important role in these diseases as well. As a result of these observations, the host immune response to arenavirus infection has been investigated extensively. Numerous studies have demonstrated that the viral nucleocapsid protein and glycoproteins serve as major targets of immune recognition at the cellular and humoral levels (see Klavinskis et al., this volume).

The cytotoxic T lymphocyte (CTL) response to LCM infection has been well characterized. Using reassortant viruses, CTL activity was mapped to the S RNA segment of LCMV (Oldstone et al., 1985; Riviere et al., 1986). Further characterization of LCM-specific CTL lead to the identification of CTL epitopes on both the nucleoprotein and glycoprotein compenents of the virion (Whitton et al., 1988a, 1988b, 1989). The recognition of individual epitopes was shown to be MHC haplotype specific. A detailed analysis of CTL recognition in C57BL/6 mice (H-2b) demonstrated the existence of two glycoprotein epitopes, one in GP-1 (now believed to be located in the signal sequence, see above) and one in GP-2 (Whitton et al., 1988a). These two glycoprotein epitopes were engineered into recombinant vaccinia viruses and expressed as "minigenes". Tissue culture cells infected with either recombinant virus was recognized by cognate CTLs, but only one construction, representing GP-C amino acids 34-43, was able to induce protection from lethal virus challenge (Klavinskis et al., 1990).

Specificity of the humoral immune response to arenavirus infections has been examined primarily by analysis of monoclonal

antibody (MAb) reactivity. Hybridoma cell lines secreting monoclonal antibodies have been generated to LCM, JUN, PIC, LAS and MOP (Buchmeier et al., 1981, 1982; Bruns et al., 1983a; Howard et al., 1985; Parekh and Buchmeier, 1986; Sanchez et al., 1989; Ruo et al., 1991). The antigenic topography of the LCM glycoproteins was mapped using MAbs. It was determined there were two epitopes capable of eliciting neutralizing antibodies, both located on GP-1 (Parekh and Buchmeier, 1986). Competitive binding assays, using these neutralizing MAbs against hyperimmune sera demonstrated that guinea pigs responded predominantly to one of these two neutralization epitopes while a battery of rat anti-LCMV neutralizing MAbs recognized only the second epitope (Parekh and Buchmeier, 1986). Two neutralization epitopes were also identified on the TAC glycoprotein by competitive binding and neutralization kinetics assays. Analysis of the kinetics of neutralization of TAC by MAb provided evidence of multi-hit neutralization kinetics (Howard et al., 1985; Howard, 1987).

To assess the role of antiviral antibody in acute LCM infection, passive protection models were investigated. Anti-glycoprotein MAbs administered passively, either before or after intra-cerebral virus challenge, protected mice from subsequent CNS disease. In contrast, passive administration of anti-NP or anti-GP-2 MAbs had no effect on the outcome of infection (Wright and Buchmeier, 1991). Likewise, mouse pups which had received maternally-derived anti-GP1 antibodies were protected from lethal challenge (Baldridge and Buchmeier, 1991, submitted). In all cases, mice which received protective levels of MAb prior to challenge had reduced CTL responses and less severe acute disease

than their unprotected cohorts. It was proposed that the presence of anti-glycoprotein antibodies limited the degree of T-cell-mediated immunopathology (Wright and Buchmeier, 1991).

The nature of a protective immune response in other arenavirus infections is less clear. Little is known concerning the CTL response to arenavirus infections other than LCM. In fatal cases of Lassa fever in humans, virus titer correlates well with prognosis; patients with high viremia have lower probability of survival than those with low levels (Johnson et al., 1987; Fisher-Hoch and McCormick, 1987). Neutralizing antiserum to PIC or LAS was shown to either block or enhance virus infectivity in a concentration-dependent manner with the U937 monocyte cell line (Lewis et al., 1988). Neutralizing antibodies can be detected in the acute phase of JUN and MAC infections (Webb et al., 1969; Howard, 1987). The use of convalescent phase sera has led to mixed results with human cases of Lassa fever, but has been used successfully with Argentine hemorrhagic fever (reviewed in Fischer-Hoch and McCormick, 1987) These antibodies may play a role in virus clearance, the establishment of persistent infection, or the selection of viral variants that are resistant to neutralization (Webb et al., 1969; Howard, 1987; Alche and Coto, 1988).

Clearly, there is much to be learned concerning the host immune response during various arenavirus infections. Not all lessons learned with the LCM infected mouse model can be extrapolated to other arenavirus infections and particularly those of humans. Future studies should provide a clearer

picture. The development of successful vaccines and prophylactic therapies will depend heavily on these data.

## V. ROLE OF THE GLYCOPROTEINS IN IMMUNOPATHOLOGY AND PERSISTENT INFECTIONS

Arenaviruses typically induce either acute or life-long persistent infections. This dichotomy is seen both *in vivo* and *in vitro* (Welsh and Oldstone, 1977; Welsh and Buchmeier, 1979; Oldstone and Buchmeier, 1982; D'Aiutolo and Coto, 1986). Factors which determine the outcome of arenavirus infections include route of inoculation, immunologic status of the host, age of the host and virus strain (for review see Buchmeier et al., 1980; Buchmeier and Knobler, 1984; Southern et al., 1986). Recently it has been demonstrated that the presence or absence of pre-existing anti-glycoprotein antibody may also play a role in the establishment of infection (Wright and Buchmeier, 1991; Baldridge and Buchmeier, 1991, unpublished data).

In the immunocompetent adult mouse, the outcome of acute intracerebral LCMV infection is either viral clearance with the development of protective immunity or fatal central nervous system (CNS) disease. The results of numerous studies strongly demonstrate the importance of CTLs in the course of acute LCM infection and the development of fatal CNS disease (reviewed in Allan et al., 1987). Adult immunosuppressed mice do not respond to LCM infection, fail to develop fatal CNS disease, and become persistently infected. Adoptive transfer of splenocytes from immune mice into such persistently infected mice may result in the development of fatal CNS disease (Gilden et al., 1972). In

contrast, virus infection in neonatally infected carrier mice is usually cleared following adoptive transfer of immune T cells. When analyzed at the cellular level in H-2b mice, it was demonstrated that CTLs were responsible for clearance (Ahmed, 1988). Whitton and co-workers demonstrated that the vast majority of CTLs in H-2b mice are reactive towards the glycoproteins (1988a; see above). It is likely that these glycoprotein reactive CTLs play a significant role in acute CNS disease. This conclusion is supported by the finding that the LCM glycoproteins, but not the nucleocapsid protein, is expressed on the surface of choroid, meningeal and ependymal cells from several strains of LCM infected mice (Oldstone and Buchmeier, 1982), targeting these cells for cytolysis by specific CTLs.

The establishment of persistent arenavirus infections has been carefully examined in animal and tissue culture systems. A major feature of the persistent infection is an apparent down-regulation of the expression of the viral glycoproteins (Welsh and Oldstone, 1977; Welsh and Buchmeier, 1979; Oldstone and Buchmeier, 1982). This decreased level of glycoprotein expression may allow persistently infected cells to escape immune surveillance (Oldstone and Buchmeier, 1982), although persistently infected animals mount specific antibody responses to both the viral nucleocapsid protein and the glycoproteins (Buchmeier and Oldstone, 1978; Oldstone et al., 1983). The antibody produced is largely IgG-1 subclass and does not neutralize virus efficiently <u>in vitro</u>.

Defective interfering (DI) virus is rapidly generated during

acute infection and has been implicated in the establishment of persistence with LCM and TAC (Welsh and Pfau, 1972; Welsh et al., 1972; Popescu and Lehmann-Grube, 1977; Dutko and Pfau, 1978; Welsh and Buchmeier, 1979; D'Aiutolo and Coto, 1986). DI virus has been characterized at both the nucleic acid and structural protein levels. No direct correlation could be made between the appearance of subgenomic viral RNAs or an under-representation of genome segments in DI virions (Francis and Southern, 1988). In comparing DI with standard LCM virions, Welsh and Buchmeier found a slight difference in bouyant density but no difference in structural protein composition (1979). In contrast, Martinez Peralta and colleagues reported that DI LCM virions lacked the S RNA segment (23 S RNA) and contained a novel 65 kDa glycoprotein instead of GP-1 and GP-2 (1981). These findings must be viewed with caution as their virus contained the 63 kDa nucleocapsid protein which is also encoded on the 23 S genomic segment. Recently it has been reported by this laboratory that the 65 kDa glycoprotein is actually a product of the S genomic segment and arises from the generation of a truncated form of the GP-C mRNA which when translated yields the 65 KDa glycoprotein (Bruns et al., 1990). Further experimentation is needed to reconcile these data.

Several vaccinia virus recombinants, containing arenavirus glycoprotein gene segments, have been recently reported (Fisher-Hoch et al., 1989; Morrison et al., 1989; Klavinskis et al., 1990). These potential vaccines reportedly generate antibody and/or CTL responses which protect vaccinees from subsequent live arenavirus challenge. In contrast Oehen and co-workers (1991)

report that a recombinant vaccinia virus of similar construction may in fact aggravate a subsequent arenavirus infection when used as a vaccine. The possible selection of resistant virus variants and differential responses with MHC haplotype variation must also be kept in mind when evaluating such potential vaccines (Alche and Coto, 1988; Klavinskis et al., 1990; Pircher et al., 1990). This is especially true when only a fragment of the glycoprotein gene will be included in the recombinant vector. The interactions between the immune system and the infected host are quite complex, but further studies of the immune responses to recombinant vaccines should provide us with a better foundation from which to design vaccine strategies.

## VI. CONCLUSIONS

Emerging evidence suggests that all members of the arenavirus family contain two structural glycoproteins, derived from a single glycoprotein precursor. Sequence data from an increasing number of arenaviruses has consistently demonstrated the ambisense nature of the S genome segment, encoding the glycoprotein precursor GP-C and nucleoprotein (NP) genes (Auperin et al., 1984, 1986; Romanowski and Bishop, 1985; Franze-Fernandez et al., 1987; Southern et al., 1987; Salvato et al., 1988; Auperin and McCormick, 1989; Wilson and Clegg, 1991). Comparison of deduced amino acid sequences predicted for the varying GP-C molecules indicates that several features of the GP-C structure are conserved among the arenaviruses. These include a long (ca. 58 amino acid) cleaved signal sequence at the amino-terminus, a

second cleavage site near a paired basic amino acid site at the middle of the GP-C ORF, and a highly hydrophobic transmembrane spanning domain near the carboxy-terminus. Moreover, observations from this laboratory have shown that GP-1, the amino-terminal cleavage fragment, is a peripheral membrane protein, whilst GP-2 is an integral membrane protein, anchored in the viral envelope by the hydrophobic membrane spanning domain and a cytoplasmic tail near its carboxy-terminus. Identification of a second structural glycoprotein in JUN and TAC (Grau et al., 1981; Burns et al., manuscript in preparation), both previously reported as containing only one structural glycoprotein, further support the conclusion that arenavirus particles contain two structural glycoproteins.

The early electron microscopic analyses reported that the arenaviruses were morphologically indistinguishable particles containing similar club-shaped surface projections, 6-10 nm in length (Murphy et al., 1970; Murphy and Whitfield, 1975; Young et al., 1981). The surface projections were shown early on to be composed of the structural glycoproteins. We have recently provided evidence that the two LCM glycoproteins each form homo-oligomeric structures, presumably tetramers (Wright et al., 1989; Burns and Buchmeier, 1991). Based on further characterization, we have proposed a schematic model of the LCM spike structure which we believe to be composed of an oligomeric GP-2 stalk component which interacts via ionic interactions with an oligomeric head assembly composed of GP-1. We have extended some of our analyses to both PIC and TAC and have obtained consistent results. Therefore, it is our belief that the structure of this

glycoprotein spike structure may be common to all members of the Arenaviridae, in agreement with the previous studies using electron microscopy. Assuming these conclusions prove accurate, attempts at X-ray crystallography of the spike and its individual glycoprotein components can begin in earnest. The determination of the three-dimensional structure of the glycoprotein spike will provide invaluable insight for future studies on the role of the glycoproteins in the biology of the virus and the host immune response.

Study of the murine-LCMV infection model has proven to be extremely productive in increasing our understanding of virus-host interactions. Recent observations concerning the in vivo generation of CTL resistant variant virus (Pircher et al., 1990), novel mechanisms of viral clearance in the CNS (Oldstone et al., 1986) and the characterization of the mechanism of CTL killing (Welsh et al., 1990) are but a few examples of the far-reaching utility of this model system in furthering our knowledge. As the viral glycoproteins have been repeatedly implicated as playing key roles in regards to immune recognition, the initiation of infection, viral maturation, persistence and as components of potential vaccine strategies, the continued study of these molecules will certainly provide us with a plethora of important observations.

ACKNOWLEDGEMENTS

We wish to thank Dr. Ronald Milligan for the cryoelectron micrographs presented in Figure 3, B and C. MJB was supported by NIH grant AI 16102 and Army Contract C6234. JWB was supported by NIH training grant GM07437. The views, opinions and/or findings contained in this publication are those of the authors and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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## FIGURE CAPTIONS

Figure 1. Structure of the proteolytic sites of the GP-C precursor glycoprotein. Topographical markers indicating signal sequence (ss), amino terminal GP-1 and carboxy terminal GP-2, and hydrophobic transmembrane spanning domains (tm) are shown above and structure of the cleavage sites of signal peptidase after amino acid 58 and a golgi-associated protease after amino acid 265 are indicated by bold arrows. N-terminal sequences of GP-1 and GP-2 as confirmed by microsequencing are underlined.

Figure 2. Sequencing indicates a high degree of conservation of GP-C cleavage sites among several Old World and New World arenaviruses. Sequences are aligned at the signal peptide-GP-1 junction and the GP-1:GP-2 junction. Underlined sequences have been determined experimentally.

Figure 3. Electron microscopy of lymphocytic choriomeningitis virions. Panel A: Thin section showing virions budding from infected BHK-21 cells. Typical 110 nm virions containing numerous electron dense 20 nm particles are evident. Panels B, C: Cryoelectron microscopy of unstained, purified LCM virions at 1.5 u (B) and 3.0 u (C) defocus levels. In Panel B the lipid bilayer is emphasized (arrow) and in the right panel the surface topography is more evident. Glycoprotein spikes are indicated by arrows. Magnification of Panel B and C is 167,100x and the inset is 232,750x. Bars are 1000A.

Figure 4. Proposed working model of the LCMV glycoprotein spike.

The disulfide linked GP-1 homotetramer forms the crossmember component of the spike which is associated via ionic interactions with the N-terminal portion of the Gp-2 transmembrane protein. The spike is anchored in the membrane by an 18-amino acid hydrophobic domain and the C-terminal internal (cytoplasmic) tail may associate with the ribonucleoprotein complex within the virion.

OUTLINE

I. INTRODUCTION

II. GLYCOPROTEIN EXPRESSION AND PROCESSING

III. STRUCTURE AND ORGANIZATION OF THE GLYCOPROTEIN SPIKE

IV. HOST IMMUNE RESPONSES

V. ROLE OF THE GLYCOPROTEINS IN IMMUNOPATHOLOGY AND PERSISTEECTIONS

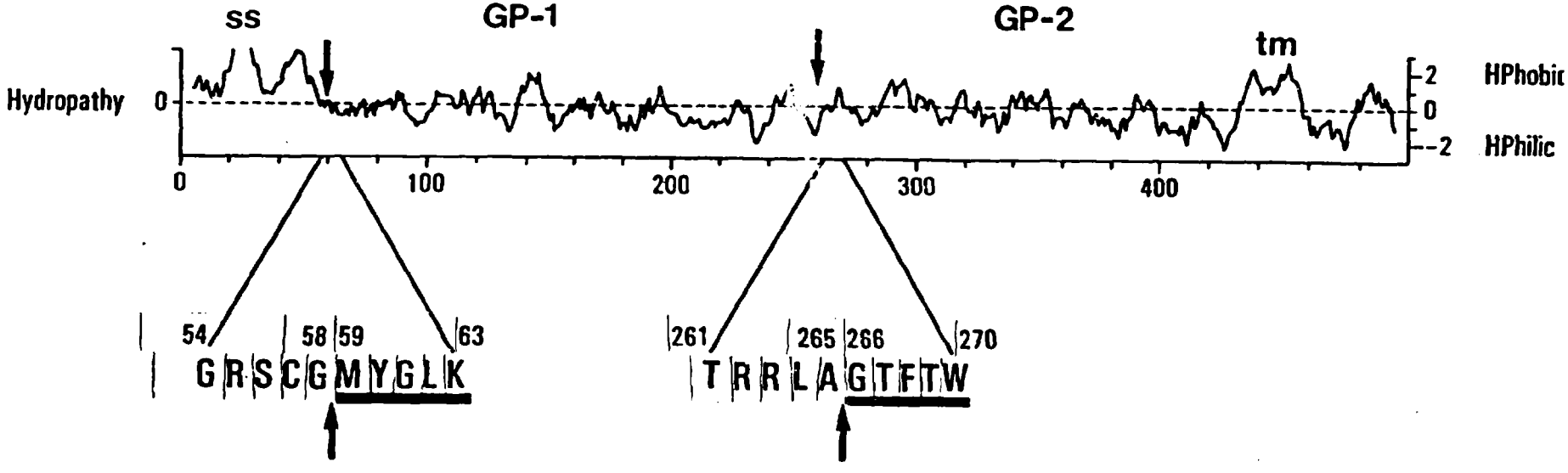
VI. CONCLUSIONS

ACKNOWLEDGEMENTS

REFERENCES

FIGURE CAPTIONS

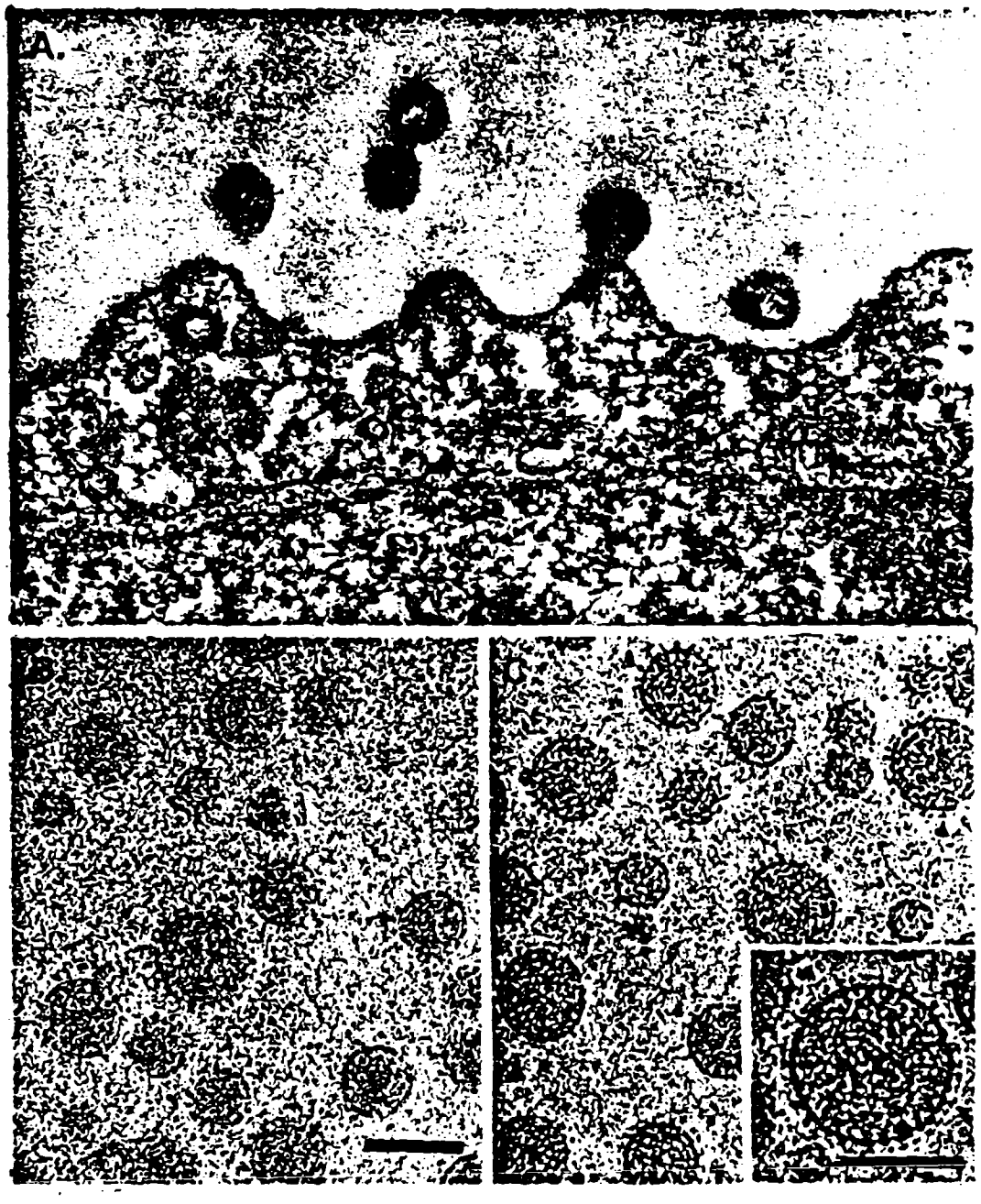
# LCMV ARMSTRONG GPC CLEAVAGE SITES



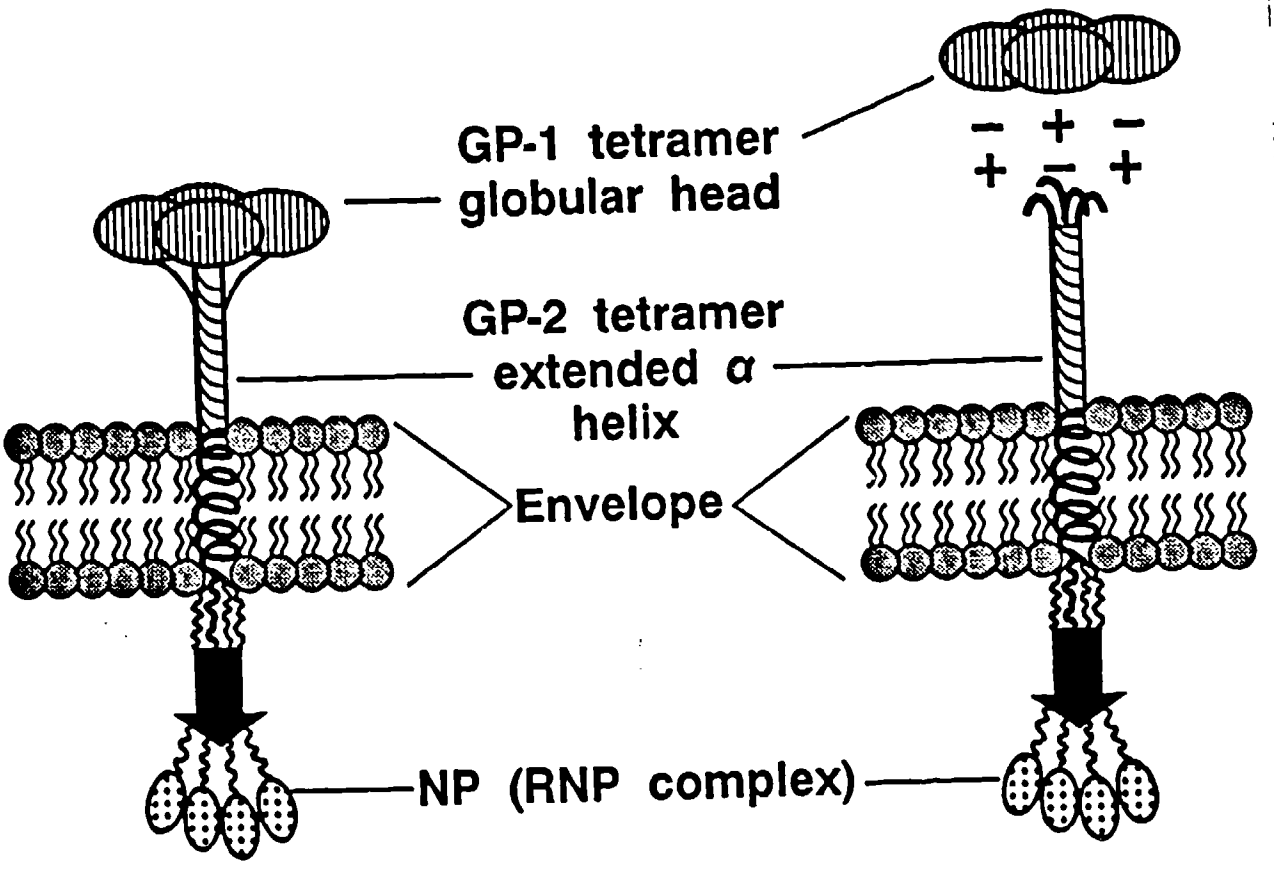
# ARENAVIRUS GPC CLEAVAGE SITES

| | | | |
|---|---|---|---|
| | | 58↓ | 265↓ |
| Arm: | P H I I D E V I N I—L L A G R S C G | M Y G L K | —F T R R L A | G T F T W | T L S D |
| | | 58↓ | 265↓ |
| WE: | P H I I D E V I N I—L L A G R S C G | M Y G L N | —L T R R L S | G T F T W | T L S D |
| | | 58↓ | 259↓ |
| Lassa: | P H V I E E V M N I—L L C G R S C | T T S L Y K | —I S R R L L | G T F T W | T L S D |
| | | 58↓ | 261↓ |
| Tacaribe: | P I F L Q E A L N I—V L A G R S C S | E E T F K | —V G R T L K | A F F S W | S L T D |
| | 16↓ | 56↓ 59↓ | 273↓ |
| Pichinde: | P E V L Q E V F N V—I L S G R S C D S | M M I D | —V S R K L L | G F F T W | D L S D |
| Consensus: | P E N L G R S C | | R L | F W | L D |

Fig. 3




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GP-1 tetramer
globular head
GP-2 tetramer
extended α
helix
Envelope
NP (RNP complex)
− + −
+ − +

JOURNAL OF VIROLOGY, Apr. 1987, p. 982–985
0022-538X/87/040982-04$02.00/0
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Vol. 61, No. 4

# Site-Specific Antibodies Define a Cleavage Site Conserved among Arenavirus GP-C Glycoproteins†

M. J. BUCHMEIER,* P. J. SOUTHERN, B. S. PAREKH, M. K. WOODDELL, AND M. B. A. OLDSTONE

*Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

Received 29 September 1986/Accepted 9 December 1986

**Arenaviruses share a common strategy for glycoprotein synthesis and processing in which a mannose-rich precursor glycoprotein, termed GP-C in lymphocytic choriomeningitis virus (LCMV), is posttranslationally processed by oligosaccharide trimming and proteolytic cleavage to yield two structural glycoproteins, GP-1 and GP-2. Mapping the orientation and proteolytic cleavage site(s) in such polyproteins has traditionally required direct protein sequencing of one or more of the cleaved products. This technique requires rigorous purification of the products for sequencing and may be complicated by amino-terminal modifications which interfere with sequence analysis. We used an alternative approach in which synthetic peptides corresponding to sequences bracketing a potential protease cleavage site were used to raise antisera which define the boundaries of the cleaved products. We found that cleavage of LCMV GP-C to yield GP-1 and GP-2 occurs within a 9-amino-acid stretch of GP-C which contains a paired basic amino acid group -Arg-Arg-, corresponding to amino acids 262 to 263 in the LCMV GP-C sequence. By comparison with the predicted amino acid sequences of a second LCMV strain, LCMV-WE, as well as with the deduced amino acid sequences of the New World arenavirus Pichinde and the Old World virus Lassa, we observed similar conservation of paired basic and flanking amino acid sequences among these viruses.**

Lymphocytic choriomeningitis virus (LCMV) contains two segments of single-stranded genomic RNA termed L and S (7). The S RNA is arranged in an ambisense coding orientation in which the 3′ half of the molecule contains the gene for viral nucleoprotein (NP; 63 kilodaltons) in a genomic complementary reading sense and the 5′ half contains the viral glycoprotein gene in a positive-stranded orientation (1; P. Southern, M. Singh, Y. Riviere, D. Jacoby, M. J. Buchmeier, and M. B. A. Oldstone, Virology, in press). The glycoprotein gene codes for a mannose-rich precursor, GP-C (75 kilodaltons) (4, 7). GP-C is posttranslationally modified by proteolytic cleavage and oligosaccharide trimming to yield the structural glycoproteins GP-1 (44 kilodaltons) and GP-2 (35 kilodaltons) (4). This pattern of glycoprotein synthesis and processing has now been shown for several other arenaviruses including Pichinde, Junin, and Lassa viruses (11, 13, 15, 20).

Attempts to determine the arrangement of GP-1 and GP-2 within GP-C and to identify the proteolytic cleavage site by direct protein sequencing have been unproductive. Using the methods of Hunkapiller et al. (18) we extracted GP-2 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and attempted microsequencing of the amino terminus. Despite the presence of sufficient quantities of protein, we were unable to sequence GP-2 by conventional techniques, presumably because the amino terminus is blocked. To resolve this problem, we developed an approach involving the use of synthetic peptides deduced from nucleotide sequences for S RNAs of two strains of LCMV, Armstrong (ARM) (Southern et al., in press) and WE (26) and also for Pichinde virus (1).

Peptides were selected from predominantly hydrophilic regions within the predicted LCMV-ARM glycoprotein-coding sequence (Southern et al., in press) and were used to raise antisera in rabbits. By their reactivity with the viral polypeptides GP-1 and GP-2, these antisera define the orientation of these glycoproteins on the precursor and the probable site of proteolytic cleavage.

## MATERIALS AND METHODS

LCMV was propagated in BHK-21 cells and purified by banding on isopycnic Renografin-76 (Squibb Diagnostics, New Brunswick, N.J.) gradients as previously described (4). Purified virus was diluted to less than 20% (vol/vol) Renografin and then pelleted. Pellets were resuspended in 0.01 M Tris–0.1 M NaCl–0.001 M EDTA (pH 7.4) and adjusted to 1 mg/ml.

**Synthetic peptides.** Peptides selected from predominantly hydrophilic regions within the LCMV-ARM glycoprotein sequence were synthesized by symmetrical anhydride chemistry on a model 430A automated synthesizer (Applied Biosystems, Redwood City, Calif.) (S. Kent and I.-C. Lewis, *in* K. Alitalo, P. Partanen, and A. Vaheri, ed., *Synthetic Peptides in Biology and Medicine*, in press), and sequential additions were monitored by ninhydrin analysis (27). Peptides were cleaved and deblocked with anhydrous hydrogen fluoride (29), and homogeneity was assessed by high-performance liquid chromatography on a reverse-phase C-18 column (Vydac Corp., Hesperia, Calif.). Peptides were linked to keyhole limpet hemocyanin at a ratio of 1 mg peptide per mg of keyhole limpet hemocyanin by using the cross-linking agent MBS (*m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; Pierce Chemical Co., Rockford, Ill.) and by coupling via either naturally occurring or added cysteine residues (14). Rabbits were initially immunized subcutaneously with 200 μg of keyhole limpet hemocyanin conjugate emulsified in complete Freund adjuvant and then 14 days later with the same dose in incomplete Freund adjuvant. Rabbits were boosted at approximately 3 and 5 weeks after the initial immunization with 100 μg of carrier-free peptide adsorbed on alum, and sample bleedings were taken at 4 and 6 weeks. Usually, rabbits showing high antipeptide antibody

* Corresponding author.

† Publication no. 4570-IMM, Scripps Clinic and Research Foundation.

TABLE 1. Summary of results of antipeptide immunization

| Peptide | Rabbit | ELISA[a] antipeptide titer | Viral polypeptide specificity[b] |
|---|---|---|---|
| GP-C 59–79 | 7542 | > 16,000 | GP-1 (IP, WB) |
| | 7543 | > 16,000 | GP-1 (IP, WB) |
| | 8827 | > 16,000 | GP-1 (IP, WB) |
| GP-C 228–239 | 791 | 15,000 | GP-1 (WB) |
| | 792 | 78,000 | GP-1 (WB) |
| GP-C 253–262 | 2341 | | GP-1 (WB) |
| | 2342 | 12,000 | GP-1 (WB) |
| GP-C 272–285 | 797 | 12,000 | GP-2 (WB) |
| | 798 | 12,000 | GP-2 (WB) |
| GP-C 378–391 | 9257 | 260,000 | GP-2 (WB) |
| | 9258 | 16,000 | GP-2 (WB) |

[a] ELISA, Enzyme-linked immunosorbent assay.
[b] IP, Immune precipitation; WB, Western blot.

titers were exsanguinated at 8 weeks. Antisera were analyzed for antipeptide titer by the enzyme-linked immunosorbent assay. A 0.1-nmol portion of peptide dissolved in 0.1 M sodium carbonate buffer (pH 9.3) was dried in wells of a 96-well plate, and the wells were rinsed with phosphate-buffered saline-Tween and blocked by incubation with 3% bovine serum albumin. Fourfold serum dilutions were added, and the mixture was incubated for 90 min. After the mixture was washed, bound antibody was detected with protein A-peroxidase and orthophenylenediamine substrate. Controls consisted of preimmune sera and irrelevant peptides not derived from the LCMV sequence. Endpoints were the last serum dilution showing 20% of maximum optical density for a given sample. Western blots were done by using purified LCMV as the target antigen. Preparative 8-cm-wide slab gels were loaded with 300 μg of purified virus and electrophoresed as described previously (3, 4). Gels were then blotted electrophoretically onto nitrocellulose membranes (5), and the blots were cut into 5-mm strips and reacted with sera at dilutions from 1/25 to 1/400. After incubation, the blots were washed four times with phosphate-buffered saline-Tween, and bound immunoglobulin G was detected by incubation with $^{125}$I-labeled protein A (specific activity, 1 μCi/μg). Specificity was shown by blocking with free peptide. In all of the reactions described in this report, the reactivity of antipeptide sera with viral proteins was abrogated when sera were preincubated with free peptide.

## RESULTS

**Linear orientation of GP-1 and GP-2 on the GP-C precursor protein.** Previous studies have shown that the LCMV and Pichinde virus GP-2 proteins are likely to be integral membrane proteins (3, 4, 5, 7, 15). Dissociation of virus by lysis with mild detergent results in a complex of GP-2 with viral nucleocapsids, suggesting that this protein may span the viral envelope. GP-1, in contrast, is highly exposed on the virion envelope, as indicated by surface iodination, and is the target of neutralizing antibody (23). Recent studies have delineated the RNA and deduced protein sequences for GP-C genes of LCMV (26; Southern et al., in press) and Pichinde (1) and Lassa (2) arenaviruses and have revealed common structural features among them. All have hydrophobic stretches of approximately 20 to 30 amino acids near the carboxyl terminus, which may serve as a transmembrane anchor. There are, however, two additional prominent hydrophobic amino acid stretches within the first 50 amino acids from the amino-terminal end of each GP-C, which could also serve as anchor sequences. Hence, using the 498-amino-acid sequence of LCMV GP-C as a model, we first sought to define the positions of GP-1 and GP-2 on the precursor. Two peptides, GP-C 59–79 and GP-C 378–391, were selected as representing sequences near the extremes of the LCMV-ARM GP-C sequence. Immunization of rabbits with each of these peptides resulted in the production of high-titer antibody to the peptide (Table 1). In Western blots, antibody to peptide GP-C 59–79 reacted with GP-1 (at dilutions of 1/50 and 1/100). This reactivity was abolished by preincubating antiserum with 1 μg of peptide 59–79 in solution (Fig. 1). Similarly, antiserum to peptide GP-C 378–391 bound to GP-2. Hence, GP-1 and GP-2 lie in the amino-terminal and carboxy-terminal domains, respectively, of the GP-C precursor.

**Identification of the site of proteolytic cleavage.** Having established the location of GP-1 and GP-2 on GP-C, we sought to define the location and structure of the proteolytic cleavage site. On the basis of size estimates derived by endoglycosidase-F deglycosylation of GP-1 and GP-2 (10), we predicted that cleavage should occur between residues 230 and 290 in the GP-C sequence. Direct amino acid sequencing of the amino terminus of GP-2 extracted from gel slices (18) was attempted but was unsuccessful in three attempts. The most probable explanation for this failure was amino-terminal modification of GP-2, since on three occasions, three successive cycles of Edman degradation yielded no detectable amino acid residues. We then turned to the synthetic-peptide approach defined here to locate the cleavage site. Alignment of predicted amino acid sequences for LCMV-ARM and Pichinde virus revealed several highly homologous stretches in the region of interest (Fig. 2). These were GP-C 228–241 and GP-C 268–286 in LCMV, corresponding to 238–251 and 277–295, respectively, in Pichinde virus. Portions of these sequences (LCMV GP-C 228–239

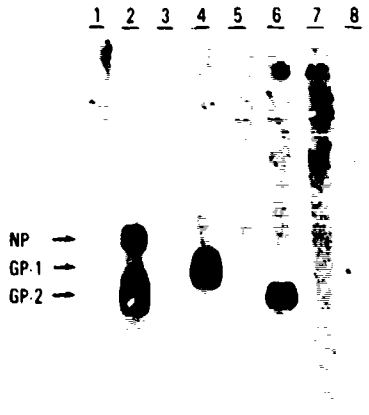



FIG. 1. Reactions of antipeptide sera with LCMV proteins. Lane 1 shows prebleed guinea pig serum; lane 2 shows guinea pig immune serum and indicates the positions of Np and GP-2 as reported elsewhere (21). Guinea pig antibody to LCMV reacts with conformational sites in GP-1 and therefore is not reactive with that protein in Western blots. Lanes 3 and 4 show prebleed and postbleed samples, repectively, of rabbit antiserum to peptide GP-C 59–79 reacting with GP-1; lanes 5 and 6 show prebleed and postbleed samples, respectively, of rabbit antiserum to GP-C 378–391 reacting with GP-2. Lanes 7 and 8 show the abrogation of reactivity of anti-GP-C 59–79 and GP-C 378–391 by preincubation with 1 μg of their respective immunizing peptides in solution prior to blotting.

and GP-C 272–285) were synthesized, and antibody was prepared. We also noted the conservation of a pair of basic amino acids, Arg-Arg at LCMV GP-C 262–263 and Arg-Lys at Pichinde virus GP-C 271–272, that represented a potential cleavage recognition site; therefore, a third peptide corresponding to LCMV GP-C 253–262 was prepared. Reactivity of all three antisera was determined by Western blotting (Fig. 3). Antisera to peptides GP-C 228–241 and GP-C 253–262 reacted with GP-1, while the antisera to GP-C 272–285 reacted with GP-2; hence cleavage of GP-C to GP-1 and GP-2 occurs between peptides 253 to 262 and 272 to 285. Within this region, the only structural feature in common to all of the arenaviruses for which sequence data are available and likely to serve as a recognition site for proteolytic cleavage is the basic sequence -Arg-Arg- at GP-C 262–263. We also attempted without success to raise antibody to the sequence GP-C 263–275 (Leu-Ala-Gly-Thr-Phe-Thr-Trp-Thr-Leu-Ser-Asp-Ser) predicted to lie at the amino terminus of cleaved GP-2 but have been unsuccessful to date owing to the hydrophobic nature of this sequence.

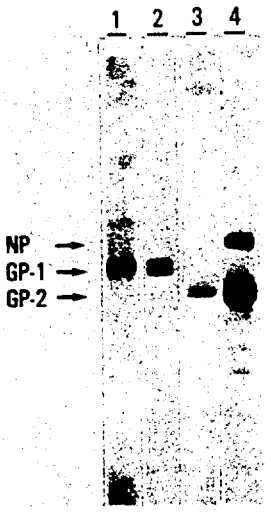



FIG. 3. Western blot of antisera against peptides flanking the putative cleavage site of GP-C. Lanes 1 (rabbit anti–GP-C 228–239) and 2 (anti–GP-C 253–262) both show reactions with GP-1, while anti–GP-C 272–285 (lane 3) reacts with GP-2. Lane 4 shows guinea pig immune serum reacting with NP and GP-2. Peptides on the amino-terminal side of the dibasic -Arg-Arg- sequence (GP-C 262–263) gave rise to antisera which reacted with GP-1, while those on the carboxyl side gave rise to antisera which reacted with GP-2.

## DISCUSSION

Proteolytic cleavage at basic amino acid sequences is well documented for viral systems including Semliki Forest virus and Sindbis virus E-2 glycoproteins (12, 25) and for yellow fever virus NS and M proteins (24), as well as for mammalian prohormone (28, 31) and proprotein (9, 16) pathways. The protease activities responsible for cleavage of viral proteins are largely uncharacterized. In influenza A viruses, cleavage of HA to $HA_1$ and $HA_2$ is accomplished by host-specific trypsinlike serine esterases and occurs at the time of or after virus maturation (19). Similarly, murine coronavirus glycoprotein E2 must be cleaved by a trypsinlike enzyme to activate its cell fusion potential (28). Cleavage in the alphaviruses and flaviviruses (12, 24, 25) is thought to involve intracellular action of a combined trypsinlike and carboxypeptidase B-like activity which functions late in the secretory pathway. The latter is consistent with our previous observations on posttranslational processing of arenavirus glycoproteins which demonstrated that only the cleaved glycoproteins reached surfaces of infected cells and virions

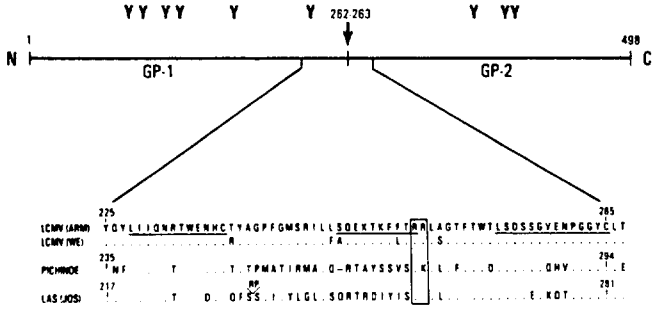



FIG. 2. Cleavage region of GP-C precursors of LCM-ARM, and LCMV-WE, Pichinde virus, and Lassa virus (Josiah). The 498-amino-acid linear sequence of LCMV ARM GP-C (Southern et al., in press) is represented schematically, with glycosylation sites marked (Y). Amino acids 225 to 285 for LCMV-ARM and WE strains, 235 to 294 for Pichinde virus (1), and 217 to 283 for Lassa virus (Josiah) (2) are shown. Conserved amino acids are indicated by dots and peptides used in the present study are underlined in the LCMV-ARM sequence. The position of the dibasic amino acid sequence (boxed) is preserved relative to the conserved flanking sequences Leu-Ile-Ile-Gln-Asn-Arg-Thr-Trp-Glu-Asn-His-Cys and Gly-Thr-Phe-Thr-Trp-Thr-Leu-Ser-Asp-Ser-Ser-Gly-Val-Glu-Asn-Phe-Gly-Gly-Tyr-Cys.

(4). Rice et al. (24) have speculated that cleavage of the yellow fever virus membrane protein is achieved by a host protease with specificity for the sequence Arg-X-Arg/Lys-Arg, postulated to be localized in the Golgi or post-Golgi vesicles. Cleavage may not be quantitative in LCMV, since van der Zeijst et al. (33) have reported that some LCMV GP-C is detectable on the surfaces of infected cells by in situ radioiodination, and we have occasionally observed traces of GP-C in purified virions (B. S. Parekh and M. J. Buchmeier, unpublished observations).

Cleavages of coronavirus, paramyxovirus, orthomyxovirus, and retrovirus membrane proteins are associated with the activation of fusion functions mediated by the cleaved products (30, 34). Such activation is thought to be due to exposure at the N terminus of predominantly hydrophobic sequences generated by cleavage. We have no evidence of similar biological function associated with cleavage of the LCMV precursor; however, the N terminus of GP-2, which would be liberated by cleavage at the Arg-Arg site, contains a hydrophobic sequence, Leu-Ala-Gly-Thr-Phe-Thr-Trp-Thr-Leu. While this sequence is not as long as the 20- to 30-amino-acid stretches generated in the above cases, it is predominantly hydrophobic and may constitute a functional domain. Availability of antisera to regions adjacent to the cleavage site of GP-C will enable us to probe further for evidence of biological function associated with cleavage.

Controlled proteolytic cleavage constitutes a fundamental regulatory mechanism in a wide variety of biological systems (22). Examples of protease control have been described for many cellular systems involving hormonal (28, 32) and enzymatic precursors and for viral systems such as picornaviruses, in which autoproteolytic cleavage of the initial polyprotein translation product VPO is a central feature of the replicative cycle (6). Furthermore, functional regulation of effector systems like the blood coagulation (8) and complement (21) pathways are also dependent on controlled proteolysis. Traditional approaches to identification of proteolytic cleavage sites have involved rigorous purifi-

cation of cleaved products and direct amino acid sequencing. With nucleotide and deduced amino acid sequences available in data banks from an increasing number of demonstrated and hypothetical viral and cellular genes, the approach we have described, involving the use of synthetic peptides and corresponding antisera to bracket cleavage signals in polyprotein gene products, should be widely applicable to other systems in which protease cleavage events activate function. With recent improvements in synthetic-peptide synthesis technology that facilitate production of large numbers of high-quality peptides at reasonable cost (17; Kent and Lewis, in press), the approach presented here may provide a valuable adjunct to gene mapping in a variety of biological systems.

## ACKNOWLEDGMENTS

We thank Richard A. Lerner and James M. Hogle for stimulating discussion and insight, Farshad Karimi for technical assistance, and Jim Johnston for manuscript preparation. We are also grateful to David Auperin for providing GP-C sequence data for Lassa virus prior to its publication.

This work was sponsored by Public Health Service grants A1-16102, NS12428 and AG04342 from the National Institutes of Health U.S. Army Medical Research Institute for Infectious Diseases contract C3013.

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# Protein Structure and Expression Among Arenaviruses

M.J. Buchmeier and B.S. Parekh

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## 1 Introduction

The proteins of arenaviruses were first studies by Smadel and his colleagues (1939, 1940, 1942), with reference to their antigenicity. These workers described the presence of a virus-specific soluble (S) antigen detectable by complement fixation (CF) in homogenates of spleen and lung from LCMV-infected guinea pigs. Soluble antigen could be separated from infectious virus by ultracentrifugation. Repeatedly washed virions reacted poorly in CF tests while the S antigen lost none of its immunoreactivity after ultracentrifugation. These studies were not extended until nearly 3 decades later when Brown and Kirk (1969), Chastel (1970), Simon (1970), and Bro-Jorgensen (1971) described antigens detectable by CF and immunodiffusion in tissues or cell cultures infected with LCMV. Bro-Jorgensen (1971) found two antigenic species by immunodiffusion using infected BHK-21 cells as the antigen source. One antigen was heat stable and resistant to proteolysis, while the second was degraded by both heating and pronase digestion. Both antigens sedimented at a rate of 3.5 *S* in rate zonal sucrose gradient centrifugation, and based on this *S* value the molecular weight of the thermolabile S antigen was estimated to be 48000.

Studies by Gschwender (1976) with LCMV established that the extractable complement-fixing antigen (ECFA) was an internal component of the virion. Antiserum directed against ECFA did not neutralize infectious LCMV, and it did not mediate complement-dependent lysis of LCMV-infected cells. Purified LCMV disrupted by detergents liberated an antigen which reacted with anti-ECFA in CF test and produced a band of identity with ECFA by immunodiffusion.

Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

Current Topics in Microbiology and Immunology, Vol 133
© Springer-Verlag Berlin Heidelberg 1987

RAWLS and BUCHMEIER (1975) arrived at similar conclusions, working with the S antigens of Pichinde virus. Antisera directed against partially purified CF antigen from cells infected by Pichinde virus were shown to react against the internal nucleocapsid protein of the virus but not with surface antigens of infected cells. Subsequent studies (BUCHMEIER et al. 1977) demonstrated that the S antigen was a degradation product of the viral nucleocapsid protein (NP). Moreover the antigenic cross-reactivity observed by CF among the new world, Tacaribe complex arenaviruses (reviewed in RAWLS and LEUNG 1979) was shown to be due to conservation of NP-related antigens (BUCHMEIER and OLDSTONE 1977).

Persistent arenavirus infections, whether in vitro (LEHMANN-GRUBE et al. 1969; WELSH and OLDSTONE 1977; WELSH and BUCHMEIER 1979; VAN DER ZEIJST et al. 1983a, b) or in vivo (TRAUB 1936; WILSNACK and ROWE 1964; OLDSTONE and BUCHMEIER 1982; RODRIGUEZ et al. 1983; see also FRANCIS et al., this volume), are characterized by the persistence of nucleoprotein, often in the absence of viral glycoprotein antigens and infectious virus production. This phenomenon, which we now recognize may be a consequence of the ambisense genomic arrangement of the S RNA segment (see BISHOP and AUPERIN, this volume), led to a great deal of confusion and ambiguity in early attempts to grow and purify arenaviruses (RAWLS and LEUNG 1979). Only when factors such as multiplicity of infection, rigorous plaque purification of the infecting virus, and time of harvest were carefully controlled did a consistent picture of the structural features of these viruses emerge.

## 2 Structural Proteins of Arenaviruses

The structural proteins of purified arenaviruses were first studied by RAMOS et al. (1972) working with Pichinde virus and by PEDERSON (1973) with LCMV. Numerous other descriptive studies of the proteins of these agents have followed, and to date the structural proteins of at least nine different arenaviruses have been examined (summarized in Table 1). Despite apparent differences, a number of common features have emerged. Characteristically, arenaviruses contain a major dominating protein which is the viral nucleocapsid protein (NP; 60–68 K). Nucleocapsid protein constitutes up to 58% of the protein in arenaviruses (VEZZA et al. 1977) and is easily detected in SDS-PAGE gels by protein staining or by radiolabeling with amino acid precursors such as [$^3$H]leucine or [$^{35}$S]methionine (Fig. 1). The viruses also contain variably either one, as reported for Tacaribe and Tamiami (GARD et al. 1977) or two, for LCMV and Pichinde virus (BUCHMEIER et al. 1978; VEZZA et al. 1977), glycopeptides of somewhat lower molecular weight than NP. Other minor proteins have also been detected but the origin of these has until recently been largely a subject of conjecture. Predominant among these quantitatively minor proteins are a 180–200 K protein termed L in Pichinde (HARNISH et al. 1981, 1983) and LCMV (BUCHMEIER, SINGH, and SOUTHERN, unpublished observation), which is a candidate for a viral polymerase, a 77 K protein termed P (VEZZA et al. 1977; GARD et al.

**Table 1.** Structural polypeptides of arenaviruses

| Virus | L proteins | | Nucleoproteins | | Envelope proteins | | Minor proteins | | References |
|---|---|---|---|---|---|---|---|---|---|
| | Molecular weight (K) | Name | Molecular weight (K) | Name | Molecular weight (K) | Name | Molecular weight (K) | Name | |
| LCMV | | | 63 | NP | 44<br>35 | GP1<br>GP2 | - | | Buchmeier et al. 1978; Buchmeier and Oldstone 1979 |
| LCMV | 200 | P 200 | 63 | p 63 | 130<br>85<br>60<br>44<br>35 | gp 130<br>gp 85<br>gp 60<br>gp 44<br>gp 35 | [illegible]<br>38[a]<br>26[a]<br>25[a]<br>19[a] | p [illegible]<br>p 38<br>p 26<br>p 25<br>p 19 | Bruns et al. 1983b |
| Pichinde | 200 | L | 64 | NP | 52<br>36 | GP1<br>GP2 | 48[a]<br>38[b]<br>28[a] | | Harnish et al. 1981 |
| Pichinde | - | | 66 | N | 64<br>38 | GP1<br>GP2 | [illegible]<br>12 | - | Vezza et al. 1977 |
| Tacaribe | | - | 68 | N | 42 | GP | 79 | | Gard et al. 1977 |
| Tamiami | - | - | 66 | N | 44 | GP | [illegible] | P | |
| Junin | - | - | 60 | N | 44<br>35–39 | | - | - | Grau et al. 1981 |
| Junin | - | - | 54 | VP-3(N) | 91<br>72<br>55<br>38 | VP-1<br>VP-2<br>VP-4<br>VP-5 | 25 | VP-6 | Martinez, Segovia, and de Mitri 1977 |
| Machupo | - | - | 68 | N | 50<br>41 | GP1<br>GP2 | 84<br>74<br>15 | -<br>- | Gangemi et al. 1978 |
| Mopeia | 180 | L | 61 | N | 48<br>35 | GP1<br>GP2 | 84 | - | Gonzalez et al. 1984 |
| Mobala | 180 | L | 60 | N | 48<br>37 | GP1<br>GP2 | | | |
| Lassa | 180 | L | 61 | N | 45<br>38 | GP1<br>GP2 | | | |
| Lassa | - | | 60 | N | 45<br>38 | GP1<br>GP2 | 76<br>68<br>25[b]<br>20[b] | -<br>- | Clegg and Lloyd 1983 |

[a] Nucleoprotein-related degradation or cleavage fragments

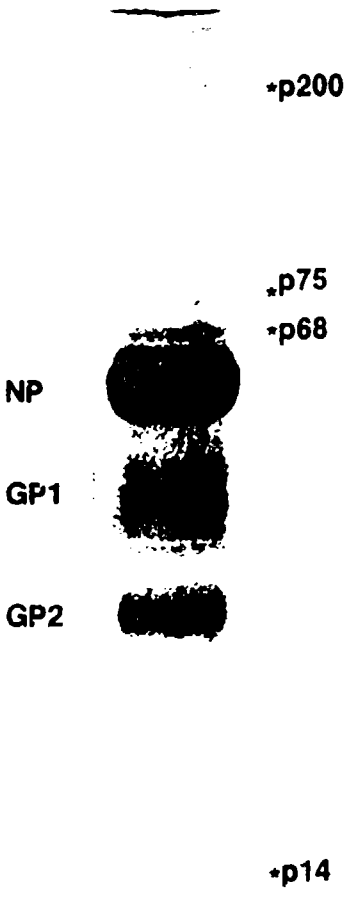



Fig. 1. [$^{35}$S]methionine-labeled polypeptides of LCMV. BHK-21 cells were infected with LCMV ARM at a multiplicity of infection of 0.1 PFU/cell then incubated for 24 h before adding 100 μCi/ml of [$^{35}$S]methionine in methionine-free medium. Supernatant medium was harvested at 40 h and virus purified and analyzed by SDS-PAGE as described (BUCHMEIER et al. 1978). Evident in the autoradiogram are the major viral polypeptides NP, GP1, and GP2, as well as quantitatively minor polypeptides (p) of 200 K, 75 K, 68 K, and 14 K. In other experiments we have shown that p 200 is encoded on the viral L RNA segment and that p 68 apparently shares tryptic peptides with NP. No definitive information is available for p 75 or p 14

1977), and several low molecular weight degradation products derived from nucleocapsid protein (HARNISH et al. 1981; BRUNS et al. 1983b; BUCHMEIER 1984). As detailed in BISHOP and AUPERIN, and SOUTHERN and BISHOP, this volume, molecular cloning approaches have definitively assigned NP and the GPC precursor of GP1 and GP2 to the S RNA segments of LCM and Pichinde

viruses. These two open reading frames leave no room on S for additional primary translation products. Moreover, HARNISH et al. (1983) have mapped the 200 K L of Pichinde virus to the viral L RNA segment using genetic reassortants, and we (BUCHMEIER, SINGH, and SOUTHERN, unpublished observation) have shown directly, using synthetic peptide antibodies, that the L RNA segment of LCMV encodes a unique ca. 180 K L. Hence it appears clear that there is not sufficient coding capacity within the genome to accommodate all of the reported additional polypeptides and glycopeptides. Thus it is more likely, for example, that the additional glycoprotein components reported for LCMV by BRUNS et al. (1983b), which include glycoproteins of 130 K, 85 K, and 60 K, in addition to the previously documented 44 K (GP1) and 35 K (GP2) species, represent products of either atypical or incomplete posttranslational processing or contaminating cellular proteins. These differences will ultimately be resolved using well-defined monoclonal and sequence-specific, antipeptide antibodies and correlating the results with cDNA sequence analysis.

Localization of the structural proteins in the virion has been studies in detail. As with other enveloped RNA viruses, the glycopeptides are displayed on the external surface of the viral envelope. VEZZA et al. (1977) demonstrated that digestion of Pichinde virus with proteolytic enzymes produced "bald" or spikeless virions which were devoid of GP1 and GP2. GARD et al. (1977) confirmed this result for the glycoproteins of Tacaribe and Tamiami viruses, and BUCHMEIER et al. (1978) also showed that the GP1 and GP2 of LCMV were susceptible to proteolysis. Moreover by surface iodination of LCMV (BUCHMEIER and OLDSTONE 1979) it was shown that GP1 was the predominant virus-specific molecule accessible to lactoperoxidase-catalyzed iodination on the plasma membranes of infected cells and on the envelope. The macromolecular arrangement of glycoproteins in the membrane has been studied by BRUNS and LEHMANN-GRUBE (1983). Based primarily on cross-linking and nearest neighbor analyses, they suggest that the glycoprotein spike of LCMV consists of a complex of 1 gp 35 molecule linked to either three gp 44 molecules or one gp 44 and one gp 85 molecule. This model awaits confirmation, however, and must be viewed with caution since it fails to take into account the essentially equimolar concentrations of GP1 and GP2 (i.e., gp 44 and gp 35) in the membrane (VEZZA et al. 1977) and also incorporates two glycoproteins, gp 85 and gp 60, which have not been confirmed by others.

The nucleoproteins of arenaviruses reside predominantly in the ribonucleoprotein (RNP) core of the virion. The basic configuration of the Pichinde virus RNP was found by YOUNG and HOWARD (1983) to be a linear array of globular subunits, or nucleosomes, 4–5 nm in diameter, that represent individual molecules of the NP. This "beaded" filament appears to fold progressively through a number of 12–15 nm helical structures and these strands in turn were twisted to form 20 nm thick fibers seen in isolated core structures.

In addition to NP, we have also found L in association with the RNP. Preparations of purified LCM virions were subjected to disruption with 1% NP-40 and 0.5 *M* KCl, then pelleted through a sucrose layer to exclude soluble components. L was detected in the pelleted RNP complex by Western blotting with antipeptide antibodies to an L-RNA specific amino acid sequence.

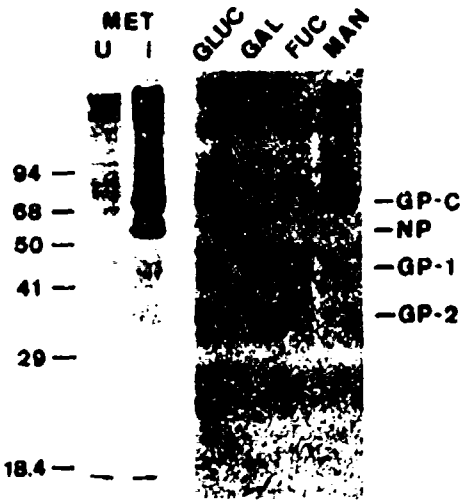



Fig. 2. Labeling of cell-associated proteins with [$^{35}$S]methionine (*MET*) and [$^{3}$H]sugars (*GLUC*, glucosamine; *GAL*, galactose; *FUC*, fucose; *MAN*, mannose). Cells were infected at a moi of 0.1 then pulse labeled for 2 h from 42–44 h after infection when virus production was maximal. Cytosols were prepared and immunoprecipitated with a hyperimmune guinea pig antiserum as described (BUCHMEIER and OLDSTONE 1979). Both GPC and NP are readily detectable using [$^{35}$S]methionine label, but only the glycopeptides label with sugar precursors. GPC is heavily labeled with mannose and glucosamine but not with galactose and fucose, demonstrating that it is of the high mannose type. Mannose residues are trimmed prior to cleavage of GPC into GP1 and GP2

A single nonstructural glycopeptide termed GPC has been described in cells infected with LCM (BUCHMEIER and OLDSTONE 1979), Pichinde (HARNISH et al. 1981), Tacaribe (SALEH et al. 1977), Lassa fever (CLEGG and LLOYD 1983), and Machupo (LUKASHEVICH and LEMESHKO 1985) viruses. As discussed in more detail in Sect. 4, GPC is the precursor of the structural glycoproteins. Intracellular GPC is an oligomannosyl rich glycopeptide which is processed posttranslationally by carbohydrate trimming and proteolytic cleavage prior to virus release (Fig. 2). Pulse-chase experiments (HARNISH et al. 1981; DIMOCK et al. 1982) have shown that the synthesis of GPC correlates closely with production of infectious virions. As GPC synthesis and consequent expression of viral glycoprotein at the plasma membrane increase, so does virus production. In turn, late in the infection when virus production wanes, GPC synthesis and surface expression of the structural glycoproteins diminish. The cellular site of GPC cleavage has not been precisely defined. In our work (BUCHMEIER and OLDSTONE 1979) we found only the mature GP1 and GP2 on the surface of infected cells and virions and suggested that cleavage occurs intracellularly; however, VAN DER ZEIJST et al. (1983a) have shown uncleaved precursor at the surface of acutely infected cells using a sensitive in situ surface iodination method. It is possible that this apparent discrepancy is a quantitative effect since we have recently observed a small quantity of uncleaved GPC in mature virions by Western blotting (PAREKH and BUCHMEIER, unpublished observation).

**Table 2.** Enzymatic activities associated with arenaviruses

| Activity | Virus | Associated with | Reference |
|---|---|---|---|
| RNA polymerase | Pichinde | Viral RNP complex | CARTER et al. 1974; LEUNG et al. 1979 |
| Poly-A polymerase | Pichinde | Ribosomes | LEUNG et al. 1979 |
| Poly-U polymerase | Pichinde | Ribosomes | LEUNG et al. 1979 |
| Protein kinase | LCM | Viral RNP complex | HOWARD and BUCHMEIER 1983; BRUNS et al. 1986 |

## 3 Enzymatic Activities Associated with Arenaviruses

Several enzymatic activities have been detected in association with purified arenaviruses (Table 2). In at least one of these, a viral RNA polymerase activity is thought to be obligatory for viral replication (CARTER et al. 1974; LEUNG et al. 1979). Polymerase activity has been studied most extensively in association with Pichinde virus, where it was shown to catalyze RNA synthesis from the viral genomic RNA template. The RNA polymerase was shown to be associated with the viral nucleocapsid complex, and was distinguishable on the basis of localization, as well as divalent cation requirements, from ribosome-associated, $Mn^{2+}$-dependent, poly-A polymerase and $Mg^{2+}$-dependent poly-U polymerase activities. The most likely candidate for the viral RNA polymerase is the nucleocapsid-associated L protein (180–200 K) which we have recently shown to be encoded by the L RNA. Characterization of the mode of transcription by this enzyme is hampered by its low activity and lability (LEUNG et al. 1979; BUCHMEIER et al. 1980b).

In addition to the RNA polymerase which appears to be virally encoded and the poly-A and -U polymerases which are ribosome associated and thus probably of host origin, HOWARD and BUCHMEIER (1983) have described a virion-associated protein kinase activity. This kinase preferentially phosphorylates serine and threonine residues in NP in the presence of a phosphate donor such as ATP (Fig. 3). The activity is internal, as indicated by its release from detergent solubilized virions, and is not stimulated by cyclic nucleotides. Kinase activity was shown by density gradient studies to be associated with the nucleocapsid core of the virion. Although it is not clear whether the kinase is a bonafide viral gene product or an encapsidated cellular constituent, it is conceivable that phosphorylation may play a role in the regulation of viral RNA polymerase activity or in virion assembly. Most attempts to demonstrate phosphorylated proteins in arenaviruses have been unsuccessful (review in COMPANS and BISHOP 1985). BRUNS et al. (1986) however, report that a fraction of the 63 K nucleoprotein is present in a soluble phosphorylated form termed p 63 E. The significance of this observation remains to be determined; these authors have suggested that p 63 E plays a regulatory role in the viral replicative process. Clearly the roles of the RNA polymerase (transcriptase) and protein kinase activities in

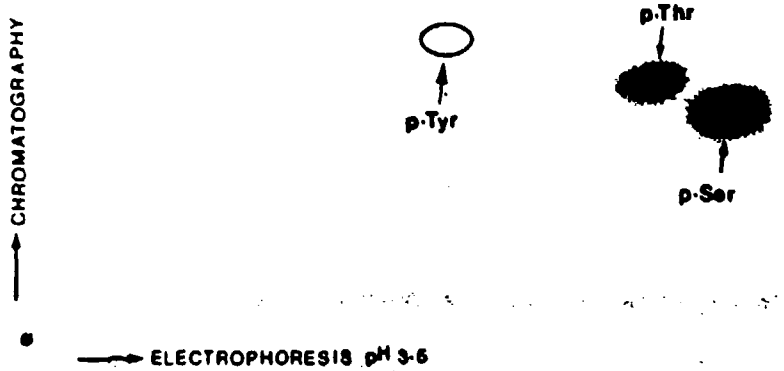



Fig. 3. Amino acids phosphorylated by the endogenous LCMV protein kinase. Phosphorylated NP was prepared as described (HOWARD and BUCHMEIER 1983) and hydrolyzed in 6 N HCl. Samples were electrophoresed in the first dimension then chromatographed in the second. Radioactive residues were identified by comparison with unlabeled markers. Only phosphoserine (*p. Ser*) and phosphothreonine (*p. Thr*) were detected as phosphorylated products of the LCMV kinase. The position of phosphotyrosine (*p. Tyr*) is indicated for reference. Reproduced from HOWARD and BUCHMEIER (1983)

the replicative cycle of the virus, as well as definitive characterization of their molecular nature, remain subjects for future investigation.

## 4 Synthesis and Expression of Proteins

Arenaviruses have in common the capacity to establish persistent infections both in vivo and in vitro. Recent studies have suggested that the inherent ability of these viruses to regulate differentially the expression of NP and surface glycoproteins may play a role in the ability of the virus to evade immune surveillance in such persistent infections (WELSH and OLDSTONE 1977; WELSH and BUCHMEIER 1979; OLDSTONE and BUCHMEIER 1982; RODRIGUEZ et al. 1983). In general, information concerning the synthesis of the viral polypeptides is limited. In cells infected with LCMV, synthesis of NP, shown by radiolabeling with [$^{35}$S]methionine, is first detected 6 h after infection (BUCHMEIER et al. 1978), coincident with the start of the exponential phase of the replicative cycle. NP is apparently synthesized as a primary translation product since the native 63 K form is observed first in the infected cell. Pulse-chase experiments have not revealed higher molecular weight forms of it which might be candidates for a precursor molecule, although recent studies in a coupled in vitro transcription translation system have shown a 73 K translation product of the Tacaribe virus S RNA, which is apparently posttranslationally modified to yield the 68 K virion form of the molecule (BOERSMA and COMPANS 1985). Both the 68 K and 73 K polypeptides

contained similar tryptic peptides, suggesting a common origin. We have observed a similar polypeptide of 68 K (Fig. 1) in LCMV and have shown in unpublished work that this molecule shares tryptic peptides with NP; caution must be exercised, however, since the 68 K protein may be contaminated with significant amounts of the more abundant 63 K NP. Nevertheless the possibility exists that a portion of NP message is translated as an elongated "read through" polypeptide or alternatively that NP (63 K) is rapidly cleaved posttranslationally off a larger primary translation product.

Several groups have shown that NP is present in both full length and degraded form in virions and infected cells. Pichinde virus was shown to encode polypeptides of 15 K and 20 K in infected cells, and these were antigenically related to NP (BUCHMEIER et al. 1977). HARNISH et al. (1981) described NP-related polypeptides of 48 K, 38 K, and 28 K in Pichinde-infected BHK-21 cells, and, after a 3-h chase period, additional species of 17 K, 16.5 K, and 14 K were also evident. All of these six quantitatively minor polypeptides shared common tryptic peptides with NP suggesting their derivation by posttranslational proteolytic cleavage, although the potential presence of premature termination products or of mutants containing deletions in the NP gene was not formally precluded.

YOUNG et al. (1985b) have observed similar NP-related cleavage products appearing late in Pichinde virus infection of Vero cells and have suggested that the intracellular level of NP plays a role in regulating genome replication transcription by committing newly transformed RNA to either nucleocapsid assembly or further rounds of replication. It was proposed that differences in cleavage patterns were related to the extent of virus regulation observed. At least one of the NP-related cleavage products, a 28 K fragment identifiable with a monoclonal antibody, was found in the cell nucleus late in the infection (YOUNG et al. 1985a). These studies suggest the possibility that NP or cleavage fragments of it may serve as regulatory molecules to modulate transcription and or replication.

Observations of cleavage fragments of nucleocapsid protein are not restricted to Pichinde virus. CLEGG and LLOYD (1983), working with Lassa virus, reported N-related polypeptides of 36 K and 24 K, termed fN1 and fN2, respectively. In this instance fN1 and fN2 were thought to be artifacts resulting from proteolysis upon disruption of Lassa-infected cells; only full length N was detected by Western blotting in cells lysed under stringent denaturing conditions. BRUNS et al. (1983b) have reported 25 K and 38 K polypeptides in LCM virions, which appeared in one-dimensional tryptic maps to be related to the NP (termed p 63 in their nomenclature). We have shown, using both peptide mapping and monoclonal antibody analyses, that LCMV contains variable amounts of NP-related polypeptides of 25 K, 38 K and approximately 40 K (BUCHMEIER, unpublished observations). The appearance and quantities of these species are highly variable and affected by factors such as the host cell used to cultivate the virus and the time after infection; hence, it is not clear at the moment whether these molecules play a functional role in virus replication.

A clearer picture of the synthesis and potential mechanisms of regulation of arenavirus glycoproteins has emerged from recent studies of their genetic

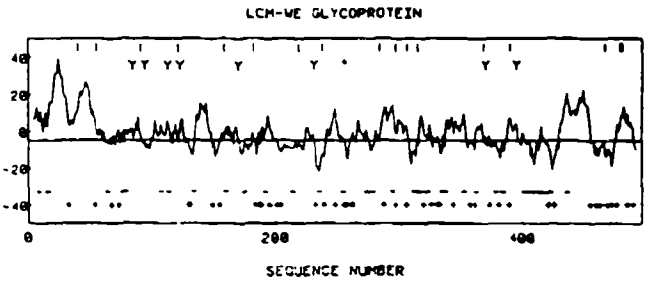



**Fig. 4.** Hydropathic plot, cysteine, and charged amino acid distribution for the GPC predicted gene product of LCMV-WE S RNA. Regions of the predicted protein with net hydrophobicity (areas *above* the center line) and those with net hydrophilicity (*below* the line) are displayed according to KYTE and DOOLITTLE (1982). As well as the positions of charged amino acids, cysteine residues (*vertical bars*) and potential asparagine-linked glycosylation sites (*Y*) are shown. Reproduced from ROMANOWSKY and BISHOP (1985)

structure (AUPERIN et al. 1984; ROMANOWSKI and BISHOP 1985; SOUTHERN et al. 1986). Previous investigation at the biochemical level demonstrated the presence of a high molecular weight (70–78 K) precursor of the virus structural glycoproteins in cells infected with LCM (BUCHMEIER et al. 1978; BUCHMEIER and OLDSTONE 1979), Tacaribe (SALEH et al. 1979), Pichinde (HARNISH et al. 1981), Lassa fever (CLEGG and LLOYD 1983), and Machupo (LUKASHEVICH and LEMESHKO 1985) viruses. Availability of nucleotide and amino acid sequence information has allowed mapping of the precursor glycoprotein, termed GPC for LCMV (BUCHMEIER et al. 1978), to the 5′ half of the virion S RNA strand (see BISHOP and AUPERIN, and SOUTHERN and BISHOP, this volume). Recent experiments in our laboratory have been directed at determining the fine structure of the GPC gene, orientation of the structural glycoproteins on the precursor, signals for proteolytic cleavage, and defining important antigenic regions of the molecule. Hydrophobicity profiles have been determined by the method of KYTE and DOOLITTLE (1982) for Pichinde virus and LCMV (Fig. 4). The GPC precursors of both LCMV WE and ARM consist of 498 amino acids in a single open reading frame. From the hydrophobicity profile it is evident that the protein contains three significant hydrophobic domains approximately spanning residues 1–32, 34–55, and 433–460 from the amino terminus. The first of these is throught by analogy with other membrane glycoproteins to constitute a signal sequence, while the carboxyl domain (433–460) is likely to constitute a membrane anchor. Amino acid sequence comparisons with the corresponding GPC genes of Pichinde and Lassa fever viruses have revealed that the gene consists of two domains. The amino terminal half is potentially highly glycosylated with six asn-x-ser thr glycosylation sites in LCMV ARM and WE, and eleven sites in the corresponding region of Pichinde GPC. Work in our laboratory has shown that at least four of these six potential sites in LCMV are actually glycosylated. Further, this domain shows only a low degree of amino acid homology between LCMV and Pichinde virus. In contrast, the carboxyl domain contains sequences which contain fewer glycosylation sites (three in LCM, five

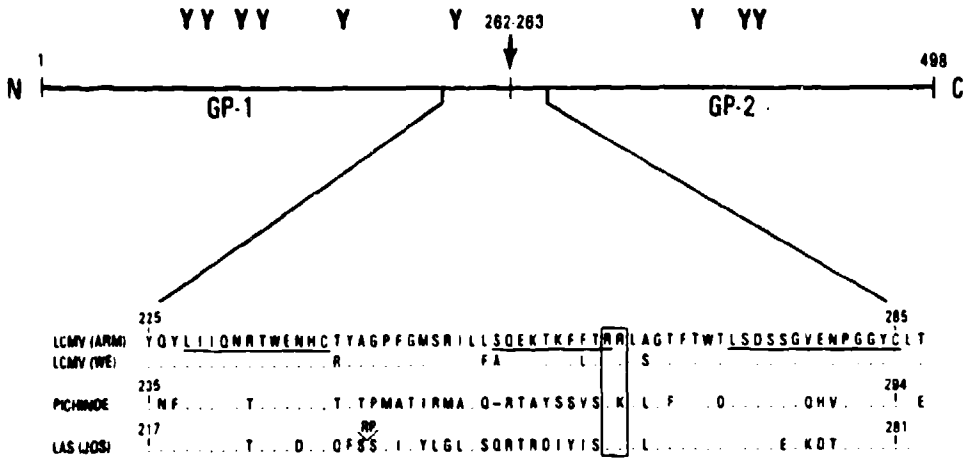



Fig. 5. Cleavage region of the GPC precursors of LCMV, Pichinde, and Lassa fever viruses. The linear sequence of LCMV-ARM GPC is represented with glycosylation sites marked (*Y*). Amino acids flanking the putative cleavage site in LCMV-ARM, LCMV-WE, Pichinde (AUPERIN et al. 1984), and Lassa fever (Josiah) (D. AUPERIN, personal communication) viruses are shown with alignment for maximum homology. Conserved amino acids relative to LCMV-ARM are indicated by *dots*, and peptides used to map the cleavage site as described in the text are *marked*. Note that the position of the conserved pair of basic amino acids relative to the *underlined* conserved flanking sequences is retained in all four viruses

in Pichinde) and share a high degree of homology with the corresponding sequences in Pichinde virus. We have used synthetic peptide and monoclonal antibodies to study the coding assignments and topography of the LCMV GPC gene. Peptides were synthesized corresponding to four regions of GPC (amino acids 59–79, 228–239, 272–285, and 378–391), and site-specific antisera raised in rabbits. Antibodies made against the more amino terminal peptides 59–79 and 228–239 reacted with native and denatured GP1, while antisera to the two peptides closest to the carboxyl end (271–285 and 378–391) reacted with GP2. Thus the gene order on the GPC message is $NH_2$-(GP1) (GP2)-COOH. Examination of the sequence between peptides 228–239 and 272–285 revealed the presence of a double basic amino acid sequence Arg-Arg at residues 262–263 flanked by hydrophobic amino acids. Alignment with the sequences of Pichinde (AUPERIN et al. 1984) and Lassa GPC (AUPERIN, personal communication) showed corresponding paired basic sequences in both of those viruses (Fig. 5). On the basis of these data it is apparent the cleavage site utilized on GPC to yield the GP1 and GP2 is defined by this conserved double basic amino acid sequence Arg-Arg on LCM and Lassa fever viruses, and Arg-Lys on Pichinde virus. Thus this site is likely to be a general feature of the arenavirus group. Moreover, analysis of sequence homology among the GPC precursors of these viruses has shown extensive conservation of sequences in GP2 predicted by the earlier demonstration of conserved antigens in GP2 (BUCHMEIER et al. 1980a, 1981, 1984) using monoclonal antibodies.

## 5 Antigenic Topography of Arenavirus Glycoproteins

Arenaviruses differ in their susceptibility to antibody-mediated neutralization. Neutralizing antibodies to LCMV have been shown to be directed against the GP1 (gp 44) glycoprotein (BUCHMEIER et al. 1981; BUCHMEIER 1984; BRUNS et al. 1983a; PAREKH and BUCHMEIER 1986). Similarly, monoclonal antibodies against the single glycoprotein of Tacaribe virus-mediated, highly efficient virus neutralization (ALLISON et al. 1984; HOWARD et al. 1985). Moreover, using competitive binding assays and analysis of neutralization resistant mutants, it was possible to map two distinct epitopes on Tacaribe G (HOWARD et al. 1985). One epitope, characterized by four monoclonal antibodies, was the target of highly efficient neutralization, while a single antibody to a second site was less efficient, leaving a large non-neutralizable persistent fraction. Failure to neutralize was not likely to be due to virus aggregation since addition of a second antibody to the alternate site resulted in further reduction in virus titer. Analysis of neutralization kinetics for the highly efficient monoclonal antibody suggested that the reaction followed double hit kinetics.

We have assessed the antigenic topography of the LCMV glycoproteins using a large library of monoclonal antibodies against GP1 and GP2 to map the epitopes on these molecules (PAREKH and BUCHMEIER 1986). Elicitation of neutralizing monoclonal antibodies to LCMV in the BALB/c mouse was a relatively infrequent event. Only 6 of 46 antibodies to the LCMV glycoproteins neutralized virus infectivity in vitro. Five of these antibodies were raised against the WE strain of virus and mapped by competition binding assay to a single conformation-dependent epitope (GP1A) shared by both ARM and WE as well as other LCMV strains (Fig. 6). The sixth neutralizing MAb was uniquely specific for the LCMV-ARM strain and its binding to that strain was only marginally affected by the other five antibodies, suggesting binding to a topographically related but not identical epitope (GP1D). Nonneutralizing MAb were found to be directed against two additional sites on GP1, (GP1B, C) as well as against three sites on GP2. The relevance of these data to the polyclonal antibody response was investigated using a potent neutralizing antiserum raised in guinea pigs. This antibody reacted predominantly with conformation-dependent structures on GP1, as indicated by its failure to bind in Western blotting, and its binding was completely inhibited by any of the five LCMV WE-specific neutralizing MAb against site GP1A. These results imply that the LCMV WE GP1 has a single immunodominant neutralizing antigenic determinant (GP1A) and that the LCMV ARM strain bears an additional topographically related but not identical site (GP1D). Attempts to neutralize other arenaviruses have met with mixed success. Patient and experimentally produced antisera show potent neutralizing activity against Junin virus; however, similar reagents from Lassa fever convalescent patients show rather low neutralizing potency unless complement is added to potentiate the effects of antibody (PETERS 1984). Virus neutralization is discussed in depth in one chapter by C.R. HOWARD in the accompanying volume in this series (134). From the brief treatment here it is evident that more information about the molecular nature of neutralizing antigenic determinants of arenaviruses is necessary before rational approaches to immunotherapy and immunization can be made. Obviously one needs to

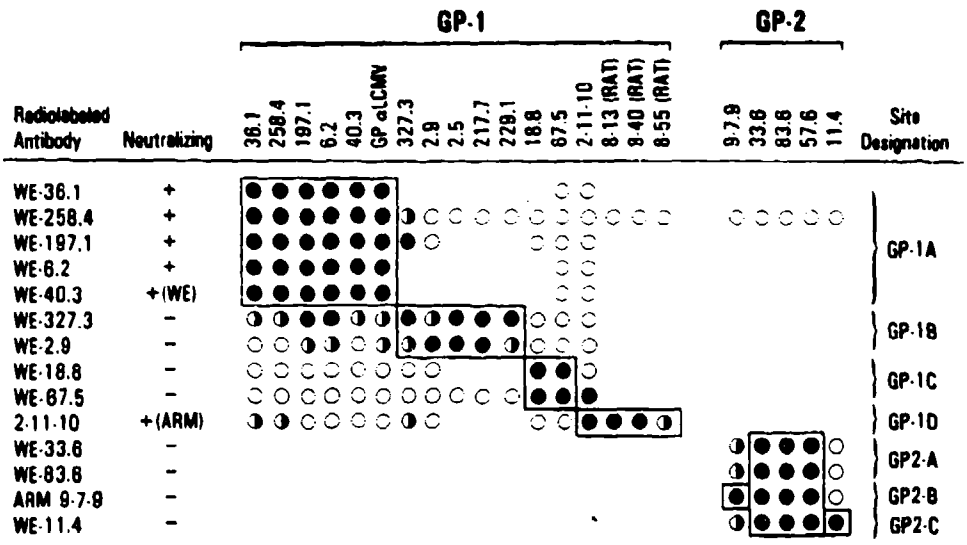



Fig. 6. Summary of results of competitive binding assay to map the epitopes on LCMV glycoproteins. Dilutions ($10^{-1}$ – $10^{-5}$) of unlabeled competing antibody were tested for their capacity to inhibit binding of constant amounts of purified, radiolabeled monoclonal antibodies. Combinations yielding greater than 80% inhibition are indicated by *filled circles*, 40%–80% by *half-filled circles* and less than 40% by *open circles*. Using this assay four epitopes on GP1 of LCMV-ARM (three on GP1 of LCM-WE) and three on GP2 have been defined. One of these, GP1A, was the major virus-neutralizing site recognized by mouse monoclonal and guinea pig polyclonal (GP anti-LCM) antibodies to LCMV. Note also that the LCMV-ARM-specific site GP1D was recognized by neutralizing monoclonal antibodies produced against that virus in the rat (8–13, 8–40, and 8–55). On GP2 antibodies WE 33.6 and 83.6 recognized a site termed GP2A which is common to both Old and New World arenaviruses (BUCHMEIER 1984)

define structure which will elicit strong protective immune responses without the risk of triggering immunopathologic disease.

## 6 Pathobiological Role of Specific Viral Gene Products In Vivo

Viral polypeptides and their degradation products trigger many of the pathobiologic manifestations observed in arenavirus infections. In the lifelong persistent infection of mice with LCMV a wasting syndrome has been well documented which is characterized by the development of immune complexes composed of viral antigen and antiviral antibody (OLDSTONE et al. 1980, 1983). These complexes lodge in the renal glomeruli where they trigger a chronic glomerulonephritis. At least one component of the virus has been identified in the glomeruli of diseased mice. Using a monospecific antibody to the NP of LCMV, BUCHMEIER and OLDSTONE (1978) demonstrated colocalization of NP antigen and the host glomerular mesangium.

A role of NP in neuronal dysfunction has also been proposed to occur during LCMV persistence. RODRIGUEZ et al. (1983) observed expression of NP in association with polyribosomes in the cytoplasm of neurons from widespread

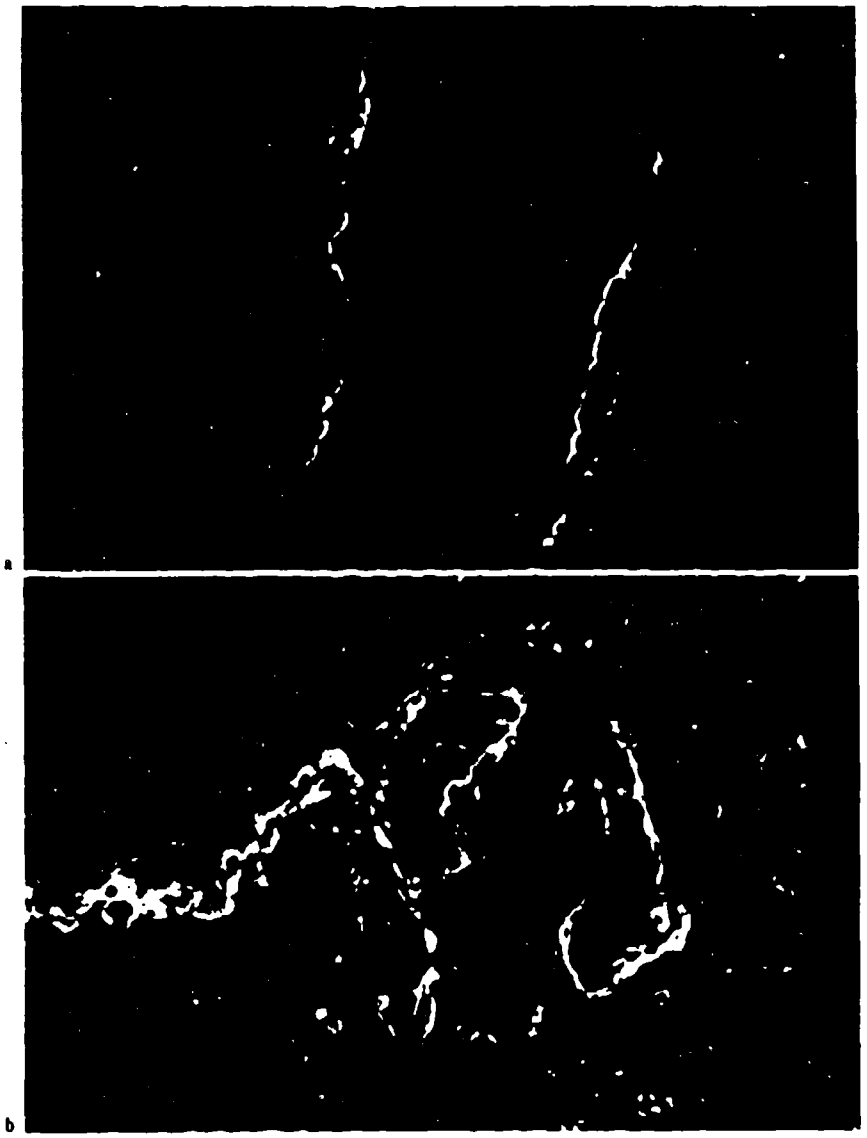


Fig. 7a, b. Immunofluorescent staining of LCMV GP1 (a) on ependymal cells lining a ventricule and (b) the choroid plexus of a C57B1·6 mouse infected 6 days earlier with LCMV-ARM. Staining with FITC-conjugated monoclonal antibody to GP1. Original magnification × 250

areas of the CNS. In contrast, these workers found no significant expression of viral glycoproteins. It was speculated that the presence of NP on the neuronal polyribosomes compromised their function (see FRANCIS et al., this volume).

Acute LCMV infection following intracerebral inoculation results in a fatal choriomeningitis (reviewed in BUCHMEIER et al. 1980b). We have used monoclonal antibodies against individual virus structural proteins to study their ex-

pression in the CNS following acute infection (BUCHMEIER and KNOBLER 1984). As is evident from Fig. 7, viral GP1 is expressed on the apical surfaces of ependymal cells in the CNS. At this site the glycoprotein (and perhaps also other virally coded proteins) triggers the well-characterized immune response which results in choriomeningitis and death.

Finally, the role of the viral L gene-encoded proteins in pathogenesis has recently been explored by RIVIERE et al. (1985, this volume) using genetic reassortants between strains of LCMV that differed in virulence for guinea pigs. These workers demonstrated that L RNA-encoded products were necessary for expression of the pathogenic potential of the virus. Each of these topics will be examined in detail in later chapters. It is clear from such studies that understanding the molecular basis of viral persistence and pathogenesis of arenaviruses is an attainable goal.

*Acknowledgements.* This is publication number 4477-IMM from the Department of Immunology, Scripps Clinic and Research Foundation. This study was supported by USPHS grant No. AI 16102 and training grant No. AG00080. We thank DONNA URANOWSKI for manuscript preparation.

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VIROLOGY 157, 145–155 (1987)

# Molecular Characterization of the Genomic S RNA Segment from Lymphocytic Choriomeningitis Virus

PETER J. SOUTHERN,[1] MANDALESHWAR K. SINGH, YVES RIVIERE,[2] DAVID R. JACOBY,[3] MICHAEL J. BUCHMEIER, AND MICHAEL B. A. OLDSTONE

*Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

*Received September 12, 1986; accepted November 5, 1986*

**We have used cDNA clones derived from the genomic S RNA segment of lymphocytic choriomeningitis virus (LCMV), Armstrong strain, as hybridization probes to monitor virus gene expression during acute infections. Our results with strand-specific probes confirm the ambisense character of the LCMV S RNA segment and document the presence of both genomic sense and genomic complementary sense RNA species over the time course of infection. We have used nucleotide sequence information to predict primary amino acid sequences for the major viral structural proteins, nucleoprotein (NP) and glycoprotein (GP-C). Antibodies raised against synthetic peptides derived from these predicted protein sequences have indicated that the gene order for the S segment is 3′ NP → 5′ GP-C and provided direct demonstration that the GP-1 portion of the GP-C precursor is encoded nearest the 5′ end of the S segment. Comparison of the predicted amino acid sequences for NP and GP-C between the Armstrong CA-1371 strain and the WE strain shows over 90% amino acid identity. This suggests that significant differences described for the pathogenic potential of the Arm and WE strains in C3H mice reside in one or a very few critical amino acid changes.** © 1987 Academic Press, Inc.

## INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) infection of its natural host, the mouse, has served as an excellent model for virus–host interactions. Key observations from this system relate to virus-induced immune response disease (Rowe, 1954), detection of thymus (T) markers on cytotoxic lymphocytes (Cole *et al.*, 1972), requirements for both viral antigen and syngeneic host major histocompatibility proteins for cytotoxic T cell recognition of virally infected cells (Zinkernagel and Doherty, 1974), virus-induced immune complex disease (Oldstone and Dixon, 1969), and lack of immunologic tolerance in persistent viral infections (Oldstone and Dixon, 1967). A novel mechanism of disease induction caused by virus disruption of differentiated cell function, but without associated cell lysis, has also emerged from studies of the LCMV system (Oldstone *et al.*, 1982). Until recently, however, there was only limited information available relating to the molecular biology of LCMV infection and the structural organization of the viral genome. We and others (Auperin *et al.*, 1984; Romanowski *et al.*, 1985; Clegg and Oram, 1985; Auperin *et al.*, 1986) have initiated cDNA cloning experiments to define the complete genetic potential of the virus and to understand viral transcription and replication mechanisms.

The genome of LCMV contains two single-stranded RNA segments (designated L and S, approximate lengths 8–9 and 3.5 kb, respectively) that encode essentially nonoverlapping genetic information. Common sequences identified at the 3′ termini of L and S RNA segments are believed to specify a recognition site for viral polymerase (Auperin *et al.*, 1982) and/or a nucleation site for formation of ribonucleoprotein (RNP) complexes. The viral S RNA contains coding regions for the major structural proteins: NP, an internal nucleoprotein involved with RNP complexes and GP-C, a precursor glycoprotein which is cleaved to produce the mature glycoproteins GP-1 and GP-2 that are present on the surface of virion particles (Buchmeier and Oldstone, 1979; Riviere *et al.*, 1985a). The L RNA is thought to encode a high-molecular-weight (>150 kDa) polymerase or replicase molecule required for viral transcription and replication. Information relating to the L RNA segment is still limited and further cDNA cloning and sequencing experiments are required to determine whether there is any additional protein coding capacity within the L segment.

Several different strains of LCMV have been described that differ with respect to (1) disease potential in experimentally infected animals (Dutko and Oldstone,

[1] To whom requests for reprints should be addressed.

[2] Present address: Institut Pasteur, Oncologie Virale, 75015 Paris, France.

[3] Present address: Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.



0042-6822/87 $3.00
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1983; reviewed by Southern and Oldstone, 1987), (2) reactivity with LCMV-specific monoclonal antibodies (Buchmeier, 1984), and (3) T1 oligonucleotide fingerprints (Dutko and Oldstone, 1983). In addition, a lymphotropic variant virus has recently been characterized that was selected by a single passage *in vivo* of the parental LCMV Armstrong virus (Ahmed *et al.*, 1984). Nucleotide sequence information from different strains of LCMV and different members of the arenavirus family allows detailed comparisons at the molecular level and may eventually lead to identification of the processes involved with disease potential and virus evolution. From reassortments of viral RNA segments, it has become clear that induction of growth hormone deficiency disease in C3H mice is associated with the Armstrong S RNA segment and that the WE S segment has no capability of inducing the same disease (Riviere *et al.*, 1985b). An explanation for this biological difference may now be forthcoming on the basis of a limited number of amino acid substitutions between the structural proteins of the Armstrong and WE strains.

## MATERIALS AND METHODS

### Virus purification

Semiconfluent (30–50% confluency) monolayers of BHK-21 cells were infected with stocks of triple plaque-purified LCMV strain Arm CA-1371 at a multiplicity of infection (m.o.i.) of 0.1. Supernatant fluids containing virus were harvested 72 hr postinfection and virus was precipitated at 4° in the presence of polyethylene glycol (PEG 6000, 7.0% w/v) and 0.375 *M* NaCl. Virus pellets were collected by centrifugation at 8000 rpm for 30 min and then resuspended in small volumes of TNE (10 m*M* Tris–HCl, pH 7.5, 1 m*M* EDTA, 100 m*M* NaCl). The concentrated virus suspension was layered on top of a discontinuous renografin gradient (50, 35, and 10% v/v renografin in TNE) and centrifuged at 4° in an SW41 rotor for 75 min at 35,000 rpm. Virus bands (clearly visible at the 35:10% interface) were collected by side puncture and diluted 1:1 with TNE before layering on top of a continuous 50–10% renografin gradient. The continuous gradients were centrifuged in an SW41 rotor at 4° for 12–16 hr at 30,000 rpm and the virus bands were again collected by side puncture. The concentrated virus bands were diluted 1:5 with TNE and the virus was pelleted by centrifugation in an SW41 rotor at 4° for 45 min at 35,000 rpm. The supernatant liquid was immediately removed and discarded and the virus pellets were resuspended in cold TNE by gentle agitation. Resuspended virus was extracted twice with preequilibrated phenol and the released viral RNA in the aqueous phase was precipitated at −20° by the addition of 2 vol of ethanol

### cDNA synthesis and cloning

Purified virion RNA preparations were used as templates for cDNA synthesis with either avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim) or Moloney murine leukemia virus reverse transcriptase (BRL) under conditions described by the manufacturers. Priming of cDNA synthesis from viral templates was achieved by (i) 3′ polyadenylation of virion RNA with polynucleotide phosphorylase (Engel and Davidson, 1978) and subsequent annealing with a synthetic $dT_{8-12}$ primer or (ii) annealing with a calf thymus random primer (Taylor *et al.*, 1976) or (iii) annealing with sequence specific oligonucleotides. Clones have been isolated successfully from each of the primers but, in general, it has been difficult to recover cDNA clones longer than about 2 kb despite careful size fractionation of the double-stranded cDNA prior to cloning (see below). After alkali treatment to remove RNA templates, second-strand synthesis was initiated by looping back within the single-stranded cDNA. Reverse transcriptase and Klenow DNA polymerase I were used sequentially in an attempt to ensure complete second-strand synthesis (Maniatis *et al.*, 1982). Double-stranded cDNA was treated with S1 nuclease to remove loops and any single-stranded tails and then treated with terminal transferase with limiting concentrations of dCTP to add homopolymer tails to 3′ termini. Tailed, double-stranded cDNA preparations were passed over small columns of sepharose CL4B to separate the cDNA according to size and only fractions with estimated sizes over 500 bp were used for subsequent cloning. Linear pBR322, cleaved at the *Pst*I site and subsequently purified from a preparative agarose gel, was similarly tailed with dGTP (Rowekamp and Firtel, 1980). The tailed cDNA samples and pBR322 vector were annealed and then used to transform competent HB101 cells. Transformants were selected in the presence of tetracycline and then colonies containing LCMV recombinant plasmids were identified by colony hybridization (Grunstein and Hogness, 1975) using hydrolyzed, 5′ end-labeled LCMV S RNA as a probe (Donis-Keller, *et al.*, 1977). Plasmid DNA was purified from positive colonies by standard Triton–lysozyme lysis of bacterial cultures followed by two successive bandings in cesium chloride–ethidium bromide gradients. LCMV specificity of individual clones was confirmed by nick-translation labeling *in vitro* and hybridization against total infected cell RNA samples and control, uninfected cell RNA (see text).

### Nucleotide sequencing

3′ and 5′ end-labeled DNA fragments were used in the chemical sequencing technique (Maxam and Gil-

bert, 1980). Sequences were determined on both DNA strands for more than 90% of the NP and GP coding regions and independent, overlapping clones were analyzed to verify the accuracy of these sequences. Computer processing of sequence information used a VAX 11/750 computer and standard programs in conjunction with the EMBL and GenBank databases.

### RNA extraction and gel electrophoresis

Cell monolayers were lysed by treatment with guanidinium thiocyanate (Chirgwin *et al.*, 1979) and chromosomal DNA was sheared by vigorous shaking. Total cell RNA was purified away from DNA by pelleting through a cushion of 5.7 *M* CsCl. RNA pellets were washed in 70% ETOH, redissolved in sterile water, and precipitated by the addition of salt and ETOH. Samples of RNA were precipitated from ETOH, redissolved in sterile water, and stored in small volumes at −70°. RNA concentrations were determined spectrophotometrically.

RNA samples (typically 10–30 μg) were denatured with glyoxal (McMaster and Carmichael, 1977) and separated in 1 or 1.5% agarose gels in 10 m*M* $NaPO_4$, pH 6.5. After electrophoresis, RNA within the gel was transferred directly to nitrocellulose filters (Thomas, 1980) by capillary diffusion of buffer (20× SSC) at room temperature. Filters were baked at 80° in a vacuum oven, prehybridized in 50% deionized formamide, 5× SSC, 2.5× Denhardt's solution with 100 μg/ml boiled, sonicated salmon sperm carrier DNA at 37°, and then hybridized in the same solution either at 37° with nick-translated probes (Rigby *et al.*, 1977) or at 55° with strand-specific RNA probes (Melton *et al.*, 1984). Filters were washed (30 min each wash) initially in 2× SSC, 0.1% SDS at 37° (twice), then at 55° in the same solution and finally at 55° in 0.1× SSC, 0.1% SDS, 0.1% Tween 20, and exposed at −70° with Kodak XAR-5 film and Cronex Lightning Fast intensification screens. After suitable exposure times, probes were stripped from the filters by washing in 0.1× SSC, 0.1% SDS, 0.1% Tween 20 at 85° for 2–3 hr, and then filters were recycled with a different probe.

### Synthesis of hybridization probes

Restriction fragments from different regions of the cDNA clones were purified from preparative agarose gels (Vogelstein and Gillespie, 1978) and then labeled *in vitro* using Klenow DNA polymerase I in a modified nick-translation reaction. Strand-specific probes were initially prepared by subcloning purified cDNA restriction fragments into M13 mp8 and mp9 vectors (Hu and Messing, 1982). A hybridization primer was used for *in vitro* labeling under standard conditions. The labeled strand was left associated with the template DNA for the hybridization reactions and we found that a cross-linking step (Brown *et al.*, 1982) could be omitted without any adverse effect on the hybridization signal. Alternatively, selected regions from the cDNA clones were subcloned into SP6 vectors (Promega Biotech) and these derivative plasmids were used as templates for *in vitro* synthesis of labeled RNA probes (Melton *et al.*, 1984). We have routinely removed G-C tails from the cDNA clones in the process of subcloning into SP6 vectors in order to minimize nonspecific background problems when using these single-stranded RNA probes.

### *In vivo* labeling of LCMV viral RNAs

Monolayers of BHK cells (75% confluency) were infected at high multiplicity (m.o.i. 2–5) with the plaque-purified stock of LCMV and allowed to grow for 12 hr at 37°. Actinomycin D (5 μg/ml) was added to the culture medium for 1 hr and then the medium was removed and replaced with 1/10 phosphate medium containing 1–2 mCi of [$^{32}$P]inorganic phosphate. The infected cells were incubated for an additional 4 hr at 37° and then the cells were trypsinized and lysed by dounce homogenization in the presence of NP40. After a clearing spin at 10K rpm to remove nuclei and cell debris, the supernatant was layered on top of a discontinuous sucrose gradient (2.5 *M*, 1.0 *M*, 0.5 *M* sucrose in 0.3 *M* NaCl, 5 m*M* $MgCl_2$, 0.05 *M* Tris–HCl, pH 7.5, plus heparin) and centrifuged in an SW41 rotor at 35K rpm for 90 min (conditions adapted from Gough and Adams, 1978). A visible band at the 2.5 *M*/1.0 *M* interface of polysomes plus viral ribonucleoprotein complexes was collected by side puncture. This material was diluted fivefold in TNE, extracted once with preequilibrated phenol and then the aqueous phase was precipitated with ETOH at −20°. Samples of these labeled RNA preparations were denatured with glyoxal and analyzed on agarose gels. After electrophoresis, the gels were dried onto DE81 paper and exposed for autoradiography.

### Peptides and antipeptide antisera

Detailed descriptions of the synthesis of peptides, coupling to inert carriers, and immunization strategies will be published elsewhere (Buchmeier *et al.*, 1987). Peptides were synthesized that correspond to regions of transition from hydrophilic to hydrophobic character and antisera were screened, initially by ELISA against disrupted purified virus and then by Western blotting. Antigen–antibody complexes were detected with iodinated protein A and visualized by autoradiography using Kodak XRP-1 film. In all cases, immunologic

specificity was demonstrated by preincubation of antisera with the corresponding soluble peptide which effectively blocked any reactivity toward the target viral antigen.

## RESULTS

### Construction and identification of LCMV specific cDNA clones

Samples of LCMV RNA extracted from purified virions routinely contained viral L and S RNAs and host ribosomal 28 and 18 S RNAs in approximately equivalent amounts (Fig. 1). A high-molecular-weight band was also sometimes observed (Fig. 1) that contained randomly sheared fragments of chromosomal DNA which were either attached to the outside of virion particles or were fortuitously incorporated into virion particles. This contaminating DNA potentially explains the source of non-LCMV, nonribosomal clones that have been recovered (P. J. Southern *et al.*, unpublished results).

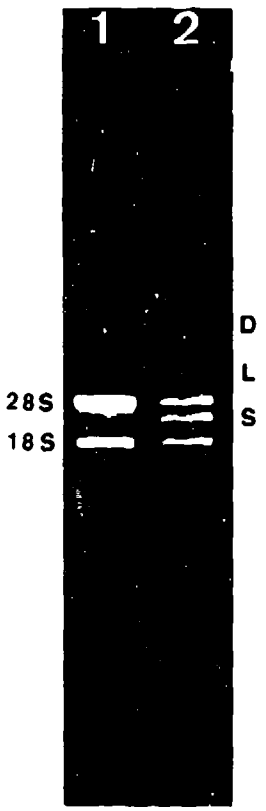



FIG. 1. Analysis of nucleic acids extracted from purified LCMV virions. Nucleic acid samples in sterile water were separated by electrophoresis in a nondenaturing 1% agarose gel formed on a microscope slide. Bands were visualized by ethidium bromide fluorescence in ultraviolet light. Lane 1: 1 μg total cell RNA from uninfected BHK cells. Lane 2: 0.9 μg of nucleic acid from purified LCMV virions. 28 S and 18 S refer to host ribosomal RNA bands; L and S refer to LCMV genomic RNA species. The high-molecular-weight band (D) contains cell DNA and consequently does not hybridize to labeled viral L or S probes (data not shown).

These purified virion RNA preparations were used as templates for reverse transcriptase under standard reaction conditions (Methods section). The cDNAs were then cloned into plasmid pBR322 and propagated by transformation of *Escherichia coli* HB101. LCMV recombinant plasmids were identified by colony hybridization (Grunstein and Hogness, 1975) using purified viral S RNA as a hybridization probe. Positive clones from this screening were rechecked for viral specificity by nick-translation labeling *in vitro* and hybridization to nitrocellulose strips containing total cell RNA from LCMV-infected and control, uninfected cells (Thomas, 1980). This hybridization analysis indicated two classes of S derived cDNA clones by virtue of differential recognition of subgenomic RNAs (see Fig. 4 for example). Overlapping DNA clones were identified by DNA–DNA hybridization and common restriction sites have now been used to reconstruct intact LCMV genes.

### Analysis of information from nucleotide sequencing

We have used the chemical sequencing method (Maxam and Gilbert, 1980) to determine the nucleotide sequences of the LCMV S coding regions and then deduced the primary amino acid sequences for these structural proteins. The nucleotide sequences were derived by >90% sequencing of both DNA strands and 30–40% of the gene sequences were confirmed by sequencing independent, overlapping cDNA clones. The complete nucleotide sequence of the LCMV Arm genomic S RNA segment will be published elsewhere (P. J. Southern *et al.*, in preparation). Direct RNA sequencing of purified LCMV virion RNA has confirmed the accuracy and representative nature of the LCMV cDNA sequence in the GP-C coding region (M. Salvato and M. B. A. Oldstone, unpublished observations).

Assignments of the NP and GP coding regions were established using synthetic peptide techniques (see below). Despite significant differences in biological properties (Dutko and Oldstone, 1983), the LCMV Arm and WE strains show extensive homology in primary amino acid sequences for both NP and GP (Fig. 2). The viral nucleoproteins contain 558 amino acid residues of which 534 are identical. Differences are scattered throughout the length of the proteins and many of the changes involve conservative substitutions. The viral glycoprotein precursors contain 498 amino acid residues of which 466 are identical. Again, differences are scattered throughout the length of the proteins and many of the changes are conservative. Changes which

appear most likely to affect the structure of the folded glycoproteins are listed in Table 1.

### Gene mapping with antipeptide antisera

Computer analysis of the nucleotide sequences identified potential protein coding regions within the cDNA clones. Synthetic peptides, corresponding to different regions of the predicted protein sequences, were used to elicit polyclonal antibody responses in rabbits. A peptide from the 3′ coding region (residues 454–462) in the S segment was shown to derive from NP, whereas antibodies raised against peptides from the 5′ coding region showed specific reactivity with the virion glycoproteins (Fig. 3). This experimental approach also demonstrated that, after cleavage, GP-1 is derived from the amino terminus and GP-2 from the carboxy terminus of the precursor GP-C molecule (Fig. 3).

### Pulse labeling of LCMV RNAs during acute infection

Replication of LCMV is believed to be confined to the cytoplasm of infected cells and there is no known DNA state. Virus replication is relatively insensitive to actinomycin D (Buck and Pfau, 1969; Rawls *et al.*, 1976), a potent inhibitor of host cell transcription, and we have taken advantage of this viral resistance to label intracellular viral RNAs selectively during an acute infection. Analysis of such labeled RNA preparations showed incorporation of $^{32}P$ into viral genomic sized L and S RNAs and significant incorporation into two subgenomic RNAs that were slightly smaller than the host 18 S ribosomal RNA (Fig. 4). These subgenomic RNAs were presumed to be of viral origin because they were not detected in the control, uninfected RNA sample and were therefore candidates for viral messenger RNA (mRNA) species. The analysis of virion nucleic acid by ethidium bromide fluorescence, however, did not show any significant accumulation of subgenomic RNAs in virion particles (Fig. 1).

### Hybridization analysis of intracellular subgenomic RNAs

The subgenomic RNAs were characterized further in RNA hybridization reactions (Thomas, 1980) using cloned cDNA probes derived from different regions of the viral genomic L and S RNAs. Figure 5 shows the results from hybridization with either a nucleoprotein or glycoprotein region-specific probe from the S segment and established that the two subgenomic RNAs were derived from essentially nonoverlapping regions of the S RNA. The larger subgenomic RNA corresponds to the NP region and may be present in somewhat greater amounts than the smaller GP region RNA (Fig. 5). (Note that Fig. 5 does not faithfully reveal the relative amounts of the two subgenomic RNAs as different sized restriction fragments were used to synthesize the respective NP and GP probes.) As the infection progressed both genomic sized and subgenomic S RNAs were present in reduced amounts in the whole cell RNA samples but the relative reduction was more marked for the subgenomic RNAs than for the full-length genomic S species.

Hybridization to purified LCMV virion RNA with NP and GP region probes produced very low levels of signal from the subgenomic RNA species (Fig. 5). This finding was reproducible with independent purified virion RNA preparations but it is not clear whether low level encapsidation of presumptive mRNAs is essential for virus infectivity or whether there is coincidental trapping of membrane-bound polysomes as virion particles form and are released from the surface of infected cells.

### Polarity of intracellular viral RNAs

Hybridization probes labeled by nick-translation incorporate label randomly into both strands of a template DNA and it is not possible to use these probes to determine the polarity of RNA species that are detected by hybridization. Therefore, strand-specific hybridization probes were prepared in order to detect the presence of full-length genomic complementary RNA (an obligatory intermediate in the replication of genomic sense S RNAs) and to examine the polarity of subgenomic RNA species. The full-length genomic complementary RNA accumulated at late times (24–48 hr postinfection using an input multiplicity of 0.1–1.0 PFU per cell) and was then present in approximately the same amount as the full-length genomic sense RNA (Fig. 6). This figure also demonstrated that the subgenomic RNA derived from the GP coding region had the same polarity as the genomic S RNA. Hybridizations with strand-specific probes from the NP region have established that the putative NP mRNA is complementary to the genome and therefore can be transcribed from the incoming genomic sense RNA in the virion (data not shown).

## DISCUSSION

The arenaviruses were originally classified as negative-strand viruses but, from cDNA cloning and sequencing and hybridization studies presented here and elsewhere (Auperin *et al.*, 1984; Romanowski *et al.*, 1985; Clegg and Oram, 1985), this now requires modification. The viral S RNA has an ambisense character in which the NP mRNA is complementary to the genome (genuine negative-strand character) whereas the presumptive GP mRNA is in the sense of the genome

A

```
           1         5         10        15        20        25        30        35        40        45        50        55
ARM        M S L S K E V K S F Q W T Q A L R R E L Q S F T S D V K A A V I K D A T N L L N G L D F S E V S N V Q R I M R
WE         M S L S K E V K S F Q W T Q A L R R E L Q G F T S D V K A A V I K D A T S L L N G L D F S E V S N V Q R I M R
Common     M S L S K E V K S F Q W T Q A L R R E L Q   F T S D V K A A V I K D A T   L L N G L D F S E V S N V Q R I M R

           56        60        65        70        75        80        85        90        95        100       105       110
ARM        K E K R D D K D L Q R L R S L N Q T V H S L V D L K S T S K K N V L K V G R L S A E E L M S L A A D L E K L K
WE         K E R R D D K D L Q R L R S L N Q T V H S L V D P K S T S K K N V L K V G R L S A E E L M T L A A D L E K L K
Common     K E   R D D K D L Q R L R S L N Q T V H S L V D   K S T S K K N V L K V G R L S A E E L M   L A A D L E K L K

           111       115       120       125       130       135       140       145       150       155       160       165
ARM        A K I M R S E R P Q A S G V Y M G N L T T Q Q L D Q R S Q I L Q I V G M R K P Q Q G A S G V V R V W D V K D S
WE         A K I M R T E R P Q A S G V Y M G N L T A Q Q L D Q R S Q I L Q M V G M R R P Q Q G A S G V V R V W D V K D S
Common     A K I M R   E R P Q A S G V Y M G N L T   Q Q L D Q R S Q I L Q   V G M R   P Q Q G A S G V V R V W D V K D S

           166       170       175       180       185       190       195       200       205       210       215       220
ARM        S L L N N Q F G T M P S L T M A C M A K Q S Q T P L N D V V Q A L T D L G L L Y T V K Y P N L N D L E R L K D
WE         S L L N N Q F G T M P S L T M A C M A K Q S Q T P L N D V V Q A L T D L G L L Y T V K Y P N L S D L E R L K D
Common     S L L N N Q F G T M P S L T M A C M A K Q S Q T P L N D V V Q A L T D L G L L Y T V K Y P N L   D L E R L K D

           221       225       230       235       240       245       250       255       260       265       270       275
ARM        K H P V L G V I T E Q Q S S I N I S G Y N F S L G A A V K A G A A L L D G G N M L E S I L I K P S N S E D L L
WE         K H P V L G V I T E Q Q S S I N I S G Y N F S L G A A V K A G A A L L H G G N M L E S I L I K P S N S E D L L
Common     K H P V L G V I T E Q Q S S I N I S G Y N F S L G A A V K A G A A L L   G G N M L E S I L I K P S N S E D L L

           276       280       285       290       295       300       305       310       315       320       325       330
ARM        K A V L G A K R K L N M F V S D Q V G D R N P Y E N I L Y K V C L S G E G W P Y I A C R T S I V G R A W E N T
WE         K A V L G A K K K L N M F V S D Q V G D R N P Y E N I L Y K V C L S G E G W P Y I A C R T S V V G R A W E N T
Common     K A V L G A K   K L N M F V S D Q V G D R N P Y E N I L Y K V C L S G E G W P Y I A C R T S   V G R A W E N T

           331       335       340       345       350       355       360       365       370       375       380
ARM        T I D L T S E K P A V N S P R P A P G A A G P P Q V G L S Y S Q T M L L K D L M G G I D P N A P T W I D I E G
WE         T I D L T N E K L V A N S S R P V P G A A G P P Q V G L S Y S Q T M L L K D L M G G I D P N A P T W I D I E G
Common     T I D L T   E K       N S   R P   P G A A G P P Q V G L S Y S Q T M L L K D L M G G I D P N A P T W I D I E G

           386       390       395       400       405       410       415       420       425       430       435       440
ARM        R F N D P V E I A I F Q P Q N G Q F I H F Y R E P V D Q K Q F K Q D S K Y S H G M D L A D L F N A Q P G L T S
WE         R F N D P V E I A I F Q P Q N G Q F I H F Y R E P T D Q K Q F K Q D S K Y S H G M D L A D L F N A Q A G L T S
Common     R F N D P V E I A I F Q P Q N G Q F I H F Y R E P   D Q K Q F K Q D S K Y S H G M D L A D L F N A Q   G L T S

           441       445       450       455       460       465       470       475       480       485       490       495
ARM        S V I G A L P Q G M V L S C Q G S D D I R K L L D S Q N R K D I K L I D V E M T R E A S R E Y E D K V W D K Y
WE         S V I G A L P Q G M V L S C Q G S D D I R K L L D S Q N R R D I K L I D V E M T K E A S R E Y E D K V W D K Y
Common     S V I G A L P Q G M V L S C Q G S D D I R K L L D S Q N R   D I K L I D V E M T   E A S R E Y E D K V W D K Y

           496       500       505       510       515       520       525       530       535       540       545
ARM        G W L C K M H T G I V R D K K K K E I T P H C A L M D C I I F E S A S K A R L P D L K T V H N I L P H D L I F
WE         G W L C K M H T G V V R D K K K K E I T P H C A L M D C I I F E S A S K A R L P D L K T V H N I L P H D L I F
Common     G W L C K M H T G   V R D K K K K E I T P H C A L M D C I I F E S A S K A R L P D L K T V H N I L P H D L I F

           551       555       560
ARM        R G P N V V T L
WE         R G P N V V T L
Common     R G P N V V T L
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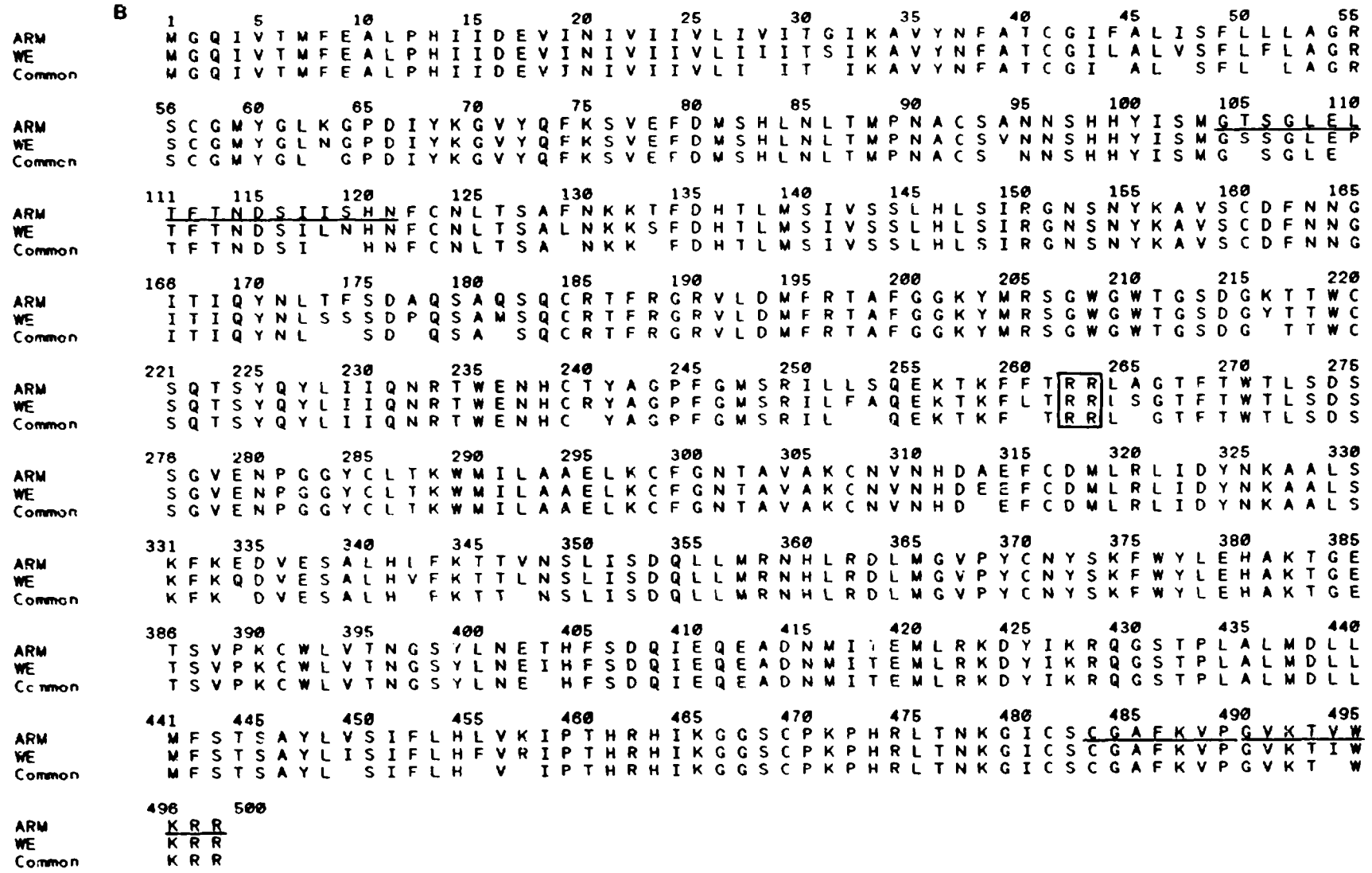



FIG. 2. Alignment of predicted amino acid sequences for the structural proteins of LCMV strains Armstrong and WE. Regions of underlined sequence represent the synthetic peptides used in Fig. 3. (A) Nucleocapsid protein (NP); (B) glycoprotein (GP-C). The cleavage site for the precursor GP-C molecule is indicated (R-R residues at positions 262-263) together with the GP-1 and GP-2 coding regions.

**TABLE 1**

SIGNIFICANT AMINO ACID CHANGES IN GP-C*

| GP-C residue | LCMV arm | LCMV WE |
|---|---|---|
| 110 | L | P |
| 133 | T | S |
| 173 | T | S |
| 174 | F | S |
| 177 | A | P |
| 181 | Q | M |
| 216 | K | Y |
| 240 | T | R |
| 253 | S | A |
| 265 | A | S |
| 313 | A | E |

* GP-C residues 1–262 = GP 1, 263–498 = GP-2.

("pseudo-positive" strand character). This gene arrangement is suggestive of a mechanism that might provide temporal regulation of arenavirus gene expression. The GP mRNA must be transcribed from a genomic complementary RNA template and, as shown in Fig. 6, there are abundant levels of full-length genomic complementary RNA at late times in acutely infected cells. Therefore, the accumulation of this full-length complementary RNA species can be explained because it is required both as a replication intermediate for the synthesis of progeny genomic sense RNAs and as a template for GP mRNA transcription. A similar ambisense coding arrangement has also been described for the genomic S RNA of the phlebovirus, Punta Toro (Ihara *et al.*, 1984).

The accumulation and subsequent reduction of steady-state levels of genomic-sized RNA and the two subgenomic RNAs may provide some indication of the molecular basis for viral persistence. After a peak of progeny virus production there is a reduction in the release of infectious virus from infected cells and eventually production of infectious virus usually ceases as the cells enter into a state of long-term persistent infection (Welsh and Oldstone, 1977; Welsh and Buchmeier, 1979). The observed reductions in release of infectious virus at late times during acute infection and during persistent infection may be a direct reflection of primary reduction in viral replication and transcription. The ambisense coding arrangement for the genomic S RNA segment suggests a mechanism whereby reduced viral replication (specifically, a reduction in the level of full-length genomic complementary RNA) would limit the availability of template RNA for glycoprotein messenger RNA synthesis (Auperin *et al.*, 1984). Clearly, formation of infectious virion particles is dependent on the presence of viral glycoprotein in the cell membrane to initiate the budding process and the absence or significant reduction (10- to 50-fold) of surface glycoprotein for persistently infected cells both *in vivo* and *in vitro* (Oldstone and Buchmeier, 1982; Welsh and Buchmeier, 1979) could now be explained in terms

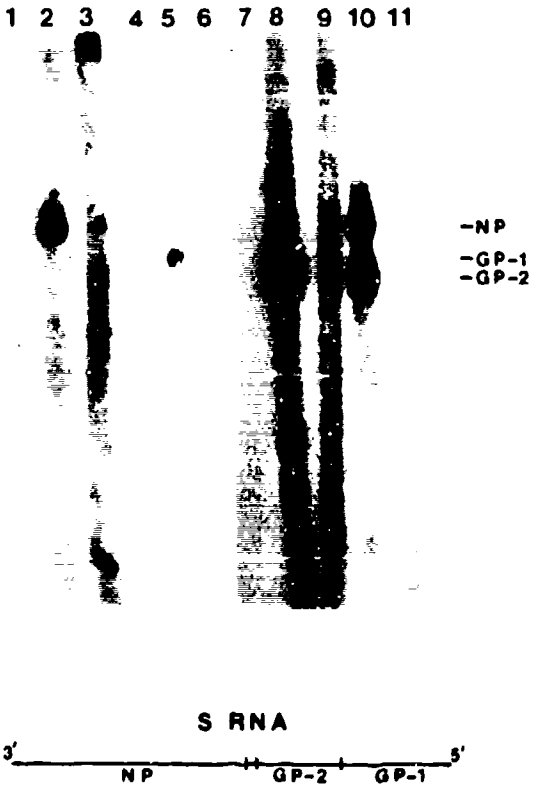



FIG. 3. Detection of LCM virion proteins in Western blotting experiments using antipeptide antibodies. Proteins from purified LCM virions were separated by electrophoresis through an SDS-polyacrylamide gel and then transferred by electroblotting to a nitrocellulose membrane. After transfer, the nitrocellulose was cut into strips and incubated with rabbit antibody preparations (1:50 dilutions) as indicated below. Prebleed serum samples were obtained from each rabbit prior to immunization with the specific peptide. Recognition of specific viral proteins was blocked by preincubating antisera with 40 μg of the homologous peptide. Antigen–antibody complexes were detected with iodinated protein A and bands were visualized using Kodak XRP-1 film. The figure represents a composite of different exposure times for the various antibody preparations but all of the strips came from the same gel and were processed simultaneously. Lane 1, prebleed; lane 2, antipeptide NP 454-462; lane 3, antipeptide NP 454-462 preincubated with the specific peptide; lane 4, prebleed; lane 5, antipeptide GP-C 104-121; lane 6, antipeptide GP-C 104-121 preincubated with the specific peptide; lane 7, prebleed; lane 8, antipeptide GP-C 483-498; lane 9, antipeptide GP-C 483-498 preincubated with the specific peptide; lane 10, polyclonal guinea pig anti-LCMV antiserum; lane 11, normal guinea pig serum. The lower part of the figure shows the positions of the coding regions for NP, GP-1, and GP-2 within the genomic S RNA segment. The actual locations of the peptides are marked on the amino acid sequences in Fig. 2.

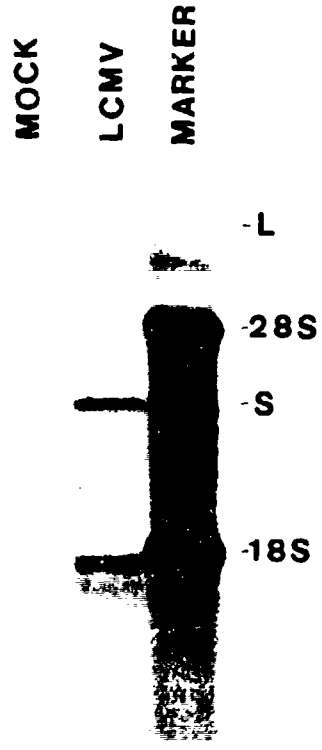



FIG. 4. Analysis of *in vivo*-labeled LCMV intracellular RNAs. Cells were pulse labeled with [$^{32}$P]inorganic phosphate for 4 hr at 12–16 hr postinfection (m.o.i. approximately 3) and intracellular RNP/polysome complexes were purified as described under Methods. RNA samples were denatured with glyoxal and then electrophoresed in an agarose gel. The gel was then dried and exposed to X-ray film. Mock: uninfected BHK cells treated with actinomycin D and $^{32}$P. LCMV: LCMV-infected BHK cells treated with actinomycin D and $^{32}$P. Marker: LCMV-infected BHK cells treated with $^{32}$P but without actinomycin D. L and S refer to viral genomic RNA segments; 28 S and 18 S refer to host ribosomal RNAs. Note the presence of two viral-specific subgenomic RNAs just below the 18 S ribosomal marker band.

of reduced viral replication. We have begun to analyze LCMV intracellular RNA in long-term persistently infected tissue culture cell lines and have evidence for genomic sized RNAs and low levels of subgenomic, presumptive mRNA species. However, we have also detected in these same cell lines, at much higher abundance than the mRNAs, novel subgenomic RNAs that are candidates for defective or defective interfering RNA species. Parallel experiments analyzing viral RNAs extracted from tissues of persistently infected mice (S. J. Francis, M. B. A. Oldstone, and P. J. Southern, manuscript in preparation) have shown a heterogeneous population of viral RNAs but with detectable levels of the subgenomic mRNA species.

The intracellular S, subgenomic RNAs that are readily apparent in acute virus infections (Figs. 4–6) are likely to be viral messenger RNAs. Auperin and colleagues (1984), working with Pichinde virus RNAs, have shown by *in vitro* translation that the larger intracellular subgenomic RNA can support NP synthesis. Equivalent, direct demonstration of coding potential for the smaller subgenomic RNA is not currently available but all the hybridization, sequencing and synthetic peptide experiments link this RNA to GP. The subgenomic RNAs are not retained on oligo(dT)-cellulose under standard conditions and there does not appear to be any polyadenylation signal within the viral RNA templates. Experiments are now in progress to examine sequences at the 5′ and 3′ termini of these subgenomic RNAs to identify precise sites of transcription initiation and termination and to evaluate the role of an intergenic hairpin region (Auperin *et al.*, 1984) in transcription termination and possible stabilization of the mRNAs at the 3′ end.

Antibodies raised against synthetic peptides have aligned open reading frames detected in the nucleotide sequences with actual viral proteins. Using these techniques, we have confirmed the S gene order as 3′ NP → GP-C 5′ and have found that GP-1 comprises the $NH_2$-terminal portion and GP-2 the COOH-terminal portion of the precursor GP-C molecule. Other exper-

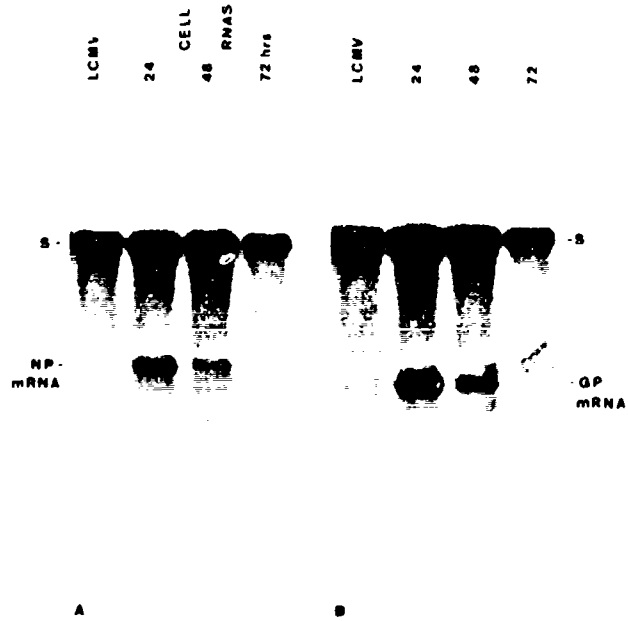



FIG. 5. Time-course analysis of intracellular S RNAs. Monolayers of BHK cells were infected with LCMV (m.o.i. approximately 1) and lysed at the indicated times by treatment with guanidinium thiocyanate. Total cell RNA was recovered by pelleting through CsCl. RNA samples (20 μg) were denatured with glyoxal, separated according to size by electrophoresis in an agarose gel, and then were transferred to a nitrocellulose filter. The filter was hybridized sequentially with nick-translated probes specific to the NP region (A) and the GP region (B). Purified virion RNA (30 ng) (LCMV) was included as a marker.

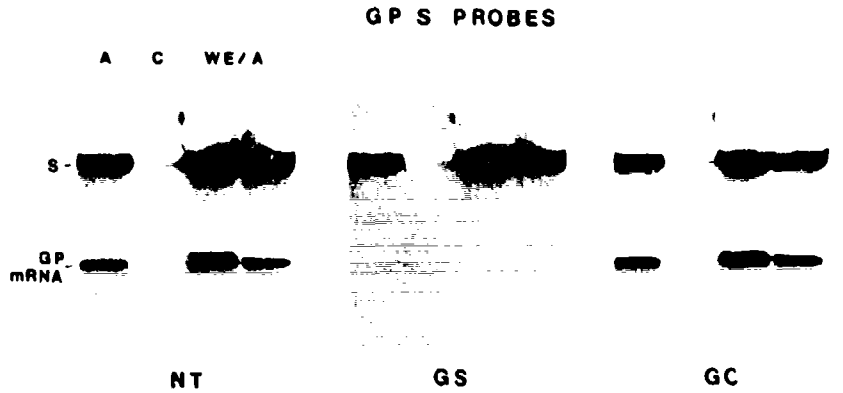



FIG. 6. Strand-specific hybridization to LCMV intracellular S RNAs. Total infected cell RNA samples were purified, electrophoresed, and transferred to a nitrocellulose filter (see Methods and legend of Fig. 4 for details). The filter was hybridized sequentially with a nick-translated probe (NT) and two M13 strand-specific probes, genomic-sense probe (GS) and genomic complementary-sense probe (GC). All three probes were derived from the same cDNA clone covering the central portion and COOH terminus of GP-1. A: LCMV Arm-infected BHK cells; C: control, uninfected BHK cells; WE/A: BHK cell RNA from infections with two independent clones of the reassortant virus L/S = WE/Arm (see Riviere *et al.* 1985b, for details).

iments have extended these peptide mapping studies to pinpoint the cleavage site within the GP-C precursor at residues 262-263 (Buchmeier *et al.*, 1987).

The extensive amino acid homology between the LCMV Arm and WE strains suggests that a limited number of amino acid changes may be sufficient to account for extensive biological differences. GP-1, the major viral glycoprotein and the target for virus neutralizing antibody, contains several significant individual amino acid changes (Table 1). The clustering of changes between residues 173 and 181 in GP-C may account for an additional neutralizing epitope that has been observed in LCMV-Arm but not in LCMV-WE (Parekh and Buchmeier, 1986). There are five potential sites for N-linked glycosylation that are precisely conserved between Arm and WE GP-1. The amino acid sequence of GP-2 is more highly conserved than GP-1. We are currently examining the various amino acid changes observed between the glycoproteins of LCMV-Arm and LCMV-WE to determine their relative involvement in differences of disease potential. We now have synthetic peptides that span the entire GP-C molecule and antibodies to such peptides may provide a mechanism for selection of site-specific viral variants with possible alterations in biological properties.

## ACKNOWLEDGMENTS

This is Publication 4398-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037. This work was supported in part by USPHS Grants AG-04342, AI-09484, and NS-12428 and by USAMRIID Contract C-3013. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents. We thank Drs. David Auperin and David Bishop for communicating information in advance of publication. We thank Ms. Nicole Nguyen, Ms. Kaleo Wooddell, and Mrs. Gay L. Schilling for excellent technical and secretarial assistance.

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# Lymphocytic Choriomeningitis, Mouse

Kathryn E. Wright and Michael J. Buchmeier

## Gross Appearance

Tissue from the central nervous system of affected mice undergoes no alteration which is recognizable macroscopically.

## Microscopic Features

The classical lesion of lymphocytic choriomeningitis occurs in adult mice only and is characterized by extensive infiltration of mononuclear cells into the meninges and choroid plexus (Rivers and Scott 1936; Lillie and Armstrong 1945). Infiltration is focal, and reports differ as to whether the third and fourth (Lillie and Armstrong 1945) or the lateral ventricles (Walker et al. 1975) are more affected. The ventricular spaces can contain both polymorphonuclear and mononuclear cells (Tosolini and Mims 1971; Walker et al. 1975). Inflammatory cells are rarely observed in the parenchyma except for some perivascular cuffing of blood vessels in mice that survive the major lesion (Rivers and Scott 1936; Walker et al. 1975). Necrosis of the neurons is equally rare (Walker et al. 1975). The spinal cord is generally free from disease, but occasionally some mononuclear infiltration of the meninges can be noted (Rivers and Scott 1936; Lillie and Armstrong 1945).

A second lesion in the central nervous system can occur in young mice if infected at 4 days of age (Cole et al. 1971; Cole and Nathanson 1974). In addition to acute choriomeningitis, cerebellar granule cell necrosis with some hemorrhage is observed. There is inflammation of neural membranes and small vessels (Cole et al. 1971; Cole and Nathanson 1974). Similar lesions can also be seen in the cerebral cortex, hippocampus, and olfactory bulb (Cole and Nathanson 1974).

Mice infected with the causative agent lymphocytic choriomeningitis virus congenitally or neonatally can be persistently infected without overt gross or microscopic lesions. Indeed this observation, first made by Traub (1936a, b), was the first description of a persistent virus infection. This virus has been the subject of a large number of experimental studies which have illuminated basic concepts in biology (Buchmeier et al. 1980).

## Ultrastructure

The major ultrastructural feature of the acute lesion is the presence of electron-dense intracytoplasmic inclusions of polyribosomes in the epithelial cells of the choroid plexus and other affected areas. Infected epithelial cells appear normal in all other respects. Enveloped virions containing multiple electron-dense granules characteristic of the virus can be observed budding into the cerebrospinal fluid from the microvillus surfaces of the choroid plexus (Walker et al. 1975). Infiltrating mononuclear cells accumulate at the endothelial basement lamina and beneath the basal margin of choroid epithelial cells and in general, are present in areas where virus is budding (Walker et al. 1975). The architectures of the meninges and choroid plexus are normal and without ultrastructurally evident lesions (Walker et al. 1975). Reports conflict regarding the presence (Doherty and Zinkernagel 1974) or absence (Walker et al. 1975) of edema.

## Differential Diagnosis

Acute lymphocytic choriomeningitis is unlikely to be confused with other diseases. The characteristic lesion develops only after intracranial infection of adult immunocompetent mice with the specific virus. Outward symptoms appear at day 4 or 5 after infection, depending on the viral dose, and include ruffling of the fur, a hunched posture, and facial edema. These symptoms worsen, the animal loses weight and becomes sluggish and sensitive to loud noises. Death occurs at day 7–9 of tonic convulsions with extended rear limbs, flexed forelimbs, and thoracic spine. Characteristic tonic convulsions can be induced in symptomatic mice by spinning them by the tail. Infection with some strains of murine hepatitis virus and anaphylactic shock can lead to similar signs (Hotchin 1962); however, no other condition results in the mononuclear infiltrate observed after intracranial infection with the virus. Inoculation of blood or tissue homogenates from infected animals intracranially to adult mice will result in the disease whereas the same material will cause asymptomatic persistent infection in neonates. Diagnosis of infection can be

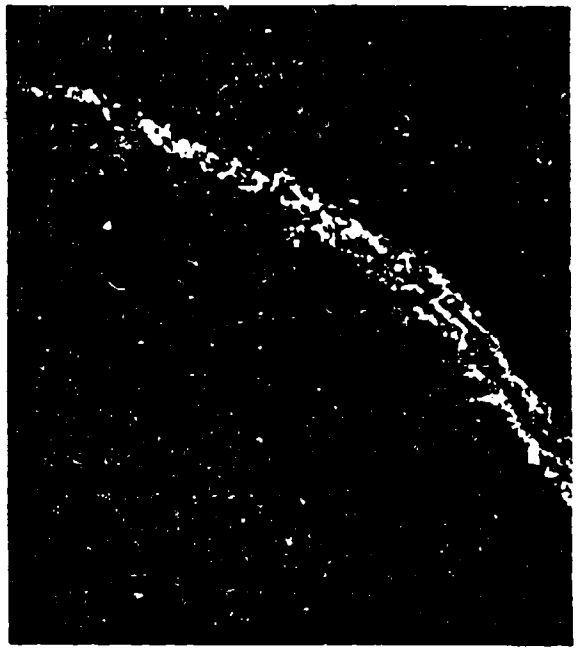




**Fig. 209** *(upper left).* Viral antigens in the brains of acutely infected mice using FITC-conjugated guinea pig antiserum to lymphocytic choriomeningitis viruses. Early expression of lymphocytic choriomeningitis viral antigen in the meninges of a mouse infected 5 days earlier. ×250

**Fig. 210** *(lower left).* Viral antigens in the brains of acutely infected mice using FITC-conjugated guinea pig antiserum to lymphocytic choriomeningitis viruses. Antigen in ependymal cells of the choroid plexus of a mouse infected 7 days earlier with the lymphocytic choriomeningitis viruses. ×250





**Fig. 211** *(upper right).* Acute lymphocytic choriomeningitis. Congestion and mild perivascular meningeal infiltrate early in disease. H and E, ×400

**Fig. 212** *(lower right).* Acute lymphocytic choriomeningitis. Moderate inflammatory focus in the choroid plexus of the third ventricle of a mouse infected 6 days earlier with lymphocytic choriomeningitis viruses. H and E, ×100

nfirmed most readily by detection of cytoplasmic viral nucleocapsid protein antigen in the brain and other tissues by immunofluorescence with virus-specific antiserum. Demonstration of characteristic intracytoplasmic inclusions in neurons and budding virions from ependymal cells are helpful, but require electron-microscopic examination.

## Biologic Features

*Natural History and Pathogenesis.* Lymphocytic choriomeningitis develops within 6 days after intracranial infection of adult, immunocompetent mice with the virus. Viral antigen is first detected in the meninges and choroid plexus around day 2 post infection (Fig. 209); by day 4 or 5 nearly all epithelial cells in both tissues are infected (Walker et al. 1975) (Fig. 210). The first signs of cellular infiltrate occur in the meninges at day 3 or 4 (Fig. 211) and extend to the choroid plexus by day 6 (Fig. 212). The ventricles may also contain inflammatory cells at this point (Lillie and Armstrong 1945; Walker et al. 1975). Death occurs at day 7–9. In surviving animals, inflammatory cells may progress into the parenchyma, but this phenomenon is never extensive (Rivers and Scott 1936; Walker et al. 1975).

Development of the lesion requires competent cellular immunity. Suppression of the cellular immune response in adult mice by irradiation (Rowe 1956), neonatal thymectomy (Rowe et al. 1963), or treatment with antithymocyte sera (Hirsch et al. 1968) or cyclophosphamide (Gilden et al. 1972) abrogated the disease and the mononuclear infiltrate associated with it. Transfer of immune T splenocytes but not immune serum to these infected mice resulted in disease and death (Cole et al. 1971, 1972; Cole and Nathanson 1974). T cells expressing cytotoxic function have been implicated in causing the disease as lymphocytes expressing the T cell antigen, Thy 1.2, and having lymphocytic choriomeningitis virus-specific cytotoxic activity in vitro have been isolated from the central nervous system of mice with lymphocytic choriomeningitis (Zinkernagel and Doherty 1973). Cells expressing Thy 1.2 can be observed in infiltrates in the ventricular spaces (Fig. 213).

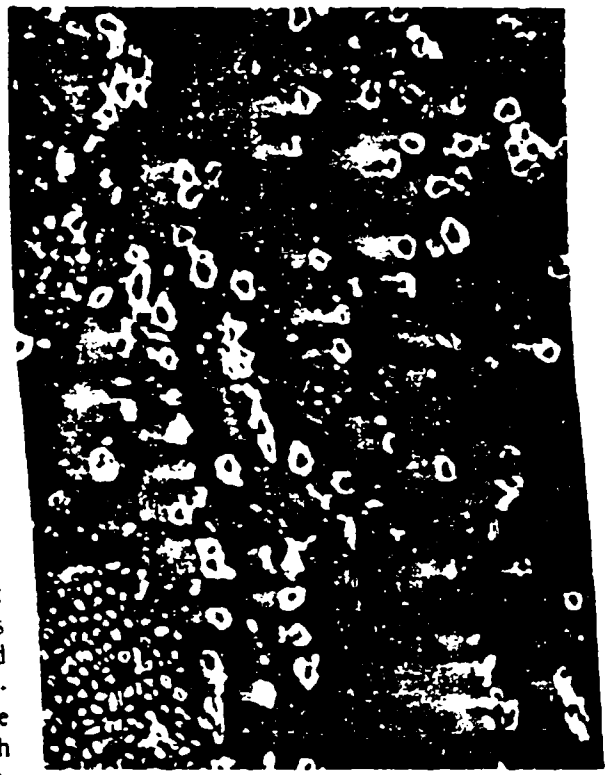


Fig. 213. Thy 1.2 positive lymphocytes in an inflammatory lesion in the ventricle of a mouse infected 7 days earlier with lymphocytic choriomeningitis virus. Rat anti-thy 1.2 staining visualized with FITC-conjugated mouse anti-rat IgG. ×400

*Etiology and Frequency.* Classic lymphocytic choriomeningitis can only be induced experimentally by inoculation of immunocompetent mice with the arenavirus, lymphocytic choriomeningitis virus by the intracranial route. Inoculation of adult mice by a peripheral route usually results in an asymptomatic infection that is rapidly cleared. However, certain strains of the virus cause extensive lesions in the viscera and death after intraperitoneal infection (Lehmann-Grube 1971), and certain strains of mice are particularly susceptible to neonatal infection and appear to die due to hormonal imbalances (Oldstone et al. 1982). Although virus is reported to reach the central nervous system in adult animals infected peripherally (Rivers and Scott 1936), only occasionally is slight meningitis or choroiditis observed (Lillie and Armstrong 1945).

Inoculation of neonatal mice by all routes results in a life-long persistent infection that mirrors the peristent infection observed in mice in the wild (Hotchin 1962; Casals 1984) Infectious virus and viral antigen can be detected in most tissues of the body, including the brain, until death. The distribution of viral antigen in the brains of carrier mice is distinct from that observed in acutely



**Fig. 214.** Persistent lymphocytic choriomeningitis virus infection in cortical neurons of a mouse infected neonatally 4 months earlier. Abundant viral antigen is evident in neuronal cell bodies throughout the cortex by this time. Monoclonal antibody to viral nucleocapsid protein detected with guinea pig peroxidase antiperoxidase (PAP). (Courtesy Dr. M. Rodriguez.) ×250

infected adult mice. There is extensive infection of neuronal cells in the parenchyma, and, as animals age, more neurons and Purkinje's cells become infected (Mims 1966; Rodriguez et al. 1983) (Fig. 214). At later stages, immune complexes can be demonstrated in the brain (Oldstone 1984). One study claims that persistently infected carrier mice display behavioral abnormalities (Hotchin and Seegal 1977), but most strains of mice show no discernable signs until late in life when they develop a wasting syndrome due to immune complex disease (Hotchin 1962). Histologically, at all ages, the brains of carrier mice are normal, with only occasional slight meningitis shortly after infection (Traub 1936a) or mild perivascular round cell infiltrate (Oldstone and Dixon 1970).

In the wild, infection is passed vertically from mother to offspring in utero or, less likely, by naso-oral infection after contact with nasal secretions and/or excreta from infected mice (Traub 1936b, 1939). The distribution of viral antigen and lack of microscopic lesions in animals infected in utero are indistinguishable from animals experimentally infected neonatally (Wilsnack and Rowe 1964).

## Comparison with Other Species

The natural reservoir for the virus is feral mice, but occasionally laboratory mice become infected through experimental or accidental introduction of the virus or virus-infected tumor cells (Parker 1986; van der Zeijst et al. 1983). Once the infection is established in a colony, it is perpetuated by congenital and/or vertical transmission to offspring. Persistent infections also occur in hamsters with both vertical and horizontal transmission (Parker et al. 1976). When young hamsters are inoculated, infection persists with prolonged viruria; older animals tend to clear infection (Smadel and Wall 1942; Lewis et al. 1965). There are no reports of pathologic lesions within the central nervous system in infected hamsters.

Humans can also become infected although they rarely transmit the disease (Parker 1986). The means of human infection is through contact with infected rodents, pet hamsters being a particular source in recent years (Buchmeier et al. 1980). Generally the virus infection is asymptomatic or produces a nonmeningeal influenza-like illness in man. Less frequent severe cases may occur as aseptic meningitis and meningoencephalitis (Scott and Rivers 1936; Buchmeier et al. 1980; Casals 1984).

Neural lesions can be induced in rats if infected at an early age (Cole et al. 1971). Rats inoculated intracranially with the virus prior to 14 days of age developed marked cerebellar hypoplasia with minimal inflammation. Treatment of suckling rats with antithymocyte serum prevented cerebellar lesions, hence the lesion appeared to be immune mediated. Guinea pigs, monkeys, and dogs have been infected experimentally, but little has been done to examine the lesions in the central nervous system after infection (Parker 1986).

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VIROLOGY 161, 448-456 (1987)

# Analysis of the Genomic L RNA Segment from Lymphocytic Choriomeningitis Virus

MANDALESHWAR K. SINGH,[1] FRANCES V. FULLER-PACE, MICHAEL J. BUCHMEIER, AND PETER J. SOUTHERN[2]

*Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

*Received May 8, 1987; accepted July 27, 1987*

**The arenavirus genomic L RNA segment represents approximately 70% of the viral genetic material but current understanding of the organization, regulation, and functioning of the viral L products remains limited. Biological studies with reassortant viruses have implicated the L RNA segment in the lethal infection of adult guinea pigs produced by LCMV-WE but no further explanation of the pathogenic process is presently available. We have initiated a detailed molecular analysis of LCMV L products based on construction and characterization of L-specific cDNA clones and synthesis of L-specific hybridization probes. Nucleotide sequencing studies have allowed the derivation of a partial amino acid sequence for a predicted L protein and antisera raised against synthetic peptides have demonstrated an L protein in Western blotting experiments. Using this approach we have identified a single high molecular weight protein (approximately 200,000 Da) in purified virions and in viral ribonucleoprotein complexes extracted from acutely infected tissue culture cells. This L protein is translated from a nonpolyadenylated, genomic complementary L mRNA and potentially represents part or all of the viral RNA-dependent RNA polymerase.** © 1987 Academic Press, Inc.

## INTRODUCTION

The genome of lymphocytic choriomeningitis virus (LCMV) contains two single-stranded RNA segments, designated L and S, of approximate lengths 8-9 and 3.4 kb, respectively (Pedersen, 1971; Vezza *et al.*, 1978). The S RNA segment encodes the major viral structural proteins: an internal nucleoprotein (NP) that is associated with the viral RNAs in ribonucleoprotein complexes (RNP) and two surface glycoproteins (GP-1 and GP-2) (Vezza *et al.*, 1980; Harnish *et al.*, 1981, 1983; Riviere *et al.*, 1985a) that are derived from post-translational cleavage of a precursor species (GP-C) (Buchmeier and Oldstone, 1979). The L RNA segment is believed to encode a high molecular weight protein, the L protein (Harnish *et al.*, 1983)—a putative viral RNA-dependent RNA polymerase. In this paper we present direct evidence for a 200-kDa L protein encoded at the 3′ end of the LCMV L RNA segment.

There have been several recent reports on the genomic organization and expression of the S RNA segment of LCMV, and other closely related arenaviruses, that have described an unusual ambisense gene coding arrangement (reviewed by Bishop and Auperin, 1987). The NP mRNA is complementary to the 3′ half of the genomic S RNA segment whereas the GP mRNA, transcribed from the 5′ half of the genomic S RNA, is in the sense of the genome (i.e., "pseudo-positive" sense arrangement). In comparison, relatively little is known about the genetic potential and expression of the genomic L RNA segment that represents approximately 70% of the total viral genetic information.

The L and S genomic RNA segments contain essentially nonoverlapping coding information. There are short common sequences extending over approximately 30 nucleotides at the 3′ termini of the L and S segments (Auperin *et al.*, 1982a, b) that are presumed to specify a recognition site for the viral polymerase and/or a nucleation site for the formation of RNP complexes. The sequences of the 5′ and 3′ termini of the genomic S RNA segment are complementary (Auperin *et al.*, 1984). This arrangement would be expected from current thoughts on the mechanism of RNA replication of single-stranded RNA viruses (reviewed by Strauss and Strauss, 1983) and the same terminal sequence arrangement is anticipated for the L segment. The sequence complementarity at the RNA termini may also have functional significance for RNA encapsidation into virions.

Recent investigations into the pathogenic potential of different LCMV strains have served to illustrate the importance of studying the genomic L segment for a complete understanding of the processes and consequences of virus infection. Riviere and colleagues succeeded in isolating genomic RNA segment reassortant viruses derived from the parental LCMV-Armstrong and LCMV-WE strains and demonstrating that the lethal disease induced in guinea pigs by LCMV-WE was associated with the WE L RNA segment (Riviere *et al.*, 1985b). At present, it is not clear whether the L

[1] Present address: Department of Virology, Karolinska Institute, Stockholm, Sweden.

[2] To whom requests for reprints should be addressed.



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gene product(s) is directly pathogenic or whether the LCMV-WE viral polymerase supports more rapid and/or more efficient virus replication in the guinea pig. The resolution of this and other unanswered questions will be greatly facilitated by molecular studies of the LCMV genomic L RNA segment.

## METHODS

### Tissue culture cells and virus stocks

Monolayer cultures of BHK-21 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. High titer preparations of LCMV were obtained by infecting semiconfluent monolayers (30–50% confluency) of BHK-21 cells at a multiplicity of infection (m.o.i.) of 0.1. The virus inoculum for these infections was a triple plaque-purified stock of LCMV strain Armstrong CA 1371 (our clone 5-3B) that had been further amplified by one cycle of infection in BHK cells. Virus in the supernatant medium was harvested after 72 hr and centrifuged to remove intact cells and any debris. Samples of this clarified supernatant were stored in small volumes at −70° for subsequent rounds of virus infection. The titers of infectious virus in these stocks were measured in plaque assays performed on CV-1 cell monolayers. Virions were purified from clarified tissue culture supernatants by precipitation with PEG 6000 followed by centrifugation in renografin gradients as described previously (Southern *et al.*, 1987).

### Construction and characterization of cDNA clones

Mixtures of L and S RNAs, extracted from purified virions, were used as templates for reverse transcriptase with a calf thymus random DNA primer (conditions described in detail in Southern *et al.*, 1987). Double-stranded cDNAs were cloned into the *Pst*I site of pBR322 and then transferred into *Escherichia coli* HB101. L-specific cDNA clones were identified in colony hybrization experiments with a $^{32}$P-labeled L probe that was prepared by partial alkaline hydrolysis of gel-purified L RNA followed by 5′ end labeling with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase. Positive colonies were picked and L broth cultures were grown for small scale plasmid DNA preparations. These DNA samples were used for analytical restriction mapping and for $^{32}$P-labeled probe synthesis by nick-translation labeling reactions *in vitro*. The probes were hybridized with nitrocellulose strips containing total cell RNA from virus-infected and control, uninfected cells (see below). Nucleotide sequencing was performed on both DNA strands using the chemical method (Maxam and Gilbert, 1980). Polynucleotide kinase with [$\gamma$-$^{32}$P]ATP and terminal transferase with [$\alpha$-$^{32}$P]ddATP were used for 5′ and 3′ end labeling reactions respectively.

Computer analysis of the nucleotide sequences and predicted amino acid sequences was performed using the Align, Search, and Relate programs distributed by the National Biomedical Research Foundation, Georgetown University Medical Center, Washington D.C.

### Anti-peptide antibodies and Western blotting experiments

Details of the experimental procedures for peptide synthesis and purification, coupling to carrier proteins, and the immunization strategies to raise polyclonal antipeptide antisera in rabbits have recently been published (Buchmeier *et al.*, 1987). Samples of purified LCM virion proteins or cytoplasmic extracts enriched for intracellular ribonucleoprotein complexes (Hill and Summers, 1982) were separated by electrophoresis in SDS–polyacrylamide gels using standard Laemmli conditions with a 3% stacking gel and a 7% separating gel. All gels were run with one end lane containing protein molecular weight markers (Bio-Rad). After electrophoresis, proteins in the gels were transferred electrophoretically onto 0.2-$\mu$m nitrocellulose filters (Schleicher & Schuell) either overnight at 250 mA or for 4–5 hr at 500 mA using a recirculation cooling system. The protein marker lane was then cut from the remainder of the filter and stained briefly in amido black to monitor the efficiency of transfer of the myosin component in the marker (approximately 200,000 mol wt). When the transfer of myosin seemed poor, then the filters were not processed further.

The filters were preblocked in 2% skimmed milk powder in PBS with 0.05% Tween 20 (Blotto; Johnson *et al.*, 1984) by incubation overnight at 4° and then treated with antibody (normally a 1:100 dilution) at 37° for 1 hr. Excess antibody was removed by washing in Blotto and bound immunoglobulin on the filter was detected with iodinated $^{125}$I-*Staphylococcus aureus* protein A. After extensive washing in Blotto to remove the excess radioactivity, the filters were dried and exposed to Kodak XAR-5 film with Cronex lightning fast intensification screens at −70°.

### Preparation of strand-specific hybridization probes

A 330-bp fragment (*Eco*RI to *Hin*dIII) from L122 was subcloned into the pSP64 and pSP65 vectors and samples of the hybrid plasmid DNAs were cleaved respectively with *Eco*RI and *Hin*dIII. $^{32}$P-Labeled RNA probes were synthesized *in vitro* according to standard conditions (Melton *et al.*, 1984). In a typical reac-

tion, 50–100 ng of DNA template produced 1–5 × $10^8$ cpm of labeled RNA.

Viral RNA samples for hybridization analysis were prepared either by phenol extraction of purified virions or by disruption of infected cells with guanidinium thiocyanate (GTC) (Chirgwin *et al.*, 1979). The RNAs were denatured with glyoxal (McMaster and Carmichael, 1977), separated by electrophoresis through agarose gels, and then transferred by capillary flow of buffer (20× SSC) onto nitrocellulose membranes (0.45 μm, Schleicher & Schuell) (Thomas, 1980). After baking at 80° *in vacuo*, the filters were prehybridized at 37° for at least 4 hr in 50% deionized formamide, 4× SSC, 2× Denhardt's solution, 150 μg/ml boiled sonicated carrier salmon sperm DNA, and 100 μg/ml yeast RNA. Hybridization reactions were performed using a fresh preparation of the same solution at 55° for 18–24 hr with 3–10 × $10^6$ cpm of $^{32}$P-labeled RNA. Filters were initially washed twice at 37° in 2× SSC, 0.1% SDS and then at 60° in the same buffer (300 ml buffer/wash, 30 min/wash). A final, stringent wash was performed at 60° for 30 min in 0.1× SSC, 0.1% SDS Tween 20. The filters were then set up for autoradiography using Kodak XAR-5 film and Cronex lightning fast intensification screens at −70°. After suitable exposure times, the hybridization signal was stripped by washing for 2–3 hr at 90° in 0.1× SSC, 0.1% SDS, 0.1% Tween 20 and then the filters were prehybridized again prior to hybridization with a different probe.

### Extraction of total cell RNA and oligo(dT) cellulose chromatography

Semiconfluent monolayers of BHK cells were infected with the LCMV-Arm virus stock (m.o.i. = 0.1) and lysed 72 hr postinfection by treatment with GTC. Total cell RNA was recovered by pelleting through a cushion of 5.7 *M* cesium chloride. The RNA was redissolved in sterile water, precipitated with ethanol, and then stored at −70° in sterile water. Samples of these RNA preparations were chromatographed on small oligo(dT) cellulose columns (Type III cellulose, Collaborative Research) under standard conditions. Part of the retained RNA fraction was set aside for gel electrophoresis while the remainder was rechromatographed over a second oligo(dT) cellulose column (see Fig. 6).

## RESULTS

### Characterization of L-specific cDNA clones

Purified preparations of LCMV virion RNA (Armstrong CA 1371 strain) were used as templates for reverse transcriptase with a calf thymus random oligonucleotide primer. Double-stranded cDNAs were cloned into the *Pst*I site of pBR322 using the G-C homopolymer tailing technique. After transformation of *E. coli* HB101, L-specific clones were identified by colony hybridization using kinase-labeled L RNA as a probe (Southern *et al.*, 1987). Individual positive colonies were picked for small-scale plasmid DNA preparations and the cDNA insert sizes were assessed by *Pst*I digestion and agarose gel electrophoresis. Recombinant plasmids were individually rechecked for L sequence content by labeling the plasmid DNA *in vitro* and subsequent hybridization to total cell RNA from acutely infected and control, uninfected cells. A small percentage of the plasmids that had been picked as positive by colony hybridization did not show the expected viral specificity in this second screening and were subsequently discarded.

The experiments presented in this paper relate to three nonoverlapping L-specific cDNA clones, L122, L39, and L123. L122 is derived from the 3′ portion of the genomic L RNA segment whereas L39 and L123 appear to come from the central portion of the L RNA. A typical hybridization pattern produced by random primer labeling (Feinberg and Vogelstein, 1983) of an L122 probe is shown in Fig. 1. This probe detected a single high molecular weight RNA in samples of acutely infected cell RNA and purified LCM virion RNA; there was no detectable hybridization to uninfected cell RNA.

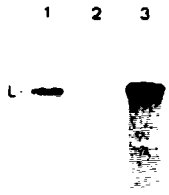



FIG. 1. Hybridization to LCMV L RNAs with an L-specific probe labeled randomly in both DNA strands. Purified RNAs were denatured with glyoxal and separated by electrophoresis in a 1.5% agarose gel (McMaster and Carmichael, 1977). RNAs within the gel were transferred to a nitrocellulose filter (Thomas, 1980) by capillary flow of buffer (20× SSC) and then the filter was hybridized with a labeled probe from clone L122 (the *Pst*I fragment, nucleotides 429–697 plus the $(dC)_n$ tail). Lane 1, 8 μg total cell RNA from BHK cells acutely infected with LCMV; lane 2, 8 μg control, uninfected BHK cell RNA; lane 3, 0.5 μg purified LCMV virion RNA. These virions were harvested from the supernatant medium at the time of extraction of the total BHK cell RNA used in lane 1.

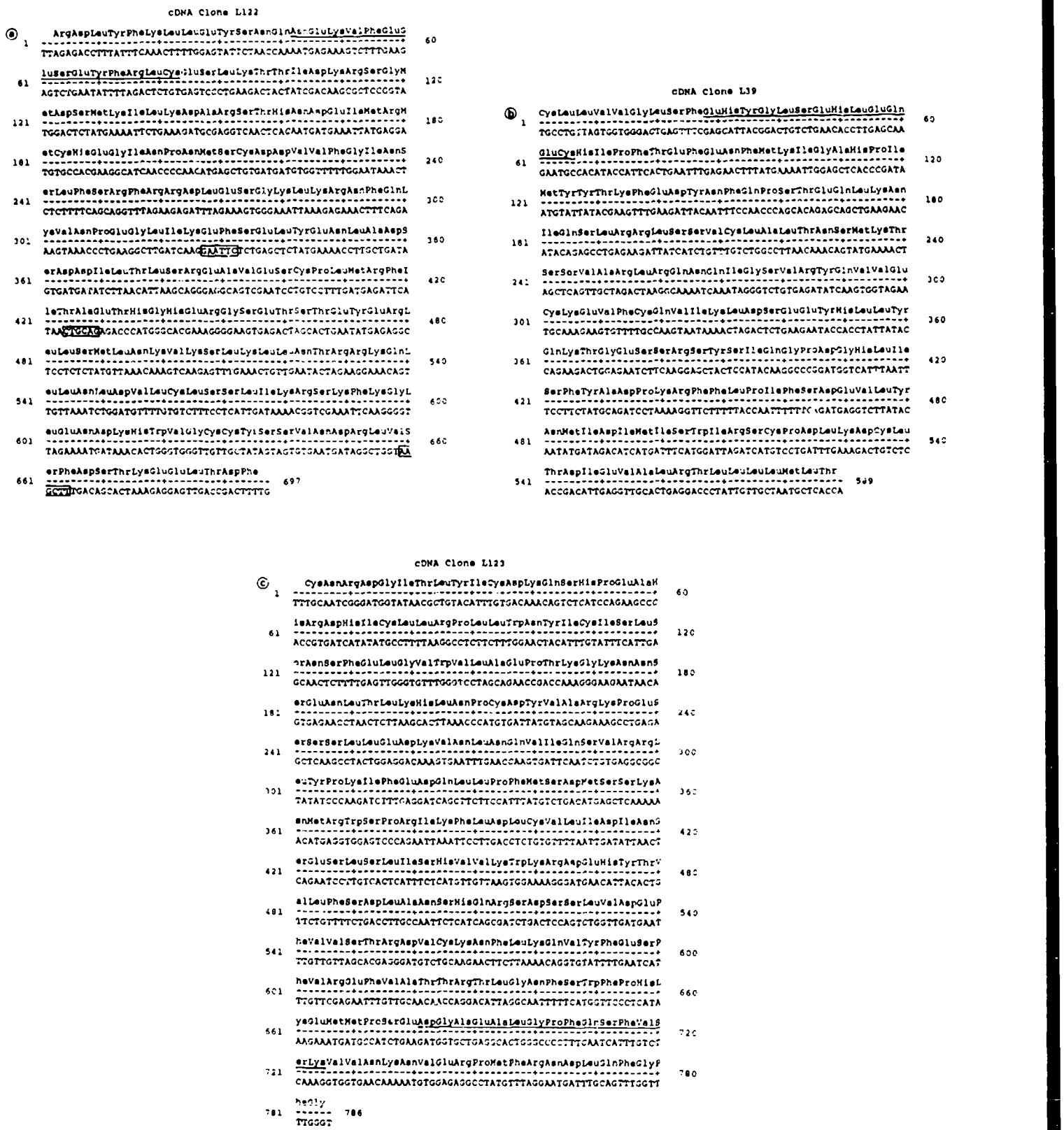



FIG. 2 Nucleotide and predicted amino acid sequences for clones L122, L39, and L123. (a) L122. The *Eco*RI [GAATTC], *Pst*I [CTGCAG], and *Hin*dIII [AAGCTT] sites mentioned elsewhere in the paper are enclosed in boxes. The synthetic peptide used in Fig. 3 is underlined in the predicted L122 amino acid sequence. (b) L39. The synthetic peptide used in Fig. 3 is underlined in the predicted L39 amino acid sequence. (c) L123. The synthetic peptide used in Fig. 3 is underlined in the predicted L123 amino acid sequence.

## Nucleotide sequencing and computer comparison with other viral sequences

We have used the chemical sequencing technique (Maxam and Gilbert, 1980) to determine the nucleotide sequences of L122, L39, and L123 (Fig. 2). These sequences were determined entirely from both DNA strands and the majority of the sequence for clones L122 and L39 was confirmed by analysis of independent, overlapping clones (data not shown). The L122 clone covered 697 bp and we immediately noticed a substantial overlap with a published sequence from the 3' end of the genomic L segment for LCMV strain WE (spanning amino acids 1-363 of a predicted L protein; Romanowski and Bishop, 1985). This overlapping region contained 500 conserved nucleotides, representing 80% overall nucleotide sequence homology and 176/203 conserved amino acid residues (87% homology) for the predicted translation products of the two virus strains. The nucleotide sequences of L39 and L123 indicated that these clones, like L122, contained single open reading frames. All three clones apparently comprise parts of the same high molecular weight viral protein (see below and Fig. 3).

## Detection of a high molecular weight protein encoded by the L RNA segment

In an attempt to identify L protein products directly, we have synthesized short peptides corresponding to different regions of the predicted protein sequence. Polyclonal rabbit antiserum, raised against a peptide - KLH carrier complex, has been reacted with proteins from purified LCM virions in Western blotting experiments. Antisera raised against the predicted amino acid residues 14-27 in L122, 10-22 in L39, and 227-241 in L123 reacted specifically with a high molecular weight protein (approximately 200,000 Da) in the virion preparations (Figs. 2 and 3). This same protein was also present in viral ribonucleoprotein complexes prepared from acutely infected BHK cells (Fig. 4). Thus, the antipeptide antibodies have provided the first specific reagents to study the distribution of L-encoded proteins in virions and in acutely infected cells. Detection of the 200,000-Da protein both in virions and in RNP complexes is consistent with the notion that this is a component of the viral polymerase.

## Hybridization studies with strand-specific L probes

Because of the occurrence of an ambisense coding arrangement for the arenavirus S RNA segments

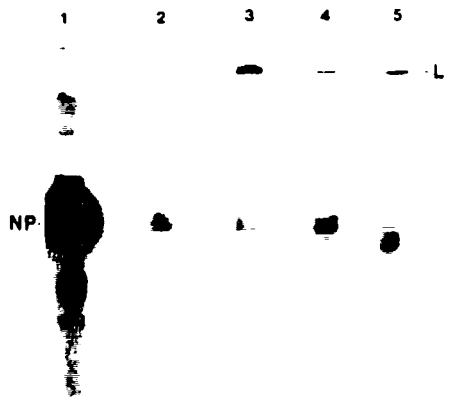



FIG. 3. Detection of an L-encoded protein in Western blotting experiments using antisera raised against synthetic peptides. Proteins from purified LCM virions were separated on a denaturing SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose filter. The filter was then sliced into narrow strips and processed with antisera followed by $^{125}$I-protein A (see Methods and Buchmeier *et al.*, 1987 for details). Lane 1, control antiserum raised against LCMV nucleoprotein (NP residues 132-145 Q-Q-L-D-Q-R-S-Q-I-L-Q-I-V-G); lane 2, control antiserum raised against a non-LCMV peptide; lane 3, antiserum against L39 peptide (E-H-Y-G-L-S-E-H-L-E-Q-E-C); lane 4, antiserum against L122 peptide (N-E-K-V-F-E-E-S-E-Y-F-R-L-C); lane 5, antiserum against L123 peptide (D-G-A-E-A-L-G-P-F-Q-S-F-V-S-K). The bands below NP in lane 1 represent reproducible cleavage or breakdown products derived from NP (Buchmeier and Parekh, 1987). Lanes 2-5 inclusive all show a minor nonspecific reaction in the NP region of the filter which is due to a direct interaction between NP and protein A.

(Bishop and Auperin, 1987), we were interested to determine whether the 200-kDa L protein was translated from a genomic sense or genomic complementary sense mRNA. We therefore inserted the 330-bp *Eco*RI to *Hin*dIII restriction fragment from L122 between the corresponding restriction enzyme recognition sites in the multiple cloning site of the pSP64 and pSP65 vectors (Promega Biotec) with the intention of synthesizing a pair of complementary $^{32}$P-labeled RNA probes *in vitro* from the SP6 promoter (Melton *et al.*, 1984). The 65-L122 probe (*Eco*RI→*Hin*dIII) hybridized stongly to total cell RNA extracted from BHK cells acutely infected with LCMV and also to purified virion RNA whereas the complementary 64-L122 probe (*Hin*dIII→*Eco*RI) showed weak hybridization to the total cell RNA sample and a barely detectable signal with the virion RNA (Fig. 5). These results indicate that the 65-L122 probe is detecting genomic sense L RNA (RNA, by definition, that is present in the purified virion) and that the 64-L122 probe detects genomic complementary L RNA. The nucleotide sequence information for L122 (Fig. 2) indicates that the coding strand is oriented *Eco*RI→*Hin*dIII and, taking account of the strand-specific hybridization results (Fig. 5), estab-

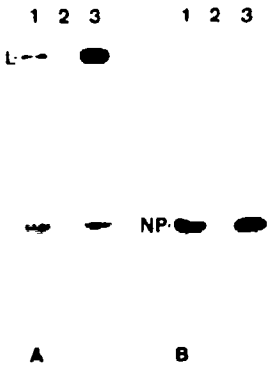



FIG. 4. Detection of viral proteins in an intracellular ribonucleoprotein fraction. Proteins from intracellular ribonucleoprotein complexes (Hill and Summers, 1982) were separated on a denaturing SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose filter. The filter was then sliced into two parts and processed with antiserum followed by $^{125}$I-protein A (see Methods and Buchmeier *et al.*, 1987 for details). (A) Antiserum against L123 peptide (Figs. 2c and 3). Exposure time 18 hr. (B) Antiserum against LCMV nucleoprotein (Fig. 3). Exposure time 3 hr. Lane 1, purified LCM virions as a marker; lane 2, RNP from uninfected BHK cells; lane 3, RNP from BHK cells acutely infected with LCMV. Note that in (A) there is a nonspecific interaction between NP and the $^{125}$I-protein A.

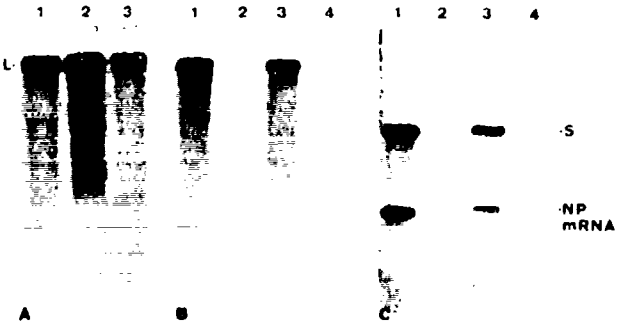



FIG. 6. Oligo(dT) cellulose chromatography of LCMV RNAs from acutely infected BHK cells. Total cell RNA was extracted with guanidinium thiocyanate at 48 hr postinfection with LCMV (m.o.i. = 0.1), purified by pelleting through cesium chloride, separated by denaturing agarose gel electrophoresis, and finally transferred to nitrocellulose (see Methods for details). (A) First passage analysis. L39 nick-translated probe. Lane 1, starting total cell RNA; lane 2, RNA not retained on column poly(A)$^+$; lane 3, RNA apparently retained on column poly(A)$^+$(?). (B) Second passage analysis. L39 nick-translated probe. Lane 1, starting total cell RNA; lane 2, uninfected BHK cell RNA; lane 3, RNA not retained on column poly(A)$^+$; lane 4, RNA retained on column (genuine poly(A)$^+$). (C) Rehybridization of the filter shown in (B) with an RNA probe from the NP coding region of the S RNA segment. This probe detected genomic complementary S RNA and NP mRNA.

lishes that the 200-kDa protein is translated from an L, genomic complementary, mRNA. Romanowski and Bishop (1985) had predicted a genomic complementary L mRNA from their analysis of the 3′ terminal sequence of LCMV, WE strain, but there was no information available at that time regarding the character of the L protein.

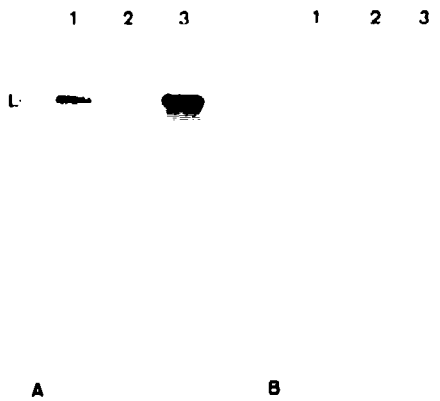



FIG. 5. Strand-specific hybridization to L RNAs. RNA samples were electrophoresed in a denaturing agarose gel and transferred to a nitrocellulose filter for sequential hybridization with RNA probes derived from opposite strands of clone L122 (see Methods and Fig. 1 legend for details). Lane 1, 8 μg total cell RNA from BHK cells acutely infected with LCMV; lane 2, 8 μg uninfected BHK cell RNA; lane 3, 0.5 μg purified LCM virion RNA. (A) The hybridization signal with the 65-L122 RNA probe which is deduced to recognize genomic sense L RNAs (exposure: 6 hr at −70° with an intensification screen). (B) The hybridization signal with the 64-L122 RNA probe which recognizes genomic complementary sense RNAs (exposure 6 days at −70° with an intensification screen).

## Oligo(dT) cellulose chromatography with intracellular viral RNAs

Total cell RNA was fractionated on an oligo(dT) cellulose column to separate poly(A)$^-$ and poly(A)$^+$ RNA species. Samples of these RNAs were analyzed in Northern blotting experiments with an L39 nick-translated probe (Fig. 6A). The majority of the L-specific signal was detected in the poly(A)$^-$ fraction but there was clear representation of an apparently full-length L RNA in the RNA fraction that had been retained on the column. This retained material was passed over a second oligo(dT) cellulose column to distinguish between genuine retention (i.e., poly(A)$^+$ RNA) and fortuitous trapping of poly(A)$^-$ RNAs. The hybridization analysis from this second column showed that there was no detectable L RNA retained on the column and that L mRNAs, like S mRNAs, do not possess 3′ poly(A) sequences that allow binding to oligo(dT) cellulose under standard conditions. The hybridization analyses in Figs. 6A and 6B also confirm the results of Figs. 1 and 5 where there was no evidence for subgenomic, L-derived RNAs in samples of RNA extracted from cells acutely infected with LCMV.

## DISCUSSION

The genomic L RNA segment represents approximately 70% of the genetic potential of the arenaviruses but, until this present study, relatively little was known about the structure and expression of the L segment. A minor, high molecular weight protein had been identified in purified preparations of $^{35}$S-labeled virions (Vezza *et al.*, 1980) and this was assigned to the L RNA segment simply by size considerations because this protein appeared to be too large to be encoded by the S RNA segment. In parallel studies, a high molecular weight protein could be immunoprecipitated by polyclonal antiserum raised against disrupted Pichinde virus (Harnish *et al.*, 1981) and was linked to the viral L RNA segment in studies of reassortant viruses (Harnish *et al.*, 1983). Most polyclonal antisera do not detect L-encoded proteins efficiently and no monoclonal antibodies have yet been reported with specificity for L proteins.

The experimental approach described here has progressed from isolation and characterization of L-specific cDNA clones to nucleotide sequencing and prediction of amino acid sequences for L-encoded proteins and then to visualization of a 200,000-Da L protein in Western blotting experiments using antisera raised against synthetic peptides. These antipeptide antibodies therefore provide the first monospecific reagents that recognize an arenavirus L protein and, as such, now permit studies on the expression and distribution of the L protein in acute and persistent infections.

We have an extensive collection of cDNA clones from the L segment of LCMV-Armstrong and are systematically compiling a complete sequence for the L segment and a reconstructed intact L cDNA. The nucleotide sequences presented here, covering 2072 bases, represent 20–25% of the total L sequence and considerably extend the previously available information. In a limited comparison with sequence from the L RNA of LCMV-WE (Romanowski and Bishop, 1985) we observed nucleotide and predicted amino acid sequence homologies (80 and 87%, respectively) that are compatible with earlier comparisons for the Arm and WE structural protein sequences, NP, GP-1, and GP-2, that are encoded by the genomic S RNA segment (Southern *et al.*, 1987). We have searched the protein sequence databases for homologies between predicted LCMV L protein sequences and the sequences of other viral proteins. We found that the L122 sequence contains an unusual pair of adjacent aspartic acid residues (-Asp-Asp-, amino acid residues 72 and 73, Fig. 2a) in a hydrophobic pocket; such amino acid arrangements have been noted previously in a wide-ranging group of viral polymerases and have been proposed to represent a conserved feature of polymerase molecules (Kamer and Argos, 1984) and have also been detected in the coding region of the L1 family of mammalian repetitive DNA sequences (Loeb *et al.*, 1986; Hattori *et al.*, 1986). In addition, we have detected an eight amino acid stretch in LCMV-Arm L protein from clone L122 (amino acid residues 78–85) that shares six identical residues with a region (residues 1006–1013) from the vesicular stomatitis virus (VSV) L protein (Schubert *et al.*, 1984) (Table 1). There is also a nine amino acid sequence in the predicted translation product from L39 (amino acid residues 169–177, Fig. 2b) that shares five identical residues with VSV L protein (residues 1287–1295). Interestingly, this same sequence from L39 exhibits even greater homology with a sequence from the *gag* polyprotein of HTLV-II (residues 395–403) (Shimotohno *et al.*, 1985; Table 1). The functional significance of these short, conserved regions remains to be elucidated.

The antipeptide antibodies, raised against predicted peptide sequences from three independent clones, all recognize a 200,000-Da protein—presumably the same protein covered by clones L122, L39, and L123. However, we cannot yet settle the question of whether this 200,000-Da protein is the only L-translation product or whether there may be additional open reading frames at the 5′ end of the genomic L segment. A 200,000-Da protein would require approximately 6 kb of coding region whereas the genomic L segment has been estimated to fall into the 8- to 9-kb size range. As there is no precedent for noncoding regions 2–3 kb in size within RNA virus genomes, it would seem that additional proteins might be encoded within the L RNA. Such proteins could be derived either from cleavage of an L polyprotein that releases the 200,000-Da species and other products or by transcription and translation of independent L coding regions. In the latter case, perhaps the ambisense coding arrangement of the S RNA segments would also appear as a feature of the genomic organization of arenavirus L RNA segments.

**TABLE 1**

SHORT AMINO ACID HOMOLOGIES BETWEEN A PREDICTED LCMV L PROTEIN AND OTHER VIRAL PROTEINS

| | Amino acid residues | |
|---|---|---|
| LCMV L122 | 78–85 | INSLFSRF |
| VSV L | 1006–1013 | INPLFPRF |
| LCMV L39 | 169–177 | W I R S C P D L K |
| VSV L | 1287–1295 | W I T S C T D H Y |
| HTLV-II *gag*-pp | 395–403 | W K R D C P Q L K |

We are currently investigating reaction conditions to assess *in vitro* activity for the viral polymerase with a view to understanding the role of the 200,000-Da protein. There have been previous reports of polymerase activity in preparations of purified Pichinde virions (Carter *et al.*, 1974; Leung *et al.*, 1979) and one report of a linked transcription/translation system for Tacaribe (Boersma and Compans, 1985). The antipeptide antibodies could be of value in defining critical domains in the L protein as assessed by *in vitro* reactions. Information is beginning to emerge on the characteristics of polymerase molecules from other negative-strand viruses including vesicular stomatitis virus (Schubert *et al.*, 1984), influenza virus (Fields and Winter, 1982; Winter and Fields, 1982; Krystal *et al.*, 1986), Sendai virus (Morgan and Rakestraw, 1986; Shioda *et al.*, 1986), and measles virus (Ray and Fujinami, 1987). In each case the polymerase represents a multifunctional protein and influenza actually requires three component polypeptide chains. The arenavirus polymerase is likely to share many characteristics with these other viral polymerases and this conclusion is supported by the identification of short stretches of conserved amino acid sequence that have been detected in computer comparisons

Our long-term interest in the viral L segment relates to an attempt to elucidate the role of the viral polymerase in the progression from acute to persistent infection. Viral gene expression is markedly reduced in persistently infected cells and potential regulatory mechanisms include reduced/altered polymerase activity and/or an unchanged polymerase enzyme being influenced by a modification to another protein or the RNA templates. As more information becomes available, it should also be possible to propose an explanation for the involvement of the genomic L RNA segment in the markedly different pathogenic potentials of the closely related LCMV-Armstrong and WE strains in infected guinea pigs.

## ACKNOWLEDGMENTS

This is Publication Number 4738-IMM from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037. This work was supported in part by USPHS Grants NS-12428 and AG-04342 and DOD contracts DAMD C-3013 and C-6234. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents. We thank Dr. Michael Oldstone for continued interest and support for these studies and Ms. Nicole Nguyen and Mrs. Gay L. Schilling for expert technical and secretarial assistance.

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Peptide Research

# High Efficiency Immunoaffinity Purification of Anti-Peptide Antibodies on Thiopropyl Sepharose Immunoadsorbants



**Bharat S. Parekh, Peter W. Schwimmbeck and Michael J. Buchmeier**
Research Institute of Scripps Clinic

## ABSTRACT

*Antibodies to peptides are routinely made by immunizing animals with peptide linked to a carrier protein such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) via a disulfide bond. The majority of such a polyclonal antibody response is directed against the carrier protein. The presence of such background antibodies often complicates efforts to characterize the desired anti-peptide antibody; hence it is desirable to isolate the specific fraction of immunoglobulin reactive against the peptide of interest. We describe here a simple and efficient technique to purify anti-peptide antibodies from such sera using commercially available reagents. Peptide antigen with a carboxy or amino terminal cysteine is coupled to thiopropyl Sepharose via a disulfide linkage. The bond between peptide and propyl group on Sepharose was stable at neutral and acidic pHs, and affinity bound anti-peptide antibodies were eluted from the column at low pH (pH 3.0). This procedure permits purification of anti-peptide antibodies, separating them from usually high-titered antibodies to the carrier protein. We describe the application of this method for purification of antibodies to two peptides derived from the glycoprotein sequence of lymphocytic choriomeningitis virus, as well as sequences derived from the human acetylcholine receptor.*

## INTRODUCTION

Antibodies to synthetic peptides have become a basic tool in the repertoire of molecular biology (8,10,11, 12). In most instances it has been necessary to couple the peptide to a carrier protein like KLH or BSA to achieve a significant immune response to the peptide under study (2,5,7,9,13). Anti-peptide antibodies thus generated are very useful for structural, immunological and other studies (2,7,13). However, the major immune response of the immunized host is directed towards the carrier protein rather than the peptide. We have encountered non-specific reactivity toward host and viral proteins which is traceable to a background anti-KLH antibody response. Although the mechanism for generation of such spurious reactions has not been studied, it seems likely to involve an element of molecular mimicry (6). Presence of such cross-reactive antibody interferes with the use of anti-peptide antibodies in various immunological assays, particularly those involving immunocytochemistry and immunofluorescence, and in inexperienced hands can lead to false conclusions. For these and other reasons it is advantageous to specifically purify the anti-peptide fraction of such antibodies.

Routinely, peptide-carrier conjugates were prepared in our laboratory using the reagent m-maleimidobenzoyl N-hydroxysuccinimide ester (MBS), which couples peptides to the carrier protein, KLH, via a cysteinyl sulfhydryl group. In studies reported elsewhere it has been demonstrated that the positioning of the coupling cysteine residue may profoundly affect the reactivity with native protein (4); hence, it is desirable to present the peptide in the immunizing conformation on the amino affinity column. Immobilizing the peptides to the solid support using a linkage analogous to that used for immunization might be expected to increase the likelihood of recognition by its antibodies. Thiopropyl Sepharose 6B (Pharmacia, Piscataway, NJ) provides such a linkage for peptides containing cysteine. We report here the results of studies involving two peptides corresponding to amino acid sequences of the GP-1 and GP-2 glycoproteins of lymphocytic choriomeningitis virus (LCMV), and to peptides derived from the sequence of human acetylcholine receptor (AChr), and their corresponding anti-peptide antibodies made in rabbits. We report the recovery of site-directed antibodies which are depleted of anti-carrier reactivity and retain a high degree of reactivity with both the immunizing peptide and the corresponding viral or receptor protein.

## MATERIALS AND METHODS

### Peptides and Anti-Peptide Antibodies

The following two peptides, forming the amino acid sequence of glycoprotein precursor (GPC) of LCMV (14), were used for the studies described here: GPC 184-205 (sequence CRTFRGRVLDMFRTAFGGKYMR) and GPC 483-498 (sequence CGAFKVPGVKTIWRKRR). The first peptide was derived from the viral glycoprotein GP-1, while the second one was derived from GP-2 (2). Peptides were made on an Applied Biosystems model 430A peptide synthesizer using symmetrical anhydride chemistry

(8). For convenience in coupling an N-terminal, cysteine was added to the peptides where it was not part of the natural sequence. Peptides were then coupled to KLH using MBS via cysteinyl sulfhydryl group (7). Antibodies were made in rabbits as described earlier (2) and assayed for reactivity by ELISA. Peptide reactive rabbit sera also reacted with LCMV GP-1 or GP-2 in Western blot analysis (3). Antibodies to amino acids 157-170 of human ACHR (sequence AINPESDQPDLSNF) were elicited by immunization with purified peptide without carrier protein as described (14). A cysteine for coupling and a Gly-Gly spacer were added at the amino terminus of this peptide for thiopropyl coupling.

### Coupling of Peptides to Thiopropyl Sepharose 6B

Typically, 1 g of thiopropyl Sepharose 6B (TPS), equivalent to 3 ml of swollen gel, was used for each peptide to be coupled. TPS was suspended in 0.1 M Tris-HCl/0.5 M NaCl/pH 7.5 (buffer A) and washed on a sintered glass filter with 200 ml of buffer A. Twenty mg of peptide (at 1 mg/ml in buffer A) was added to the washed gel and mixed end-over-end for 1 h at room temperature (24° C). Uncoupled peptide was separated from the beads by centrifugation at 1000 rpm for 5 min and supernatant containing free peptide was removed. An initial approximation of the amount of peptide coupled was made in two ways:

a) A decrease in $A_{214nm}$ of supernatant compared to $A_{214nm}$ of peptide solution at 1 mg/ml.

b) An increase in $A_{343}$ of supernatant which indicated 2-thiopyridone release, this being molar equivalent to the amount of peptide coupled ($E_{343}$ for 2-thiopyridone = $8.08 \times 10^3$ $M^{-1}$-$cm^{-1}$). The pelleted gel was washed two times with 50 ml of buffer A, and the remaining reactive sites were blocked by mixing the gel with 3-fold excess of 2-mercaptoethanol in 0.1 M citrate buffer, pH 4.5, for 1 h at 24° C. Subsequently, the gel was washed on a sintered glass filter with 200 ml of buffer A and then with 200 ml of 10 mM phosphate buffer, pH 7.4, 0.15 M NaCl (PBS) and packed into a column. Approximately 2-4 mg of peptide was coupled per gram of TPS.

**Table 1. Coupling Efficiency of Three Peptides on Thiolpropyl Sepharose**

| Peptide | Peptide Used (μmol) | Coupled[a] (μmol) | % Efficiency |
|---|---|---|---|
| GPC 104-121 | 12.60 | 2.33 | 18.5 |
| GPC 184-205 | 8.25 | 0.37 | 4.5 |
| GPC 483-498 | 14.2 | 1.73 | 12.2 |

[a]Estimated from the increase in $A_{343}$ (2-thiopyridone release) in supernatant.

### Purification of Anti-Peptide Antibodies

Rabbit sera containing anti-peptide antibodies were used directly after clarification, or gamma globulins were concentrated by 50% saturated ammonium sulfate precipitation and dissolved in PBS. The sample (usually equivalent to 2 ml of serum) was loaded slowly onto a 3 ml column at the rate of 6 ml/h and elution was continued with PBS, monitoring the effluent at 280 nm. The majority of serum proteins (80-99%) did not bind to the column and eluted in the void volume (Pool 1). The column was continually washed with PBS until no more protein was eluted and base line protein concentration measured as $A_{280}$ was stable.

Bound anti-peptide antibodies were then eluted with 0.1 M citrate buffer, pH 3.0. In one instance pH 3.0, elution was not sufficient to release highly avid antibody, so elution at pH 1.0 was also performed to recover this material. Elution profiles exhibited a peak of $OD_{280}$ absorbing material, which varied depending on the anti-peptide antibody being purified. This eluted Ig was referred to as Pool 2. Pool 2 was immediately neutralized upon collection to pH 7.0 with 1 M Tris. The column was subsequently washed and reequilibrated with PBS prior to storage or re-use.

Pools 1 and 2 were dialyzed against PBS and concentrated to the original serum volume by reverse dialysis. Anti-peptide antibodies were assayed by ELISA using microtiter wells coated with 1.0 μg of peptide per well as described (2).

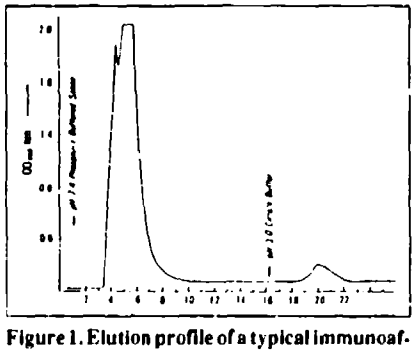


**Figure 1. Elution profile of a typical immunoaffinity purification of rabbit antibody 2704 against peptide GP-C 483-498.** Two ml of antiserum was applied to the column in phosphate buffered saline, pH 7.4, and 2 ml fractions collected as indicated. A small peak ($OD_{280}$ <0.3) of eluted protein is evident in fractions 20-22. These fractions were pooled and concentrated to 2 ml.

## RESULTS

Each gram of thiopropyl Sepharose 6B gel contains 60 μmol of bound 2-pyridyl disulfide, which can be replaced by an equivalent amount of peptide. Theoretically, for a typical 10-residue peptide (MW *ca.* 1100 dalton), 66 mg of peptide can be coupled per gram of gel. Here, we intentionally used suboptimal quantities of the peptide to be coupled (20 mg/g of gel), and the coupling ranged from 2-4 mg (5-10% of capacity); thus a substantial portion of the peptide (90-95%) remained uncoupled and was recoverable from the supernatant. Results of a representative coupling are presented in Table 1. The peptide bound to the Sepharose matrix had a very high capacity to bind to specific antibody on a molar basis. For example, only 2.2 mg of the 10-residue peptide (*ca.* 2 μmol) can bind up to 150 mg of bivalent antibody. On that basis, it seemed prudent to assume that sufficient antigen was coupled to the solid support.

Purification of anti-peptide antibody from rabbit sera was carried out as described in Materials and Methods. The effluent was monitored at 280 nm for eluting proteins, and a typical elution profile of one such run is shown in Figure 1. The flow-through portion of the run (Pool 1) contained unbound serum proteins and represented 80-

99% of the total proteins in individual runs. When bound proteins were eluted with low pH as described, a comparatively small protein peak (Pool 2) was observed. This represented 1-20% of the initially applied protein. After dialysis and concentration, the pooled fractions were analyzed for anti-peptide reactivity by ELISA and compared to the starting sera (Figure 2). Pool 1, representing the flow-through fraction, had 80-99% of the total protein, but had little or no reactivity against the respective peptides. Pool 2 had all or most of the anti-peptide activity and the titer was comparable to the serum (Table 2). The affinity-purified antibodies were checked for their activity against the parent proteins (LCMV GP-1 or GP-2) and were found to be fully active in Western blot analyses (Figure 3). Moreover, by performing an ELISA on KLH substrate, it was demonstrated that >90% of the anti-KLH antibodies did not bind to the column and were eluted in Pool 1. Trace amounts of anti-KLH antibodies occasionally present in Pool 2 were easily removed by a second pass over the peptide-affinity column.

Such peptide-affinity columns were repeatedly used for antibody purification and found to be stable and functional for at least 6 runs. When not in use, peptide-coupled solid matrix was stored in pH 7.4 buffer containing 0.02% sodium azide at 4° C.

### Elution of High Affinity Immunoglobulins

In one instance during the course of studies of peptide antibodies against sequences derived from the human acetylcholine receptor (AChr), we encountered difficulty in eluting specific antibody. Sera known to contain high titered antibody to AChr amino acids 157-170 and reactive with the parent protein were not recovered by pH 3.0 elution. However, when the pH of the eluting buffer was adjusted to 1.0, 80-90% of the applied activity was recovered, suggesting that this immunization protocol, which did not employ a carrier protein, elicited a high-affinity immunoglobulin (Table 3). Based on our experience with LCMV and other viral systems the need for pH 1.0 elution is not commonly observed. Fortunately, despite the low pH required for elution, the recovered immunoglobulin showed excellent retention of binding activity against native AChr following neutralization and concentration.

**Table 2. Summary of Antibody Reactivity**

| Peptide | Rabbit Number | Antibody Units (%)[a] Serum | Pool 1 | Pool 2 |
|---|---|---|---|---|
| GPC 184-205 | 2713 | 4530 | 0 (0%) | 3850 (85%) |
| GPC 483-498 | 2704 | 350,000 | 20,000 (5.7%) | 315,000 (90%) |

[a]Expressed as (titer x volume)

**Table 3. Reactivity of Purified Antipeptide Antisera with Acetylcholine Receptor**

| Reactivity with | Serum: 3026-9[a] | pH 3 Eluent | pH 1.0 Eluent |
|---|---|---|---|
| Peptide[b] | 1:25,600 (100%) | 1:20 (0.08%) | 1:23,000 (89.8%) |
| AChr | 1:6,400 (100%) | 1:10 (0.16%) | 1:5,000 (78.1%) |

[a]Serum 3026-9 = Anti-hu AChr serum 157-170 = AINPESDQPDLSNF

## DISCUSSION

We have described a simple and efficient technique to purify anti-peptide antibodies in a single step. Coupling of peptide to the commercially available affinity matrix can be accomplished in a few steps and may be performed simultaneously for several peptides if necessary. However, the bonding chemistry requires the presence of free cysteine sulfhydryl in the peptide. Although in our studies we have incorporated a terminal cysteine in each sequence, any cysteine residue with free sulfhydryl group, whether internal or terminal, may be adequate for its coupling to thiopropyl Sepharose. Success of this procedure may result because

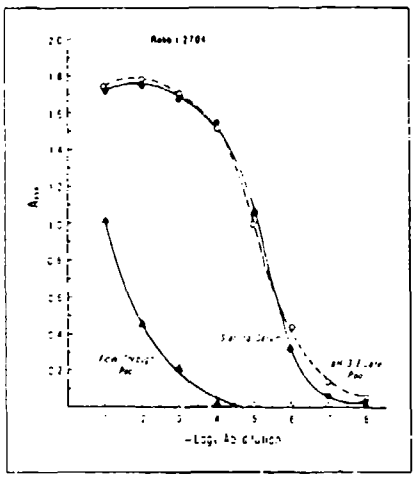


**Figure 2. Immunoreactivity by ELISA of Starting Serum No. 2704 (●-●), flow through (fractions 4-7, ▲-▲) and pH 3 eluate (fractions 20-22, ○-○) from Figure 1.** Peptide GP-C(483-498) was adsorbed (0.1 μg/well) onto polystyrene microliter plates. Each of the indicated column fractions were concentrated to the same volume (2 ml) as the starting serum and titered as described in Materials and Methods. The major portions of antigen binding activity of the starting serum was recovered in the pH 3 eluate.

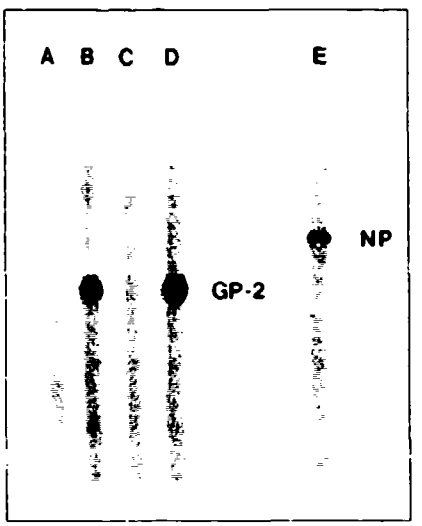



**Figure 3. Western blotting of immunoaffinity purified rabbit antibody to peptide GP-C(483-498) against LCM viral polypeptides separated by SDS-PAGE.** Lane A, prebleed rabbit 2704; B, final postimmunization bleeding serum applied to the immunoaffinity column; C, column fraction 1, unbound material; D, column fraction 2, immunoglobulin eluted at pH 3. Binding to viral glycoprotein GP-2 is evident only in lanes B and D. All fractions were adjusted to the same volume as the initial serum, then diluted 1/50 for Western blotting. The position of migration of LCM viral nucleoprotein is indicated in strip E blotted with a mouse monoclonal antibody (1-1.3) directed against the nucleocapsid protein.

the coupled peptide is presented in a configuration similar to that of the peptide-KLH conjugates used for immunization, although applicability to antisera produced by immunization with free peptide has also been shown. Advantages of thiopropyl Sepharose as the solid support of choice over other commonly used matrices, like CNBr activated agarose (1) or Affi-Gel (Bio-Rad Laboratories, Richmond, CA), may be several fold. In both these cases the linkage of peptide to the solid support is through primary and secondary amines. We have observed that anti-peptide antibodies raised by immunization with MBS-coupled peptides often fail to bind to such coupled peptides. Moreover, in the case of Affi-Gel we observed a constant bleeding of peptide from the column as monitored using an iodinated peptide (data not shown). In contrast, with thiopropyl-linked peptides we have not observed bleeding of coupled peptide during the purification procedure, the disulfide bond being stable at neutral and acidic pH under the conditions used.

It should be noted that free sulfhydryl groups of cysteine residues in peptides may become oxidized during storage, resulting in low efficiency of coupling. Storage of peptide under nitrogen, or reduction prior to the coupling reaction, may increase efficiency. To monitor this spontaneous oxidation, we routinely perform Ellman reactions to detect free cysteine-SH prior to use of specific peptides. This consideration may be more theoretical than practical, since in all cases the amount of peptide coupled was adequte to allow successful affinity chromatography.

The method described here is very useful for purifying peptide-specific antibodies directly from sera despite the presence of high concentrations of anti-carrier antibody. Further, we have demonstrated, using peptides and corresponding antibodies derived from the sequence of human acetylcholine receptor, that the methodology is useful for purification of high affinity antibody raised by injection of free peptide without carrier protein. The coupling chemistry is simple, the activated gel matrix commercially available, and purification rapid and efficient. In view of these advantages, the technique is likely to be applicable to a wide range of peptide antigen-antibody combinations.


## ACKNOWLEDGMENTS

We thank Farshad Karimi and Kaleo Wooddell for technical assistance and Jim Johnston for manuscript preparation. Supported by NIH grant AI 16102. B.S. Parekh was supported by USPHS training grant AG00080-05.


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**Address correspondence to:**

M.J. Buchmeier
*Dept. of Immunology*
*Research Institute of Scripps Clinic*
*10666 North Torrey Pines Rd*
*La Jolla, CA 92037*

VIROLOGY 164, 30–38 (1988)

# Fine Mapping of a Peptide Sequence Containing an Antigenic Site Conserved among Arenaviruses

ELBA LAURA WEBER[1] AND MICHAEL J. BUCHMEIER[2]

*Department of Immunology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, California 92037*

*Received October 26, 1987; accepted January 6, 1988*

**The lymphocytic choriomeningitis virus (LCMV) structural glycoproteins GP-1 (*M*, 44K) and GP-2 (*M*, 35K) are encoded on a single intracellular proteolytic cleavage precursor glycoprotein, GP-C (*M*, 76K). We have used a series of synthetic peptides derived from the deduced amino acid sequence of LCMV GP-C to define an antigenic site containing two topographically overlapping epitopes. Three mouse monoclonal antibodies directed against two epitopes on GP-2 were assayed for binding in solution phase blocking and solid-phase enzyme-linked immunoadsorbant assays to a series of peptides representing the sequence of the intracellular precursor glycopeptide GP-C. Both epitopes were initially localized to a single peptide GP-C 370–382 (Cys–Asn–Tyr–Ser–Lys–Phe–Trp–Tyr–Leu–Glu–His–Ala–Lys) in the GP-2 segment of GP-C. Futher analysis demonstrated that both epitopes were contained within a nine amino acid segment, GP-C 370–378, which contains five residues conserved among LCMV, Lassa, Pichinde, and Tacaribe viruses. Assays with N-terminal deletions from this sequence suggested that the minimal epitope recognized by the broadly cross-reactive monoclonal 33.6 (epitope GP-2a) consisted of five amino acids, GP-C 374–378 (Lys–Phe–Trp–Tyr–Leu). Reactivity of a second monoclonal, 9-7.9 (epitope GP-2B) but not 33.6, was abolished when substitution of tyrosine for phenylalanine was made at position 375 in the antigenic sequence corresponding to a naturally occurring sequence difference between LCM and Lassa viruses. Polyclonal sera from human cases and from animals experimentally infected with Junin, LCM, and Lassa viruses, respectively, bound to the antigenic peptide GP-C 370–382 but not to control peptides. As was the case with the monoclonals, this binding activity was abrogated by blocking with the antigenic peptide but not with control peptides in solution.** © 1988 Academic Press, Inc.

## INTRODUCTION

The Arenavirus family contains a number of significant human pathogens including Lassa, Junin, and Machupo viruses, etiologic agents of Lassa fever, Argentine and Bolivian hemorrhagic fevers, respectively (reviewed in Howard, 1986; Peters *et al.*, 1987). Arenaviruses are classified in two major subdivisions. The Old World viruses include the prototype lymphocytic choriomeningitis virus (LCMV), which is widely distributed in Europe, Asia, Africa, and North and South America, and the highly pathogenic Lassa fever virus, as well as the related nonpathogenic Mopeia, Mobala, and Ippy viruses which have been isolated in Africa (Gonzalez *et al.*, 1984, 1986). New World arenaviruses include the pathogens Junin virus, agent of Argentine hemorrhagic fever (Weissenbacher *et al.*, 1987), and Machupo virus, responsible for Bolivian hemorrhagic fever, as well as other viruses including Pichinde, Tacaribe, Tamiami, Amapari, Parana, and Latino, which are not associated with human disease. These viruses are confined to the South American continent and the region around the Caribbean. Among members of each group, intratypic antigenic relationships are predominant, although group-specific reactivities exist and constitute one of the definitive characteristics of the taxon. Antigenic relationships among pathogenic and nonpathogenic members of the group have been extensively studied to define epitopes of potential use as diagnostic tools or as immunogens (Casals *et al.*, 1975). Using polyclonal reagents it was possible to define group-specific antigens detectable by complement fixation on the nucleocapsid proteins (NP) of New and Old World arenaviruses (Rowe *et al.*, 1970; Peters *et al.*, 1973). Availability of monoclonal antibodies to specific viral proteins allowed demonstration of conserved antigens not only on NP but also on viral glycoproteins (Buchmeier *et al.*, 1980; Buchmeier, 1984). Currently available synthetic peptide technology makes it possible to study these antigenic relationships at the level of primary amino acid sequences. In the present study we describe a group-specific antigenic determinant on the native GP-2 glycoprotein of LCMV which is conserved across a wide range of arenaviruses representing both Old and New World subgroups. We have localized this native site to a stretch of nine amino acids within GP-2. Moreover, by analyzing peptide substitutions within this region we show

[1] Present address: Catedra de Microbiologia, Facultad de Medicina U.B.A., Paraguay 2155 Piso 11, (1121) Buenos Aires, Argentina.

[2] To whom request for reprints should be addressed.



0042-6822/88 $3.00
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that two GP-2-specific MAbs, which differ in their reactivity with a panel of eight arenaviruses and map to topographically overlapping epitopes, both recognize the same nine amino acid segment of GP-2 but can be differentiated by internal amino acid substitutions corresponding to the sequences of naturally occurring arenaviruses. We also present evidence suggesting that this site on GP-2 is immunogenic in the context of native glycoprotein in several different animal species infected with arenaviruses.

## MATERIALS AND METHODS

### Virus

Attenuated Junin virus, strain XJ-Clone 3, was provided as a suckling mouse brain suspension by C. J. Peters (USAMRIID) and was passaged once in this laboratory in Vero E6 cells (m.o.i. ~0.1). Tissue culture fluids were harvested at 72 hr, clarified by centrifugation for 10 min at 2000 rpm, and stored at −70°. Tacaribe and LCMV Arm (CA1371) were grown in Vero or BHK-21 cells, respectively, as previously described (Howard *et al.*, 1985; Buchmeier and Oldstone, 1979).

### Antisera

Human anti-Junin antiserum was from a recovered laboratory-acquired case of Argentine hemorrhagic fever, and was obtained 7 years after the onset of the disease. Anti-LCM polyclonal serum was prepared by priming adult Hartley guinea pigs with $2 \times 10^4$ PFU of the Armstrong strain of virus, and then challenging 3–4 weeks later with the WE strain. Postinfection serum was harvested 10–15 days after second inoculation and had neutralizing titers of greater than 1/5000 measured by plaque reduction assay (Parekh and Buchmeier, 1986). Rhesus monkey anti-Lassa convalescent serum was provided by Dr. P. Jahrling.

### Monoclonal antibodies

The generation and characterization of mouse MAbs against LCM virus are detailed elsewhere (Buchmeier *et al.*, 1981; Parekh and Buchmeier, 1986). MAbs 33.6, 83.6, and 9-7.9 all reacted with the glycoprotein GP-2, while MAb 2-11.10 was specific for GP-1.

### Synthetic peptides

Peptides corresponding to predicted sequences of regions of LCM GP-C, the cellular precursor of the glycoproteins GP-1 and GP-2, were made as described elsewhere (Buchmeier *et al.*, 1987; Southern *et al.*, 1987) utilizing an Applied Biosystems Model 430 peptide synthesizer (ABI, Inc., Redwood City, CA).

### ELISA assays

Linbro/Titertek polystyrene plates were coated with 0.1 or 1.0 μg of peptide in PBS per well, dried overnight at 37°, and blocked with 2% milk powder in PBS containing 0.05% of Tween 20. When assayed with polyclonal antibodies the plates were coated with 1 μg of peptide per well. For assays with LCM virions the plates were coated with 1–1.5 μg of purified virus in PBS per well, incubated overnight at 4°, and blocked with 3% bovine serum albumin in PBS, as described (Parekh and Buchmeier, 1986).

For Tacaribe and Junin viruses, Vero cells were infected with an m.o.i. ≃1, and when the cytopathic effect was 50% the monolayers were fixed with cold methanol for 10 min, air dried, and stored at −20° until used. In all cases binding of antibody was detected with protein A–peroxidase, using *o*-phenylenediamine as the chromogenic substrate, and optical density at 492 nm was quantitated.

### Western blotting

Purified LCM virus was electrophoresed in a 10% polyacrylamide gel and the proteins were transferred electrophoretically to nitrocellulose sheets (Burnette, 1981). Monoclonal or polyclonal antibody dilutions were made in PBS–Tween-20 containing 2% skim milk powder, reacted with the nitrocellulose, and washed, and bound immunoglobulin was detected by incubation with $^{125}$I-protein A.

### Immunofluorescence assays

Immunofluorescence assays were carried out on acetone-fixed Vero (Tacaribe and Junin viruses) or L929 (LCM virus) cell substrates, using sheep anti-mouse FITC as conjugate, as previously detailed (Buchmeier *et al.*, 1981).

In order to demonstrate binding in solution, polyclonal or monoclonal antibody dilutions were incubated with peptides for 1 hr at room temperature or for 30 min at 37°, and residual reactivity of the mixture was titrated by immunofluorescence, ELISA, or Western blotting as indicated. Reaction of antibodies with peptides in solution was considered positive when binding of antibodies to the viral glycoprotein was blocked after incubation with peptide.

## RESULTS

### Monoclonal antibodies to GP-2 define group-specific antigens

Three monoclonal antibodies raised by immunization with LCMV and showing cross-reactivity by indi-

**TABLE 1**

REACTIVITY OF LCM MONOCLONAL ANTIBODIES WITH DIVERSE OLD AND NEW WORLD ARENAVIRUSES

| | Polypeptide specificity | Old World | | | New World | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | LCMV | Lassa | Mopeia | Pichinde | Tacaribe | Junin | Amapari | Parana |
| 33.6 | GP-2 | + | + | + | + | + | + | + | + |
| 83.6 | GP-2 | + | + | + | + | + | + | + | + |
| 9-7.9 | GP-2 | + | − | + | − | − | − | − | − |
| 2-11.10 | GP-1 | + | − | − | − | − | − | − | − |

*Note.* + indicates positive reaction in immunofluorescence at an ascites dilution of ≥1:100. − indicates no reaction at 1:100.

rect immunofluorescence with heterologous arenaviruses were chosen for the present study. These monoclonals have been shown by competitive binding studies to map to two overlapping epitopes on the LCMV GP-2 molecule (Parekh and Buchmeier, 1986). We assayed these same antibodies for reactivity with a panel of arenavirus antigens representing Old World (LCMV, Lassa, Mopeia) and New World (Pichinde, Tacaribe, Junin, Amapari, Parana) viruses and found that antibodies 33.6 and 83.6 reacted positively against all of these viruses. Monoclonal 9-7.9, in agreement with previous studies (Buchmeier *et al.*, 1980), reacted only with LCM and Mopeia viruses (Table 1).

### Reactivity of anti-GP-2 monoclonals with synthetic peptides

We have recently constructed a library of synthetic peptides representing over 90% of the LCMV-ARM GP-C open reading frame which is encoded at the 5′ end of the viral S RNA (Southern *et al.*, 1987). Glycoprotein-2 is encoded in the carboxyl half of GP-C and spans amino acids 263–498. We therefore assesed the reactivity of each of the three cross-reactive GP-2 monoclonals with each of 18 synthetic peptides spanning GP-2. Only one, corresponding to GP-C residues 370–382, reacted specifically with those antibodies (Table 2). No binding was observed against the overlapping flanking peptide sequences GP-C 353–370 or 378–391, suggesting that the epitope recognized by MAbs required the unique sequence at the amino terminal end (370–377) of the peptide. By comparison with the published sequences of three other arenaviruses, Pichinde (Auperin *et al.*, 1984), Lassa (Auperin *et al.*, 1986), and Tacaribe (Franze-Fernandez *et al.*, 1987), we found extensive conservation of amino acid sequence among all four viruses in this region.

### Peptide 370–382 specifically inhibits binding of the MAbs to the glycoprotein of Old and New World arenaviruses

To determine whether the binding sequence in GP-2 corresponded to GP-C 370–382, and to establish whether binding occurred in solution, MAbs 33.6 and 9-7.9 were incubated with GP-C peptides 353–370, 370–382, and 378–391, as indicated under Materials and Methods, and then used to detect GP-2 in Western blots of LCMV. Figure 1 illustrates the reactions observed when the monoclonals were first reacted with these peptides and then with GP-2. Preincubation of either monoclonal with peptide 370–382 completely blocked binding to GP-2, indicating that the peptide reacted with antibody in solution, and that the sequence recognized by the MAbs in the denatured viral glycoprotein is contained in the peptide sequence. Specificity of the reaction in solution was confirmed by the lack of blocking by peptides 353–370 and 378–391.

Since MAb 33.6 was found to bind to both Old and New World arenaviruses, we sought to further confirm the validity of the GP-C 370–382 sequence by blocking reactivity of the antibody with Tacaribe and Junin viruses. Antibody binding to each of these viruses was measured by ELISA assay before and after preincubation with peptides 353–370, 370–382, or 378–391.

**TABLE 2**

ELISA REACTIVITY OF LCM MAbs WITH PEPTIDES CORRESPONDING TO GP-C AMINO ACIDS 353–370, 370–382, AND 378–391

GPC[a] $^{353}$DGLLMRNHLRDLMGVPYCNYSKFWYLEHAKTGETSVPKC$^{391}$

| | Reciprocal ELISA titer against | | | |
|---|---|---|---|---|
| MAbs | LCM virions | 353–370 | 370–382 | 378–391 |
| 33.6 | 83,176 | <100[b] | 25,600 | <100 |
| 83.6 | 32,768 | <100 | 6,400 | <100 |
| 9-7.9 | 28,526 | <100 | 1,638,400 | <100 |
| 2-11.10 | $>1 \times 10^6$ | <100 | <100 | <100 |

[a] The sequence shown corresponds to residues 353–391 of the LCMV-Arm strain (P. J. Southern, and D. H. L. Bishop, 1987).

[b] Reciprocal of highest dilution showing positive reactivity in ELISA. <100 indicates no significant reaction at 1:100.

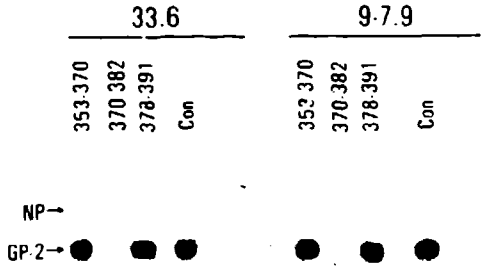



FIG. 1. Blocking activity of GP-2 peptides against monoclonal antibodies 33.6 and 9-7.9. Purified LCM virus was disrupted in SDS-2-mercaptoethanol and then electrophoresed on a 10% SDS gel. Proteins were electrophoretically transferred to nitrocellulose and 0.5-cm strips were cut. MAbs at a 1/50 final dilution were preincubated with buffer (Con) or with 50 μg of the indicated peptide for 30 min at 37°, and then used in a standard Western blot protocol to detect GP-2 on the indicator strips. Binding was visualized using $^{125}$I protein A as described under Material and Methods. Only peptide 370-382 blocked binding of 33.6 and 9-7.9 to GP 2.

Aliquots of MAb 33.6 were preincubated with each of the three peptides and then titered by ELISA against LCM, Junin, or Tacaribe antigens (Fig. 2). Again, reactivity of the antibody with each of these substrates was abrogated after incubation with peptide 370-382. Futher evidence that the peptide contained a conserved epitope was obtained by Western blotting and by immunofluorescence assays in which peptide 370-382 alone blocked binding of MAbs 33.6 and 9-7.9 to Tacaribe and Junin viral antigens (data not shown).

## Fine mapping of epitopes on peptide 370-382

Previous studies (Parekh and Buchmeier, 1986) have shown that MAbs 33.6 and 83.6 recognize a common epitope, GP-2A, while MAb 9-7.9 reacts with a topographically overlapping epitope, GP-2B; however, present results indicated that both epitopes are contained in a nine amino acid segment within peptide GP-C 370-382. In order to establish the limits of these epitopes and the molecular basis of their differences in virus specificity we constructed a series of peptides with a common carboxyl terminus corresponding to Lys 382 and spanning 368-382, 370-382, 372-382, 374-382, and 376-382 and quantitated antibody binding to each by ELISA. These data are presented in Table 3. It is evident that ELISA titers decreased from >40,960 to 2560 for both monoclonals when residues 370 and 371 (Cys-Asn) were deleted, and all residual reactivity was lost with omission of residues 372 and 373 (Tyr-Ser). To establish the C-terminal limit of the epitope an additional series of nested peptides was synthesized utilizing Leu 378 at the carboxy terminus and extending toward the amino terminus as above. In this instance, titers of >40,000 were once again observed with GP-C 368-378 diminishing to 6400 with 370-378. Deletion of residues 370 and 371 further diminished ELISA reactivity of 33.6 and virtually eliminated that of 9-7.9. Thus the minimal sequence containing both the 33.6 and 9-7.9 epitopes encompasses GP-C 370-378 when measured by solid-phase ELISA assay. Moreover, there may be a significant contribution to reactivity of the short peptide with amino terminal extension to include two additional conserved residues Tyr 369 and Pro 368.

To minimize the possibility of spurious results due to the use of the solid-phase assay we assayed each truncated peptide for capacity to block binding of 33.6 to GP-2 in Western blots. All the peptides shown in Table 3 containing the seven amino acid core sequence Tyr-Ser-Lys-Phe-Trp-Try-Leu (372-378) exhibited blocking activity (data not shown); however, peptide 376-382 not containing the entire 372-378 core sequence did not block binding of 33.6 to GP-2 (Fig. 3). Peptide 374-382 was of particular interest since it completely blocked binding of 33.6 but only partially inhibited 9-7.9 binding to GP-2.

## Molecular nature of species specificity of MAb 9-7.9

We found it surprising that MAbs 33.6 and 9-7.9 both recognized a common nine amino acid peptide in view of previous results, indicating differences in their recognition of diverse arenaviruses (Table 1) and their assignment to separate although overlapping epitopes

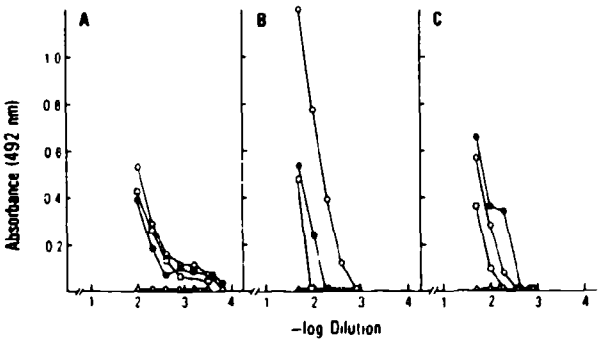



FIG. 2. Blocking of binding of MAb 33.6 to Old and New World arenaviruses by peptide 370-382. MAb 33.6 was incubated with peptides as indicated in Fig. 1 and used in ELISA against (A) LCM virions, (B) Tacaribe-infected cells, (C) Junin-infected cells. LCM: Old World arenavirus. Tacaribe and Junin: New World arenavirus. (●) MAb 33.6 alone. (○) 33.6 + peptide 353-370; (△) 33.6 + 370-382; (□) 33.6 + 378-391.

TABLE 3

EFFECT OF AMINO AND CARBOXY TERMINAL DELETIONS ON REACTIVITY OF MAbs 33.6 AND 9-7.9 WITH CONSERVED GP-2 SEQUENCES

| GP-C | Peptide | ELISA titer | | | Blocking activity | |
|---|---|---|---|---|---|---|
| | | 33.6 | 9-7.9 | GP > LCM[a] | 33.6 | 9-7.9 |
| Expt 1 | | | | | | |
| 368-382 | PYCNYSKFWYLEHAK | >40,960[b] | >40,960 | 160 | +[c] | + |
| 370-382 | CNYSKFWYLEHAK | >40,960 | >40,960 | 40 | + | + |
| 372-382 | YSKFWYLEHAK | 2,560 | 2,560 | <10 | + | + |
| 374-382 | KFWYLEHAK | 40 | 10 | 10 | + | ± |
| 376-382 | WYLEHAK | <10 | <10 | <10 | − | − |
| Expt 2 | | | | | | |
| 368-378 | PYCNYSKFWYL | >40,960 | >40,960 | ND[d] | + | + |
| 370-378 | CNYSKFWYL | 6,400 | 6,400 | ND | + | + |
| 372-378 | YSKFWYL | 1,600 | 100 | ND | + | + |

[a] Hyperimmune guinea pig antiserum to LCMV.
[b] Reciprocal of highest titer showing positive reactivity in ELISA.
[c] + indicates that peptide in solution blocks reactivity of indicated monoclonal antibody in Western blots.
[d] ND, not done.

(Parekh and Buchmeier, 1986). We sought then to probe these differences in more detail by constructing peptides with single amino acid substitutions consisting of Ser → Thr at residue 373 and Phe → Tyr at residue 375. These changes correspond to the sequences in this region of Pichinde and Lassa viruses, respectively. The carboxy terminal sequence Leu–Glu–His–Ala–Lys which is found only in LCMV was left intact for convenience. In other experiments in which the exact sequences of LCMV and Pichinde which differ in this region were tested no differences in binding attributable to this region were observed (data not shown). As evident in Table 4, substitution of Thr for Ser at position 373 had little effect on antibody binding; however, the Lassa-specific Phe → Tyr change at position 375 resulted in almost total loss of binding by MAb 9-7.9 without loss of 33.6 binding. Since 33.6 reacts with Lassa virus and LCMV and 9-7.9 reacts only with LCMV, the results with these peptides suggest that the basis for this difference in specificity is the Phe → Tyr substitution.

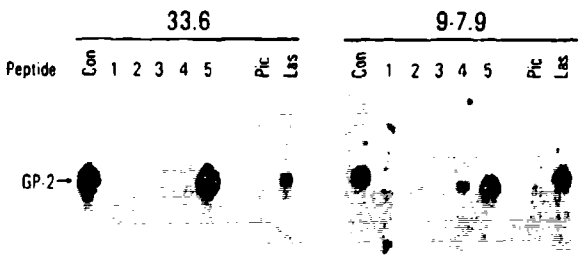



FIG. 3. Blocking effect of amino terminus deleted and substituted and peptides on binding of MAbs 33.6 and 9-7.9 to LCMV GP-2. MAbs were incubated with peptides as indicated (Fig. 1) and used in Western blots with LCM proteins.

| | GP-C Sequence | |
|---|---|---|
| 1: | 368-382 | PYCNYSKFWYLEHAK |
| 2: | 370-382 | CNYSKFWYLEHAK |
| 3: | 372-382 | YSKFWYLEHAK |
| 4: | 374-382 | KFWYLEHAK |
| 5: | 376-382 | WYLEHAK |
| Pic: | Thr 373 → Ser | PYCNYTKFWYLEHAK |
| Las: | Phe 375 → Tyr | PYCNYSKYWYLEHAK |

Reactivity of these MAbs and substituted peptides in solution was tested as indicated before by incubation of antibodies with peptides and immunoblotting with purified LCM to detect GP-2. The Lassa-specific substitution of Phe for Tyr only slightly diminished the blocking activity reaction with 33.6 as indicated by weak binding to LCMV GP-2 in the Western blot. The Lassa equivalent substitution abolished the blocking activity of the peptide for MAb 9-7.9, as indicated by the full reactivity of that monoclonal against GP-2 remaining after incubation with peptide (Fig. 3).

## Polyclonal antibodies against Old and New World arenaviruses bind specifically to peptide 370–382

Having established that peptide 370-382 contains the conserved epitope in the arenavirus glycoprotein, it was of interest to determine whether GP-C 370–382 was recognized in the context of native protein in the infected host. Polyclonal antisera against LCM, Lassa, and Junin were tested in ELISA with peptides 353–370, 370–382, and 378–391 (Table 5). Specific

**TABLE 4**

EFFECT OF AMINO ACID SUBSTITUTIONS CORRESPONDING TO LASSA AND PICHINDE SEQUENCES ON REACTIVITY OF MABS 33.6 AND 9-7.9

| Viral sequence | Peptide | Reciprocal ELISA titer | | |
|---|---|---|---|---|
| | | 33.6 | 9-7.9 | GPaLCM[a] |
| LCMV GPC 368-382 | PYCNYSKFWYLEHAK | >40,960 | >40,960 | 160 |
| Ser (374) → Thr[b] | PYCNYTKFWYLEHAK[d] | 40,960 | 10,240 | 10 |
| Phe (376) → Tyr[c] | PYCNYSKYWYLEHAK | 10,960 | 160 | 10 |

[a] GPaLCM guinea pig anti-LCM serum.
[b] Substitution corresponding to Pichinde virus.
[c] Substitution corresponding to Lassa virus.
[d] Amino acids LEHAK are unique to the sequence of LCMV and are not found in Lassa or Pichinde viruses.

binding of all of these antisera was observed with peptide 370–382. As above, binding in solution was determined by incubation of the LCMV immune guinea pig serum with peptides, and assaying by immunoblotting (Fig. 4). Maximum inhibition of binding to GP-2 was approximately 70% at both 1/50 and 1/100 dilutions as determined by quantitation of the $^{125}$I radioactivity in the excised GP-2 bands from the nitrocellulose strips.

Polyclonal guinea pig serum was also tested for reactivity against the truncated and substituted peptides described in Table 3 and 4. As in evident from the data in those tables, deletion of the amino terminal residues or substitution resulted in loss of reactivity of the polyclonal antiserum.

## DISCUSSION

We have employed a series of synthetic peptides derived from the deduced amino acid sequence of the LCMV-ARM GP-2 (Southern *et al.*, 1987) to map the binding site of three monoclonal antibodies against two topographically distinguishable epitopes on native GP-2 (Parekh and Buchmeier, 1986). Both direct solid-phase ELISA and binding assays in solution have shown that this site consists of a stretch of nine amino acids spanning residues 370-378 of LCMV GP-2 and is largely conserved among four other arenaviruses for which sequence is available (Fig. 5) including LCMV strain WE (Romanowski *et al.*, 1985), Lassa (Auperin *et al.*, 1986), Pichinde (Auperin *et al.*, 1984), and Tacaribe (Franze-Fernandez *et al.*, 1987). The observation of conservation of this sequence across a broad representation of arenavirus from the New and Old World groups (Howard, 1986) suggests that it represents an important functional or structural component of the virus. We have not as yet been able to assign functional significance to this site. Neither MAb 33.6 nor 9-7.9 mediated complement independent virus neutralization in our hands although 9-7.9 shows modest neutralizing activity when guinea pig complement is

**TABLE 5**

REACTIVITY OF POLYCLONAL ANTISERA TO ARENAVIRUSES WITH PEPTIDES 353-370, 370-382, AND 378-391 IN ELISA

GPC ... DQLLMRNHLRDLMGVPYCNYSKFWYLEHAKTGETSVPKC ...
(353 and 391 mark the peptide boundaries)

| Serum | 353-370 | 370-382 | 378-391 |
|---|---|---|---|
| Anti-LCMV (guinea pig) | <5[b] | 320 | <5 |
| Anti-Junin[c] (human) | <5 | 160 | <5 |
| Anti-Lassa[c] (rhesus monkey) | <5 | 20 | <5 |
| Normal human[d] | <5 | <5 | <5 |

[a] LCMV-Arm GP-C amino acids 353-391 as described by Southern and Bishop (1987).
[b] Reciprocal of highest dilution showing reactivity in ELISA is indicated.
[c] Late convalescent serum from a laboratory infection.
[d] Lassa convalescent sera obtained from Dr. P. Jahrling, USAMRIID (Fort Detrick, Frederick, MD).
[e] Five normal human sera were tested.

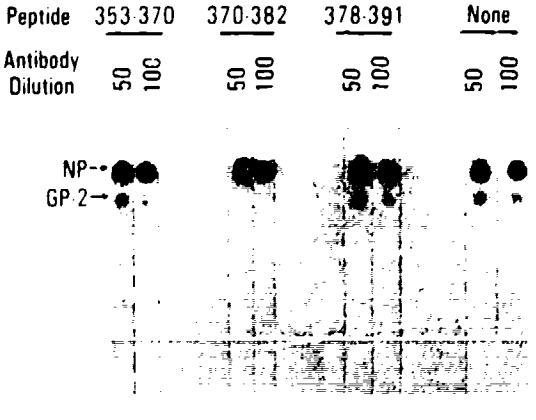



FIG. 4. Immunoblotting of polyclonal anti-LCM serum with LCMV protein. Serum dilutions were incubated with 50 μg peptides (Fig. 1) and assayed by Western blotting.

added to the reaction (Buchmeier *et al.*, 1981). In contrast, several monoclonals against GP-1 have been shown to be strongly neutralizing in the absence of complement (Buchmeier *et al.*, 1981; Bruns *et al.*, 1983; Parekh and Buchmeier, 1986). The question of structural significance is a subject of current investigation. We and others have suggested that LCMV GP-2 is a transmembrane glycoprotein which interacts inside the virion with the nucleocapsid complex (reviewed in Buchmeier and Parekh, 1987). If this interpretation is correct then in all likelihood the 370–378 site lines on the external side of the membrane since antibodies 33.6 and 9-7.9 both react with surface components of intact virus-infected cells (Buchmeier, unpublished observations). We are currently extending our studies to determine whether the 370–378 site participates in recognition by virus-primed helper or cytotoxic T cells.

Mapping of antibody-binding epitopes on viral proteins has proven valuable in interpreting the nature of antigen recognition and of mutational changes which allow the pathogen to escape neutralization. We have previously shown (Buchmeier *et al.*, 1980) cross-reactivity of one of our monoclonals, 9-7.9, with the nonpathogenic arenavirus Mopeia (previously Mozambique) but not with the pathogenic Lassa virus. In contrast, MAb 33.6 reacts avidly with both of these viruses as well as with LCMV and a variety of South American New World arenaviruses. In view of this difference in specificity of virus recognition it was of interest to us that both antibodies mapped to a common nine amino acid segment of GP-2. Previous work (Parekh and Buchmeier, 1986) showing that 33.6 and 9-7.9 overlap topographically coupled with the results obtained in this study with peptides containing single amino acid substitution at positions 373 and 375 provided a theoretical basis for this specificity and further evidence that the sequence 370–378 contained two epitopes. A Ser → Thr change at 373 (corresponding to the Pichinde virus sequence) had no effect on binding of either monoclonal, but a Phe → Tyr change at 375 (corresponding to the sequence of Lassa virus) rendered MAb 9-7.9 unreactive without affecting binding of 33.6, suggesting that Phe 375 is an important contact residue for the former but not the latter. Estimates of the shortest continuous stretch of sequence which retained reactivity were made by ELISA and by blocking assays. By ELISA the minimal peptides retaining significant reactivity with both 33.6 and 9-7.9 contained the nine amino acid core sequence GP-C 370–378 (Cys–Asn–Tyr–Ser–Lys–Phe–Trp–Tyr–Leu). The actual epitope is likely to be somewhat shorter since peptides containing the seven amino acid core sequence GP-2 372–378 blocked the activity of 33.6 when incubated in solution and subsequently assayed by Western blotting. The 33.6 epitope may be contained within the five amino acids spanning 374–378 since peptide 374–382 inhibited 33.6 binding in the Western blot assay (Fig. 3). This size is consistent with those of continuous epitopes mapped in other viral systems (Elder *et al.*, 1987; Houghten, 1985). The discrepancy between the results obtained by solid-phase ELISA and by solution phase blocking may reflect constraints imposed on antigen–antibody interaction in the solid-phase system. Data presented in Table 3 support this notion. Comparing binding titers of 33.6 and 9-7.9 against peptides 370–382 and 370–378, we observed a decrease in titer against the shorter peptide (>40,960 vs 6400) which was restored with the addition of two additional amino acids at the N-terminus (peptide 368–378; titer 40,960). Amino acids 379–382 are unlikely to contribute to antigenicity since in other experiments where the LCMV sequence (Cys–Asn–Tyr–Ser–Lys–Phe–Trp–Tyr–Leu–Glu–His–Ala–Lys) was compared with the homologous sequence from Pichinde virus (Cys–Asn–Tyr–Thr–Lys–Phe–Trp–Tyr–Ile–Asn–Asp–Thr–Ile), where the five carboxyl terminal amino acids are not conserved, both peptides reacted equally with MAbs 33.6 and 9-7.9. Binding the peptide antigen to plastic would be expected to restrict mobility and therefore freedom to react with antibody. The effect of this restriction is likely to become more severe as the peptide approaches the size of the minimal epitope.

Alternatively the difference in results between solution and solid-phase assays may be in part quantitative. In solution blocking assays, approximately 50 μg (0.05 μmol) of peptide 372–378 was offered to 0.5 μg of monoclonal IgG (ratio of $10^{-1}$ μmol peptide/μg IgG). In solid-phase, plates were coated with 0.1 μg/well ($10^{-4}$ μmol) and a 1/10 initial dilution of MAb contained 0.25 μg IgG, thus the ratio was $4 \times 10^{-4}$ μmol peptide/μg IgG. If difference in peptide concentration was

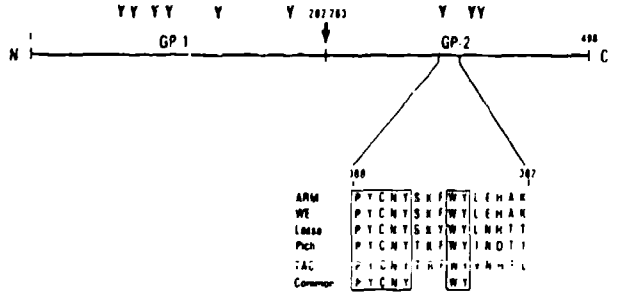



FIG. 5. Schematic diagram of GP-C showing the position of the conserved antigenic site. The proteolytic cleavage site of GP-C to yield GP-1 and GP-2 is shown at amino acids 262–263 (Buchmeier *et al.*, 1987). Potential N-linked glycosylation sites (Y) are indicated.

solely responsible, however, increased binding with dilution of the MAb would be expected. Such a prozone effect was never observed.

We cannot exclude the possibility that residues from other portions of the GP sequence contribute to antibody binding to native GP-2. Barlow and colleagues (1986) have suggested that all epitopes are discontinuous to some extent. Thus although binding to the 370–378 sequence is clearly demonstrable, other residues may contribute to binding or stability of the antigen–antibody complex in the context of native protein. It must be emphasized that the monoclonals used in this study were prepared and selected against native protein (Buchmeier *et al.*, 1981), yet they show substantial reactivity against short peptides. MAb 9-7.9 in particular binds at higher titer against the peptide antigen than against native virus (Table 2).

One aspect of our data remains enigmatic. In Table 1 we show that MAb 9-7.9 (representing site GP-2B) reacts with native LCM and Mopeia viruses but not Pichinde virus, yet from the data presented in Table 4 it is apparent that substitution of Ser → Thr at position 373, corresponding with the sequence of Pichinde virus, only slightly decreases reactivity of this antibody. Two possible explanations are suggested by the data. Both peptides contain a consensus N-linked glycosylation site of the form Asn–Tyr–Ser (LCMV) or Asn–Tyr–Thr (Pichinde). It is possible that this site is glycosylated in Pichinde virus but not in LCMV resulting in masking of the epitope in the former. Our observations argue against this possibility; binding of 33.6, which maps to the same nine amino acid peptide segment, is similar with both native viruses. Other influences such as differences in local conformation around the antigenic sequence may also account for the observed differences, but further structural information is necessary to fully explain the observation.

The present studies may prove to be of practical importance. As described, we have observed binding of polyclonal convalescent serum directed against LCM, Junin, and Lassa Viruses with the synthetic peptide, suggesting that it may be of use as a diagnostic antigen to detect antibody against arenaviruses. Such a peptide diagnostic reagent has several potential advantages over the currently utilized viral or infected cell derived antigen preparations. Among these are low cost, stability, and the ability to rapidly provide sequences corresponding to those of naturally occurring viruses. Our current efforts are directed toward evaluating the potential utility of the sequences we have discribed as diagnostic tools.

## ACKNOWLEDGMENTS

This is Publication Number 5058-IMM from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037. This work was supported by USPHS Grants AI-16102 and DOD Contracts DAMD C-6234. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents. The authors thank Farshad Karimi, Gretchen Bloom, Leslie Igarashi, Rebecca Day, and Kaleo Wooddell for technical support, Peter Southern for helpful comments, and Jim Johnston for manuscript preparation.

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**Immunobiology and Pathogenesis of Persistent Virus Infections**
Edited by **Carlos Lopez**
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1911 I Street, N. W.
Washington, DC 20006

*Chapter 7*

# Structure and Expression of Arenavirus Proteins

*M. J. Buchmeier, K. E. Wright, E. L. Weber, and B. S. Parekh*

## INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV), like all arenaviruses, has the capacity to establish persistent infection in vivo. Such persistent infection both guarantees survival of LCMV from one generation to the next and poses the challenging intellectual puzzle of unraveling the mechanism of virus persistence in vivo. Studies described below have focused on two primary aspects of arenavirus infections, i.e., their protein synthesis and expression and their antigenicity, and we have attempted to correlate in vitro biochemical and immunologic data with biological observations during acute and persistent infections. In this brief review, recent findings which highlight some of the important features of the protein structure of LCMV and other arenaviruses are described and related to biological and immunological phenomena observed during acute and persistent virus infections.

## STRUCTURAL PROTEINS OF ARENAVIRUSES

The structural proteins of purified arenaviruses were first studied by Ramos et al. (22) with Pichinde virus and by Pedersen (20) with LCMV. Numerous other descriptive studies of the proteins of these agents followed, and to date, structural proteins of at least nine different arenaviruses have been examined (summarized in reference 9). Despite differences, a number of common features have emerged. Arenaviruses

M. J. Buchmeier, K. E. Wright, E. L. Weber, and B. S. Parekh • Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037.

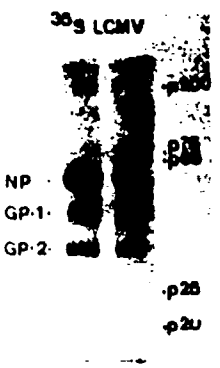



Figure 1. [$^{35}$S]methionine-labeled LCMV separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In addition to NP, GP1, and GP2, the positions of migration of several minor proteins are marked. One of these, p200, has been shown by us to correspond to the L gene-encoded 200-kilodalton protein (see text).

all contain a major dominating protein which is the viral nucleocapsid protein (NP; 60 to 68 kilodaltons). NP constitutes up to 58% of the protein in arenaviruses (28) and is easily detected in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels by protein staining or by radiolabeling with amino acid precursors such as [$^{3}$H]leucine or [$^{35}$S]methionine (Fig. 1). The viruses also contain either one, as reported for Tacaribe and Tamiami viruses (11), or two, as reported for LCMV and Pichinde virus (4, 28), glycoproteins of somewhat lower molecular weight than NP. Other minor proteins have also been detected, but their origin has until recently been largely a subject of conjecture. Predominant among these quantitatively minor proteins is a 180- to 200-kilodalton protein termed L in Pichinde virus (13) and LCMV (26a), which is likely to be the viral RNA polymerase.

Recent advances in our understanding of the molecular genetics of arenaviruses have cleared up some of the ambiguity surrounding the identity and derivation of the viral proteins. As recently detailed in reviews by Bishop and Auperin (2) and Southern and Bishop (27), molecular cloning approaches have definitively assigned NP and the glycoprotein precursor (GPC) of glycoproteins GP1 and GP2 to the S RNA segments of LCMV and Pichinde virus. These two open reading frames, which are arranged in an ambisense orientation (2), completely account for the coding capacity of S.

We sought to establish definitively the linear orientation of GP1 and GP2 within the GPC-coding region at the 5′ end of the viral S RNA. Using the methods described by Hunkapiller et al. (16), we first attempted to determine the N-terminal sequence for each of the glycoproteins isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation and extraction from the gel matrix. Despite the presence of sufficient

quantities of protein, we were unable to degrade the amino terminus, presumably because it was blocked. To resolve this problem, we developed an alternative strategy involving the use of synthetic peptides deduced from the nucleotide sequences of two strains of LCMV, strains ARM and WE.

Peptides were selected from predominantly hydrophilic stretches within the predicted LCMV ARM glycoprotein-coding sequence and were used to raise antisera in rabbits. By their reactivity with the viral glycoproteins GP1 and GP2, these antisera defined the orientation of the glycoproteins on the precursor and the probable site of proteolytic cleavage.

Previous studies (1, 4, 22) showed that the GP2 proteins of LCMV and Pichinde virus were probably integral membrane proteins. Dissociation of virions with mild detergents resulted in a complex of GP2 with viral nucleocapsid complexes, suggesting that the former spanned the membrane. GP1, in contrast, is highly exposed on the virion envelope, as indicated by its susceptibility to surface iodination and its targeting by neutralizing antibodies. Recent studies have delineated the sequences of several arenavirus GPC genes, and a number of common features among them have emerged (Fig. 2). All have prominent hydrophobic stretches of 20 to 30 amino acids near their carboxy termini; these stretches are thought to serve as membrane, and possibly transmembrane, anchor sequences. There are, however, two additional hydrophobic sequences within the amino terminal 50 amino acids. The first of these, spanning approximately the first 30 amino acids, is likely to be a signal sequence by analogy with other membrane glycoproteins. The second, spanning amino acids 30 to 50 of GPC, may provide an alternative membrane anchor sequence for the precursor or cleaved products or both.

Considerable structural homology is evident among arenaviruses in the carboxyl half of GPC, whereas much divergence is found in the amino-terminal domain (27). In particular, a motif appearing between LCMV GPC amino acids 225 and 285 was found to be repeated in Pichinde and Lassa viruses (Fig. 3). This motif consisted of the amino acid sequences $_{228}$LIIQNXTWEXHC$_{239}$ and $_{272}$ISDSXGXX XPGGYCL$_{280}$, bracketing a pair of basic amino acids, RR or RK, which appeared to be likely targets for membrane-bound or extracellular proteases. Antisera were prepared to each of the flanking conserved peptides and to a third peptide immediately to the left of the RR pair. The peptide closest to the carboxyl terminus elicited an antiserum reactive with GP2, whereas the two peptides to the left of the RR pair elicited GP1-specific antibodies (Fig. 4). Thus, it appears highly likely that the RR doublet at GPC amino acids 262 to 263 constitutes the site of proteolytic cleavage to

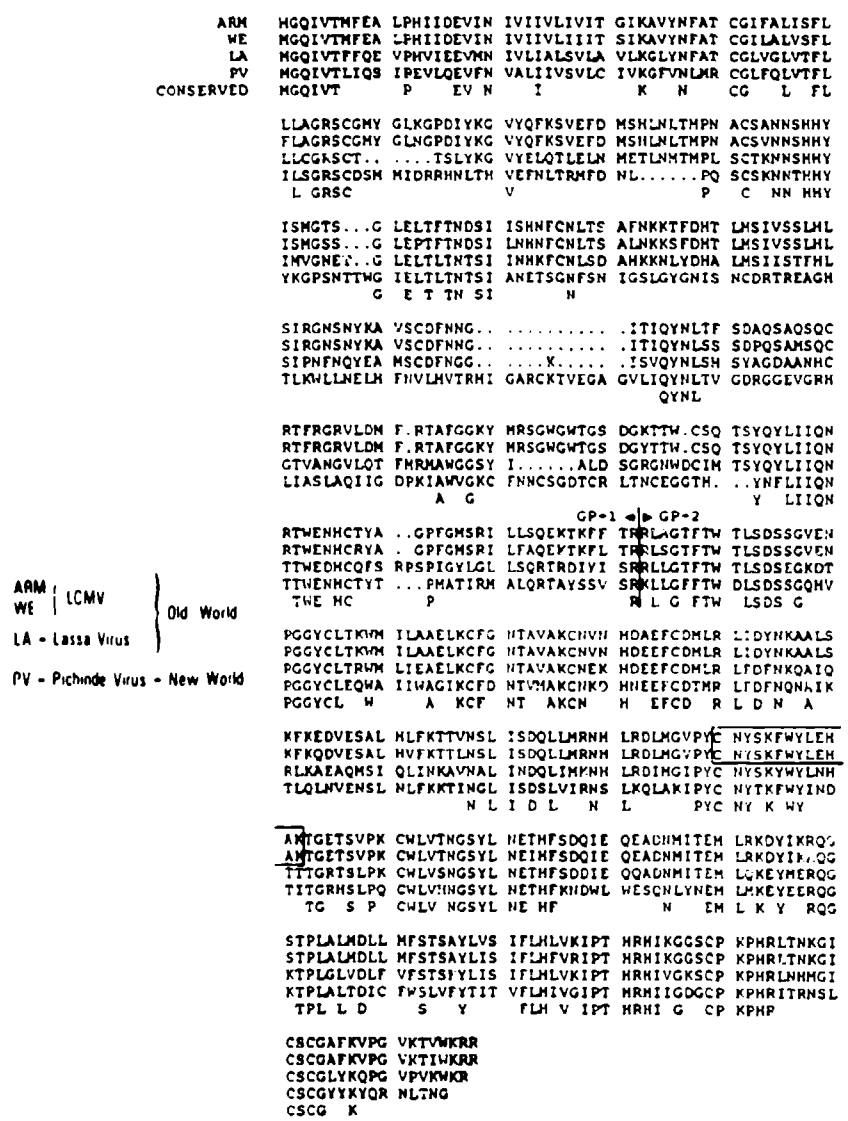



**Figure 2.** Deduced polypeptide sequences of LCMV ARM and WE, as well as Lassa (LA) and Pichinde (PV) viruses. Conserved amino acids are more frequent in the carboxyl half of GPC, which corresponds to the GP2 glycoprotein after proteolytic cleavage. The peptide sequence identified as containing a group-specific antigen in LCMV is boxed.

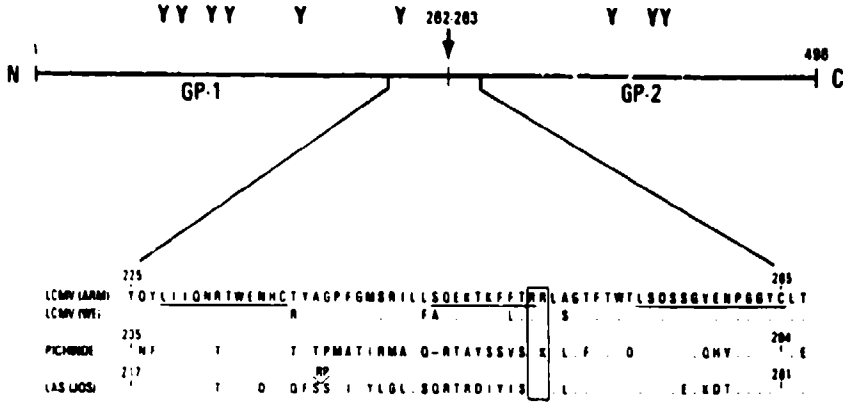



Figure 3. Structure around the GPC cleavage site of LCMV, Pichinde virus, and Lassa virus. Consensus N-linked glycosylation sites (N × S or N × T) are indicated as Y. Peptides synthesized to make antisera as described in the text and shown in Fig. 4 are underlined.

produce the mature glycoproteins from the GPC precursor. From other studies (4), we know that this cleavage occurs rapidly and is probably coincident with oligosaccharide processing of the mannose-rich GPC to the mannose-depleted viral proteins. We also have preliminary evidence that glycosylation is a prerequisite for this proteolytic processing. Such results are consistent with findings with a number of enveloped virus systems, including Sindbis and Semliki Forest virus E2 glycoproteins (12, 24) and for yellow fever virus NS and M proteins (23). Still, the




Figure 4. Western blot reactivity of peptide antisera showing the proteolytic cleavage site of LCMV GPC. Lanes 1 to 4 represent the reaction of antisera to LIIQNRTWENHC, SQEKTKFFTR, and LSDSSGVENPGGYC and polyclonal antiserum to LCMV, respectively.

proteolytic enzymes responsible for affecting these cleavages are largely uncharacterized. In the alphaviruses and flaviviruses in particular, cleavage is thought to involve intracellular action of a combined trypsin and carboxypeptidaselike activity functioning late in the secretory pathway (23, 24). Our results with LCMV are consistent with this model. We previously reported that only the fully processed glycoprotein products reach the cell surface (4).

Extracellular or postviral maturation cleavages of coronavirus, paramyxovirus, orthomyxovirus, and retrovirus glycoproteins are associated with the activation of cell fusion potential mediated by one of the products of cleavage. We have no evidence yet for biological activity associated with the cleaved LCMV glycoproteins; however, the amino terminus of GP2 liberated by cleavage at the RR site consists of a predominantly hydrophobic stretch of amino acids, LAGTPTWTL. Currently, we are studying this and other regions of the LCMV glycoproteins, taking advantage of synthetic peptide technology to define important functional regions. We have also used these techniques to definitively identify the gene product of the L RNA of LCMV as a 200-kilodalton polypeptide associated with the ribonucleoprotein complex (Singh et al., in press).

One might reasonably ask whether these studies of LCMV glycoprotein structure and processing are relevant to infection in vivo. During the transition from acute to persistent infection following neonatal inoculation of mice, we have observed a down-regulation or modulation of the expression of LCMV glycoproteins in infected cells (17). This down-regulation occurs predictably following either in vivo or in vitro infections and ultimately results in a cell or tissue that expresses little viral glycoprotein but that has abundant viral nucleoprotein within its cytoplasm and no cytopathology. This state provides potential advantages for persistence, since the load of viral antigen is low and cells are competent to perform normally enough for survival. Therefore, it is clear that understanding the mechanism of glycoprotein gene regulation in acute and persistent states is a significant piece of the puzzle of persistence.

## ANTIGENICITY OF THE ARENAVIRUSES

Arenaviruses differ in their susceptibility to antibody-mediated neutralization. Neutralizing antibodies to LCMV have been shown to be directed against the GP1 glycoprotein. Similarly, monoclonal antibodies against the single glycoprotein of Tacaribe virus mediated highly efficient virus neutralization (15). Moreover, by using competitive binding assays and analysis of neutralization-resistant mutants, it was possible to map two distinct epitopes on Tacaribe virus type G. One epitope, character-

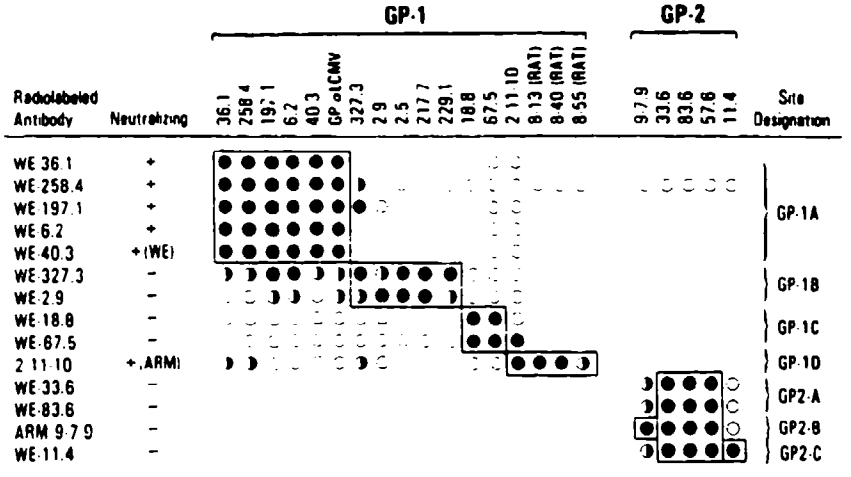



Figure 5. Summary of epitope mapping of LCMV monoclonal antibodies to GP1 and GP2 delineating four epitopes on GP1 and three on GP2. Symbols: ●, competitive binding greater than 90%; ◕, competitive binding less than 20%; ◑, competitive binding of 40 to 80%. GP αLCMV indicates guinea pig immune serum (from reference 19).

ized by four monoclonal antibodies, was the target of highly efficient neutralization, whereas a single antibody to a second site was less so, leaving a large nonneutralizable persistent fraction. Failure to neutralize was not likely to be due to virus aggregation, since addition of a second antibody to the alternate site resulted in further reduction in virus titer. Analysis of neutralization kinetics for the highly efficient monoclonal antibody suggested that the reaction followed double-hit kinetics.

We have assessed the antigenic topography of the LCMV glycoproteins by using a large library of monoclonal antibodies against GP1 and GP2 to map the epitopes on these molecules (19). Elicitation of neutralizing monoclonal antibodies to LCMV in the BALB/c mouse was a relatively infrequent event. Only 6 of 46 antibodies to the LCMV glycoproteins neutralized virus infectivity in vitro. Five of these antibodies were raised against LCMV WE and were mapped by competition binding assay to a single conformation-dependent epitope (GP1a) shared by both ARM and WE strains and other LCMV strains (Fig. 5). The sixth neutralizing monoclonal antibody was uniquely specific for LCMV ARM, and its binding to that strain was only marginally affected by the other five antibodies, suggesting binding to a topographically related, but not identical, epitope (GP1d). Nonneutralizing monoclonal antibodies were found to be directed against two additional sites of GP1, (GP1b and

GP1c), as well as against three sites on GP2. The relevance of these data to the polyclonal antibody response was investigated by using a potent neutralizing antiserum raised in guinea pigs. This antibody reacted predominantly with conformation-dependent structures on GP1, as indicated by its failure to bind in Western immunoblotting, and its binding was completely inhibited by any of the five LCMV WE-specific neutralizing monoclonal antibodies against site GP1a. These results imply that the LCMV WE GP1 has a single immunodominant neutralizing antigenic determinant (GP1a) and that LCMV ARM bears an additional topographically related but not identical site (GP1d). Attempts to neutralize other arenaviruses have met with mixed success. Antisera collected from patients and antisera produced experimentally show potent neutralizing activity against Junin virus, whereas similar reagents collected from patients convalescent with Lassa fever show rather low neutralizing potency unless complement is added to potentiate the effects of antibody (21). Virus neutralization is discussed in greater depth by Howard (14), but from the brief treatment here it is evident that more information about the molecular nature of neutralizing antigenic determinants of arenaviruses is necessary before rational approaches to immunotherapy and immunization can be made. Obviously, one needs to define a structure which will elicit strong protective immune responses without the risk of triggering immunopathologic disease.

Toward this end, we have recently studied in detail a group-specific antigen conserved on the glycoproteins of all arenaviruses. In previous studies (6) we described a monoclonal antibody, 9-7.9, which cross-reacted between LCMV and the African arenavirus Mopeia (Mozambique) virus. Subsequently we isolated several more group-reactive monoclonal antibodies, and one of these, 33.6, is of particular interest. This monoclonal antibody reacts with GP2 glycoprotein of LCMV and cross-reacts with both New World (Pichinde, Junin, Tacaribe, Amapari, Parana, and Machupo) and Old World (LCMV and Lassa, Mopeia, and Mobala) viruses. Given this promiscuous cross-reactivity, we sought to define at the molecular level the binding site of 33.6. During this and other studies, we synthesized a series of nested peptides corresponding to over 90% of the GPC sequence. Monoclonal antibody 33.6 was screened for reactivity against this panel, and we found that it reacted only with one peptide, corresponding to GPC (GP2) residues 370 to 382 (Table 1). Further analysis demonstrated that both the 33.6 epitope and the previously described 9-7.9 epitope mapped within a nine-amino-acid segment spanning residues 370 to 378, which contains five amino acids conserved among LCMV, Lassa virus, and Pichinde virus (Fig. 6).

Assays with N-terminal deletions from this sequence suggested that

Table 1. Reactivity of Monoclonal Antibodies with Peptides 353 to 370, 370 to 382, and 378 to 391[a] in Enzyme-Linked Immunosorbent Assay

| Monoclonal antibody | Reactivity with following peptide: | | |
|---|---|---|---|
| | 353–370 | 370–382 | 378–391 |
| 33.6[b] | <100 | 25,600 | <100 |
| 83.6[b] | <100 | 6,400 | <100 |
| 9-7.9[b] | <100 | 1,638,400 | <100 |
| 2-11.10[c] | <100 | <100 | <100 |

[a] The peptide sequence is as follows

GPC 353 DQLLMRNHLRDLMGVPYCNYSKFWYLEHAKTGETSVPKC 391...

[b] Monoclonal antibodies 33.6, 83.6, and 9-7.9 bind specifically to peptide 370 to 382.

[c] Negative control

the minimal epitope recognized by the broadly cross-reactive monoclonal antibody 33.6 (epitope GP2a) consisted of five amino acids, GPC 374 to 378 (Lys-Phe-Trp-Tyr-Leu). Reactivity of a second monoclonal antibody, 9-7.9 (epitope GP2b), but not 33.6, was abolished when tyrosine was substituted for phenylalanine at position 375 in the antigenic sequence corresponding to a naturally occurring sequence difference between LCMV and Lassa virus.

The possibility of a single peptide antigen containing a universal arenavirus antigenic determinant was raised by these observations; therefore, we assayed convalescent-phase serum samples for evidence of reactivity with the peptide. Polyclonal serum samples from human patients and from animals experimentally infected with Junin virus,

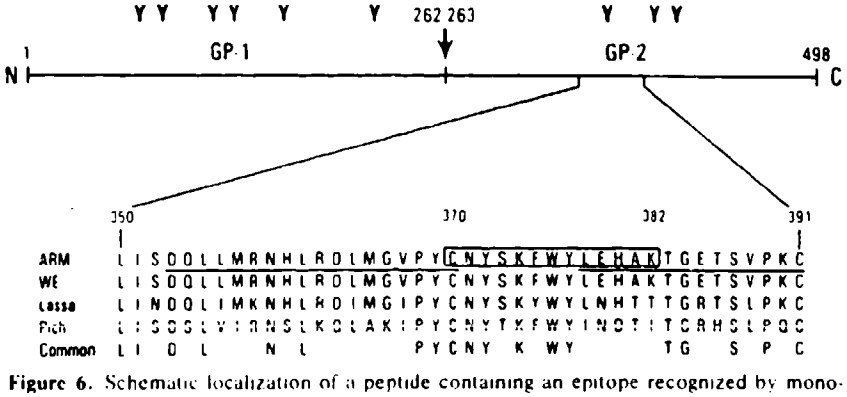



**Figure 6.** Schematic localization of a peptide containing an epitope recognized by monoclonal antibody 33.6 on all arenaviruses (box). Flanking peptides (underlined) were used as controls and were not reactive with 33.6 (see Table 1).

LCMV, and Lassa virus bound to the antigenic peptide GPC 370 to 382 but not to control peptides. As was the case with the monoclonal antibodies, this binding activity was abrogated by blocking with the antigenic peptide, but not with control peptides in solution.

The observation of conservation of this sequence across a broad spectrum of arenaviruses from the New World and Old World groups suggests that it represents an important functional or structural component of the virus. We have not as yet been able to assign functional significance to this site. We found that neither 33.6 nor 9-7.9 mediated complement-independent virus neutralization, although 9-7.9 showed modest neutralizing activity when guinea pig complement was added to the reaction mixture (7). In contrast, several monoclonal antibodies against GP1 have been shown to be strongly neutralizing in the absence of complement (3, 7, 19). The question of structural significance is a subject of current investigation.

In studies reported elsewhere in this volume, Whitton et al. have mapped at least one cytotoxic T-lymphocyte recognition epitope to LCMV GP2 amino acids 272 to 293. We are currently extending our studies of the sequence from 370 to 382 to determine whether it plays a significant role in either induction of or recognition by virus-primed helper or cytotoxic T lymphocytes.

The present studies may prove to be of practical importance. As described above, we have observed binding of polyclonal convalescent-phase serum samples directed against LCMV, Junin virus, and Lassa virus with the synthetic peptide, suggesting that it may be of use as a diagnostic antigen to detect antibody against arenaviruses. Such a peptide diagnostic reagent has several potential advantages over the currently used virus- or infected-cell-derived antigen preparations. Among these are low cost, stability, and the ability to rapidly provide sequence variants corresponding to naturally occurring viruses. We are currently exploring the potential utility of the sequences we have described and corresponding antibodies as diagnostic tools for arenavirus infections.

## PATHOBIOLOGICAL ROLE OF SPECIFIC VIRAL GENE PRODUCTS IN VIVO

Viral polypeptides and their degradation products trigger many of the pathobiologic manifestations observed in arenavirus infection. In the lifelong persistent infection of mice with LCMV, a wasting syndrome characterized by the development of immune complexes composed of viral antigen and antiviral antibody has been well documented (8, 18). These complexes lodge in the renal glomeruli, where they trigger a



Figure 7. Immunofluorescent localization of LCMV GP1 along the ependymal cell layer of a BALB/c mouse infected 6 days earlier with virus. Monoclonal antibody 2-11.10 against GP1 was used.

chronic glomerulonephritis. At least one component of the virus has been identified in the glomeruli of diseased mice. Using a monospecific antibody to the NP of LCMV, we demonstrated colocalization of NP antigen and the host glomerular mesangium (8).

A role of NP in neuronal dysfunction during LCMV persistence has also been proposed. Rodriguez et al. (26) observed expression of NP in association with polyribosomes in the cytoplasm of neurons from widespread areas of the central nervous system. In contrast, no significant expression of viral glycoproteins was seen. Therefore, it was proposed that the presence of NP on the neuronal polyribosomes compromised their function.

Acute LCMV infection following intracerebral inoculation of the virus results in a fatal choriomeningitis (reviewed in reference 10). We have used monoclonal antibodies against individual viral structural proteins to study their expression in the central nervous system following acute infection (5). Viral GP1 is expressed on the apical surfaces of ependymal cells in the central nervous system (Fig. 7). At this site the glycoprotein (and perhaps also other virally encoded proteins) triggers the

well-characterized immune response which results in choriomeningitis and death; this response appears to depend on the presence of virus-directed cytotoxic T lymphocytes.

Finally, the role of the viral L gene-encoded proteins in pathogenesis has recently been explored by Riviere et al. (25), who used genetic reassortants between strains of LCMV that differed in virulence for guinea pigs. They demonstrated that L RNA-encoded products were necessary for expression of the pathogenic potential of the virus. It is clear from such studies that understanding the molecular basis of viral persistence, regulation of viral gene products, and pathogenesis of arenavirus infections is an attainable goal.

*Acknowledgments.* We thank Rebecca Day, Gretchen Bloom, and Kaleo Wooddell for excellent technical assistance and Jim Johnston for manuscript preparation.

Portions of this work were supported by Public Health Service grant A116102 from the National Institutes of Health and U.S. Army contract C6234. K. E. Wright was supported by a fellowship from the Arthritis Society of Canada.

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*J. gen. Virol.* (1988), 69, 1893–1902. *Printed in Great Britain*



Key words: *LCMV/hybridization/interfering activity*

# Deleted Viral RNAs and Lymphocytic Choriomeningitis Virus Persistence *in vitro*

By STEPHEN J. FRANCIS AND PETER J. SOUTHERN*

*Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California 92037, U.S.A.*

(*Accepted 20 April 1988*)

SUMMARY

Lymphocytic choriomeningitis virus (LCMV) infection of most tissue culture cell lines results in a non-cytopathic persistent infection. Persistent infections *in vitro* share many characteristics with persistent LCMV infection of mice; both are associated with decreased titres of infectious virus, restricted accumulation of viral glycoproteins at the surface of infected cells and the generation of interfering particles. We have used gel electrophoresis and hybridization techniques to analyse LCMV gene expression during persistent infection of a number of tissue culture cell lines. Our study has demonstrated that, although deleted viral RNAs can be detected during persistent LCMV infection *in vitro*, there may not be an obligatory association between deleted RNAs and persistence. In addition, we have found that LCMV interfering activity can be produced in the apparent absence of deleted intracellular viral RNAs.

## INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) readily establishes persistent infections in tissue culture cell lines and mice (Buchmeier *et al.*, 1980; Lehmann-Grube *et al.*, 1980). Persistent infections are characterized by reduced viral replication, limited accumulation of viral glycoproteins at the surface of infected cells and the generation of LCMV-specific interfering particles that appear very rapidly after initiation of infection (Buchmeier *et al.*, 1980; Lehmann-Grube *et al.*, 1980; Oldstone & Buchmeier, 1982; Popescu & Lehmann-Grube, 1977; Welsh *et al.*, 1972, 1977; Welsh & Pfau, 1972; Welsh & Oldstone, 1977; Welsh & Buchmeier, 1979). The net result is a significant reduction in the production of infectious particles.

Tissue culture cell lines infected with LCMV at either high or low m.o.i. exhibit minimal cytopathic effects during acute or persistent infection. Two cell lines, MDCK and P15, however can be lytically infected by LCMV (Dutko & Pfau, 1978; Jacobson *et al.*, 1979). These cell lines do not generate interfering activity but can be protected from lysis, and persistent infections can be established, when standard virus is co-administered with preparations of interfering particles that have been generated in another cell line. These observations suggest that the non-cytopathic character of LCMV infection is dependent on the rapid generation of interfering particles and that interfering and/or defective interfering particles are involved with the establishment and maintenance of persistent infections (reviewed in Barrett & Dimmock, 1986; Huang, 1973; Perrault, 1984).

Viral proteins appear to be qualitatively unchanged in purified virion preparations and cells during persistent LCMV infection *in vitro* (Welsh & Buchmeier, 1979). To date, however, there is only limited information concerning the state of the viral genome. Martinez Peralta *et al.* (1981) examined LCMV RNA (strain WE) in preparations of purified interfering particles and were able to detect the viral L segment and host 28S and 18S ribosomal RNAs but not the viral S segment. Van der Zeijst *et al.* (1983) detected a number of novel intracellular subgenomic RNAs of presumed viral origin during persistent infection of BHK cells. Gimenez & Compans (1980)

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and Dutko *et al.* (1976), who studied the related arenaviruses Tacaribe and Pichinde respectively, suggested that, during persistence, a number of novel subgenomic RNAs appear in conjunction with loss of the S segment. Therefore, the presence of novel subgenomic viral RNAs and/or the loss of a viral genomic RNA segment have been associated with arenavirus persistence.

The LCMV model is of particular interest because (i) it is one of the few systems in which interfering particles can be detected during a persistent infection in animals (Jacobson & Pfau, 1980; Lehmann-Grube *et al.*, 1980; Popescu & Lehmann-Grube, 1977) and (ii) there are extensive similarities between the persistent infections of tissue culture cells and mice (Buchmeier *et al.*, 1980; Oldstone & Buchmeier, 1982). The availability of a good *in vitro* model system for persistent infection of animals creates the opportunity for more controlled biochemical manipulations than are possible *in vivo*.

The LCMV genome has recently been cloned and sequenced (Romanowski & Bishop, 1985; Singh *et al.*, 1987; Southern *et al.*, 1987) and specific probes are now available to characterize viral RNA species and the transcription and replication strategy of the virus during acute (Fuller-Pace & Southern, 1988; Singh *et al.*, 1987; Southern *et al.*, 1987) and persistent infection. We were particularly interested in evaluating whether changes in intracellular viral RNA species could be demonstrated in the context of persistent LCMV infections.

## METHODS

*Virus stocks and infection of tissue culture cell lines.* Persistent infections were initiated either with uncloned preparations of LCMV Armstrong CA1371 or with a triply plaque-purified stock (clone 53B) derived from the uncloned stock. Semi-confluent monolayers of cells were infected with virus at multiplicities of 0·01 to 0·1 p.f.u. per cell and the cultures were trypsinized and replated when confluent. In all cases there was no reduction in the rate of cell division as a consequence of LCMV infection and the persistent infections became stabilized, as judged by reduced production of infectious virus and cell surface glycoprotein (GP) accumulation, within a few weeks. Infectious LCMV was quantified by plaque assay on Vero cell monolayers (Welsh & Buchmeier, 1979). LCMV interfering activity was determined by inhibition of plaque formation (Welsh & Buchmeier, 1979).

*Virion concentration.* A simplified scheme for concentration of virions was used to avoid the loss or inactivation of virus commonly observed during purification on Renografin gradients. Tissue culture cell supernatants were centrifuged in 30 ml Corex tubes at 8000 r.p.m. for 30 min in an HB-4 rotor in an RC28 Sorvall high speed centrifuge to remove cells and clarify the supernatant. The supernatant was decanted and centrifuged for 1 h at 30000 r.p.m. in a TY35 rotor in a Beckman ultracentrifuge (L8-M). The supernatant was decanted, discarded, and the pelleted material resuspended in Eagle's minimum essential medium (concentrated virion preparation). Using this concentration scheme a 12-fold, and in a later experiment a 120-fold, concentration of supernatant virus was obtained. The total titre of infectious virus calculated for the concentrated and stock supernatants was approximately the same.

*Vesicular stomatitis virus (VSV) plaque assay.* VSV stocks were titrated on L929 cell monolayers. A standard dilution that gave approximately 50 plaques/well was used for interference assays. Interference mediated by concentrated virion preparations (from GH3 cells) and by interferon gamma was determined by reduction in VSV plaque formation.

*RNA purification from tissue culture cells.* Total intracellular RNA was isolated by a modification of the method of Chirgwin *et al.* (1979). Cell monolayers were washed with sterile phosphate-buffered saline pH 7·4, and then 10 ml of GTC (4 M-guanidinium thiocyanate, 25 mM-sodium citrate, 0·5% Sarkosyl, 0·1 M-2-mercaptoethanol) was added. The resultant viscous solution was vortexed for approximately 45 to 60 s, layered over 2 ml of diethyl pyrocarbonate-treated 5·7 M-caesium chloride (Sigma), 100 mM-EDTA, and centrifuged in an SW41 rotor for 16 h at 35000 r.p.m. at 18 °C in a Beckman ultracentrifuge. The RNA pellet was washed with 70% ethanol and then repelleted.

*Nucleic acid concentration.* Purified RNA pellets were resuspended in sterile water and the concentration and purity were determined by absorbance measurements at 260, 280 and 320 nm. In addition, the amount and integrity of ribosomal RNA was assessed by electrophoresis of total intracellular RNA on non-denaturing 1% agarose mini gels.

*RNA electrophoresis.* RNA was denatured with glyoxal (1 M) and DMSO (50%) as previously described (Francis & Southern, 1988; McMaster & Carmichael, 1977). A standardized amount of total intracellular RNA (5 or 10 μg) was electrophoresed on a 1·5% agarose gel in 10 mM-$NaH_2PO_4$/$Na_2HPO_4$ pH 6·5 (Francis & Southern, 1988; Maniatis *et al.*, 1982). Electrophoresis was performed at 14 mA constant current (approx. 2·5 V/cm) for 16 h with continual recirculation of the phosphate buffer.

*RNA transfer and hybridization conditions.* Transfer of RNA from agarose gels to nitrocellulose or Nytran filters was performed under standard conditions (Thomas, 1983). Filters were air-dried and baked for 2 h at 80 °C. Prehybridization and hybridization with nick-translated probes were performed in 50% formamide (deionized by treatment with Bio-Rad AG 501-X8), 5 × SSC, 2·5 × Denhardt's solution, 50 mM-phosphate buffer pH 6·5, 0·1% SDS, and 100 μg/ml of sonicated salmon sperm DNA for 24 and 48 h, respectively. Hybridization with radiolabelled RNA probes was for 24 h at 55 °C.

After hybridization, the filters were washed three times in 2 × SSC, 0·1% SDS for 30 min at 37 °C and once in 0·1 × SSC, 0·1% SDS for 30 min at 55 °C. The wash in 0·1 × SSC, 0·1% SDS was repeated for 30 min at a temperature higher than 55 °C if necessary as indicated by an excessive background.

*Synthesis of hybridization probes.* Purified LCMV cDNA sequences were labelled with [α-$^{32}$P]dATP and [α-$^{32}$P]dCTP to a specific activity of $\geq 2 \times 10^8$ c.p.m./μg by the procedure of Rigby *et al.* (1977). Single-stranded RNA probes were synthesized in the presence of [α-$^{32}$P]UTP using the SP6 promoter and polymerase system (Melton *et al.*, 1984). All LCMV hybridization probes contained LCMV sequences exclusively.

## RESULTS

### *Novel subgenomic viral RNAs are detectable during persistent infection of tissue culture cell lines*

Total intracellular RNA was analysed from four cell lines [GH3, rat pituitary cells (Tashjian *et al.*, 1968); RIN, rat insulinoma cells (Gazdar *et al.*, 1980); L, mouse fibroblasts; B-113, a B cell hybridoma (M. B. A. Oldstone *et al.*, unpublished results)] that were persistently infected with LCMV Armstrong CA1371. After size-fractionation on denaturing agarose gels and transfer to nitrocellulose filters, the RNAs were hybridized with radiolabelled virus-specific nick-translated probes from the nucleoprotein (NP) and GP coding regions of the S segment (Fig. 1*a*, *b*, respectively). Uninfected cells did not show any virus-specific hybridization but the persistently infected cell lines demonstrated a signal from the S segment and from subgenomic viral mRNAs transcribed from the NP- and GP-coding regions (Southern *et al.*, 1987). In addition, multiple new subgenomic viral RNAs were evident in the GH3, RIN and B-113 cells (using NP and GP segment probes) but no such subgenomic RNAs could be detected in the L cell RNA.

### *Detection of viral L segment RNAs during persistent infection*

We analysed the persistently infected cells for the presence of the full length L segment and any subgenomic L-derived RNAs. Fig. 2 compares the results obtained with persistently infected GH3 cells with those from acutely infected BHK cells. The filter was sequentially hybridized with S segment (Fig. 2*a*) and L segment (Fig. 2*b*) nick-translated probes. Two faint subgenomic L bands, present in both the acutely and persistently infected cell RNA samples, have not been associated with expression of the L RNA segment and may be minor degradation products or representative of low-level cross-hybridization to viral S sequences (see legend to Fig. 2).

The L segment was readily detected during both acute and persistent infection but without any indication of unique, L segment, subgenomic RNAs (Singh *et al.*, 1987). The acute and persistent infections were not identical because the relative levels of genomic S and L RNAs changed and the latter was under-represented in persistent infections (compare the signal ratios of L and S in lanes 2 and 4, Fig. 2*a*, *b*).

### *Systematic analysis of novel subgenomic RNAs found during persistent infection of GH3 cells*

We extended the characterization of novel subgenomic S RNAs in GH3 cells by examining the sequence content and polarities of the intracellular viral RNAs. Samples containing equivalent amounts of persistently infected GH3 cell RNA or BHK cell RNA (48 h after acute infection with LCMV) were separated on a denaturing agarose gel (see Methods). After transfer of the RNA to a nitrocellulose filter, the filter was cut into strips and incubated with single-stranded RNA probes that detected genomic sense or genomic complementary sense sequences from either the 3′ terminal NP region [approx. nucleotides (nt) 35 to 450], the internal NP region (approx. nt 1200 to 1600), or the 5′-terminal GP region (approx. nt 2300 to 3400) of the S segment (Fig. 3*a*).

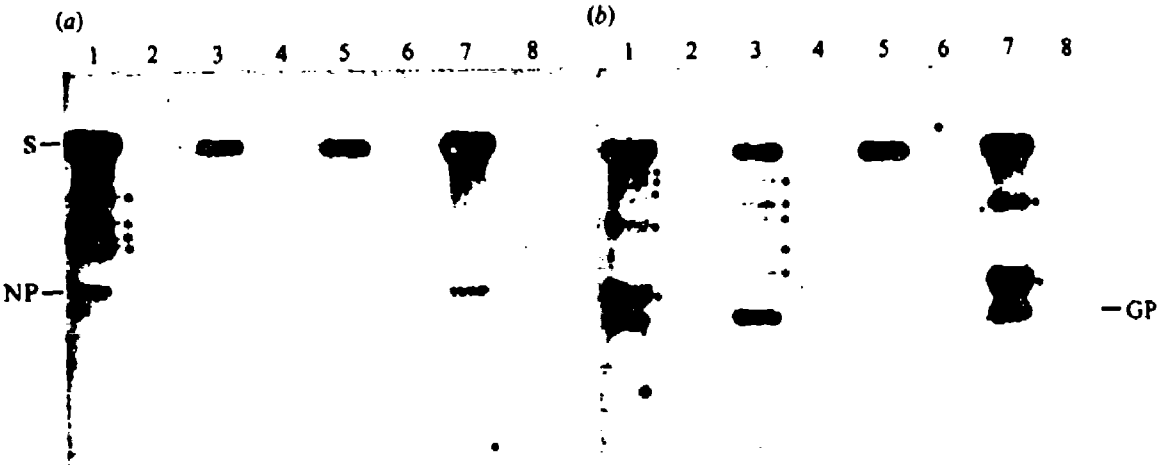



Fig. 1. Analysis of intracellular S RNAs from cell lines persistently infected with LCMV. Total intracellular RNA from four cell lines, persistently infected with LCMV for a minimum of 1 year, was size-fractionated by agarose gel electrophoresis under denaturing conditions. After transfer of the RNAs to a nitrocellulose filter, hybridization reactions were performed using radiolabelled nick-translated probes from the viral S segment. The filter was hybridized with an NP region probe (*a*), completely stripped of signal, and rehybridized with a GP region probe (*b*). RNAs from uninfected cell lines were used as negative controls (lanes 2, 4, 6 and 8). Lanes 1 and 2, GH3 cells; lanes 3 and 4, RIN cells; lanes 5 and 6, L cells; lanes 7 and 8, B-113 cells. The NP and GP mRNAs are indicated and prominent subgenomic RNAs have been marked with asterisks.

The results of the hybridizations to detect genomic sense sequences are shown in Fig. 3(*b*). The acutely infected BHK cell RNA (lane 1) displayed the expected signal from the S segment and the GP mRNA with the GP region probe. The probes from the NP and GP regions detected different virus-specific RNAs in the GH3 RNA samples (see also Fig. 1) and, by extending this analysis, we could begin to assemble maps of the genomic S RNA sequences that were present or absent from individual subgenomic RNAs. In most cases, a given subgenomic RNA appeared to hybridize with probes of each polarity, providing evidence for intracellular replication of the subgenomic species.

### *Interference during persistent LCMV infection of GH3 cells*

The GH3 intracellular RNA used in Fig. 1 and 2 had been extracted from an LCMV Armstrong CA1371 persistently infected cell line but, after storage in liquid nitrogen, viable cells could not be recovered. We therefore examined other GH3 cells and expanded a cloned GH3 cell line, GH3-21, that had been persistently infected with LCMV Armstrong CA1371 (clone 53B) to test whether these cells produced any interfering activity. Multiple experiments were performed in which stock supernatant from GH3-21 cells was stored (at −70 °C) and the remaining supernatant (approx. 300 ml, equivalent to culture medium from 10 T175 culture vessels) was concentrated (see Methods). T. stock supernatant and the concentrated virion preparations were titrated for infectious virus, and the virus preparations were tested for interference with standard LCMV (SV) replication and for the presence of interferon (see Methods). The stock supernatant showed a sequential reduction in virus titres at all dilutions (approximate titre $1{\cdot}8 \times 10^5$ p.f.u./ml) whereas infectious virus could be detected only in the concentrated virus preparations at a 1 : 2000 dilution, but not at a 1 : 2 or 1 : 20 dilution. The titre of infectious virus in the concentrated virion preparation was approximately $4 \times 10^6$ p.f.u./ml (as shown in Fig. 4*a*) and, since the virion preparation was originally concentrated 12-fold, this was in reasonable agreement with the titre of virus in the stock supernatant. The concentrated virion preparation interfered with plaque formation by SV (Fig. 4*b*) and there was no role for interferon in this process (Fig. 4*c*). The interfering activity in the concentrated GH3 stock was subsequently shown to be virus-specific (Table 1). A $10^{-5}$ dilution of SV produced 200 p.f.u./ml.

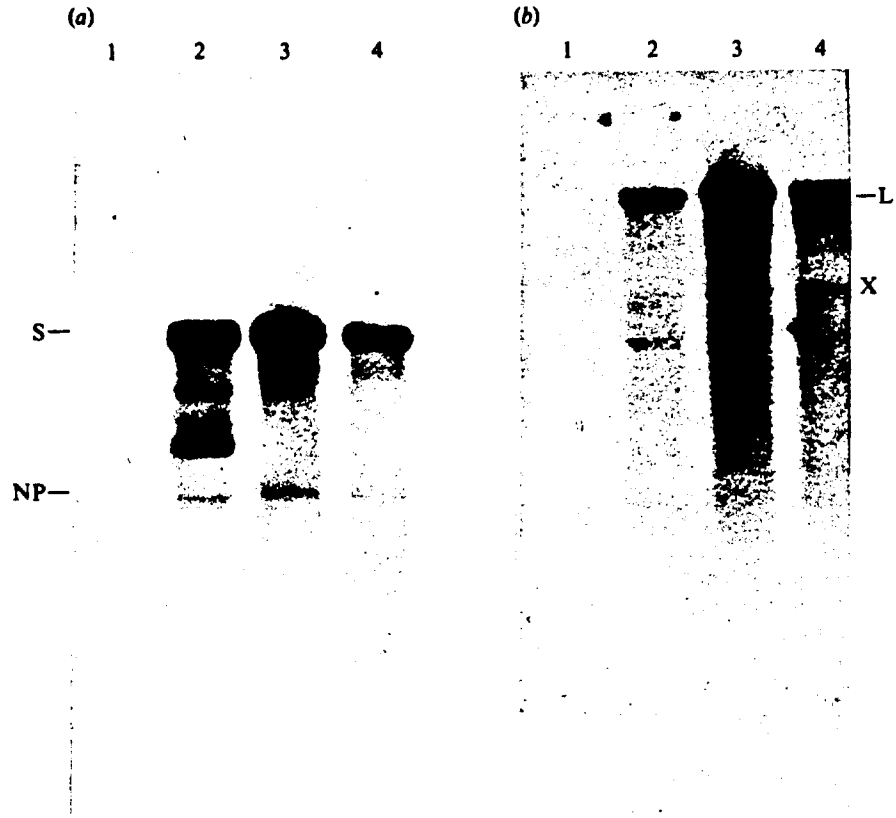



Fig. 2. Detection of L-derived RNAs during acute and persistent LCMV infection. Total intracellular RNAs extracted from uninfected GH3 cells (lane 1), persistently infected GH3 cells (lane 2) and two independent acute infections of BHK cells (lanes 3 and 4) were hybridized with (*a*) S segment (NP region) and (*b*) L segment nick-translated probes. The filter was first hybridized with the S segment probe, completely stripped, and then rehybridized with the L segment probe. The S segment and NP mRNAs are indicated in (*a*) and the L segment RNA in (*b*). Faint low $M_r$ bands detected with the L probe may represent breakdown products and/or demonstrate low-level cross-hybridization to the genomic S RNA segment. The appearance of one band (X) may also be due to a high concentration of 28S ribosomal RNA that produces a local distortion in the gel.

Table 1. *Detection of LCMV interfering activity*

| Dilution | SV stock (p.f.u./ml) $10^{-5}$ | $10^{-6}$ | $10^{-7}$ | Treatment |
|---|---|---|---|---|
| | 200 | 20 | 0 | None |
| | 50 | ND* | ND | Heat† |
| | 0 | 0 | 0 | Heat† and neutralization‡ |
| | 0 | 0 | 0 | Heat† and concentrated virions§ |
| | 125 | ND | ND | Neutralized, concentrated virions‖ |

* ND, Not determined.

† Virus incubated for 30 min at 37 °C as a control for treatments below.

‡ Guinea-pig anti-LCMV, 1:100 dilution.

§ Concentrated virion preparation from persistently infected GH3-21 cells, 1:2 dilution.

‖ Concentrated virion preparation, 1:2 dilution, neutralized with anti-LCMV, 1:100 dilution, for 30 min at 37 °C before being added to the Vero cells with the SV stock (which was not heated).

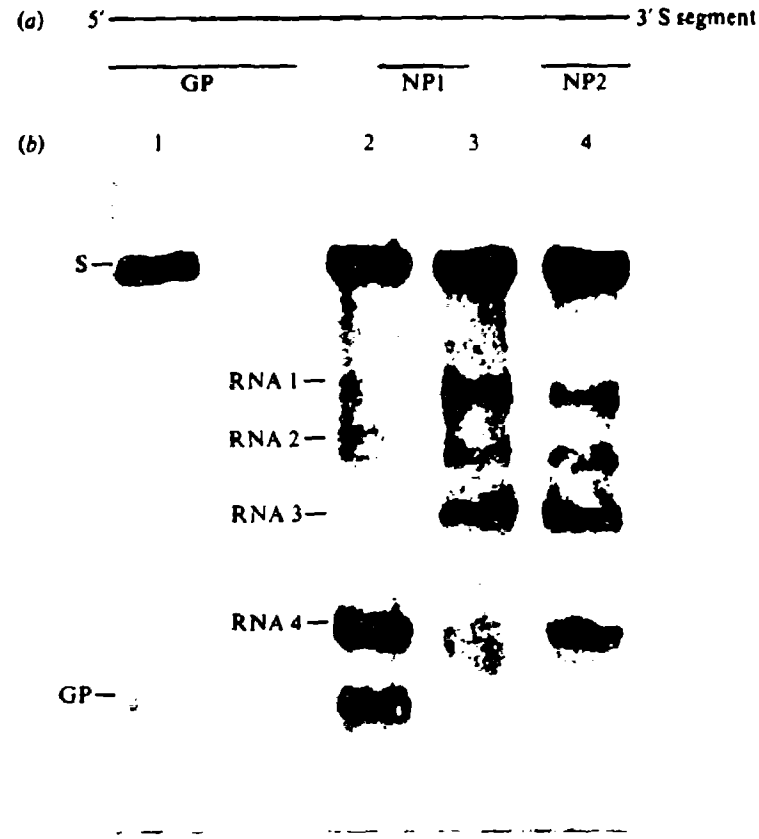



Fig. 3. Hybridization analysis of GH3 cell subgenomic RNAs. Individual strips of nitrocellulose containing RNA from either acutely infected BHK cells or persistently infected GH3 cells were hybridized with radiolabelled single-stranded RNA probes from the 5' GP region (nt 2300 to 3400) and the NP coding region (NP1, nt 1200 to 1600; NP2, nt 35 to 450). (*a*) A diagrammatic representation of the location of these probes within the S segment and (*b*) the hybridization patterns with probes detecting genomic sense sequences. Lane 1. RNA from acutely infected BHK cells, lanes 2 to 4, RNA from persistently infected GH3 cells. The probes used for hybridization were: lanes 1 and 2, GP; lane 3, NP1, lane 4, NP2. The GP mRNA, genomic S segment and four novel subgenomic RNAs, designated RNAs 1 to 4, are indicated. The NP mRNA was not detected by any of these single-stranded probes.

whereas inclusion of either antiserum to LCMV or the preparation of GH3 particles (1:2 dilution) completely prevented the formation of virus plaques. Pretreatment of the particle preparation with anti-LCMV antibody eliminated most or all of the interfering activity (Table 1).

*Evidence for subgenomic viral RNAs in persistently infected cell lines exhibiting interference*

Total intracellular RNA from persistently infected GH3-21 cells (this study) and long term persistently infected BHK and L cells (cells kindly provided by Ray Welsh, University of Massachusetts Medical School, Worcester, Mass., U.S.A.) were analysed by hybridization with virus-specific single-stranded RNA probes. The persistently infected BHK and L cell lines (initiated with an uncloned LCMV Armstrong CA 1371 stock) had previously been shown to exhibit interference and decrease expression of surface GP (R. Welsh, personal communication). The L cells did not contain any subgenomic viral RNAs, except GP mRNA, but the BHK cells exhibited four viral RNA molecules, a GP mRNA, two non-identical but approximately S segment size RNAs and a distinct subgenomic RNA of approximately 2300 nt (Fig. 5). The persistently infected GH3-21 cells showed weak, diffuse subgenomic RNA bands at the time samples were removed for biological assays (Fig. 4 and Table 1) but these RNA species

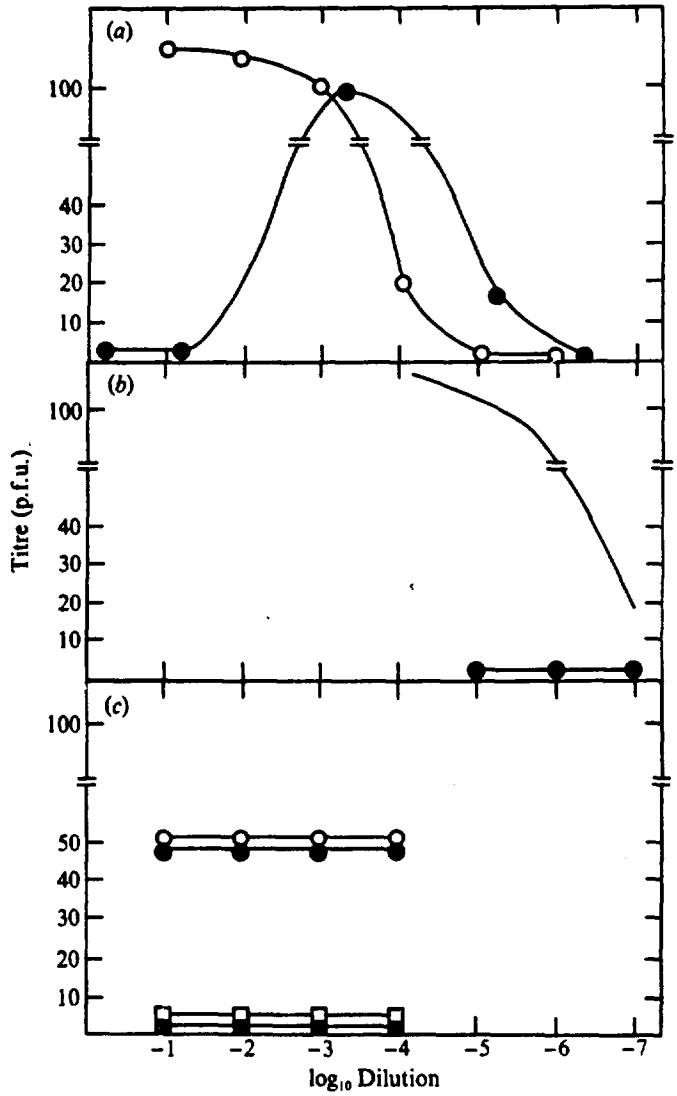



Fig. 4. Detection of interfering activity in supernatant medium collected from cultures of GH3-21 cells persistently infected with LCMV. (*a*) Virus titration with the supernatant (○) and a 12-fold concentrated supernatant (see Methods) of persistently infected GH3 cells (●) at various 10-fold dilutions (unconcentrated GH3 cell supernatant dilutions $10^{-1}$ to $10^{-6}$; GH3 concentrated cell supernatant dilutions 1 : 2, 1 : 20, 1 : 2000, 1 : 200000, 1 : 2000000). Titres (p.f.u.) are the number of virus plaques per well (six-well plates) per ml of stock. Titres of $1{\cdot}8 \times 10^5$ p.f.u./ml for the GH3 SN and $4 \times 10^6$ p.f.u./ml for the GH3 conc. preparations were observed in this experiment. (*b*) Titration (p.f.u.) of a stock of LCMV clone 53B (SV) alone (upper line) and after co-incubation with a 1 : 2 dilution of the GH3 conc. (SV + GH3 conc. 1 : 2; ●). A titre of $1{\cdot}8 \times 10^8$ p.f.u./ml was measured for SV in this experiment and there was no detectable p.f.u. for SV + GH3 conc. (*c*) Assays for interferon activity. Titration (p.f.u.) of a VSV stock alone (○), VSV treated with 20 units of interferon gamma (□), VSV treated with GH3 conc. (●) and a control without any added virus (■).

appeared to be more heterogeneous than in the analysis performed several months earlier (compare Fig. 3 and 5).

## DISCUSSION

We have demonstrated that a number of cell lines (GH3, RIN, B-113 and BHK) may exhibit one or more novel subgenomic viral RNAs when persistently infected with LCMV. We did not detect the elimination of either the genomic L or S RNA segments or viral mRNA species

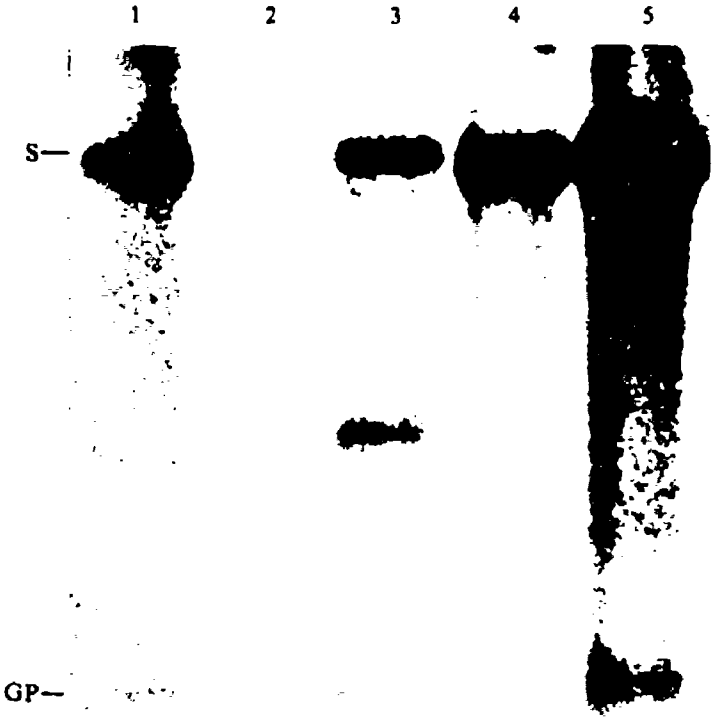



Fig. 5. Analysis of intracellular viral RNAs from persistently infected cell lines that produce LCMV interfering particles. Total intracellular RNA from acutely infected BHK (lane 1), uninfected BHK (lane 2) and persistently infected cells: BHK (lane 3), L (lane 4) and GH3-21 (lane 5, purified in April 1987 at the time of the biological interference assays) was analysed using a single-stranded RNA probe to detect genomic sense sequences from the GP coding region of the S segment. The genomic S segment and GP mRNA are indicated.

during the course of persistence, although the L segment does appear to be selectively under-represented in comparisons of persistent and acute infections. The viral NP and GP mRNAs were both readily detected during persistent infection of all cell lines we examined. In other studies, where intracellular viral RNAs have been analysed during persistent arenavirus infection, van der Zeijst *et al.* (1983) purified viral nucleocapsids and found a number of viral RNA species present that were different from those normally found in LCMV. None of these RNAs could be translated *in vitro*. Dutko *et al.* (1976) were able to detect a 15S RNA, which is approximately the size of the viral mRNAs, in Pichinde virions from acutely but not persistently infected BHK cells but it is unclear what this 15S RNA represents. Immunofluorescence and immunoblotting analyses of viral GP expression in persistently infected cells reveal different levels of GP expression that are dependent on the particular cell line examined (S. J. Francis, unpublished results). Our detection of GP mRNA both *in vitro* (this study) and *in vivo* (Francis & Southern, 1988) suggests that post-transcriptional regulation of GP must account for at least part of the decreased expression of GP. This is consistent with the observation of Welsh & Buchmeier (1979) that a large percentage of persistently infected cells contain cytoplasmic GP but that surface accumulation of GP is decreased relative to acute infections.

The generation of new subgenomic viral RNAs appears to depend on both the particular cell line and the viral stock used to initiate infection. Persistent infection of BHK cells initiated by

cloned isolates of LCMV Armstrong CA1371 may or may not give rise to subgenomic RNAs (Fig. 5). Differences in the virus stocks and/or the BHK cells may be responsible for this variability. The use of the uncloned stock, LCMV Armstrong CA1371, often appears to result in the accumulation of subgenomic S segment RNAs. The RNAs which we studied were extracted from cell lines that had been persistently infected for various but extensive times (> 1 year) prior to analysis. Therefore we cannot comment on whether the initial pattern of subgenomic RNAs found in any particular cell line was similar to that in other cell lines or was related to those, if any were present, contained in the LCMV Armstrong CA1371 virion preparations. Since each cell line appears to contain unique viral subgenomic RNAs there must have been either a selection and/or evolution of the original RNA species of the LCMV Armstrong CA1371 stock. Interestingly, not all persistent infections (i.e. in L cells) initiated with LCMV Armstrong CA1371 contained subgenomic viral RNAs yet they still exhibited interfering activity. This could be explained either by an inability of our routine analytical methods to detect subgenomic RNAs of less than 400 to 500 bases or by the presence of viral segments with minor deletions or sequence aberrations that were not large enough to produce a shift in size that was detectable by denaturing agarose gel electrophoresis. Although most defective interfering RNAs described in other viral systems (Barrett *et al.*, 1984; Huang, 1973; Perrault, 1984; Rao & Huang, 1982; Weiss *et al.*, 1983) have contained readily detectable deletions, copy-backs, and/or rearrangements, there may not be an absolute requirement for such major defects (for example see Schubert *et al.*, 1984).

Our description of novel LCMV intracellular subgenomic RNAs during persistent infections agrees with prior observations made with closely related arenaviruses. Dutko & Pfau (1978) and Gimenez & Compans (1980) studying Pichinde and Tacaribe viruses, respectively, reported the appearance of new RNAs in virions purified from persistently infected BHK cells. We have been unable to detect any subgenomic viral RNAs in virions from persistently infected BHK cells. A newly initiated persistent infection of BHK cells has not yet generated any novel subgenomic RNAs. Virion particles are released by these cells (F. Fuller-Pace, unpublished observations) but there is no detectable infectious virus, even when titrated out to a $10^{-9}$ dilution. We have found that the viral genomic S and L segments are present intracellularly and in virions that are formed during the persistent infection of these BHK cells.

Persistent infection with arenaviruses appears to be a highly complex phenomenon and is associated with different alterations in viral gene expression which depend on the cell type and the virus stock used to establish persistence. Interfering viruses that arise during persistent infections may or may not be correlated with intracellular subgenomic viral RNAs. The significance of variation in the relative levels of the genomic L and S segments during acute and persistent infection remains uncertain. At this time it is unclear whether the changes we have described are causal or consequential to the state of LCMV persistence.

We would like to thank Michael Oldstone for his continued interest and support of this work and Ray Welsh for many useful discussions. We would also like to thank Gay Schilling for excellent secretarial assistance. S.J.F. received a postdoctoral fellowship from the Juvenile Diabetes Foundation. This is Publication Number 5037-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037, U.S.A. This work was supported in part by USPHS grants NS-12428, AG-04342, AI-25224 and USAMRIID DOD contracts DAMD C-3013 and C-6234. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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*(Received 19 October 1987)*

VIROLOGY 162, 260–263 (1988)

# Temporal Analysis of Transcription and Replication during Acute Infection with Lymphocytic Choriomeningitis Virus

F. V. FULLER-PACE[1] AND P. J. SOUTHERN

*Department of Immunology, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, California 92037*

*Received June 29, 1987; accepted September 18, 1987*

We have analyzed the accumulation of viral genomic and messenger RNAs in tissue culture cells during the first 24 hr of acute infection with lymphocytic choriomeningitis virus (LCMV). This has allowed comparison of the relative amounts of the genomic L and S RNAs (both genomic sense and genomic complementary sense) and of nucleoprotein (NP) and glycoprotein precursor (GP-C) mRNAs. Using these techniques NP mRNA was detected simultaneously with genomic S RNA, but the amount of NP mRNA accumulating during this period of infection was higher than that of GP-C mRNA. This is consistent with a model for ambisense RNA transcription and replication proposed by D. D. Auperin, V. Romanowski, M. Galinski and D. H. L. Bishop (*J. Virol.* 52: 897–904, 1984). The accumulation of S RNA exceeded that of L RNA and, for both L and S RNAs, the amount of genomic sense RNA was higher than that of genomic complementary RNA. © 1988 Academic Press, Inc.

Lymphocytic choriomeningitis virus (LCMV) has been studied extensively as a model system for virus–host interactions (*1–4*). Until recently, however, very little was known about the molecular structure of the viral genome (*5–8*) and information is only now emerging on potential regulatory mechanisms that may influence viral gene expression. The LCMV genome consists of two segments of single-stranded RNA: a large segment, designated L, 8–9 kilobases (kb) in length and a small segment, designated S, of approximately 3.4 kb. The L segment encodes a high-molecular-weight protein (approximately 200,000) which is thought to be part or all of the viral RNA-dependent RNA polymerase (*8, 9*). The S segment encodes the three major structural proteins: the internal nucleoprotein (NP; MW 63,000) which is associated with the genomic RNA, and the two glycoproteins GP-1 (MW 43,000) and GP-2 (MW 36,000) (*10*) which are derived from cleavage of a common precursor, GP-C (*11*).

Sequence analysis of cDNA clones (derived from the S segment of LCMV) has shown that the S segment is ambisense (*6, 7*); i.e., the NP mRNA is complementary to the genomic RNA whereas the GP-C mRNA is in the sense of the genome. Strand-specific RNA probes derived from the S segment confirmed the ambisense character of the S RNA and showed the presence of both genomic sense and genomic complementary RNA species during infection, including subgenomic RNAs corresponding to NP mRNA and GP-C mRNA (*7*). Such a coding strategy was first described for Pichinde, a related arenavirus, by Auperin *et al.* (*12*), who also identified an intergenic region in the S RNA, between the open reading frames for GP-C and NP, which could form a hairpin structure. A stable hairpin in this region of the genome could act as a transcription terminator for the GP-C and NP mRNAs and may also be involved in the regulation of replication.

Auperin *et al.* (*12*) have proposed a hypothetical model for the regulation of S transcription and replication in which NP mRNA could be synthesized directly using the viral genome as template, while the synthesis of GP-C mRNA must await the formation of full-length genomic complementary RNA, to serve as a template. Thus, the first event after viral infection would involve the synthesis of NP mRNA which may be concomitant with, or followed by, the production of full-length genomic complementary RNA (i.e., the replication intermediate). The genomic complementary RNA could then be used as a template for either transcription of GP-C mRNA or replication of full-length genomic sense RNA. Relatively little is known about the expression and functions of the LCMV L segment. Sequencing and hybridization studies with cDNA clones derived from the L segment indicate that at least the major part of this segment is of negative polarity (*8*). However, the complete coding potential of the L segment is not yet known.

We have followed the steady-state accumulation of intracellular viral RNAs during the early stages of acute infection using the combined techniques of denaturing agarose gel electrophoresis (*13*), RNA transfer to nitrocellulose filters (*14*), and hybridization with viral-specific probes. The experimental results have predominantly been derived by sequential hybridization

[1] To whom requests for reprints should be addressed



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reactions with individual nitrocellulose filters in order to eliminate variability between samples or gels. The hybridization probes were prepared from viral-specific cDNA clones (*7*) either by nick-translation (both DNA strands labeled equally (*15*)) or by *in vitro* synthesis of a single strand of labeled RNA (*16*) (see Fig. 1). These strand-specific RNA probes were necessary to distinguish between full-length genomic sense and genomic complementary sense L and S RNAs (see below).

Monolayers of BHK 21 cells (75% confluency) were infected with LCMV (Armstrong strain CA1371) at a multiplicity of infection (m.o.i.) of 5 and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Total-cell RNA was extracted from individual T175 flasks at different times postinfection by guanidine thiocyanate and purified by pelleting through cesium chloride (*17*). Samples of RNA were analyzed by electrophoresis in denaturing agarose gels and then transferred to nitrocellulose filters. Hybridization with a nick-translated probe (NP:A, Fig. 1a) demonstrated that the infecting viral RNA was evident at 0 hr postinfection, but was no longer detectable at 3 hr postinfection, possibly due to degradation (Fig. 2a). The NP mRNA and genomic size S RNAs appeared to accumulate at approximately the same time (i.e., 6–9 hr) after infection. The filter was subsequently hybridized with strand-specific RNA probes (NP:A1 and NP:A4; Figs. 1a, 2c, and 2d) to monitor the appearance of the genomic complementary NP mRNA and to distinguish between the accumulation of full-length genomic sense and genomic complementary sense S

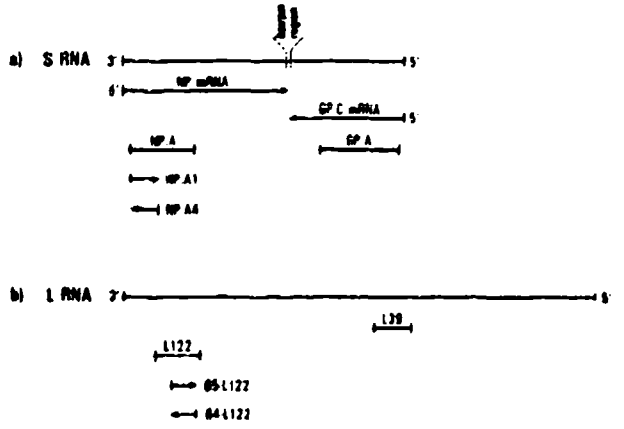



FIG. 1. Regions of LCMV S and L segments covered by nick-translated cDNA and strand-specific probes. (a) S segment: nick-translated cDNA probes—NP:A; GP:A. Strand-specific RNA probes—NP:A1 (detects genomic sense RNA), NP:A4 (detects genomic complementary RNA). (b) L segment: nick-translated cDNA probes—L122, L39. Strand-specific RNA probes—65-L122 (detects genomic sense RNA); 64-L122 (detects genomic complementary RNA). Arrows indicate 5′ to 3′ direction for RNA probes

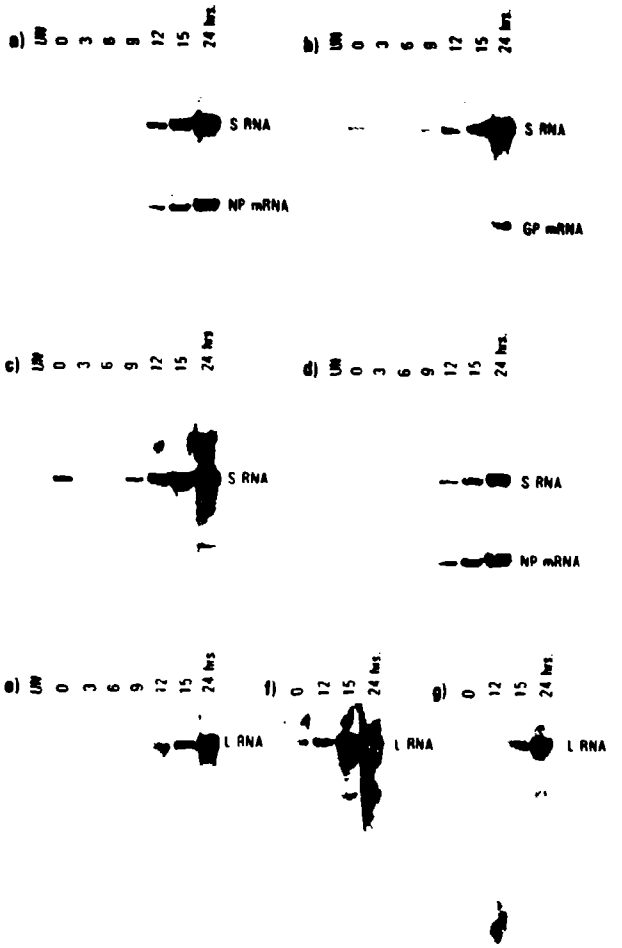



FIG. 2. Hybridization of total-cell RNAs with probes from the LCMV genome. (a) NP:A nick-translated S cDNA probe. (b) GP:A nick-translated S cDNA probe. (c) NP:A1 strand-specific probe to detect genomic sense S RNA. The genomic complementary sense NP mRNA does not hybridize with this probe. (d) NP:A4 strand-specific probe to detect genomic complementary S RNA, including the NP mRNA. (e) L39 nick-translated L cDNA probe. (f) 65-L122 strand-specific probe to detect genomic sense L RNA. (g) 64-L122 strand-specific probe to detect genomic complementary L RNA. UN—uninfected cell RNA; S RNA—genomic S RNA; L RNA—genomic L RNA; NP mRNA—nucleoprotein messenger RNA; GP mRNA—glycoprotein messenger RNA. Times shown (0–24) are hours postinfection. Films were exposed for 24 hr at −70°. The unidentified faint, low-molecular-weight bands seen at later times postinfection may represent breakdown products of viral genomic RNAs; their derivation is currently under investigation.

RNAs. The NP:A1 probe hybridized with genomic sense RNA and therefore detected the infecting virus RNA (time 0 hr) and the appearance of genomic sense RNA. The NP mRNA, which is complementary to the genome (see Fig. 1a), did not hybridize to this probe. The NP:A4 probe is in the sense of the genomic S RNA and therefore indicated the accumulation of genomic complementary S RNA and the NP mRNA. The hybrid-

ization patterns obtained with these probes (Figs. 2c and 2d) showed that the accumulation of genomic sense S RNA exceeded that of genomic complementary S RNA. (The two RNA probes were made in the same way using equal amounts of template DNA; equal counts per minute (cpm) were used for each hybridization and films were exposed for equivalent times.) The nitrocellulose filter was then hybridized with GP:A, a nick-translated cDNA probe derived from the glycoprotein coding region of the S segment (see Fig. 1). As is seen from Figs. 2a and 2b, although low levels of the GP-C mRNA were detected at approximately the same time as the NP mRNA and genomic S RNAs, the levels detected for the first 24 hr postinfection were significantly lower than those for NP mRNA. Densitometric analysis of the autoradiographs shown in Fig. 2 indicates that the ratios of NP mRNA and GP mRNA to genomic S RNA remained approximately constant over this time course of infection. This suggests that, although there is apparently a five-fold excess of NP mRNA over GP mRNA, the mRNAs initially accumulate at similar rates.

There have been several independent indications that the LCMV genomic L and S RNA segments are not present in equimolar amounts (reviewed in (*1*)). Therefore, we have used these total-cell RNA preparations to monitor expression of the viral L RNA segment and the relative accumulation of L- and S-derived RNA sequences. In order to achieve internal consistency, the nitrocellulose filter used in the S RNA analysis was hybridized with L39, a nick-translated cDNA probe derived from the L segment (Figs. 1b, 2e). Comparison with the hybridization pattern seen for S RNA (see Fig. 2) showed that newly synthesized genomic size L RNA was first detected at approximately the same time as genomic S RNA although the amount of L RNA, particularly at the earlier times, was lower. There was significant accumulation of L RNA 12–15 hr postinfection, as compared with 9–12 hr for S RNA. The relative amounts of genomic sense and genomic complementary L RNA were estimated from further Northern blotting experiments using L-derived strand-specific RNA probes—64-L122 (to detect genomic complementary sense RNAs) and 65-L122 (to detect genomic sense RNAs) (*8*) (see Figs. 1b, 2f, and 2g). As in the case of the S RNA hybridizations, the amount of genomic sense L RNA accumulating over the first 24 hr of acute infection was considerably higher than that of genomic complementary L RNA (see Fig. 2). (The probes were again made using equal amounts of DNA and equal cpm were used for hybridization.) Therefore the replication of the LCMV L segment appears to follow the same pattern as that of the S segment.

We have also used a method for pulse labeling of viral intracellular RNAs *in vivo* as a second approach to determine the ratio of genomic L:S RNA sequences during acute infection. Monolayers of BHK 21 cells (75% confluency in T175 flasks) were infected with LCMV at a m.o.i. of 5 and allowed to grow for 12 hr at 37°. Newly synthesized viral RNAs were then labeled with $^{32}P$ inorganic phosphate (1 mCi/flask) for 4 hr in the presence of 5 $\mu$g ml$^{-1}$ actinomycin D to block host cell transcription. Cytosol extracts from such labeled cells were centrifuged on discontinuous (2.5, 2.0, 1.0, 0.5 *M*) sucrose gradients (*18*) and two peaks of radioactivity were obtained. RNAs were recovered from the peak fractions by phenol extraction and ethanol precipitation and 25-$\mu$g samples were separated by agarose gel electrophoresis under denaturing conditions (*13*). As shown in Fig. 3, peak 1 (0.5 *M*/1.0 *M* interface) contained viral ribonucleoprotein complexes (RNP) formed almost exclusively with genomic sized S RNA whereas peak 2 (2.0 *M*/2.5 *M* interface) contained viral RNP complexes with both L and S genomic RNAs and cellular polysomes including polysome-bound viral NP and GP-C mRNAs. Based on uniform incorporation of label into L and S RNA species, the peak 2 fraction appeared to contain less L RNA than S RNA and this observation, together with the detection of an S-RNP apparently lacking any L RNA component, confirmed

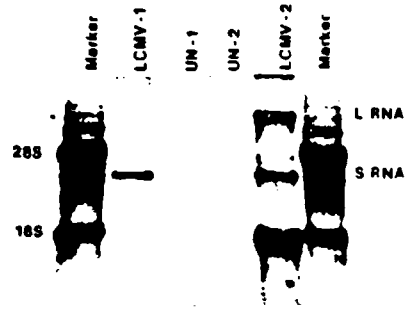



FIG. 3. *In vivo* labeling of LCMV RNAs. RNAs were extracted from intracellular RNP complexes and separated by denaturing agarose gel electrophoresis. Marker. Total infected cell RNA extracted with guanidine thiocyanate. These cells were labeled with $^{32}P$ in the absence of actinomycin D to give 28 and 18 S ribosomal RNAs as markers. LCMV-1 and LCMV-2. Fractions from the 0.5 *M*/1.0 *M* and 2.0 *M*/2.5 *M* sucrose gradient interfaces, respectively, from LCMV-infected cells. UN-1 and UN-2. Fractions from the 0.5 *M*/1.0 *M* and 2.0 *M*/2.5 *M* interfaces, respectively, from uninfected cells. The positions of the 28 and 18 S ribosomal RNAs in the marker lanes are indicated, as are the genomic L and S viral RNAs which align with bands in the LCMV-2 lane. In this sample the NP and GP mRNAs are visible just below the position of 18 S RNA in the marker. This figure is an autoradiograph of a dried gel and was obtained from a 24-hr film exposure at −70°.

the preferential accumulation of genomic S RNA over genomic L RNA during acute infection.

The ambisense coding strategy of the LCMV S segment allows independent regulation of transcription of the mRNAs corresponding to the major structural viral proteins NP, GP-1, and GP-2. With the experiments described in this paper and the limits of sensitivity of the hybridization techniques used, it was not possible to show a temporal separation of LCMV transcription and replication, because NP mRNA (the first mRNA to accumulate in appreciable amounts) appeared at approximately the same time as newly synthesized virus genomic sense RNA (Fig. 2a). The synthesis of newly replicated genomic sense RNA requires the prior synthesis of the genomic complementary RNA as an intermediate; therefore from the results shown above, we could not establish that synthesis of NP mRNA and subsequent translation to form NP are prerequisites for replication, although this may be anticipated by analogy with other viral systems (*19–21*). The amount of NP mRNA present at 24 hr postinfection was approximately 5-fold higher than that of GP-C mRNA (Figs. 2a and 2b), suggesting that synthesis of NP mRNA occurs in the early stages of infection. This was also found to be the case for Pichinde virus (*12*).

The results reported in this paper suggest that LCMV replication is itself under various other controls. For both L and S segments the amount of genomic sense RNA during the first 24 hr after infection is considerably higher than that of genomic complementary RNA (see Fig. 2). The genomic complementary RNA is the replication intermediate and the relatively low amount of this RNA suggests that it is used repeatedly as a template for the replication of genomic sense RNA during the early stages of infection.

## ACKNOWLEDGMENTS

This is Publication Number 4857-IMM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037. We thank Drs. John Holland, David Meek, and Michael Oldstone for their constructive comments on this manuscript and Michael Oldstone for his continuing interest and support. We thank Ms. Nicole Nguyen and Mrs. Gay L. Schilling for excellent technical and secretarial assistance. This work was supported in part by USPHS Grants AI-09484, NS-12428, and AG-04342 and by USAMRIID Contracts C-3013 and C-6234. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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JOURNAL OF VIROLOGY, May 1989, p. 1938–1944
0022-538X/89/051938-07$02.00/0
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Vol. 63, No. 5

# Detection of Virus-Specific RNA-Dependent RNA Polymerase Activity in Extracts from Cells Infected with Lymphocytic Choriomeningitis Virus: In Vitro Synthesis of Full-Length Viral RNA Species†

FRANCES V. FULLER-PACE* AND PETER J. SOUTHERN

*Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037*

Received 17 November 1988/Accepted 19 January 1989

**We have developed an in vitro assay for the lymphocytic choriomeningitis virus (LCMV) RNA-dependent RNA polymerase with ribonucleoprotein complexes extracted from acutely infected tissue culture cells. The RNA products synthesized in vitro corresponded in size to the full-length genomic L and S RNAs and subgenomic NP and GP mRNAs normally produced in vivo during acute LCMV infection. In a temporal analysis spanning the first 72 h of acute infection, the in vitro polymerase activity of ribonucleoprotein complexes was maximal at 16 h and declined significantly at later times. In contrast, the intracellular levels of the viral L protein (the putative polymerase protein) appeared to be maximal at 48 to 72 h postinfection. Our results suggest that the accumulation of L protein correlates with reduced viral replication and transcription at later times in acute infection and may be involved in the transition from acute to persistent LCMV infection.**

Lymphocytic choriomeningitis virus (LCMV), the prototype arenavirus, has been studied extensively as a model system for virus-host interactions (8, 9, 18). Recent interest in the molecular details of infection by LCMV and the other arenaviruses has prompted studies on the structure and organization of the viral genome and on regulatory mechanisms that influence viral gene expression (30–32, 34). The LCMV genome consists of two single-stranded RNA segments, designated L and S, with approximate lengths of 7.2 and 3.4 kilobases, respectively (26, 36). The S RNA segment has an ambisense coding arrangement (2) that directs synthesis of the three major structural proteins: an internal nucleoprotein (NP; molecular weight 63,000) that is associated with the genomic RNA, and two surface glycoproteins, GP-1 (molecular weight 43,000) and GP-2 (molecular weight 36,000) (5), that are derived by posttranslational cleavage of a precursor polypeptide, GP-C (6). The L RNA segment encodes a high-molecular-weight protein (molecular weight ca. 200,000), thought to be part or all of the viral RNA-dependent RNA polymerase (14, 32), and the possible presence of a second, L-encoded protein is currently under investigation (M. Salvato, personal communication).

Genetic mapping studies have clearly implicated the viral L RNA segment in lethal LCMV infection of adult guinea pigs (29) and in the altered biological properties of variant viruses recovered from the spleens of persistently infected mice (1). In the guinea pig infection, it is not clear whether an L-encoded protein is directly pathogenic or whether the LCMV (WE strain) polymerase supports more rapid initial virus replication to precipitate a lethal infection via unidentified secondary mechanisms. Similarly, precise molecular explanations are not currently available for altered immune responses in mice infected with the spleen-variant viruses, but the altered phenotype maps to the L RNA segment and may be associated with replication of the variant viruses in lymphoid cells (1, 25).

To date, characterization of the RNA-dependent RNA polymerase of LCMV and other arenaviruses has been confined to virion-associated enzymes. Leung et al. (19), using purified Pichinde virions, described an in vitro polymerase activity that synthesized a heterogeneous population of RNAs complementary to virion RNA. With Tacaribe virus, Boersma and Compans (3) coupled a viral in vitro transcription reaction with a rabbit reticulocyte lysate translation system to demonstrate the synthesis of virus-specific polypeptides. For LCMV, Bruns et al. (4) have reported an RNA polymerase activity that is associated with nucleocapsids derived from purified virions. In the present study, we have begun to examine the properties of the LCMV RNA-dependent RNA polymerase and have developed an in vitro assay for polymerase activity that uses extracts from acutely infected cells. Using this system, we have demonstrated synthesis in vitro of full-length genomic L and S RNAs and subgenomic NP and GP mRNAs and have monitored apparent changes in the polymerase activity during the course of an acute LCMV infection. In previous studies, we have analyzed the steady-state levels of intracellular viral RNA species that accumulate over the time course of acute infection (13, 34) and can now make comparisons between these intracellular species and the in vitro reaction products synthesized from cell extracts harvested at various times postinfection. Our eventual goal is to determine whether changes in polymerase activity are involved in the regulation of viral replication and transcription during various stages of infection.

## MATERIALS AND METHODS

Cells and virus. BHK-21 cells were grown in Falcon T175 flasks in Dulbecco-Vogt modified Eagle medium supplemented with 5% fetal calf serum. The 50% confluent monolayers were infected with LCMV (Armstrong strain CA-1371) at a multiplicity of infection of 5, and extracts were

* Corresponding author.

† Publication No. 5492-1MM from the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

prepared for Western blotting (immunoblotting) and polymerase assays at various times after infection.

**Preparation of cell extracts.** Extracts enriched for ribonucleoprotein (RNP) complexes were prepared as described by Hill and Summers (16). Monolayers (in T175 flasks) were washed with phosphate-buffered saline, scraped off into phosphate-buffered saline, and collected by centrifugation at 1,000 × *g* for 5 min. The cell pellet was suspended in lysis buffer (10 mM KCl, 1.5 mM magnesium acetate, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], 0.5 mM dithiothreitol) (1 ml per T175 flask), allowed to swell for 5 min on ice, and then disrupted with 30 strokes of a Wheaton B Dounce homogenizer. Nuclei, cell membranes, and other cell debris were removed by centrifugation at 10,000 × *g* for 20 min at 4°C. NaCl and Triton X-100 were added to 0.5 M and 1%, respectively, and RNP complexes were pelleted by centrifugation through 2.5 ml of 50% glycerol, in the above lysis buffer, for 2.5 h at 45,000 rpm in a Beckman SW50.1 rotor, at 4°C. The pellet was suspended in cold 2× reaction buffer (200 mM KCl, 100 mM Tris hydrochloride [pH 7.5], 10 mM magnesium acetate, 4 mM dithiothreitol) (50 μl for each T175 flask) and used in polymerase assays immediately.

**Western blotting.** Proteins in the cell extracts were separated by electrophoresis in 7% polyacrylamide–sodium dodecyl sulfate (SDS) gels (17) and transferred electrophoretically to nitrocellulose filters (0.2 μm pore size) overnight at 250 mA, with a recirculation cooling system. The filters were incubated with antipeptide antibodies directed against regions of NP and L (kindly provided by M. J. Buchmeier). Bound immunoglobulin was detected with $^{125}$I-labeled *Staphylococcus aureus* protein A as described previously (32).

**Assays for RNA polymerase activity.** Unless specified otherwise, assays were carried out for 1 h at 30°C in 100-μl volumes containing 50 μl (200 μg of protein) of cell extract in 2× reaction buffer, 1 mM each unlabeled ATP, CTP, and UTP, 10 μM unlabeled GTP, and 50 μCi of [α-$^{32}$P]GTP (Du Pont NEN Products; 600 Ci/mmol). Where necessary, multiple 100-μl reaction mixes were used. These gave higher incorporation than reactions performed in larger volumes. Reactions were stopped by the addition of unlabeled GTP and EDTA (final concentrations, 1 and 10 mM, respectively), followed by the addition of 100 μl of 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA–100 mM NaCl–0.1% SDS. Unincorporated nucleotides were removed with a Sephadex G-50 spin column (20), and after phenol extraction, RNAs in the reaction mixture were precipitated in ethanol and suspended in sterile distilled water for subsequent analysis.

**RNA gel electrophoresis and Northern blots.** RNAs in the polymerase reaction mixes were denatured with glyoxal and separated by agarose gel electrophoresis in 10 mM $NaPO_4$, pH 6.5 (21). Unless specified otherwise, the gels were dried onto DE81 paper at 80°C under vacuum and exposed for autoradiography with Kodak XRP-1 film. In some cases, after electrophoresis, the RNAs were transferred to nitrocellulose filters by capillary diffusion of 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature (35) and baked for 2 h at 80°C under vacuum. After autoradiography, the $^{32}$P was allowed to decay, and the filters were then hybridized with LCMV probes to confirm the specificity of the labeled polymerase reaction products.

**Southern blots.** Plasmids containing cDNA fragments from LCMV L and S segments were digested with appropriate restriction enzymes to give discrete LCMV-derived DNA fragments and transferred to nitrocellulose (33). cDNAs derived from 28S rRNA were used as controls. After being baked at 80°C under vacuum, the filters were hybridized with the polymerase reaction products as described below.

**Alkaline hydrolysis.** Partial hydrolysis of RNAs in the polymerase reaction mixes was achieved by incubation for 1 h at 40°C in 50 mM $Na_2CO_3$, followed by neutralization with 150 mM sodium acetate (pH 5). This procedure gave RNA fragments ranging in size from 100 to 300 nucleotides.

**Synthesis of hybridization probes.** For hybridization with LCMV-derived probes, restriction fragments from the LCMV L and S segment cDNAs were purified from preparative agarose gels (37) and then labeled in vitro with Klenow DNA polymerase I in a modified version of the original nick translation reaction (28).

**Hybridization.** For hybridization with cDNA probes, the filters were prehybridized at 37°C for 3 h in 50% deionized formamide–5× SSC–2.5× Denhardt solution (1× Denhardt solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone) with 100 μg of denatured sonicated salmon sperm carrier DNA per ml; for hybridization with polymerase assay reaction products, the filters were prehybridized for 3 h at 55°C in 50% formamide–4× SSC–2× Denhardt solution, with 100 μg of denatured salmon sperm DNA per ml and 100 μg of yeast tRNA per ml as carriers. In each case, hybridization was carried out for 20 to 24 h under the conditions used for prehybridization. Filters were washed twice in 2× SSC–0.1% SDS at 37°C, then at 60°C in the same solution, and finally at 60°C in 0.1× SSC–0.1% SDS–0.1% Tween-20. All washes were for 30 min. Autoradiography was carried out at −70°C with Kodak XAR-5 film and Du Pont Cronex Lightning Fast intensifying screens. Before subsequent hybridizations, probes were stripped from filters by washing in 0.1× SSC–0.1% SDS–0.1% Tween-20 for 2 h at 85°C. Filters were then prehybridized as before.

## RESULTS

**Detection of viral proteins in intracellular RNP complexes.** Intracellular RNP complexes were prepared from cultures of acutely infected BHK cells (see Materials and Methods) at various times postinfection. Viral proteins were detected by Western blotting with antipeptide antibodies specific for NP, GP-2, or L (the putative polymerase) protein (7, 32). The amounts of both NP and L protein increased for 24 to 72 h (Fig. 1), whereas GP-2 could not be detected within the intracellular RNP complexes (data not shown). Analysis of different cellular fractions by this Western blotting approach showed that L protein was only detectable in the RNP fraction (data not shown). Based on these results, cell extracts enriched for viral RNP complexes were initially prepared at 72 h postinfection in order to ensure a high concentration of L protein for the in vitro polymerase reactions.

**Synthesis of virus-specific RNAs in vitro.** Concentrated preparations of intracellular RNP complexes extracted from acutely infected BHK cells 72 h postinfection and parallel cultures of mock-infected BHK cells were suspended in reaction buffer (see Materials and Methods) and incubated in the presence of [α-$^{32}$P]GTP at 30°C. We observed a linear, time-dependent increase in the incorporation of radioactivity into trichloroacetic acid (TCA)-precipitable counts in a 1-h incubation but could find no reproducible difference between the infected and uninfected RNP preparations in this system.

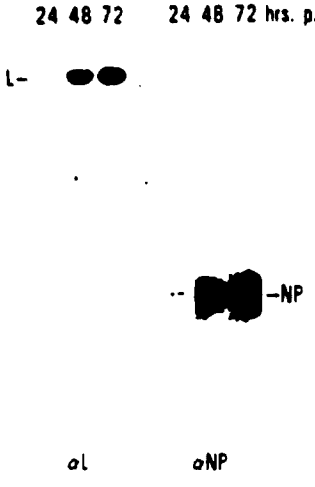



FIG. 1. Western blot showing accumulation of viral L protein and nucleoprotein (NP) in RNP complexes extracted from cells at various times postinfection (p.i.). In each case, 25 μg of protein was loaded on an SDS-polyacrylamide gel and separated by electrophoresis. Viral proteins were detected by using monospecific rabbit anti-L (αL) or anti-NP (αNP) antipeptide antibodies, followed by treatment with $^{125}$I-labeled *S. aureus* protein A. Relative exposures of autoradiographs: L, 72 h; NP, 3 h.

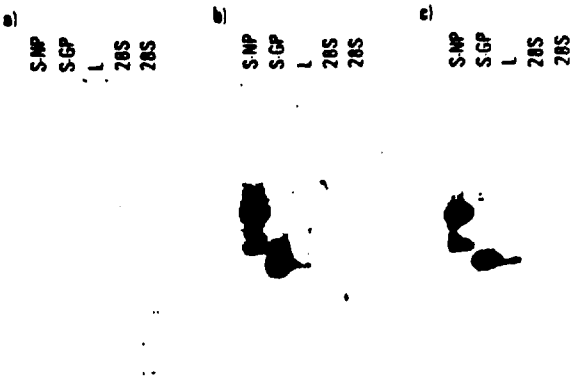



FIG. 2. Hybridization reactions with $^{32}$P-labeled RNAs syn sized in vitro. Plasmid DNAs were digested with restriction zymes to release different cDNA target sequences derived from genomic S or L RNAs or host 28S RNA. The DNA fragments v separated by agarose gel electrophoresis, transferred to nitroce lose filters, and hybridized with the following probes: (a) $^{32}$P-labe RNAs synthesized with an extract from uninfected cells; (b) labeled RNAs synthesized with an extract from infected cells; (c in panel b, but the labeled RNAs were partially hydrolyzed treatment with alkali before the hybridization reaction was initia Target cDNA sequences are identified as follows. S-NP, cD from bases 1665 to 3342 in LCMV S sequence (31). This gives fragments, 1,043 and 634 base pairs, on digestion with *Pst*I. S-( cDNA from bases 11 to 435 in LCMV S sequence (31). L, L cDNA clone from LCMV L segment (32); bases 511 to 1208 sequence (M. Salvato, E. Shimomaye and M. B. A. Oldst submitted for publication). This gives two fragments of 426 and base pairs on digestion with *Pst*I. 28S, cDNAs from mouse rRNA as non-LCMV-specific control. First 28S lane, bases 385 4147; second 28S lane, bases 1499 to 1917 (P. J. Southern, unp lished observations).

Furthermore, extraction of nucleic acid from the reaction mixtures prior to TCA precipitation and several variations in the conditions for the actual TCA precipitation still did not provide any numerical discrimination between the infected and uninfected reactions (data not shown).

As an alternative approach to demonstrate virus-specific RNA synthesis, we used the in vitro reaction products as hybridization probes against target cDNAs derived from the viral genome or, as a control, cDNAs from host 28S rRNA sequences. With the extract harvested from infected cells 72 h postinfection, there was strong hybridization to viral target sequences from the genomic S RNA segment (NP and GP coding regions) and a low level of hybridization to an L-derived target sequence, but no hybridization to the 28S rRNA target sequences (Fig. 2b). None of the target sequences was detected when the reaction products from the uninfected cell extract were used as hybridization probes (Fig. 2a). As an additional control, similar hybridization reactions were repeated, and identical results were obtained with probes that had been partially hydrolyzed by treatment with alkali. This control was included because in preliminary experiments with the cell extracts and [α-$^{32}$P]UTP, we found that preexisting rRNAs in the reaction could become labeled at the 3′ terminus (data not shown). Cellular poly(U) polymerases are known (19), but there are no reports of endogenous cellular poly(G) polymerase activity; thus, we chose labeled GTP to follow the reaction. Nevertheless, for the results from the hybridization assays to be valid, it was critical to distinguish between terminal labeling of preexisting full-length viral RNAs and de novo synthesis of RNAs in vitro. If the full-length RNAs present in the enriched RNP preparations were being end-labeled in the in vitro reactions, alkaline hydrolysis of the probe would only allow hybridization with cDNA targets corresponding to the 3′ ends of the RNAs. In these experiments, the cDNAs were derived from internal regions of the viral genomic or rRNAs, and the observed hybridization after alkaline hydrolysis was therefore consistent with de novo synthesis of uniformly labeled RNAs. In addition, we subsequently observed that neither viral nor 28S rRNAs were labeled if [α-$^{32}$P]GTP was used the absence of any other ribonucleotides (data not show

**Temporal analysis of polymerase activity during LC infection.** The L protein was not detected in infected extracts at 24 h after infection but had accumulated sign cantly by 48 to 72 h postinfection (Fig. 1). In contrast, accumulation of LCMV mRNAs in vivo and the releas infectious virions were maximal at 16 to 24 h postinfec under our standard conditions for virus infection (mu plicity of infection of 5) (8; our unpublished observatio This suggested that there may be a disparity between levels of L protein and the intracellular activity of the polymerase. Therefore, in vitro reactions were perfor with infected-cell extracts harvested at 24, 48, and 7 postinfection, and the reaction products were analyzed denaturing agarose gel electrophoresis (Fig. 3). Separatio the in vitro reaction products by gel electrophoresis p vided an immediate method for monitoring polymerase tivity. Although the hybridization method had the advant of showing virus-specific RNA synthesis, the electropho sis approach was considerably less cumbersome and allow direct analysis of the reaction products.

Individual in vitro reactions were performed immedia after extraction and concentration of the RNP comple and then the labeled RNA products were recovered stored for gel analysis. These results indicated that infect cell extracts, harvested at 24 h postinfection, were capa of synthesizing both genome-sized RNA and subgeno mRNAs in vitro and that, at later times, the polymer activity was markedly diminished. The labeled RNAs s thesized in vitro corresponded to the genomic and mR

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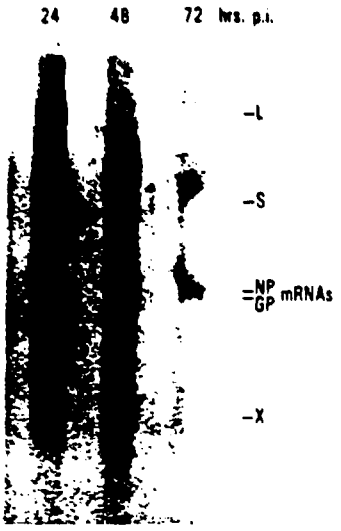



FIG. 3. Autoradiograph of a denaturing agarose gel showing the $^{32}$P-labeled RNA products that were synthesized in vitro with extracts harvested at various times postinfection (p.i.). The total products from three 100-μl polymerase reaction mixes were loaded on the gel for each time point. RNAs corresponding to LCMV RNAs are indicated. L, genomic L RNA; S, genomic S RNA; NP, nucleoprotein mRNA; GP, glycoprotein mRNA; X, novel subgenomic RNA.

species normally produced during an acute infection of tissue culture cells (see below and Fig. 6). There was one novel product formed during the in vitro reaction (Fig. 3, band marked X) that may represent a premature termination product. The derivation of this discrete RNA species (length, approximately 300 to 500 bases) is currently under investigation.

**Determination of optimal time for extraction of RNP from infected cells.** The relatively high polymerase activity observed at 24 h after infection prompted us to study changes in the in vitro activity of infected cell extracts at earlier times following LCMV infection. The accumulation of reaction products was clearly maximal in the RNP fraction extracted

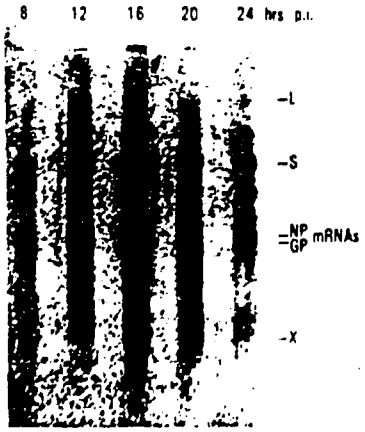



FIG. 4. Autoradiograph of a denaturing agarose gel showing the $^{32}$P-labeled RNA products that were synthesized in vitro with extracts harvested between 8 and 24 h postinfection (p.i.). Products from three 100-μl polymerase reaction mixes were loaded on the gel for each time point. RNAs corresponding to LCMV RNAs are indicated. See Fig. 3 legend for details.

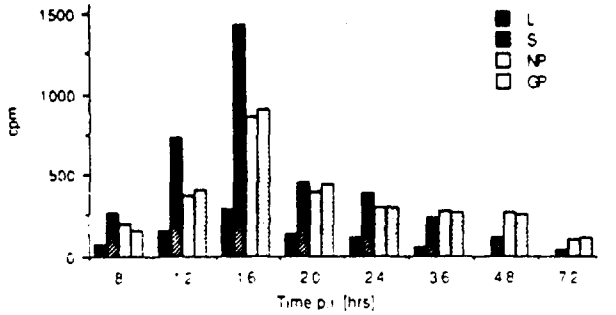



FIG. 5. Relative levels of incorporation of [$^{32}$P]GMP into individual viral RNA species. Reactions were performed in vitro with extracts harvested at various times during the first 72 h of acute LCMV infection. Incorporation of radioactivity is expressed as counts per minute in each RNA species and was obtained by direct scanning of dried agarose gels with an AMBIS β scanner. Scanning was carried out overnight, and the counts obtained were individually corrected for background within each lane of the gel. The standard deviation as calculated by the scanner was 1 to 3%. Average counts from two or three assays (three 100-μl reaction mixes in each case) are represented for each time point. L, genomic L RNA; S, genomic S RNA; NP, nucleoprotein mRNA; GP, glycoprotein mRNA.

at 16 h postinfection (Fig. 4). The unidentified RNA, X, was also most highly labeled in the 16-h sample. None of the labeled RNAs synthesized in vitro, including X, was produced in reactions with RNP complexes from uninfected cells.

In a subsequent experiment, the polymerase activity over the first 72 h after infection was monitored to compare the synthesis of the individual viral RNAs. RNA products from reactions carried out at various times postinfection were separated by gel electrophoresis, and the incorporation of [α-$^{32}$P]GMP into the individual RNAs was quantitated directly by scanning the dried agarose gels with an AMBIS β scanner (Fig. 5). The patterns of synthesis of L and S genomic RNA were parallel over the 72-h period of infection, although L RNA was synthesized in three- to fivefold-lower amounts. Likewise, NP and GP mRNAs were synthesized in parallel throughout this time course and at each time point were found in approximately equal amounts. The synthesis of both genome-sized RNAs (L and S) and subgenomic mRNAs (NP and GP mRNAs) increased coordinately and peaked at 16 h postinfection. From 16 to 20 h postinfection, both decreased sharply in parallel. After 20 h, the decrease was considerably more gradual but the synthesis of genome-sized RNAs declined more rapidly; this is particularly evident in Fig. 3.

**Comparison between viral RNAs synthesized in vitro and viral RNAs synthesized in vivo during acute infection of BHK cells.** The labeled RNAs from in vitro reactions, performed with either infected or uninfected extracts, were separated by denaturing agarose gel electrophoresis and transferred to a nitrocellulose membrane. A sample of RNA, extracted from the intracellular RNP fraction prior to initiating the in vitro reactions, was denatured, electrophoresed, and transferred to the same nitrocellulose membrane. Direct autoradiography of the filter indicated that genome-sized L and S RNAs, subgenomic NP and GP mRNAs, and the novel subgenomic RNA (X) were all synthesized in vitro (Fig. 6a). After the $^{32}$P was allowed to decay (approximately 2 months), the filter was hybridized sequentially with nick-translated probes specific to the GP and NP coding regions

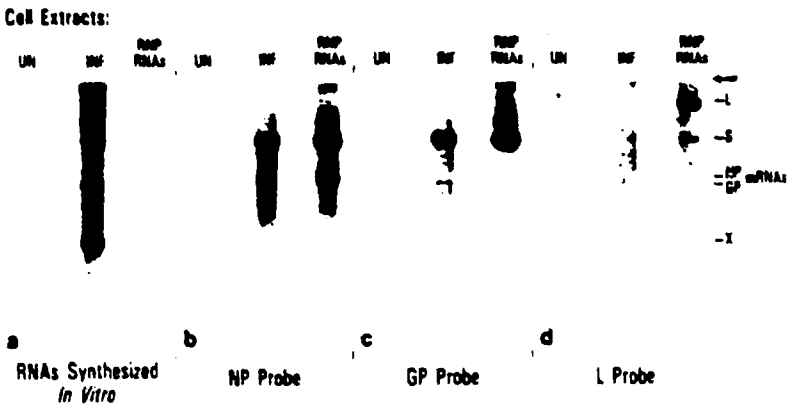



FIG. 6. Comparison between viral RNAs synthesized in vitro and viral RNAs that accumulate within acutely infected tissue culture c (a) $^{32}$P-labeled RNA products from in vitro LCMV polymerase reactions carried out with RNP complexes harvested 16 h after infection RNA species were separated by electrophoresis in a denaturing agarose gel and then transferred to a nitrocellulose filter for autoradiograr After the $^{32}$P was allowed to decay, the nitrocellulose filter was hybridized sequentially with LCMV-specific cDNA probes. (b) NP p derived from the 3' end of the S segment; bases 2299 to 3342 in S RNA sequence (31). This detects genomic S RNA and NP mRNA. (c probe derived from the 5' end of the S segment, bases 11 to 435 in S RNA sequence (31). This detects genomic S RNA and GP mRNA. low level of hybridization to GP mRNA reflects the low amount of this RNA present in infected cells at this time post infection (13). ( probe derived from the L segment; L122 cDNA clone (32), bases 511 to 1208 in L RNA sequence (Salvato et al., submitted). This det genomic L RNA. This autoradiograph was exposed for 72 h, compared with 24 h for the NP and GP probes). UN, Extract from uninfe cells; INF, extract from infected cells; RNP RNAs, infected-cell extract prior to polymerase reaction. Arrow, Gel origin.

of the S RNA segment and an L probe (Fig. 6b, c, and d, respectively). This analysis clearly demonstrated that the in vitro reaction products were indistinguishable in size from the intracellular viral RNAs that accumulate during acute LCMV infection.

**Time course of polymerase-dependent incorporation of label into RNA products.** Reactions performed with cell extracts harvested at 16 h postinfection were allowed to proceed for various lengths of time ranging from 0 to 4 h. In this experiment, the synthesis of L and S genomic RNA and NP and GP mRNA products increased linearly for the first hour

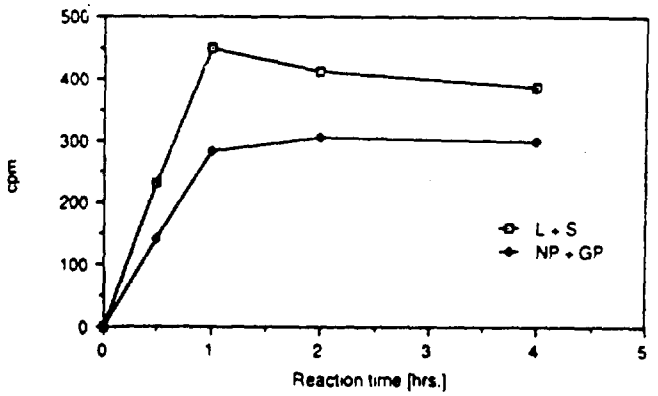



FIG. 7. Time course for incorporation of label into in vitro reaction products. The incorporation of [$^{32}$P]GMP into L and S genomic RNAs and NP and GP mRNAs was measured in in vitro reactions. The products from individual 100-μl reaction mixes were separated by denaturing agarose gel electrophoresis, and after drying, the gel was scanned. For these comparisons, counts from L RNA were considered to represent genomic L RNA synthesis. To date, we have been unable to identify a discrete L mRNA species, and it may be electrophoretically indistinguishable from genomic L RNA. Therefore, some of the counts in L RNA may be due to L mRNA rather than genomic L RNA synthesis. However, as the overall incorporation of $^{32}$P into L RNA was relatively low, any incorporation resulting from L mRNA synthesis would not have affected the overall genomic/mRNA synthesis ratio significantly.

of the polymerase reaction, and then essentially no fur incorporation of radioactivity occurred (Fig. 7). Theref to obtain some estimate of the specific activity for polymerase in cell extracts, reactions were stopped aft min—a time point well within the linear portion of the g (Fig. 7). Under these assay conditions, the specific act of the LCMV polymerase was calculated by estimatin total [$^{32}$P]GMP incorporation into full-length LCMV nomic and mRNAs. An average of 10 assays gave a spe activity of approximately 50 fmol of nucleotide incorpo per mg of cell extract protein per h at 30°C.

**Titration of protein concentration for the in vitro poly ase reaction.** Reaction mixes with different amounts fected cell extract (0 to 240 μg of total protein), harvest h after infection, included a compensatory amount of fected-cell extract to bring the total protein concentrat all the reaction mixes to 240 μg. The constant p concentration was maintained in order to minimize po problems that could occur at low protein concentration to either dissociation of viral RNP complexes or los cellular cofactor. Analysis of the reaction products indi that the synthesis of both genome-sized RNAs and mR increased linearly with increasing concentration of p from infected cells (Fig. 8). Moreover, addition of da mycin and α-amanitin, which inhibit cellular RNA pol ases, did not affect the synthesis of these RNAs (da shown). This suggests that the observed polymerase a was due to a viral, not a cellular, polymerase.

## DISCUSSION

The initial reports of viral polymerase activity assc with arenaviruses had involved the use of purified vir the source of viral polymerase and RNA templates (3. In contrast, we chose to use RNP complexes extracte acutely infected cells for two reasons: (i) the study of polymerase activity in cellular extracts will facilitate comparisons of the polymerase activities in acute persistently infected cells and may provide valuable mation on the role of the viral polymerase in the mo

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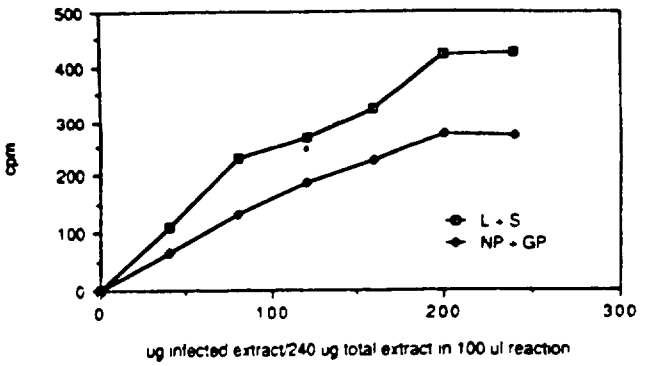



FIG. 8. In vitro RNA synthesis as a function of the protein concentration derived from infected-cell extracts. The total incorporation of [$^{32}$P]GMP into L and S genomic RNAs (see legend to Fig. 7) and NP and GP mRNAs was determined over a range of infected-cell extract concentrations. All in vitro reactions were carried out at 30°C for 40 min. In each case, the total protein concentration was 240 μg. Data were obtained by scanning a dried agarose gel (see Fig. 7). Counts are for individual 100-μl reaction mixes.

mechanism of viral persistence, and (ii) optimal replication and transcription may depend on interaction of the viral polymerase with cellular factors that may not be present in purified virions. It is not known whether cellular proteins are involved in arenavirus replication and transcription, but there have been several reports of cellular proteins enhancing or being required for in vitro RNA synthesis in other RNA viruses, including vesicular stomatitis virus (VSV), Sendai virus, and poliovirus (15, 23, 24). In the case of measles virus, polymerase activity was found to be 10-fold higher in RNP complexes from infected cells than in purified virions, although no direct role for a cellular cofactor has yet been identified (27).

In our in vitro assay for the LCMV polymerase, we observed synthesis of full-length, LCMV-specific RNAs corresponding in size to the genomic L and S RNAs and the NP and GP mRNAs (Fig. 4 and 6). A similar assay system, with cytoplasmic RNP complexes, demonstrated the synthesis of full-length genome-sized RNA and mRNA in vitro for influenza virus (10). The RNAs synthesized in vitro hybridized specifically with LCMV-derived cDNAs (Fig. 2). Other hybridization experiments with strand-specific M13 subclones derived from various regions of the LCMV L and S segments have shown that full-length genomic sense and genomic complementary-sense RNAs were synthesized in this in vitro assay system (our unpublished observations). The absence of RNA synthesis in extracts from uninfected cells (Fig. 2 and 6), the correlation of RNA synthesis with the concentration of infected-cell RNP extract (Fig. 8), and the insensitivity of the polymerase to dactinomycin and α-amanitin strongly suggest that the polymerase is viral.

The level of in vitro polymerase activity in extracts harvested at various times during the first 72 h of acute infection approximately mirrors the accumulation of intracellular viral RNAs that are present at equivalent times postinfection (13, 34). The rates of both in vitro synthesis and in vivo accumulation of viral RNA were reduced after 16 to 24 h; this correlates with a previously observed decrease in the production of infectious virus (8). The reduction in in vitro polymerase activity contrasts with the obvious accumulation of L (the putative polymerase) protein in these extracts (Fig. 1) and suggests a loss or inhibition of activity at high concentrations of L protein. A similar observation was made in VSV complementation experiments, where replication of a temperature-sensitive L mutant virus could be supported in host cells expressing low levels of L protein but replication of the mutant was inhibited in the presence of high levels of L protein (21). Likewise, high levels of L protein also inhibited replication of wild-type VSV. The reduced replication may be due to formation of aberrant complexes involving other viral proteins that are required for replication. It is not known which viral proteins play a direct role in LCMV replication or transcription, but the higher amount of L protein at later times in infection might affect the association of L with the RNA-NP complex and therefore the replication and transcription efficiency. This type of regulatory mechanism may reflect a common feature in the control of negative-strand and ambisense RNA virus polymerases.

The in vitro synthesis of L and S genomic RNAs and NP and GP mRNAs closely paralleled each other between 8 and 20 h after infection, with a pronounced peak at about 16 h (Fig. 4 and 5). After 16 h there was a biphasic decrease in polymerase activity, with a rapid fall between 16 and 20 h followed by a more gradual decline. By 48 h, however, the synthesis of genome-sized RNAs fell to virtually undetectable levels, while that of subgenomic mRNAs decreased much more slowly (Fig. 3 and 5). This suggests an uncoupling of the two processes and may reflect changes in the polymerase, perhaps caused by accumulating levels of L protein or interaction with another viral protein or a cellular factor.

LCMV readily establishes persistent infections in cultured cells and in mice infected within the first 24 h of life. Persistent infections are characterized by reduced viral replication, decreased accumulation of viral glycoproteins at the surface of infected cells, the generation of interfering particles, and the appearance of novel viral RNAs. However, it is not known what is responsible for the switch from acute to persistent infection. The reduction in the generation of infectious virus (8), appearance of novel subgenomic viral RNAs (11, 12), and generation of interfering particles (18) may be due to an altered RNA polymerase or replicase activity. We are currently investigating whether there are any differences in the viral polymerase activity or in the RNAs synthesized in vitro with RNP extracts from persistently infected cells.

## ACKNOWLEDGMENTS

We are grateful to John Fazakerley, David Meek, Michael Oldstone, and Jacques Perrault for constructive comments on the manuscript and to Michael Oldstone for continuing interest and support. We thank Charles Frankel and David Meek for help with the AMBIS β scanner and Gay Schilling for excellent secretarial assistance.

This manuscript was supported in part by Public Health Service grants NS-12428, AG-04342, and AI-25224 from the National Institutes of Health. F. V. Fuller-Pace is a recipient of a postdoctoral fellowship from the Juvenile Diabetes Foundation.

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VIROLOGY 171, 417–426 (1989)

# Neutralizing Epitopes of Lymphocytic Choriomeningitis Virus Are Conformational and Require Both Glycosylation and Disulfide Bonds for Expression

K. E. WRIGHT,[1] M. S. SALVATO, AND M. J. BUCHMEIER[2]

*Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037*

*Received August 4, 1988; accepted April 10, 1989*

**Lymphocytic choriomeningitis virus (Armstrong strain) bears two overlapping epitopes, GP-1A (A) and GP-1D (D), recognized by neutralizing antibodies on the major surface glycoprotein GP-1. Both are discontinuous conformational epitopes that require prior formation of disulfide bridges and addition of N-linked oligosaccharides. Using monoclonal antibodies specific for each of these epitopes, as well as for conformation-independent epitopes, we have investigated the requirements for biosynthesis and folding of the epitopes. The carbohydrate residues themselves do not appear to comprise critical informational components of these epitopes, but are required for proper folding of the nascent glycopeptide chain within the rough endoplasmic reticulum. These epitopes differ in their resistance to denaturation, epitope D is retained when denatured with SDS under nonreducing conditions, whereas epitope A is lost. Monoclonal antibodies to epitope A cross-react with several strains of LCMV. However, epitope D is detected in only a subset of isolates derived from the Armstrong strain of LCMV. By RNA sequence analysis, we have mapped a single amino acid change distinguishing those virions containing epitope D. Acquisition of binding activity of the epitope D-specific monoclonal correlates with a Thr → Ala or Thr → Lys mutation at amino acid 173 of the GP-1 molecule and concomitant disruption of a consensus N-linked glycosylation site.** © 1989 Academic Press, Inc.

## INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV) is the prototype of the arenaviridae, and as such its biology and structure have been extensively studied. LCMV infection of the mouse has proved to be a model system in which it has been possible to examine multiple aspects of persistent infection (Traub, 1936; Mims, 1970) including tolerance and immune-complex disease (Oldstone and Dixon, 1967, 1969, 1970) and the concept of alteration of "luxury" functions of cells while vital functions are normal (Oldstone *et al.*, 1977, 1982, 1984; Klavinskis *et al.*, 1988). Acute infection of mice with the virus has been used to demonstrate a major role for T cells both in immune-mediated pathology (Cole *et al.*, 1972; Gilden *et al.*, 1972; Allen and Doherty, 1985) and in clearance of the virus (Zinkernagel and Welsh, 1976; Anderson *et al.*, 1985).

Structurally LCMV is the best characterized of the arenaviruses. There are three major structural proteins, a minor 10- to 14-kDa polypeptide and a putative RNA polymerase encoded by a two-segmented RNA genome. The 10- to 14-kDa polypeptide is consistently observed in preparations of purified LCMV and other arenaviruses, but its function is unknown. The structural proteins include a nucleoprotein (NP) ($M_r$ 63K) associated with the viral genome, and two surface glycoproteins, GP-1 ($M_r$ 44K) and GP-2 ($M_r$ 35K), that are derived from a precursor glycoprotein, GP-C, found in infected cells (Buchmeier *et al.*, 1987; Buchmeier and Oldstone, 1979). This precursor is a molecule of 75,000 Da and is cleaved at a defined site (Buchmeier *et al.*, 1987) to yield GP-1 and GP-2. GP-2 appears to be less accessible on the virion surface than GP-1 (Buchmeier *et al.*, 1978) and is highly conserved among the strains of LCMV and among the other arenaviridae (Southern and Bishop, 1987; Buchmeier *et al.*, 1981; Weber and Buchmeier, 1988). On the other hand, GP-1 is the major surface glycoprotein of the virion (Buchmeier *et al.*, 1978) and is more polymorphic than GP-2 (Buchmeier, 1984). GP-1 has also been shown to be the target of neutralizing antibody (Buchmeier, 1984; Buchmeier *et al.*, 1981; Bruns *et al.*, 1983; Parekh and Buchmeier, 1986).

Two strains of LCMV were used in our laboratory to generate monoclonal antibodies (MAbs) (Buchmeier *et al.*, 1980, 1981; Buchmeier, 1984), and these MAbs have been used to define the antigenic topography of GP-1 and GP-2. Competitive binding assays demonstrated the presence of three B-cell epitopes on GP-2, and four on GP-1 (Parekh and Buchmeier, 1986). Two of the GP-1 epitopes, A and D, were targets of neutralizing MAbs. However, epitope D appeared to be unique to the Armstrong strain of LCMV (LCMV Arm) and was absent from LCMV WE (Parekh and Buch-

[1] Present address: Department of Microbiology, University of Ottawa, Ottawa, Canada.

[2] To whom requests for reprints should be addressed.



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meier, 1986). Epitopes A and D are not identical but overlap, as shown by reciprocal partial inhibition of binding of MAbs to each of these determinants (Parekh and Buchmeier, 1986).

For the present studies we have focused on the neutralizing epitopes of LCMV Arm because of their biological relevance. In related studies we have established that monoclonal antibodies to both epitopes A and D passively protected mice from acute infection and disease following intracranial challenge with virus (manuscript in preparation). Furthermore, preimmunization of guinea pigs with LCMV Arm protected them against subsequent challenge with a more virulent strain of LCMV, LCMV-WE (Riviere *et al.*, 1985; Peters *et al.*, 1987). While the basis of this protection has not been determined, the dominant B-cell response in protected animals is directed against the neutralizing epitope A (Parekh and Buchmeier, 1986). Moreover, immunization with LCMV Arm has also been reported to cross-protect guinea pigs from lethal challenge with Lassa virus (Peters *et al.*, 1987), an arenavirus highly pathogenic for humans. These findings suggest that information derived from studies of the basis of neutralization of LCM virus may be applicable to other human arenavirus pathogens. For this reason, we were interested in further examining the physical relationship of epitopes A and D on GP-1, as well as defining the conditions required for the proper folding of these conformational epitopes.

## MATERIALS AND METHODS

### Virus and cell culture

The various isolates of LCMV, Arm-4, Arm-5, Arm-3 and Arm-10, were triply plaque-purified from a parental stock of Armstrong CA-1371 (Parekh and Buchmeier, 1986). Working stocks were grown in BHK-21 cells infected at a m.o.i. of 0.1 and harvested 48 hr later. These cloned isolates have been phenotypically stable over more than 10 subcultures in a 5-year period. Virus was purified by banding on 10–40% (v/v) Renografin-76 (Squibb Diagnostics) gradients in 0.01 *M* Tris, 0.1 *M* NaCl, 0.001 *M* EDTA, pH 7.4 (TNE), as previously described (Buchmeier and Oldstone, 1979).

### Immunofluorescence and enzyme-linked immunosorbent assays (ELISA)

Indirect immunofluorescence was carried out with monoclonal antibodies on acetone-fixed coverslips of infected BHK-21 or Vero cells as described (Buchmeier *et al.*, 1981). For ELISA assays, purified virus was coated in the cold on 96-well flat-bottomed plates (Flow Laboratories) in PBS at a concentration of 0.5 μg/well. In some experiments viral antigen was treated for 30 min at 37° in 0.2% 2-mercaptoethanol (2-ME) (Bio-Rad Laboratories) prior to coating on plates. After coating, plates were blocked for 3 hr with 2% skim milk powder in PBS containing 0.05% Tween-20 (PBS–Tween). Antibodies were titered in fourfold dilutions in a volume of 100 μl. After a 60-min incubation, the plates were washed and bound antibody was detected with protein A–peroxidase (Sigma) and orthophenylenediamine substrate (Bio-Rad Laboratories) as described elsewhere (Parekh and Buchmeier, 1986).

### Glycosylation inhibitors

Tunicamycin (TUN), deoxymannojirimycin (DMJ), and swainsonine (SSN) were purchased from Boehringer-Mannheim and castanospermine (CSP) and *N*-methyldeoxynojirimycin (N-DNJ) were from Genzyme. Inhibitors were prepared as stock solutions at 2 mg/ml in DMSO and were added at the indicated final concentrations to media of infected coverslips for the last 16 hr of culture. Control cultures received equivalent concentrations of DMSO without drug.

### Polyacrylamide gel electrophoresis (PAGE) and Western blotting

SDS–PAGE was performed on 10% slab gels as described by Laemmli (1970). For Western blots purified virus was loaded, without heating, at a concentration of 10 μg/well, in 1% sodium dodecyl sulfate (SDS) sample buffer either containing 1% 2-ME (reducing conditions) or not (nonreducing conditions). Electrophoresis was carried out in the cold as described by Swack *et al.* (1987). Gels were electrophoretically transferred at a temperature of 4° onto 0.2-μm nitrocellulose paper (Schleicher and Schuell) (Burnette, 1981). Blots were blocked with 2% milk powder in PBS–Tween for 3 hr, incubated with antibody diluted 1/100 in PBS–Tween for 1 hr, and washed three times before bound antibody was detected with $^{125}$I-labeled protein A.

### Immunoprecipitation

BHK-21 cells were infected at a m.o.i. of 1.0 for 48 hr, then labeled for 1 hr in methionine-free Dulbecco's minimal essential medium (Flow Laboratories) containing 60 μCi/ml L-[$^{35}$S]methionine (Amersham). Lysates were prepared in a buffer containing 20 m*M* Tris, 137 m*M* NaCl, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, 1% (v/v) aprotinin and then cleared by centrifugation at 14,000 rpm for 15 min. Equal volumes (cell equivalents) of control and test lysates were incubated with monoclonal antibody at a final dilution of 1:500 for 45–60 min, after which 80 μl of washed protein A–Sepharose CL-4B beads (Sigma)

was added. Tubes were further incubated for 30 min with continuous shaking. Precipitates were collected by pelleting beads which were washed three times in wash buffer (100 m*M* Tris, 500 m*M* LiCl) before resuspension in 20 μl sample buffer with 1% 2-ME, 2% SDS. Samples were boiled at 100° for 2 min before loading onto polyacrylamide gels as described above. $^{14}$C-methylated protein markers (Amersham) were run on each gel. After electrophoresis gels were fixed in 7% acetic acid, 20% methanol, washed with distilled water, incubated in Autofluor (National Diagnostics) for 30 min, then dried and exposed at −70°.

### Sequencing of LCMV glycoprotein genes

Genomic RNA was isolated from purified virus stocks by extraction with phenol, followed by phenol/chloroform before ethanol precipitation as described elsewhere (Salvato *et al.*, 1988). Briefly, the genomic RNA was sequenced by annealing a radioactive oligonucleotide primer to viral RNA and then extending enzymatically using a protocol described by Hamlyn *et al.* (1981). Reaction mixtures contained avian myeloblastosis virus (AMV) reverse transcriptase (Life Sciences) and deoxy- and dideoxynucleotide triphosphates from Sigma and Pharmacia, respectively. Sequences were resolved on thin (0.4 mm) gradient polyacrylamide gels (Biggin *et al.*, 1983). Samples from the different virus isolates were run simultaneously and loaded on the sequencing gels in adjacent lanes to ensure valid comparisons.

## RESULTS

### Epitopes A and D are dependent on the presence of disulfide bonds

We wished to examine the effects of reducing conditions on the neutralizing epitopes in the absence of denaturing detergents. Purified virus was treated with 0.2% 2-ME or PBS prior to use as antigen in ELISA assays. In Fig. 1, it can be seen that the binding of MAb 2.11.10 to epitope D and MAbs 197.1 and 6.2 to epitope A were abrogated by 2-ME treatment of viral antigen, while binding of MAb 67.2 to the linear epitope GP-1C was unaffected.

### Epitopes A and D are differentially sensitive to denaturation with detergent

Our initial attempts to detect these two epitopes by standard Western blot protocols failed, confirming their conformational nature. We then tried a modified Western blotting procedure using conditions that had been successful in retaining conformational epitopes in other systems. Swack *et al.* (1987) were able to detect

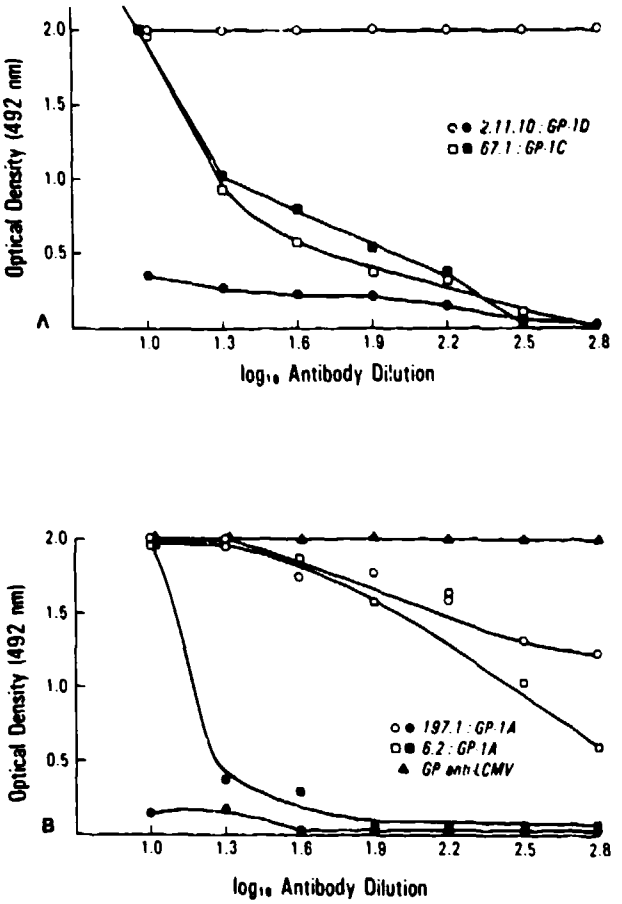



FIG. 1. Dependence of conformational epitopes in the presence of disulfide bridges. Untreated (open symbols) or reduced (solid symbols) virus was coated on ELISA plates. (A) Epitope D 1D detected by MAb 2.11.10 (circles); control epitope C detected by MAb 67.1 (squares). (B) Epitope A detected by MAbs 197.1 (circles) and 6.2 (squares); control: polyclonal guinea pig antiserum (triangles).

HLA Class II epitopes by Western blotting when they eliminated reduction and boiling of the antigen and performed the SDS–PAGE and transfer to nitrocellulose in the cold. Using their methods, we were able to consistently detect epitope D in Western blots (Fig. 2). Under nonreducing conditions we also observed higher-molecular-weight species that appeared to be homopolymers of GP-1. In contrast to these results, MAbs against epitope A were only slightly reactive, even when the SDS concentration was reduced from 1 to 0.1% in the sample buffer and the protein concentration was increased as suggested by Cohen *et al.* (1986) (data not shown).

### Epitopes A and D are dependent on N-linked glycosylation

There have been several reports of carbohydrates influencing the antigenicity of viral glycoproteins, and

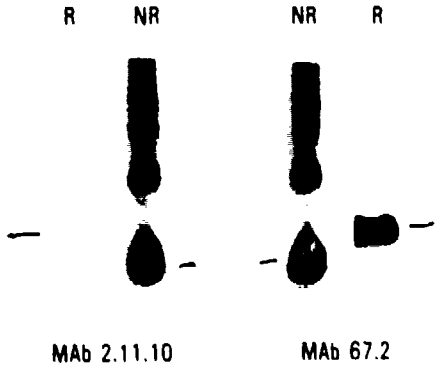



FIG. 2. Detection of epitope D by Western blot. Virus in reducing (R) or nonreducing (NR) sample buffer was electrophoresed in the cold, transferred to NC, and then incubated with MAb 2.11.10 to epitope D or to a linear determinant (MAb 67.2).

many conformational determinants cannot be detected when N-linked glycosylation is prevented with TUN (Pierotti *et al.*, 1981; Long *et al.*, 1986; Sugawara *et al.*, 1988; Hongo *et al.*, 1986; Bruck *et al.*, 1984; Kaluza *et al.*, 1980). We examined the effect of TUN on conformational epitopes of Arm-4 by indirect immunofluorescence on infected BHK coverslips after growth for 16 hr in 0.5 μg/ml TUN. Monoclonal antibodies detecting epitope D (MAb 2.11.10), epitope A (MAb 197.1), or a control epitope on GP-2 (MAb 33.6) were used. Figure 3 shows that in the presence of TUN both A and D were undetectable, although there was viral protein present as indicated by positive staining with the control antibody. Identical results were obtained in infected Vero cells. The absence of epitope D in TUN-treated infected cells has been confirmed by immunoprecipitation of metabolically labeled lysates (Fig. 4). In these experiments the epitope on the precursor molecule, GP-C, is detected. Using a control antibody to another epitope on GP-C (MAb 33.6) we visualized a doublet band of ca. 55 kDa that represented unglycosylated GP-C. We have confirmed that this doublet is not glycosylated by demonstrating its resistance to digestion with Endoglycosidase H and O-Glycanase (data not shown). The 55-kDa band could not be detected after immunoprecipitation with antibody to epitope D (2.11.10). We were unable to compare immunoprecipitates of epitope A with epitope B, even using several MAbs directed to epitope A, consistent with the sensitivity of this epitope to detergent disruption.

### Conformational epitopes are dependent on addition of core oligosaccharides but require no further trimming or processing

Because the neutralizing epitopes were undetectable in TUN-treated LCMV-infected cells, there was a possibility that oligosaccharide side chains contributed directly to the structure of the epitopes. To formally examine the role of carbohydrates in the structure of

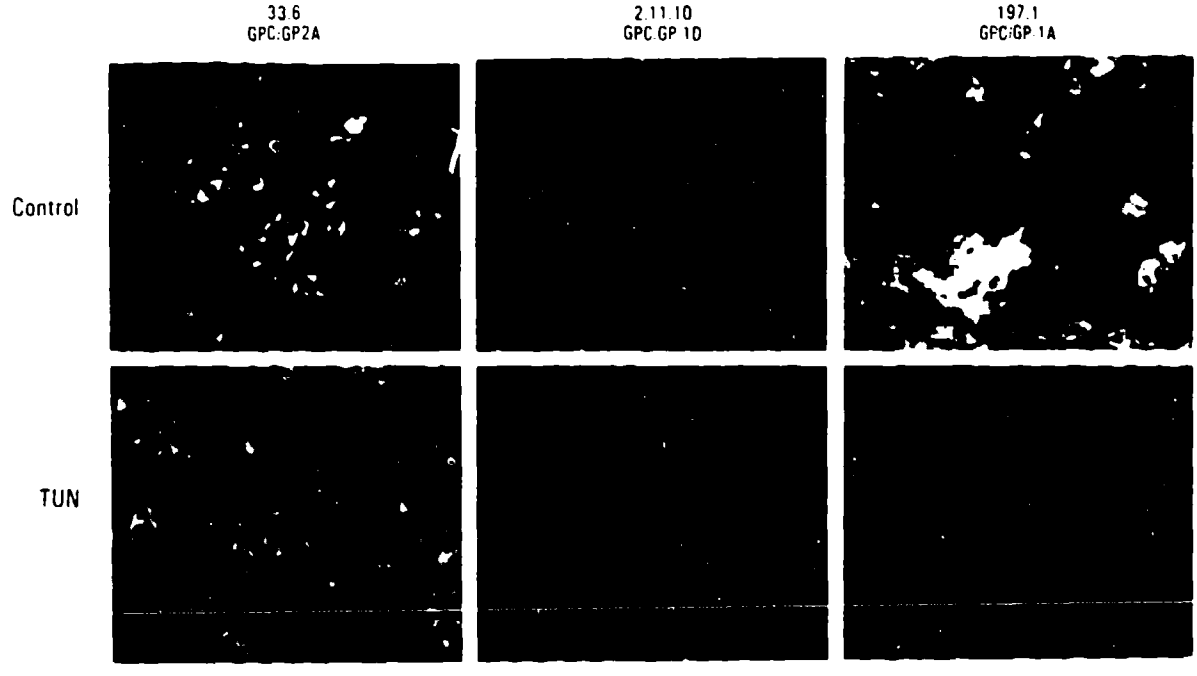



FIG. 3. Dependence of conformational epitopes on N-linked glycosylation. BHK cells were mock-treated with 0.25% DMSO (control) or TUN (0.5 μg/ml) for the last 16 hr of infection and then fixed and stained as described. Neither epitope A nor D was detected in the TUN-treated cells.

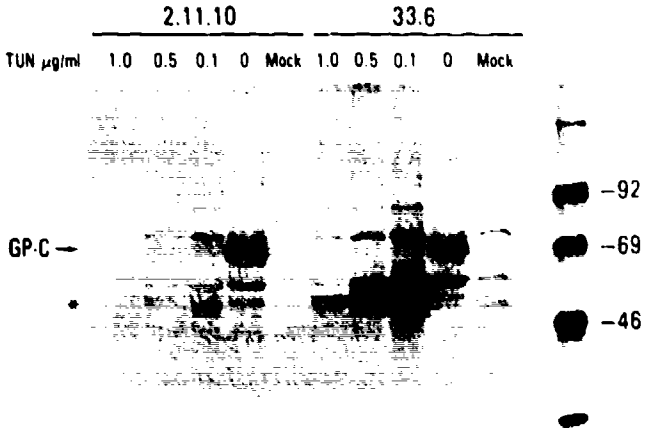



FIG. 4. Failure of MAb 2.11.10 to immunoprecipitate epitope D in absence of glycosylation. Infected BHK cell lysates were immunoprecipitated with MAb 2.11.10 or a control GP-2 epitope (33.6) after incubation in varying concentrations of TUN for 24 hr. Mature GP-C is precipitated by both MAbs; unglycosylated GP-C (*) is precipitated only by MAb 33.6.

these epitopes, we used several inhibitors that block various stages of trimming of the core oligosaccharide following *en bloc* addition to the protein backbone (for review see Elbein, 1987). We reasoned that if the composition of the carbohydrate side chain was responsible for the detectable antigenicity, we might expect differential reactivity at various stages of processing. Castanospermine (CSP) prevents the first stage of carbohydrate processing, trimming of terminal glucose residues by glucosidases I and II. Swainsonine (SSN) inhibits mannosidase II, a Golgi enzyme that completes the final trimming of mannoses before the addition of terminal residues that occurs in the formation of complex carbohydrates. We fixed infected coverslips grown in the presence of these drugs for the duration of culture and then proceeded with indirect immunofluorescence as before. Both epitopes A and D were undetectable in the presence of TUN, but appeared in the presence of CSP and remained in the presence of SSN. The data for epitope A are presented in Fig. 5. We have confirmed these results for D by immunoprecipitating infected BHK cell lysates with MAb 2.11.10 (Fig. 6). In this experiment two additional inhibitors that affect trimming at intermediate steps between CSP and SSN were also included. These were N-DNJ, which inhibits glucosidases I and II, and DMJ, which inhibits the Golgi enzyme mannosidase I. MAbs to epitope D precipitated GP-C in the presence of all the trimming inhibitors, but failed to do so in the presence of TUN. Electrophoretic mobility of GP-C was retarded after growth in CSP and N-DNJ, consistent with the presence of the three additional terminal glucose residues on each oligosaccharide side chain. These residues are trimmed in the presence of DMJ and SSN, as indicated by the shift in migration of GP-C.

## Epitope D is disrupted by the presence of one extra N-linked carbohydrate

The isolate of LCMV used in our laboratory, Arm-4, is one of 10 clones plaque-purified in 1981 from a parental stock of LCMV Arm (CA 1371), which was used to generate our monoclonal antibodies (Buchmeier *et al.*, 1980, 1981). Of these 10 clones, all possessed the major neutralizing epitope, A, but only 5 reacted with monoclonal antibody specific for epitope D as detected by ELISA, indirect immunofluorescence, or neutralization assays. We sequenced the GP-1 gene of Arm-4 (epitope A+D+), Arm-5 (epitope A+D−), and two other isolates, Arm-3 (epitope A+D−) and Arm-10 (epitope A+D+), to search for mutations correlating with the presence or absence of the epitope. Two nucleotide changes were found which resulted in amino acid changes in GP-1. By the numbering of Salvato *et al.* (1988), $A_{594}$ (Arm-5) is $G_{594}$ (Arm-4) and $C_{831}$ (Arm-5) is $T_{831}$ (Arm-4) (Southern and Bishop, 1987; Salvato *et al.*, 1988). Isolates Arm-5 and Arm-3 matched the published sequence of GP-1. The changes at nucleotide position 594 result in amino acid changes from Thr 173 → Alanine 173 in Arm-4, and those at position 831 result in Leu → Phe in Arm-4. The latter amino acid substitution was also seen in the WE strain of LCMV which, unlike Arm-4, lacks epitope D (Parekh and Buchmeier, 1986). On the other hand, Arm-4 and Arm-10, both of which expressed epitope D, had Thr → Ala and Thr → Lys substitutions respectively at amino acid position 173 of the GP-1 gene (Table 1). The presence of the Thr 173 in Arm-3 and Arm-5 completes the consensus sequence for a potential N-linked glycosylation site, $_{171}$Asn-Leu-Thr$_{173}$. Hence, isolates of LCMV Arm lacking epitope D bear six potential N-linked glycosylation sites within the GP-1 sequence, whereas isolates expressing epitope D only have five such sites. In other studies we have confirmed by controlled deglycosylation that all of the five potential asparagine-linked glycosylation sites in GP-1 are utilized when LCMV Arm-4 is grown in BHK-21 cells.

To confirm that the sixth glycosylation site was utilized in isolates lacking epitope D, we performed immunoprecipitation of metabolically labeled BHK lysates infected with either Arm-4 or Arm-5 in the presence or absence of TUN. Fully glycosylated GP-C of Arm-5 was approximately 3 kDa larger than that of Arm-4, consistent with the expected molecular weight of one carbohydrate side chain (Nakamura and Compans, 1979).

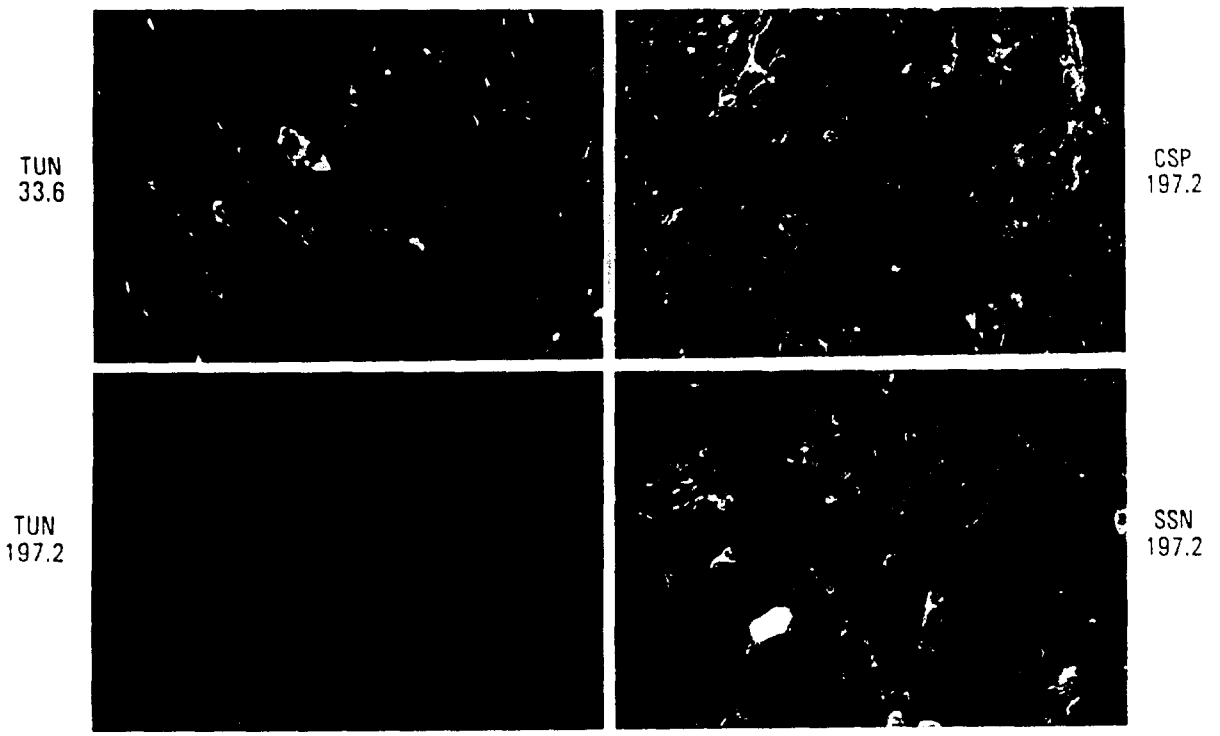



FIG. 5. Addition but not trimming of N-linked sugars is required for the formation of conformational epitopes. Infected BHK cells were treated with 0.25% DMSO (control), TUN (0.5 μg/ml), CSP (80 mg/ml), or SSN (0.5 μg/ml) for the whole time of infection and then fixed and stained as described with MAb 197.2 detecting epitope A. TUN-treated cells stained with MAb 33.6 directed to an epitope on GP-2 are shown as a control.

Bands corresponding to unglycosylated GP-C immunoprecipitated from TUN-treated cultures infected with the two viruses were the same size (Fig. 7A). These data indicate that the polypeptide backbone of GP-C is the same size in both virus isolates, and the observed difference in size of GP-C is the result of differential N-linked glycosylation. Analysis of the mature viral glycoproteins by Western blotting demonstrated that the difference in apparent molecular weight resides in GP-1 (Fig. 7B).

### Prevention of N-linked glycosylation does not expose epitope D on Arm-5

Carbohydrate moieties have been shown to directly block the binding of antibodies to regions of the influenza hemagglutinin known to be B-cell epitopes (Skehel *et al.*, 1984; Alexander and Elder, 1984). Because epitope D was a discontinuous, conformational epitope and was dependent on the presence of N-linked glycosylation, it seemed unlikely that prevention of all N-linked glycosylation would expose the epitope on the GP-1 of Arm-5 and Arm-3, which normally bear one additional oligosaccharide at Asn 171. To explore this, both Arm 4 and Arm 5 were grown in the presence of TUN and metabolically labeled, then infected cell lysates were immunoprecipitated with a control antibody (MAb 33.6) and with MAb 2.11.10 directed to epitope D. As can be seen in Fig. 8, the control antibody precipitated fully glycosylated and unglycosylated GP-C of both Arm-4 and Arm-5, but epitope D was not detected on unglycosylated GP-C of Arm-4, and did not appear on unglycosylated Arm-5. Similarly, the epitope did not appear on Arm-5 in the presence of any of the trimming inhibitors CSP, N-DNJ, DMJ, or SSN.

## DISCUSSION

For most animal viruses quantitative immunochemical analyses of neutralizing epitopes have been described, but characterization of the physical structure of these epitopes is less common. From those studies that have been published, there appears to be no general rule whether neutralizing epitopes are linear or conformational; multiple examples of each class have been reported (Bruck *et al.*, 1984; Alexander and Elder, 1984; Long *et al.*, 1986; Wimmer *et al.*, 1986). In the context of our studies of the structure and function of arenavirus glycoproteins we sought to explore the nature of the neutralizing epitopes of LCM virus.

The isolate of LCMV Armstrong used in our laboratory, Arm-4, bears two partially overlapping epitopes recognized by our panel of neutralizing monoclonal antibodies (Parekh and Buchmeier, 1986). Data pre-

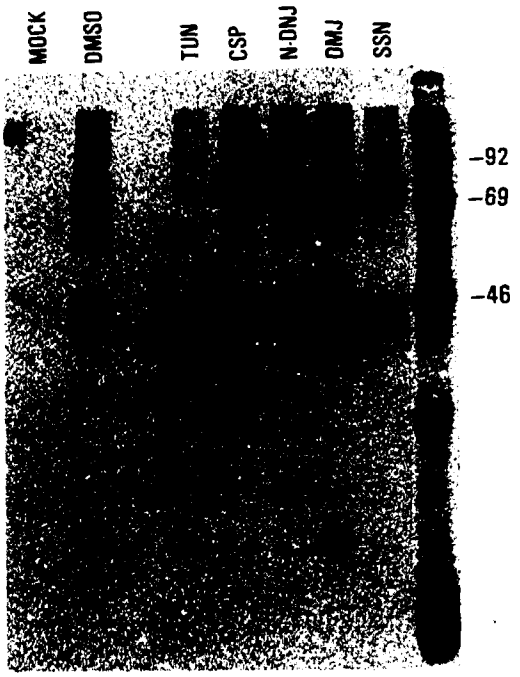



FIG. 6. Immunoprecipitation of GPC precursor with MAb to epitope D in the presence of inhibitors of glycosylation trimming. Immunoprecipitation of BHK cell lysates infected with Arm-4 was carried out as described after growth in the presence of TUN (0.5 μg/ml), CSP (80 mg/ml), N-DNJ (2 m*M*), DMJ (2 m*M*), or SSN (0.5 μg/ml) for 16 hr.

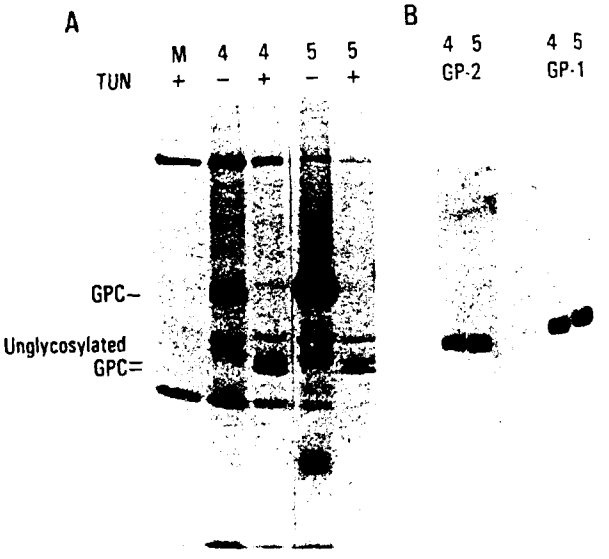



FIG. 7. LCMV isolates lacking epitope D have one extra carbohydrate on GP-1. (A) Arm-4 and Arm-5 were grown in the presence of TUN, then lysates were immunoprecipitated with MAb 33.6. (B) Purified Arm-4 and Arm-5 were separated by SDS-PAGE, transferred to NC, and then blotted with MAbs specific for GP-1 (67.2) or GP-2 (33.6).

sented in this study demonstrate that both of these epitopes are conformational and are dependent both on the presence of disulfide bridges and on N-linked glycosylation. These two epitopes can be distinguished on a genetic basis. By sequencing virus isolates which differ in their reactivity with a MAb recognizing epitope D, we have identified amino acid residue 173 of GP-1 as critical for expression of epitope D. Sequence comparison of four such isolates has determined that two isolates (Arm-3 and Arm-5) which have potential N-linked glycosylation sites Asn × Thr at residues 171–173 lack epitope D and two isolates (Arm-4 and Arm-10) which lack the potential for glycosylation at that site express epitope D.

Dependence of these conformational epitopes on N-linked glycosylation is perhaps not unexpected. Oligosaccharide precursor molecules are added *en bloc* to asparagine residues of the protein in the lumen of the rough endoplasmic reticulum almost as soon as the asparagine residue emerges through the ER membrane and prior to folding of the protein and disulfide

TABLE 1

SEQUENCE COMPARISON AND ANTIBODY REACTIVITY OF GP-1 OF LCMV ARM ISOLATES

| LCMV isolate | GP-C sequence[a] nucleotides 582–620 amino acids 169–181 | Epitope[b] A | Epitope[b] D |
|---|---|---|---|
| | CAATACAACTTGACATTCTCAGATCGACAAAGTGCTCAG | | |
| Arm-5 | GlnTyrAsnLeuThrPheSerAspArgGlnSerAlaGln | + | − |
| | GCA | | |
| Arm-4 | ----------Ala---------- | + | + |
| | ACA | | |
| Arm-3 | ----------Thr---------- | + | − |
| | AAA | | |
| Arm-10 | ----------Lys---------- | + | + |

[a] Sequences were determined by primer extension.
[b] Reactivity with MAbs to epitope A (MAbs 197.1 and 6.2) and epitope D (2.11.10) is scored. + indicates a positive reaction.

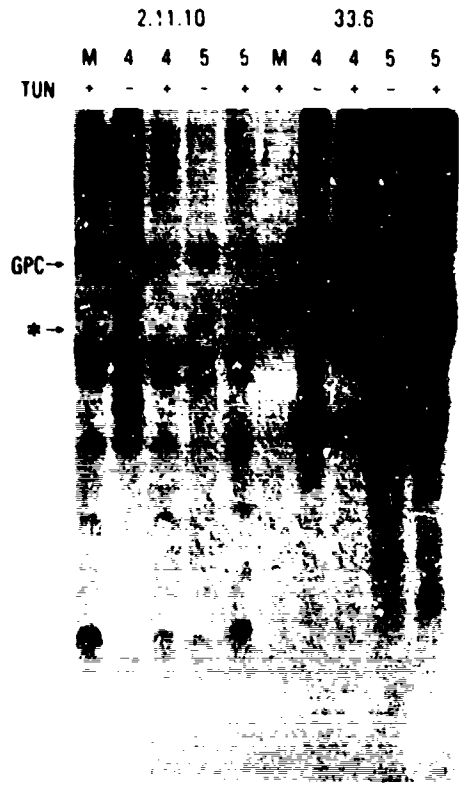



FIG. 8. Prevention of glycosylation does not expose epitope D on Arm 5. Arm 4 and Arm 5 were grown in the presence or absence of TUN and then lysates were immunoprecipitated with MAb specific for epitope D (2.11.10) or a control GP-2 MAb (33.6). Asterisk (*) denotes ungiycosylated GP-C.

bond formation. For this reason it has been suggested that glycosylation may play a role in directing and maintaining folding of some proteins (Gibson *et al.*, 1978, 1980; Kaluza and Pauli, 1975; van Grunen-Littel-van den Hurk, and Babiuk, 1985).

Direct support for this concept has come from studies of the intracellular folding of human immunodeficiency virus (HIV-1) gp 120 (Fennie and Lasky, 1989). Folding of gp 120 to a conformation which was able to bind the viral receptor CD-4 molecule required addition of high-mannose precursor sugars and was completely blocked by tunicamycin inhibition of oligosaccharide addition. In a similar manner, addition of high-mannose oligosaccharides prior to disulfide bond formation was shown to be a requirement for correct folding of the Sendai virus glycoproteins to a conformation recognized by conformation-dependent MAbs (Vidal *et al.*, 1989).

A role for N-linked glycosylation in the formation of neutralizing epitopes of other viruses has also been described. Bovine (Bruck *et al.*, 1984) and murine (Pierotti *et al.*, 1981) leukemia viruses, Newcastle disease virus (Long *et al.*, 1986), and influenza C (Sugawara *et al.*, 1988) all possess conformational neutralizing epitopes that are absent after growth of the viruses in tunicamycin. For one of these, removal of carbohydrates from mature viral proteins does not eliminate the epitope (Pierotti *et al.*, 1981), confirming that cotranslational additional of carbohydrates is required for appropriate folding, but not for maintaining protein conformation. In each of these instances the carbohydrate moieties themselves were not directly involved as recognition structures in the epitopes. Our data using inhibitors of trimming also suggest a role for the carbohydrates in the folding of GP-1 rather than having a direct role in the epitope. By controlling the extent of trimming using glycosylation inhibitors we have demonstrated that addition of any oligosaccharide from high mannose to fully trimmed complex resulted in restoration of full reactivity. The observation that the presence of one more carbohydrate residues on GP-1 at Asn 171 also disrupts epitope D strengthens our argument that the process of glycosylation and its influence on protein folding rather than the composition of the carbohydrates is important. On the basis of the evidence at hand we cannot distinguish whether addition of a sixth sugar at Asn 171 alters the folding of GP-1 or directly sterically hinders antibody binding. One approach to this problem is to determine whether the epitope remains intact on Arm-4 after removal of the carbohydrates from mature virions. Were this the case removal of the carbohydrates from Arm-5 might also reveal the epitope, indicating that folding of the protein was the same in both viruses, and the sixth sugar directly blocked the epitope. If the epitope was not revealed, it would suggest that folding differed. Unfortunately, using gentle treatment of native GP-1 with either Endoglycosidase F or N-Glycanase we were unable to remove all of the carbohydrate, and more rigorous treatment required to produce a ladder of partially deglycosylated GP-1 molecules disrupted the epitope even in the absence of enzymes. Thus we were unable to satisfactorily resolve this issue.

Conformational epitopes relying on disulfide bridges have also been described for tick-borne encephalitis virus (Winkler *et al.*, 1987), Semliki Forest virus (Kaluza and Pauli, 1975), rabies virus (Dietzschold *et al.*, 1982), and herpes simplex virus-1 (Wilcox *et al.*, 1988). We know that disulfide bonds are formed in the presence of tunicamycin (data not shown), but cannot distinguish whether appropriate or inappropriate pairings are obtained in the absence of glycosylation. Evidence has been presented for Sendai virus glycoproteins which suggests that incorrect interchain disulfide bonds are formed under conditions of tunicamycin inhibition (Vidal *et al.*, 1989).

Because the neutralizing epitopes are conformational it is unlikely that linear synthetic peptides can be used successfully as mimotopes for immunization against native viral GP-1. We already know that none of our monoclonal antibodies directed to epitopes A and D bind preferentially to any of a series of synthetic peptides spanning GP-1 of LCMV Arm (Parekh and Buchmeier, unpublished data). Neither do MAbs against epitope A react with recombinant GP-C produced by a baculovirus expression vector (Buchmeier and Bishop, unpublished observation). Whereas epitope D is found only on LCMV Arm substrains, epitope A is the immunodominant neutralizing epitope recognized on all strains of LCMV by both monoclonal and polyclonal antibodies (Parekh and Buchmeier, 1986), and thus is more crucial. The sensitivity of this epitope to even mildly denaturing conditions suggests that retention of reactivity may be difficult. These findings are consistently observed with several independently derived MAbs, hence are unlikely to stem from the use of a single MAb.

Our data do not identify what specific amino acid sequences of GP-1 make up these neutralizing epitopes. We know from sequence data that there are eight highly conserved cysteine residues in GP-1 and find no evidence of interchain disulfide linkage between GP-1 and GP-2. We are currently analyzing intrachain disulfide bond pairings and attempting to identify cleavage fragments of GP-1 that retain reactivity with the antibodies defining epitopes A and D.

## ACKNOWLEDGMENTS

This is Publication No. 5496-IMM from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037. This work was supported by USPHS Grant AI-16102 (M.B.) and AI-25522-01 (M.S.). K.E.W. is a Fellow of The Arthritis Society, Toronto, Canada. We thank Robert Spiro for advice with glycosylation inhibitors; Mary Paine, Elaine Shimomaye, and Rebecca Day for technical assistance; and Jim Johnston for manuscript preparation.

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VIROLOGY 177, 175-183 (1990)

# Post-translational Processing of the Glycoproteins of Lymphocytic Choriomeningitis Virus

K. E. WRIGHT,[1] R. C. SPIRO,[2] J. W. BURNS, AND M. J. BUCHMEIER[3]

*Department of Neuropharmacology, Research Institute of Scripps Clinic, La Jolla, California 92037*

*Received April 25, 1989; accepted March 14, 1990*

**Intracellular events in the synthesis, glycosylation, and transport of the lymphocytic choriomeningitis virus (LCMV) glycoproteins have been examined. We have shown by *N*-glycanase digestion that LCMV strain Arm-4 bears five oligosaccharides on GP-1 and two on GP-2. By pulse-chase labeling experiments in the presence of drugs which inhibit N-linked oligosaccharide addition and processing we demonstrate that addition of high mannose precursor oligosaccharides is necessary for transport and cleavage of the viral GP-C glycoprotein. Moreover, in the presence of tunicamycin which inhibits *en bloc* addition of these mannose-rich side chains, virus budding was substantially decreased and infectious virions were reduced by more than 1000-fold in the supernatant medium. Incubation in the presence of castantospermine, which permits addition of oligomannosyl-rich chains but blocks further processing, restored transport and cleavage of GP-C and maturation of virions. Finally, by temperature block experiments we have determined that maturation of GP-C oligosaccharides to an endoglycosidase H resistant form precedes cleavage to GP-1 and GP-2. The latter process is most likely to occur in the Golgi or post-Golgi compartment.** © 1990 Academic Press, Inc.

## INTRODUCTION

Lymphocytic choriomeningitis virus (LCMV), the prototype member of the Arenaviridae, has provided investigators with a wealth of information about virus–host interaction. In the mouse, LCMV can establish a range of diseases from acute, lethal choriomeningitis to lifelong persistent infection. Through the study of these diseases several fundamental concepts have evolved including tolerance and immune complex disease (reviewed in Buchmeier *et al.*, 1980), virus alteration of specialized or luxury functions of differentiated cells (Oldstone *et al.*, 1984; Klavinskis *et al.*, 1988), and the requirement for major histocompatability complex in expression of antiviral cytotoxic T-cell killing and virus clearance (Zinkernagel and Doherty, 1974; Zinkernagel and Welsh, 1976).

The structure of LCMV is the best characterized of the arenaviruses. There are three major structural proteins, a nucleocapsid protein (NP, $M_r$ 63 kDa), and two glycoproteins, GP-1 ($M_r$ 44 kDa) and GP-2 ($M_r$ 35 kDa). In addition, there are at least two quantitatively minor proteins, L ($M_r$ 200 kDa) which is presumed to be a viral RNA dependent RNA polymerase, and a minor 12–14,000 $M_r$ polypeptide, termed Z, which may constitute a zinc binding protein (Salvato *et al.*, 1989; Buchmeier and Parekh, 1987). Glycoproteins GP-1 and GP-2 are post-translationally cleaved from a cell-associated mannose-rich precursor, GP-C ($M_r$ 75–76 kDa) (Buchmeier and Oldstone, 1979). Most of our work to date has focused on the antigenic structure of these glycoproteins. The major glycoprotein, GP-1, has at least four B-cell epitopes, two of which bind neutralizing antibodies. GP-2 has three overlapping epitopes (Parekh and Buchmeier, 1986), of which two are conserved among the arenaviruses (Weber and Buchmeier, 1988; Buchmeier *et al.*, 1981).

Little is known about post-translational processing of arenavirus glycoproteins. The proteolytic cleavage site of GP-C is apparently a paired basic amino acid sequence, Arg–Arg at amino acids 262–263 (Buchmeier *et al.*, 1987), and cleavage is mediated by a cellular protease. A similar precursor glycopeptide has also been identified for Pichinde (Harnish *et al.*, 1981), Lassa (Clegg and Lloyd, 1983), and Tacaribe viruses (Gimenez *et al.*, 1983; Franz-Fernandez *et al.*, 1987); however, Tacaribe contains only one structural glycoprotein.

An understanding of the biosynthesis, processing, and transport of the LCMV glycoproteins may aid in interpreting aspects of the viral biology. For example, in persistent LCMV infections selective modulation of glycoprotein expression has been reported in infected cells (Welsh and Buchmeier, 1979) and tissues (Oldstone and Buchmeier, 1982), but the mechanism of regulation remains unclear. Moreover, recent studies have described cytotoxic T-cell epitopes on the glycoproteins of LCMV (Whitton *et al.*, 1988). Based on current knowledge of the role of class I MHC in the endogenous pathway of antigen presentation, it is likely that association between class I and glycoprotein or a frag-

[1] Present address: Department of Microbiology and Immunology, School of Medicine, University of Ottawa, Ottawa, Canada K1H 8M5.

[2] Department of Immunology, Research Institute of Scripps Clinic.

[3] To whom requests for reprints should be addressed at IMM5, 10666 N. Torrey Pines Road, La Jolla, CA 92037.



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ment derived from it occurs within the intracellular transport pathway. Finally, little is known about interaction between the LCMV glycoproteins and cell-surface viral receptors and the mechanism of viral entry into cells. Studies reported here address two basic aspects of post-translational processing of LCMV: N-linked glycosylation of the glycoproteins, and requirements for and kinetics of subsequent transport, trimming and proteolytic cleavage of GP-C, GP-1 and GP-2.

## MATERIALS AND METHODS

### Virus and cell culture

The virus used throughout these experiments, LCMV Armstrong clone 4 (Arm-4), was plaque purified from a stock of Armstrong CA-1371 (Parekh and Buchmeier, 1986; Wright *et al.*, 1989). Working stocks were prepared by infecting BHK-21 cells at a multiplicity of infection (m.o.i.) of 0.1 and harvesting the supernatants 48 hr later. Virus was purified by polyethylene glycol precipitation followed by banding on 10–40% (v/v) renograffin-76 (Squibb Diagnostics) gradients (Buchmeier and Oldstone, 1979).

### Glycosylation inhibitors

Tunicamycin (TUN), deoxymannojirimycin (DMJ), and swainsonine (SSN) were purchased from Boehringer-Mannheim, and castanospermine (CSP) and *N*-methyldeoxynojirimycin (NM-DNJ) from Genzyme. Stock solutions of TUN were prepared in DMSO. Stock solutions of DMJ, SSN, and NM-DNJ were prepared in culture medium. In immunofluorescence experiments inhibitors were added at the time of infection and maintained for the duration. For metabolic labeling, inhibitors were added to infected cultures for 24 hr before labeling and retained throughout the labeling and chase periods. Optimal drug concentrations used were predetermined by titration of their inhibitory activity in BHK-21 cells. Concentrations used were TUN, 0.5 μg/ml; CSP, 80 μg/ml; NM-DNJ, 2 m*M*; DMJ, 2 m*M*; SSN, 0.5 μg/ml. Control cultures for TUN were incubated in the presence of equivalent concentrations of DMSO.

### Immunofluorescence

Indirect immunofluorescence of permeabilized cells was done on BHK-21 cell coverslips infected 24 hr earlier at an m.o.i. of 1.0 (Buchmeier *et al.*, 1981). For surface immunofluorescence, infected BHK cells were trypsinized, then stained with anti-LCMV monoclonal antibodies (MAb) and fluorescein-labeled sheep anti-mouse IgG. Other studies have established that LCMV antigens on the surfaces of infected cells resist trypsin treatment (Buchmeier *et al.*, 1981).

### Immunoprecipitation and polyacrylamide gel electrophoresis (PAGE)

BHK-21 cells were infected at an m.o.i. of 1.0 for 48 hr, then pulse labeled for 1 hr in methionine-free Dulbecco's medium (Flow Laboratories) containing 60 μCi/ml L-[$^{35}$S]methionine (Amersham). Cells were pretreated for 24 hr with glycosylation inhibitors at the indicated concentrations in methionine-free media before labeling. Cells were chased as indicated in medium containing glycosylation inhibitors and a 10-fold excess of cold L-methionine. Lysates were prepared in a buffer containing 20 m*M* Tris, 137 m*M* NaCl, 1 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 1% (v/v) NP-40, 10% (v/v) glycerol, and 1% (v/v) aprotinin, then cleared by centrifugation at 14,000 rpm for 15 min. Cell equivalents of control and test lysates were incubated with antibody at a final dilution of 1:500 for 45–60 min at 22°, after which 80 μl of washed protein A–Sepharose CL-4B beads (Sigma) were added. Tubes were incubated for 30 min with continuous shaking, then precipitates were collected by centrifugation and washed three times in wash buffer (100 m*M* Tris, 500 m*M* LiCl) before resuspending in 20 μl buffer containing 1% 2-ME, 2% SDS. Endoglycosidase H (EH) digestion was done on immune-precipitated viral proteins. Briefly, each precipitated sample was digested for 2 hr at 37° with 0.005 units of EH. Samples were heated to 100° for 2 min, then loaded onto 10% polyacrylamide gels (Laemmli, 1970). $^{14}$C-methylated protein markers (Amersham) were run on each gel. After electrophoresis gels were fixed in 7% acetic acid, 20% methanol, washed with distilled water, incubated in Autofluor (National Diagnostics) for 30 min, then dried and exposed to film at −70°. For temperature block experiments, chases were carried out at the indicated temperatures in individual water baths.

### Western blotting

Purified virus (200 μg) was digested with 0.5 units peptide *N*-glycosidase F (PNGF), a gift from Dr. J. Elder, Scripps Clinic and Research Foundation, for varying lengths of time to remove N-linked carbohydrates. Samples were then separated by SDS-PAGE, electrophoretically transferred to nitrocellulose, and immunoblotted with MAb specific for either GP-1 (67.5) or GP-2 (33.6).

### Electron microscopy

Virus-infected and uninfected control monolayer cultures of BHK-21 cells were detached by scraping with a rubber policeman, then pelleted at 500 *g*. Pellets were fixed in 2.5% buffered glutaraldehyde and epon embedded. Thin sections were prepared, stained with osmium tetroxide, and examined in an Hitachi electron microscope.

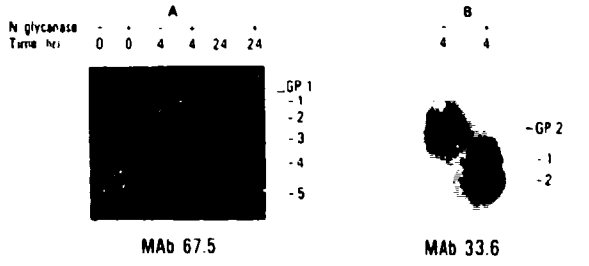



FIG. 1. Enumeration of oligosaccharide side chains on the LCMV GP-1 and GP-2 glycoproteins. Purified LCMV Arm-4 was digested with PNGF as described under Materials and Methods. Samples of the digest were removed immediately after adding all reagents (0 time) and at 2, 4, and 24 hr as indicated. Polypeptides were resolved on 10% (A) or 12.5% (B) SDS-PAGE gels and viral proteins visualized by Western blotting using GP-1-specific (MAb 67.5) or GP-2-specific (MAb 33.6) MAb.

## RESULTS

### Glycosylation of the major glycoprotein of LCMV

We have previously observed that the precursor molecule, GP-C, was labeled heavily with 2-[$^3$H]mannose, whereas the mature GP-1 and GP-2 glycoproteins contained relatively little mannose but were labeled with [$^3$H]galactose and [$^3$H]fucose (Buchmeier and Oldstone, 1979; Buchmeier and Parekh, 1987). These results suggested that GP-C contained high mannose oligosaccharides which were trimmed to a complex form prior to post-translational proteolytic cleavage to GP-1 and GP-2. In order to determine the extent of glycosylation of each glycoprotein, purified virus was digested with PNGF for various intervals before separation by SDS–PAGE and Western blotting with GP-1- or GP-2-specific antisera. Digestion produced a ladder of bands representing polypeptide chains with successively fewer oligosaccharide side chains. By this method we were able to demonstrate a total of six bands which reacted with GP-1 antibody, indicating that Arm-4 GP-1 contained five N-linked carbohydrates (Fig. 1A). One of these bands, labeled -1 on Fig. 1A, was present only momentarily after addition of PNGF and comigrated with the fastest migrating portion of the undigested GP-1 band, suggesting that native GP-1 may actually be a mixture of polypeptide core chains with four and five oligosaccharides.

### Duration between synthesis and cleavage of GP-C

In order to estimate the time between synthesis and cleavage of GP-C, replicate cultures were pulsed for 5 min with [$^{35}$S]Met, then chased for intervals of 15, 30, 60, 90, 120, 180, 270, or 360 min before immunoprecipitation with antibody to GP-2. The results of such a pulse–chase experiment are illustrated in Fig. 2. GP-C was evident immediately after pulsing but GP-2, indicative of GP-C cleavage, was not evident until 90 min chase had elapsed. In additional experiments focusing on the 60- to 120-min chase interval we have observed the first appearance of GP-2 at 75 min (data not shown).

### Post-translational cleavage of GP-C requires prior glycosylation

In experiments carried out to characterize the two neutralizing epitopes on GP-1, it was found that folding of the GP-C precursor required N-linked glycosylation (Wright *et al.*, 1989). In order to investigate the role of glycosylation in post-translational processing, we utilized the glycosylation inhibitors TUN, DMJ, SSN, CSP, and NM-DNJ to examine the requirement for N-linked glycosylation in more detail. BHK-21 cells were infected with Arm-4, then incubated with the indicated inhibitor for 24 hr prior to pulse labeling for 1 hr with [$^{35}$S]methionine. Labeled cells were then chased in the presence of drug for 4 hr and immunoprecipitated with MAb 33.6 as above. In the presence of TUN there was only a slight reduction of the band representing unglycosylated GP-C and no concomitant appearance of a faster migrating band indicative of unglycosylated GP-2. We next examined the effect of inhibition of oligosaccharide trimming on cleavage using the inhibitors CSP, NM-DNJ, DMJ, and SSN. CSP and NM-DNJ inhibit glucosidases I and II and prevent trimming of terminal glucose residues from the core oligosaccharide. DMJ and SSN inhibit mannosidases I and II, respectively, and prevent trimming events that occur in the Golgi (for review see Elbein, 1987). In the presence of these trimming inhibitors, cleavage of GP-C was observed (Fig. 3, data for SSN not shown). Quantitative estimates of the proportion of the incorporated radioactivity in GP-C and GP-2 in pulse and chase samples were made on the basis of densitometer scans of exposed films. Table 1 summarizes the analysis of the experiment de-

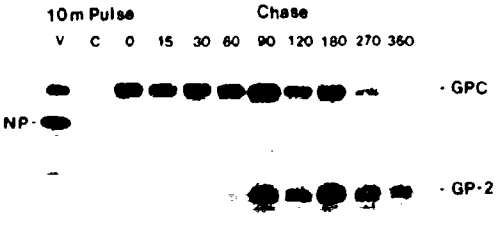



FIG. 2. Pulse-chase labeling of LCMV GP-C. BHK-21 cells were infected with LCMV-Arm (m.o.i. 0.1). After 24 hr cultures were pulse labeled for 10 min with $^{35}$S-translabel (200 μg/ml). GP-C and GP-2 were immunoprecipitated using MAb 33.6 as described under Materials and Methods and analyzed on a 10% SDS-polyacrylamide gel. Cleavage of GP-C to yield GP-2 was first evident at 90 min in this experiment. V, pulse-labeled lysate incubated with guinea pig polyclonal antiserum to LCMV.

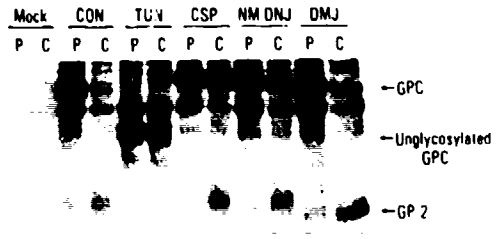



FIG. 3. GP-C is not cleaved when glycosylation is prevented by incubation in the presence of TUN. Infected BHK cells were pulsed and chased in the presence of various inhibitors of glycosylation as described under Materials and Methods, then immunoprecipitated with MAb 33.6 that binds to both glycosylated and unglycosylated GP-C and GP-2. Appearance of GP-2 was indicative of cleavage of the GP-C precursor polypeptide.

picted in Fig. 3. The scan confirmed the observation that cleavage failed to occur in the TUN-treated cultures. Cleavage occurred in the presence of CSP, NM-DMJ, and DMJ, although CSP appeared to exhibit a slight inhibitory effect on processing as indicated by greater retention of label in GP-C in the chase samples. Thus, while addition of N-linked oligosaccharides is necessary for cleavage, processing is tolerant of variations in the extent of trimming.

## Cleavage of GP-C occurs in the Golgi or post-Golgi compartment

The data indicated that in the absence of glycosylation, cleavage failed to occur because the protein was not transported to the site of cleavage, or perhaps because the protein, although transported, was not folded in a conformation appropriate for proteolysis. To determine the intracellular location of GP-C cleavage we examined the temporal relationship between cleavage and trimming by pulse–chase under conditions of reduced temperature which have been shown to halt the transport of viral proteins in defined compartments of the cell (Matlin and Simons, 1983; Saraste and Kuismanen, 1984; Saraste *et al.*, 1986; Balch and Keller, 1986; Copeland *et al.*, 1988). At 15°, proteins exit the rough endoplasmic reticulum, but are halted at a pre-Golgi compartment. At 20° proteins reach the trans-Golgi, as determined by the transition to complex carbohydrate, but are not transported to the cell surface. Infected cells were pulsed for 1 hr at 37°, then chased at 15, 20, and 37° for 4 hr. Lysates were then immunoprecipitated with MAb 33.6 to determine whether cleavage had taken place. There was no evidence for cleavage at either 15 or 20° (Table 2). At both temperatures uncleaved GP-C remained EH sensitive, indicating that GP-C did not reach the medial Golgi. To define the location more precisely, we repeated the experiments chasing at 15, 26 and 37° in order to find the lowest temperature where GP-C acquired EH resistance, and this occurred at 26°. As evident in Fig. 4, there was a small amount of GP-2 present when cells were chased at 15°, and this was likely due to cleavage of GP-C during the 1-hr pulse period. The bands increased markedly at 26 and 37°. During the pulse, and at both 15 and 26°, GP-C was sensitive to EH, as indicated by a shift in migration relative to control lanes. However, at 37°, GP-C was EH resistant, indicating addition of terminal sugars. At all temperatures, GP-2 was resistant to EH. These results indicate that cleavage of GP-C to GP-1 and GP-2 must occur after trimming, that is, in the medial or trans-Golgi or later. Although cleavage had occurred at 26°, a portion of GP-C was still EH sensitive, indicating that transport past the medial Golgi compartment was incomplete. At all temperatures, some full-length GP-C acquired EH resistance, suggesting that cleavage was not essential for transport of GP-C to the medial Golgi.

## Production of infectious virus is reduced in the presence of TUN

The results of the above experiments indicated that GP-C was cleaved in the medial or trans-Golgi or later, and that in the absence of N-linked glycosylation GP-C was not cleaved. It seemed likely that this was because the unglycosylated proteins were not transported to the site of cleavage, but we could not exclude the possibility that abnormal folding precluded recognition by

TABLE 1

QUANTITATIVE DISTRIBUTION OF LABEL BETWEEN GP-C AND GP-2 IN THE PRESENCE OF INHIBITORS OF GLYCOSYLATION

| Inhibitor | Sample | Percentage of GP-C + GP-2 Label[a] | |
|---|---|---|---|
| | | GP-C | GP-2 |
| Control | Pulse[b] | 98 | 2 |
| | Chase[c] | 40 | 60 |
| TUN | Pulse | 100[d] | 0 |
| | Chase | 100 | 0 |
| CSP | Pulse | 100 | 0 |
| | Chase | 75 | 25 |
| NM-DNJ | Pulse | 100 | 0 |
| | Chase | 50 | 50 |
| DMJ | Pulse | 99 | 1 |
| | Chase | 25 | 75 |

[a] Distribution of label in GP-C and GP-2 was estimated by quantitative densitometry of two autoradiographic exposures of the gel shown in Fig. 3. Total area under GP-C + GP-2 bands was normalized to 100%. Percentage distribution of exposure under each peak is shown.

[b] Pulse label was for 1 hr with [$^{35}$S]methionine (60 μCi/ml) in Met-free medium.

[c] Chase was for 4 hr in the presence of a 10-fold excess of unlabeled methionine.

[d] Present as unglycosylated GP-C.

TABLE 2

QUANTITATIVE DISTRIBUTION OF LABEL BETWEEN GP-C AND GP-2 AT VARIOUS TEMPERATURES

| Sample | Temperature | Endo H | Percentage of GP-C and GP-2 Label[a] | |
|---|---|---|---|---|
| | | | GP-C | GP-2 |
| Pulse[b] | 37° | — | 100 | 0 |
| Chase[c] | 15° | — | 94 | 6 |
| Chase | 20° | — | 95 | 5 |
| Chase | 26° | — | 83 | 16 |
| Chase | 37° | — | 54 | 46 |
| Pulse | 37° | + | 96 | 4 |
| Chase | 15° | + | 94 | 6 |
| Chase | 20° | + | 93 | 7 |
| Chase | 26° | + | 78 | 22 |
| Chase | 37° | + | 54 | 46 |

[a] Distribution of label in GP-C and GP-2 was estimated by quantitative densitometry of two autoradiographic exposures of the gel shown in Fig. 4. Total area under GP-C + GP-2 bands was normalized to 100%. Percentage distribution of exposure under each peak is shown. Data were pooled from two experiments.

[b] Pulse labeling was for 1 hr with 60 μCi/ml [$^{35}$S]methionine in Met-free medium.

[c] Chase period was 4 hr at the indicated temperature with 10-fold excess of [$^{35}$S]methionine.

the appropriate protease. As another measure of the effect of glycosylation inhibitors on transport, we assayed the production of infectious virus grown in the presence of TUN, SSN, or CSP. Supernates from infected BHK-21 cells were titered 48 hr after infection. Medium containing the drugs was changed once at 24 hr, so the data represent virus released in the last 24 hr of infection. Only TUN significantly reduced the amount of virus produced (Fig. 5).

## TUN prevents transport of LCMV glycoproteins

To determine whether the reduction in infectivity was due to the production of noninfectious particles, or a

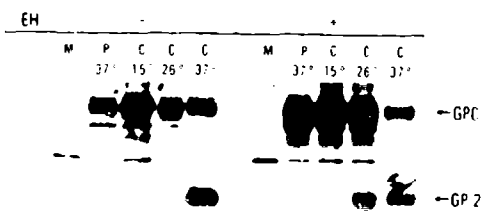



FIG. 4. Cleavage of GP-C occurs in the Golgi or post-Golgi compartment. Infected BHK cells were pulsed for 1 hr at 37°, then chased for 4 hr at the specified temperatures, before immunoprecipitation with MAb binding GP-C and GP-2. Half of each sample was treated with EH after immunoprecipitation as described under Materials and Methods. The other half was incubated in EH buffer without enzyme for the same time before SDS–PAGE.

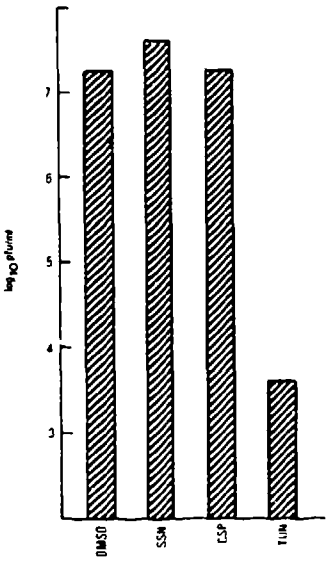



FIG. 5. Replication of LCMV is reduced in the presence of TUN. BHK cells were infected with LCMV, then incubated for the duration of infection with TUN, CSP, SSN, or DMSO (control). Media were changed once after 24 hr and supernates were collected at 48 hr and assayed for infectious virus.

failure to bud, we attempted to purify virus particles from infected cultures grown in TUN. We were unable to band virus in renografin gradients prepared from supernatants of TUN-treated cultures. We also examined virus budding by electron microscopy and found that budding was inhibited in the presence of TUN, suggesting that the unglycosylated viral proteins were not reaching the surface of the cell (Fig. 6). This result was confirmed by immunofluorescence staining of viral proteins on the surfaces of infected BHK cells. We used a MAb against GP-1 which stained control virus-infected cells and also reacted with unglycosylated glycoproteins in permeabilized cells treated with TUN. This MAb failed to detect any viral protein on the surfaces of TUN-treated cells (Fig. 7). Viral glycoproteins were evident at the surfaces of infected cells treated with the trimming inhibitors CSP and SSN in agreement with infectivity data. Thus we concluded from these studies that transport of the unglycosylated proteins to the cell surface was blocked.

## DISCUSSION

Results presented in this paper address basic aspects of glycosylation and proteolytic cleavage of the LCMV glycoprotein precursor, GP-C, and how these processes affect transport of viral proteins within the infected cell. We have shown that the mature structural glycoproteins, GP-1 and GP-2, of the Arm-4 strain of LCMV bear five and two N-linked complex carbohy-

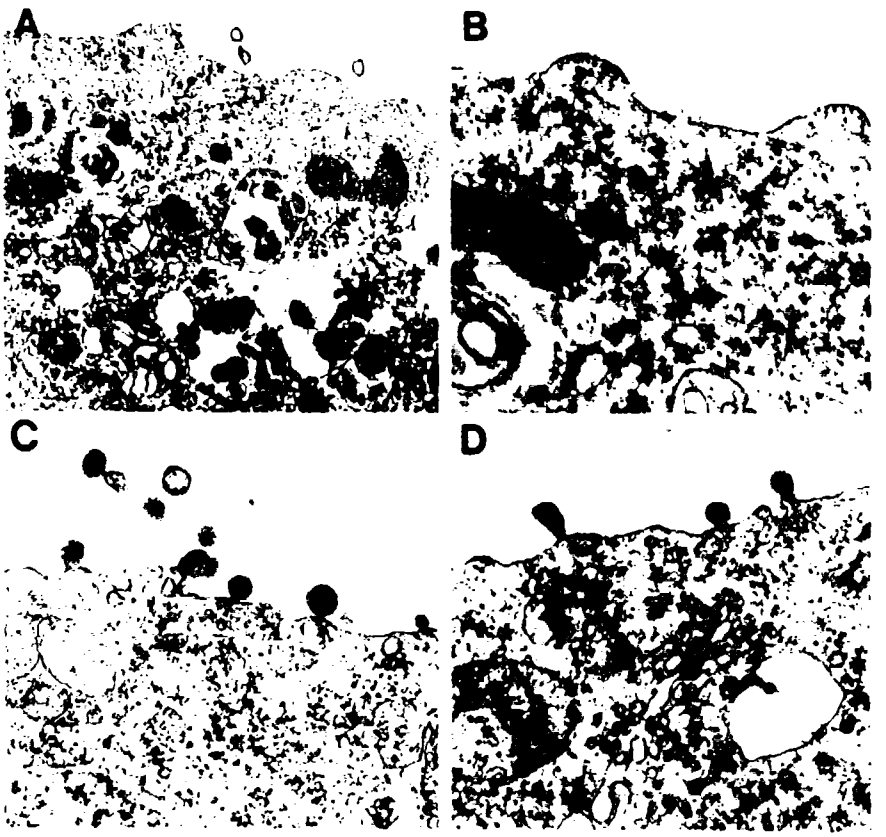



FIG. 6. Budding of LCMV is inhibited in the presence of TUN. Monolayers of BHK cells were infected with LCMV and grown for 24 hr in the presence of TUN or DMSO (control) before fixation and examination by EM. No virions were evident budding from tunicamycin-treated cells (panels A and B), while numerous budding virions were seen in control cultures (panels C and D). Magnified 30,000×.

drates, respectively. In previous studies we have shown that radiolabeled mannose and glucosamine were preferentially incorporated into GP-C, while the GP-1 and GP-2 cleavage products were labeled with glucosamine, galactose, and fucose but contained little residual mannose, suggesting that GP-C was a high mannose precursor (Buchmeier and Oldstone, 1979; Buchmeier and Parekh, 1987). When the role of glycosylation in post-translational events was examined, we found that appropriate folding of the glycoprotein, indicated using MAb which bind to conformational epitopes, failed to occur without glycosylation (Wright *et al.*, 1989). In the present study, we have demonstrated that cleavage and transport of GP-C also failed to occur when glycosylation was blocked by TUN.

To determine how prevention of glycosylation affected cleavage, we needed to establish the temporal sequence of oligosaccharide trimming and proteolytic cleavage of GP-C. As noted, GP-1 and GP-2 bear complex carbohydrates, but GP-C contains predominantly mannose-rich core sugars. A quantitatively minor fraction of GP-C does however contain fucose and galactose, indicative of mature N-linked oligosaccharides. Transition from high mannose carbohydrate to complex carbohydrate, measured by loss of sensitivity to EH and acquisition of terminal sugars, occurs in the medial Golgi (Balch and Keller, 1986). The results of our pulse–chase experiments suggest that cleavage occurs approximately 75–90 min after synthesis and after transport to the medial Golgi. These conclusions were strengthened in temperature block pulse–chase experiments. When infected cells were incubated at temperatures known to halt protein transport at defined compartments within the cell, we never observed EH sensitivity in the cleaved GP-2 molecule, indicating that transition to complex carbohydrates occurred before cleavage. Moreover, by temperature block experiments we localized the site of cleavage to the Golgi apparatus. In cells pulsed and then chased at 20°, no cleavage of GP-C was observed. When the chase was performed at 26°, a temperature which halts transport in the medial/trans-Golgi, we observed cleavage similar to that seen in cells chased at 37°. The reported sites of cleavage of precursor glycoproteins of other enveloped RNA viruses vary. In this respect, LCMV resembles most closely the paramyxoviruses, where the

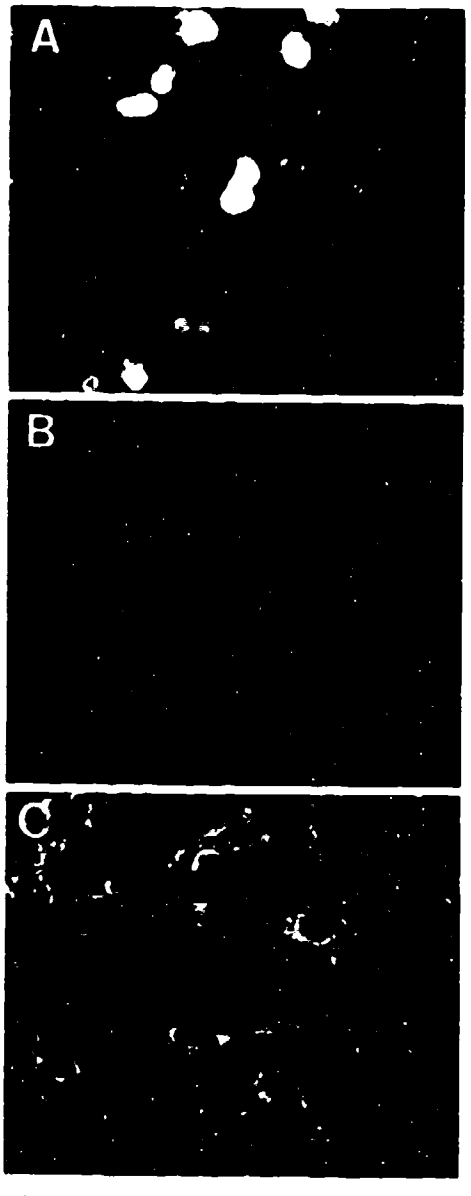



FIG. 7. LCMV glycoproteins are not transported to the cell surface when glycosylation is blocked. BHK cells were infected, then incubated in the presence of TUN for the duration of infection. Living cells were assayed for surface expression of GP-1 using MAb 67.2. Control cells expressed GP-1 (A); TUN-treated cells did not (B). Infected BHK monolayers also incubated in TUN were permeabilized with acetone, then stained to visualize intracellular GP-C (C).

F proteins are cleaved in the trans-Golgi or the immediate trans-Golgi compartment (Sato *et al.*, 1988; Yamada *et al.*, 1988; Morrison *et al.*, 1985; Nagai *et al.*, 1976). However, for measles virus, unlike LCMV, cleavage apparently continues at the cell surface (Yamada *et al.*, 1988).

There are two likely possibilities to explain how prevention of glycosylation interferes with cleavage of GP-C. Either the unglycosylated GP-C polypeptide chain aggregates in the RER and does not reach the site of cleavage or cleavage is dependent on a conformation which is not expressed in the unglycosylated molecule. Our results indicate that under conditions of TUN block, unglycosylated, uncleaved GP-C does not reach the cell surface. Prevention of GP-C transport is reflected by both lack of viral glycoprotein staining at the cell surface and a failure to produce virions by budding at the plasma membrane. Similar findings have been reported for feline and murine leukemia virus (Polinoff *et al.*, 1982; Pinter *et al.*, 1984), Mason-Pfizer monkey virus (Chatterjee *et al.*, 1981), Junin virus (Padula and de Martinez Segovia, 1984), bovine herpes virus (van Grunen-Littel-van den Hurk and Babiuk, 1985), and vesicular stomatitis virus (VSV) (Leavitt *et al.*, 1977). For the latter, it was suggested that failure of transport was due to protein aggregation in the RER (Gibson *et al.*, 1978, 1979). Unglycosylated envelope proteins of measles virus (Sato *et al.*, 1988) and Sendai virus (Mottet *et al.*, 1986) also may remain in the RER. We observed accumulation of unglycosylated GP-C within cells in a form which was not recognized by antibodies to conformational epitopes on GP-C and GP-1 (Wright *et al.*, 1989). We attempted to demonstrate transport of unglycosylated GP-C in cells held at 30°, conditions under which unglycosylated VSV G protein is transported, but neither GP-C transport nor virion production was observed. On the basis of the accumulated evidence, it is likely that unglycosylated GP-C aggregates in the RER or pre-Golgi compartment and thus never reaches the site of proteolysis.

To determine whether transport and cleavage were dependent upon oligosaccharide chain structure, we assessed cleavage in the presence of a variety of inhibitors of oligosaccharide trimming. For some viruses, addition of the core oligosaccharides alone is not adequate for transport, and additional trimming of sugar moieties is required. Specifically, prevention of trimming of the outermost glucose moieties on the core oligosaccharide ($Glc_3$ Man 5–9 $GlcNAc_2$) with either CSP or DNJ inhibits transport of the envelope glycoproteins of murine retroviruses (Pinter *et al.*, 1984), murine hepatitis virus (Repp *et al.*, 1985), and VSV (Schlesinger *et al.*, 1984), but has no effect on the transport of HA of influenza (Romero *et al.*, 1983; Burke *et al.*, 1984; Elbein *et al.*, 1984) or of the envelope glycoprotein of RSV (Bosch and Schwarz, 1984). The same drugs reduced infectivity of Sindbis not by preventing transport but by preventing cleavage of the glycoprotein precursor (Schlesinger *et al.*, 1985; McDowell *et al.*, 1987). For LCMV, we found that addition of the core oligosaccharide alone without any further processing was sufficient to allow transport, cleavage, and production of infectious particles.

Although we can correlate the absence of cleavage with reduced infectivity in these experiments, we do not know whether cleavage is necessary for transport to the plasma membrane. We do occasionally see small amounts of a glycoprotein comigrating with GP-C associated with mature virions, and others have reported a molecule the same size as GP-C on the surfaces of infected cells (van der Zeijst *et al.*, 1983), suggesting that cleavage may not be an absolute requirement for viral maturation. Relatively few experiments have been conducted in viral systems where the need for cleavage has been examined in the absence of drugs such as TUN or monensin that also effect transport. When cleavage of a glycoprotein precursor activates fusion activity of the subunits, as is the case for many enveloped RNA viruses, absence of cleavage would reduce infectivity. This is true for Sendai virus (Scheid and Choppin, 1974, 1976), mammalian influenza viruses grown in avian cells (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975; Kawaoka *et al.*, 1984), and HIV-1 (McCune *et al.*, 1988). Heretofore, the glycoproteins of LCMV have not been associated with membrane fusion activity. Efforts to further define the biosynthetic pathways of the LCMV glycoproteins and their interaction in the virion structure are under way.

## ACKNOWLEDGMENTS

This is Publication 5646-IMM from the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California. This work was supported by NIH Grant AI-16102 and a fellowship to K.E.W. from the Arthritis Society of Canada. J.W.B. was supported by Postdoctoral Training Grant 07078. We thank Rebecca Day and Mary Paine for excellent technical assistance, Jim Johnston for manuscript preparation, and John Elder and John Harper for helpful comments.

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# High resolution *in situ* hybridization to determine the cellular distribution of lymphocytic choriomeningitis virus RNA in the tissues of persistently infected mice: relevance to arenavirus disease and mechanisms of viral persistence

**John K. Fazakerley,[1]*† Peter Southern,[2] Floyd Bloom[1] and Michael J. Buchmeier[1]**

[1]*Department of Neuropharmacology, Research Institute of Scripps Clinic, Imm5, 10666 North Torrey Pines Road, La Jolla, California 92037 and* [2]*Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455, U.S.A.*

By the application of *in situ* hybridization to thin sections of paraffin-embedded tissues we have been able to determine with high resolution the cell types containing lymphocytic choriomeningitis virus nucleic acid in the tissues of persistently infected mice. We confirm and extend previous observations of virus persistence in the brain, lung, liver, kidney, pancreas, thyroid and reticuloendothelial system. In addition, we demonstrate for the first time persistence of viral nucleic acid in specific cell types in the thymus, lymph nodes, testes and bladder, and the adrenal, parathyroid and salivary glands; the cell types infected were observed in several animals. In lymphoid tissue, viral nucleic acid was predominantly located in the T cell-dependent areas of the spleen and lymph nodes; it was also present in cells of the thymic medulla. This has important implications for the deficiency in T cell function observed in persistently infected mice. In the testes, viral nucleic acid was detected in spermatogonia but not differentiating spermatocytes and therefore, in this tissue at least, persistence is related to the state of differentiation of the cell. Endocrine and exocrine dysfunctions have been described in persistently infected mice and we report that the highest levels of viral nucleic acid were found in the adrenal gland. The infection of endocrine and exocrine tissue was not pantropic, specific cell types expressed viral nucleic acid in each tissue. In the adrenal cortex, cells of the zona reticularis and zona fasciculata but not the zona glomerulosa were positive, whereas in the adrenal medulla viral nucleic acid was predominantly localized to adrenalin-secreting cells. Infection of the renal tubules, transitional epithelium of the bladder and the ducts of the salivary gland indicates the likely sites of virus production for the dissemination of arenavirus infections.

## Introduction

Since the original isolations of lymphocytic choriomeningitis virus (LCMV) were made from mouse brains (Armstrong & Lillie, 1934; Traub, 1935), LCMV has been studied in many laboratories, becoming one of the best understood models of virus persistence and virus-induced immunopathological disease (Buchmeier *et al.*, 1980; Lehmann-Grube, 1984; Oldstone *et al.*, 1985*a*). LCMV is an arenavirus, a family which also includes Lassa, Junin and Machupo viruses, the aetiological agents of Lassa fever, and Argentine and Bolivian haemorrhagic fevers respectively. These viruses are endemic in Africa and South America, being maintained in various species of rodents (Peters *et al.*, 1987). Human disease may range from subacute to fatal and results from contamination of human food and water supplies with rodent excreta. LCMV infection of man is also an important cause of aseptic meningitis spread by persistently infected mice, in this case *Mus musculus*.

Following experimental intracerebral inoculation of suckling or adult mice, LCMV replicates in the meninges and choroid plexi and the animals usually die of immune-mediated choriomeningitis which is dependent upon the presence of virus-primed cytotoxic T lymphocytes (Byrne & Oldstone, 1984). Virus also replicates in the reticuloendothelial system (Lehmann-Grube, 1971), including cells of the lymph nodes (Traub & Kesting, 1964), spleen and liver (Mims, 1966; Mims & Subrahamanyan, 1966), as well as peripheral blood lymphocytes (Doyle & Oldstone, 1978).

Mice infected with LCMV *in utero* or neonatally

† Present address: Department of Pathology, Tennis Court Road, Cambridge CB2 1QP, U.K.

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(Hotchin & Cinits, 1958), or experimentally immunosuppressed adult animals, become persistently infected (Gilden *et al.*, 1972). Persistently infected mice generally have undetectable levels of LCMV-specific T cells (Mims & Blanden, 1972), but remain responsive at the B cell level and produce antibody to viral antigens resulting in immune complex disease (Oldstone & Dixon, 1967; Buchmeier & Oldstone, 1978). Persistently infected mice demonstrate changes in endocrine homeostasis associated with infection of various tissues (Oldstone *et al.*, 1985*b*; Klavinskis & Oldstone, 1987*a*; Tishon & Oldstone, 1987). In persistent infection, viral antigens have been detected in neurons of the central nervous system (CNS) (Nathanson *et al.*, 1975; Rodriguez *et al.*, 1983), renal tubules (Accinni *et al.*, 1978), thyroid (Klavinskis & Oldstone, 1987*a*), pancreas (Rodriguez *et al.*, 1985) and in T and B lymphocytes, and macrophages in the spleen, thymus and lymph nodes (Doyle & Oldstone, 1978; Popescu *et al.*, 1979). Most of these studies have used immunofluorescence techniques on frozen sections to detect viral antigens, but immunocytochemistry and electron microscopy have also been employed. More recently the tissue, but not the cellular distribution, of viral nucleic acid has been examined by *in situ* hybridization of whole body sections (Blount *et al.*, 1986).

In this study, we report the results of an *in situ* hybridization study of the cellular distribution of viral nucleic acids during persistent LCMV infection. By application of this technique to thin sections of paraffin-embedded tissue we have been able to determine cell types containing viral nucleic acids at high resolution. This technique has greater resolution than previous studies on frozen tissues or whole animal sections and an advantage over immunostaining, because expression of specific viral proteins can be down-regulated during persistent infection (Oldstone & Buchmeier, 1982). We report for the first time the cell types infected in the thymus, adrenal, parathyroid and salivary glands, and the testes, and extend the observations of previous studies to cell types infected in other tissues.

## Methods

*Virus.* The origin and passage history of the Armstrong CA 1371 (clone 53B) of LCMV have been described previously (Dutko & Oldstone, 1983).

*Mice.* BALB/c mice from the Scripps Clinic and Research Institute breeding colony were inoculated intracerebrally with 100 p.f.u. of LCMV within 18 h of birth.

*In situ hybridization.* Neonatally infected mice, 6 to 8 months of age, were killed by metaphane anaesthesia and tissues were removed and placed in 10% neutral phosphate-buffered formalin. Tissues from five different mice were examined, except in the case of the brain where eight animals were examined, brains were divided down the mid-line and sections were cut sagittally. Tissues were processed and embedded in paraffin, and 5 μm sections cut onto polylysine-coated slides. The technique for *in situ* hybridization was based on that described by Reynolds-Kohler & Nelson (1990). Briefly, sections were dewaxed, hydrated and treated sequentially with 0·2 M-HCl (20 min), 1% Triton X-100 (15 min) and 10 μg/ml proteinase K (15 min, 37 °C), and then acetylated in 0·25% acetic anhydride in 0·1 M-triethanolamine, pH 8·0 (10 min). Sections were washed twice for 3 min between each treatment with sterile PBS. The PBS wash after proteinase K digestion contained 0·2% glycine and 5 mM-EDTA to stop the reaction.

Before hybridization, sections were prehybridized for 1 h at 37 °C in hybridization solution without dextran sulphate. The hybridization solution consisted of 50% deionized formamide, 5 × Denhardt's solution, 10% dextran sulphate, 0·1% SDS, 0·75 M-NaCl, 0·025 M-PIPES, 0·025 M-EDTA, 500 μg/ml sonicated salmon sperm DNA (boiled before addition), 250 μg/ml yeast tRNA, 20 units/ml heparin, pH 6·8, and was made freshly before use. Slides were drained and 25 μl hybridization solution containing $2 \times 10^5$ c.p.m./μl of probe was added to each. The sections were covered with gel bond (FMC), hydrophobic side down, and sealed with rubber cement. Hybridization was carried out at 37 °C for approximately 16 h. Sections were washed sequentially in SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-sodium citrate), 2 × SSC (three times for 15 min each at 20 °C), 0·2 × SSC (twice for 15 min each at 20 °C), 0·2 × SSC (twice for 15 min each at 37 °C) and 0·2 × SSC (once for 15 min at 50 °C). Sections were air-dried and exposed to high resolution Cronex film (DuPont). Sections were then dipped in Kodak NTB2 emulsion, diluted 50% with 0·66 M-ammonium acetate. After 5 days, slides were developed, counter-stained with haematoxylin and eosin, and examined by dark and bright field microscopy.

The probe was a $^{35}$S-labelled DNA molecule, produced by random hexanucleotide priming of a gel-purified 1164 bp *Bgl*II fragment of the nucleocapsid gene of the S RNA of LCMV (Southern *et al.*, 1987; Salvato *et al.*, 1988). This probe gives maximum sensitivity because random priming generates a size range of fragments and both strands of the probe can hybridize, either to the viral genomic RNA (negative sense) or the nucleoprotein gene mRNA. The probe was used at a final concentration of $10^5$ c.p.m./μl and had a specific activity $> 10^9$ c.p.m./μg.

The specificity of the probe was established by inclusion of control, uninfected sections of each tissue type. In addition, sections from mouse brains infected with mouse hepatitis virus (MHV) type 4 were included in each experiment. In parallel experiments in this laboratory using the same techniques on paraffin sections, these MHV-infected brains gave an intense signal with an MHV-specific probe (J. K. Fazakerley *et al.*, unpublished results). No hybridization to control, uninfected tissues or to MHV-infected brains was observed with the LCMV probe.

To examine the different areas of the brain, brains were removed, cut down the mid-line into two equal halves and the two hemispheres were embedded in paraffin in opposite orientations. For the brains of five mice, sections close to the mid-line were examined and three other brains had several sections cut from each of 10 representative areas, which were examined to determine precisely the areas infected. In these sections, virus distribution was first determined by exposure to Cronex film, the same sections were then dipped in emulsion and examined microscopically.

A comparison of *in situ* hybridization on 5 μm paraffin-processed sections and 20 μm cryostat sections demonstrated an equal level of detection of viral nucleic acid in persistently infected brains. The paraffin-processed sections showed better morphological preservation than the cryostat sections and allowed more detailed resolution of the signal distribution.

*Immunostains.* Brain sections from paraffin-processed tissue were




Fig. 1. Images from Cronex film of five representative, sagittal sections from progressively lateral areas of one half of a mouse brain persistently infected with LCMV. The mouse was inoculated intracerebrally within 48 h of birth and sampled 6 months later. The top panel is the closest to the midline. Viral nucleic acid was detected by *in situ* hybridization and shows black. Infected cells are found throughout the brain but are more numerous in the midbrain and hindbrain than in the forebrain. Signal is particularly apparent at all levels in the thalamus (t) and brain stem (b). The most intense signal is in the choroid plexus (cp) and a distinct region on the dorsal face of the thalamus (below the x), this is probably the brachium of the superior colliculus. The meninges (m) are infected as are cells in the olfactory bulb (o). The frontal cortex (fc), caudate (c), hippocampus (h) and cerebellum (cb) have the least number of infected cells.

also stained with an LCMV nucleoprotein-specific antibody, 1-1.3 (Buchmeier *et al.*, 1981). Sections were dewaxed, treated with 0·3% $H_2O_2$ in methanol (30 min) and digested with proteinase K (20 μg/ml, 37 °C, 20 min). Sections were incubated (20 °C, 30 min) in PBS with 20% normal goat serum (NGS) before incubation (37 °C, 2 h) in antibody diluted 1:20 in PBS with 1% NGS. Sections were then washed (twice for 5 min each) in PBS, incubated (37 °C, 1 h) with a biotinylated, affinity-purified goat anti-mouse antibody (Tago Immunologicals), rewashed in PBS (three times for 5 min each) and incubated with ABC-peroxidase reagent (Vector Laboratories), using diaminobenzidine tetrahydrochloride (Polysciences) as the substrate.

## Results and Discussion

### *Central nervous system*

Previous observations on the distribution of LCMV in the CNS derive from studies of protein expression by immunofluorescence (Mims, 1966) and immunostaining (Rodriguez *et al.*, 1983), and *in situ* hybridization studies on whole animal sections (Blount *et al.*, 1986; Lipkin *et al.*, 1989). While confirming the main observation of these previous studies, that virus persists in neurons scattered throughout the CNS, this report also extends these findings by determining the neuroanatomical distribution of the virus, which includes intense signal in the brachium of the superior colliculus, and by observing that long-term persistence can lead to a change in virus tropism with the infection of putative glial cells.

Eight brains and spinal cords were examined from mice with well established infections. Numerous cells containing viral RNA detectable by hybridization were observed scattered throughout the brain. The majority of these cells could be clearly identified morphologically as neurons, predominantly large neurons, and were most numerous in the thalamus, superior colliculus, inferior colliculus, pons, dentate gyrus, brain stem and deep cerebellar nucleus (Fig. 1). Silver grains covered the cytoplasm and not the nucleus as expected for an RNA virus (Fig. 2*a*) and, using an antibody to the viral nucleocapsid, only the cytoplasm was stained (Fig. 4*h*). Infected neurons were often adjacent to uninfected neurons (Fig. 2*a*), which could result from the preferential infection of neuronal subpopulations, transaxonal spread of virus or the transient nature of the infection. In

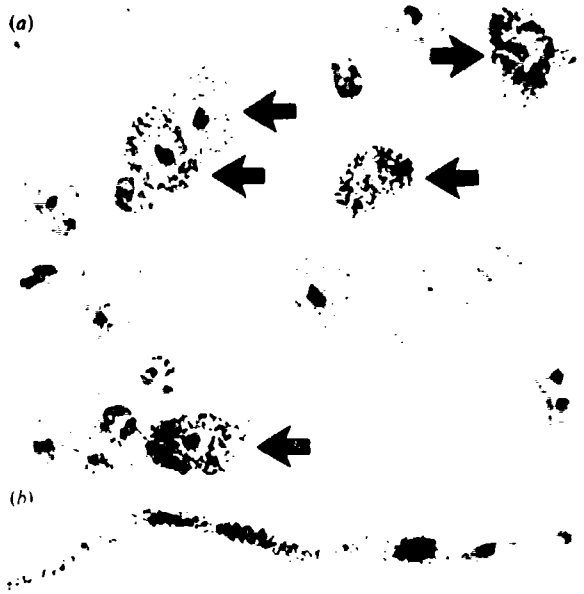



Fig. 2. (*a*) LCMV RNA-positive neurons in the thalamus detected by *in situ* hybridization (arrowed). These are large neurons with prominent, dark staining nucleoli. Signal is distributed over the cytoplasm but not the nucleus. Note that despite infected surrounding neurons, many neurons remain uninfected. (*b*) Positive meningeal cells.

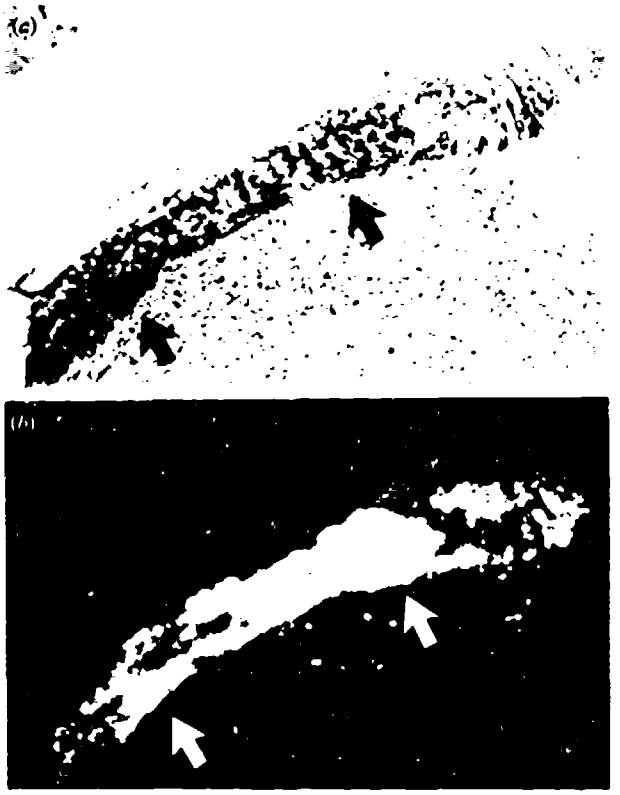



Fig. 3. The strongest positive *in situ* signal in the brain was observed on the dorsal face of the thalamus, projecting into the ventricle. This structure is probably the brachium of the superior colliculus (arrowed) and is shown in light (*a*) and dark field (*b*) microscopy. In dark field microscopy, scattered infected neurons can be seen in the underlying thalamus.

this regard, LCMV has been shown to infect somatostatin- but not cholecytokinin-producing neurons (Lipkin *et al.*, 1988). The cortex, caudate, hippocampus and outer layers of the cerebellum showed less intense signal (Fig. 1), but scattered neurons were infected in all these areas. In the olfactory lobe the periglomerular cells, some cells in the external plexiform layer and scattered cells in the mitral cell layer were positive (Fig. 4*g*). Some positive cells were observed in many of the white matter tracts, including those in the cerebellum and spinal cord.

By far the strongest signal and highest percentage of positive cells observed in any area of the brain was in a distinct region on the dorsal face of the thalamus across the 3rd ventricle from the dentate gyrus, probably the brachium of the superior colliculus (Fig. 1 and 3). The number of infected cells and the intensity of the signal over each cell were greater than observed elsewhere in the brain and were similar to that seen in endocrine tissues, such as adrenal medulla or pancreatic islets.

In addition to the observation of signal associated with neurons and white matter cells, all eight brains demonstrated signal over meningeal (Fig. 2*b*), choroid plexus (Fig. 4*k*) and endothelial cells. These are the cell types known to be infected in the acute LCMV infection (Walker *et al.*, 1975). Comparison with brain sections from an animal acutely infected with LCMV demonstrated a more intense signal in these cell types in acute than in persistent infection (Fig. 4*a*). No pyknosis of infected cells or other pathological changes were observed in any of the brains, nor was any inflammatory infiltrate evident in the persistent infections observed. The distribution of virus in the brain was the same by immunostaining, using a monoclonal antibody to the viral nucleocapsid protein, and *in situ* hybridization with a probe to the nucleocapsid gene.

Predominantly neuronal infection, with the distribution described above, was observed in six of eight mice in the present study. In the remaining two mice, numerous positive cells were found throughout both the grey and white matter, the white matter involvement being far more extensive than in the other six animals examined. In these two animals, there was minimal involvement of the cortex, thalamus, hypothalamus and basal ganglia, and infection was principally confined to the white matter of the brain stem, cerebellum and spinal cord. This distribution was observed both by *in situ* hybridization and immunostaining. Morphologically it was not possible to distinguish the cell types that were labelled in the white matter, but the location of some of these cells in chains of adjacent nuclei in the white matter (Fig. 4*i*, *j*)

suggested that at least some of these cells were oligodendrocytes. Interestingly, persistence of LCMV within putative glial cells of the white matter tracts was not associated with any morphological abnormality of these cells nor were any signs of demyelination evident.

The explanation for this differential distribution in these two mice is not readily apparent, but one possibility is the selection of a variant virus with an enhanced ability to infect glial cells. Variants of LCMV that differ in their ability to persist in adult mice and in their induction of cytotoxic T lymphocytes have been isolated from the brains of persistently infected mice (Ahmed & Oldstone, 1988), indicating that tissue-specific virus variants can arise during persistence.

*Reticuloendothelial system*

Infected endothelial cells were observed in all tissues examined including brain, salivary gland, thymus, thyroid, parathyroid, heart, liver, spleen, kidney, adrenal gland, pancreas, skeletal and smooth muscles, and testes. In this respect, infection of the endothelial system is similar to that described for the related Pichinde virus in hamsters (Murphy *et al.*, 1977).

Virus infection of T cell-dependent areas of the spleen has been shown previously by immunofluorescence (Mims, 1966). The present study confirms these findings and extends the observations to the lymph node and the thymus. In the spleen (Fig. 5*a*, *b*), viral nucleic acid was found predominantly in the T cell areas of the white pulp. We could not determine whether these were lymphocytes or dendritic cells. Few positive cells were seen in the surrounding B cell-dependent areas or in germinal centres, but many positive macrophages and lymphocytes were evident in the red pulp. Infection of T cell areas was also observed in the lymph nodes (Fig. 5*c*, *d*), although again we could not definitively identify the cells as lymphocytes, macrophages or dendritic cells; only rare positive cells were observed in germinal centres or surrounding B cell-dependent areas. These findings are consistent with the demonstration of viral nucleic acid in approximately 2% of peripheral blood lymphocytes (Oldstone *et al.*, 1988); others have identified these cells as being predominantly $CD4^+$ cells (Ahmed *et al.*, 1987). No positive cells were observed on examination of the bone marrow from one mouse.

*The thymus*

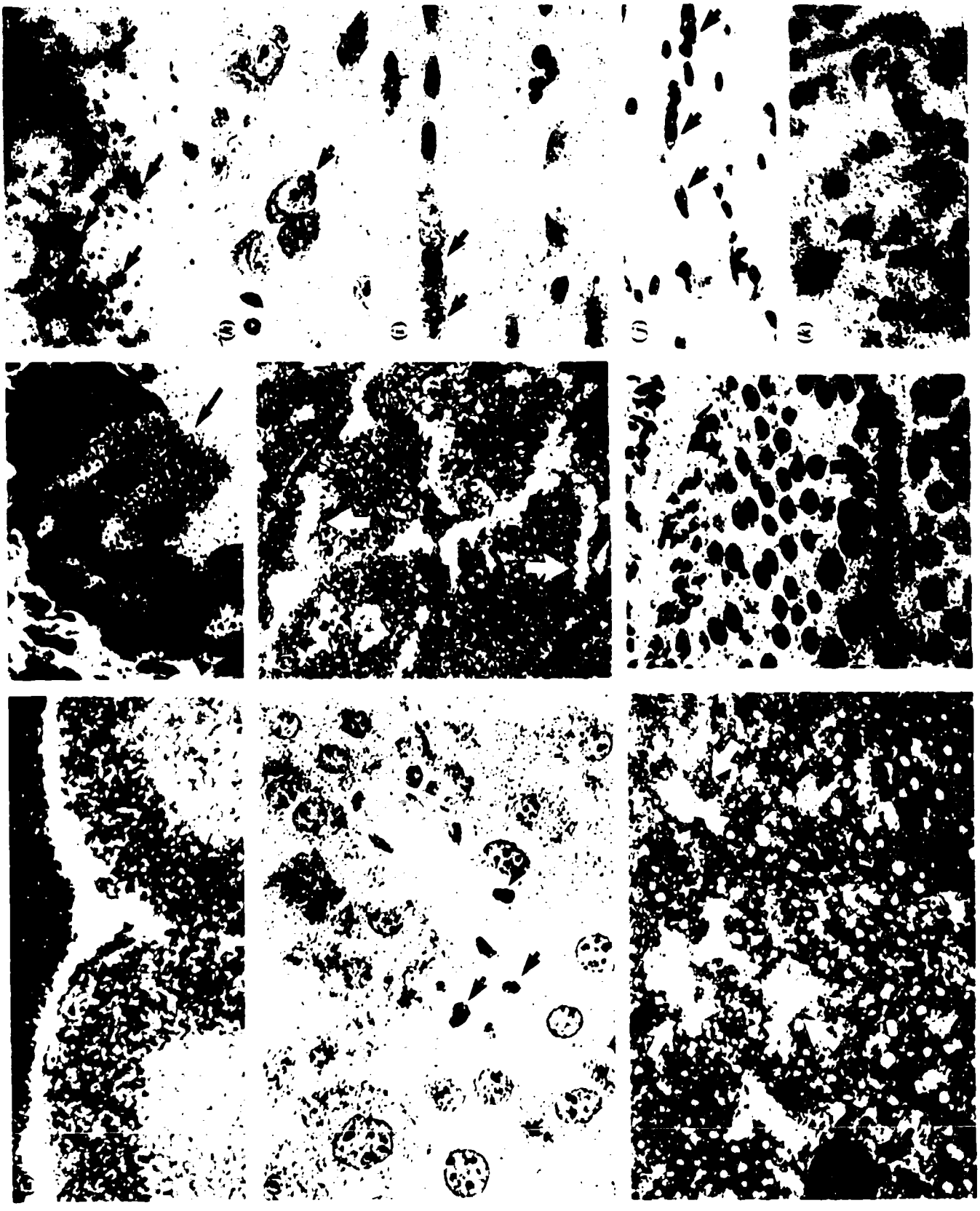
There are no previous reports of viral persistence in the thymus and here we demonstrate for the first time the persistent infection of cells in this tissue. Isolated positive cells were found scattered throughout the gland (Fig. 5*e*), but were most numerous in the medulla (Fig. 5*f*); both mononuclear and epithelial cells were positive. In some areas, a greater number of positive cells appeared to be present at the cortico-medullary junction, an area which contains many macrophage-like cells. Occasional, isolated positive cells, mostly epithelial cells and rarely lymphocytes, were present throughout the cortex.

The mechanism by which LCMV persists in the mouse without elimination by the immune system is not completely understood but appears to involve the selection of immunosuppressive lymphotropic variant viruses, impairment of T lymphocyte responses and decreased cell surface expression of viral glycoproteins (Ahmed *et al.*, 1984; Gilden *et al.*, 1972; Oldstone & Buchmeier, 1982). Infection of the thymus, where the majority of infected cells are in the medulla, could be highly relevant to the impairment of LCMV-specific T lymphocyte responses. In animals persistently infected from birth, infection of the thymus could be involved in maintaining LCMV-specific T cell unresponsiveness by continual elimination or inactivation of newly arising LCMV-specific T cell precursors. Consistent with this is the ability of animals from which the persistent infection has been cleared to generate cytotoxic T cell responses (Jamieson & Ahmed, 1988).

*Endocrine and exocrine tissues*

Abnormalities of endocrine and exocrine systems have previously been observed in mice persistently infected with LCMV (Oldstone *et al.*, 1984*a*, 1985*b*). Viral nucleic acid has been demonstrated by dot blot analysis in adrenal, pituitary, pancreas and thyroid tissues of adult BALB/Wehi, C3H/st and SWR/J mice persistently infected within 18 h of birth with the Armstrong strain of LCMV (Klavinskis & Oldstone, 1987*b*). However, the cellular distribution of viral nucleic acid within these tissues has not been previously determined. Our results confirm that the virus infects specific cell types within these tissues and that this is probably the basis for the dysfunction of specific endocrine systems.

The highest percentage of positive cells in all of the tissues examined was in the adrenal medulla. Viral nucleic acid was detectable in most chromaffin cells, although occasional cells were negative despite being surrounded by intensely positive cells (Fig. 6*e*, *f*). The adrenal medulla is composed of two types of chromaffin cell, one producing adrenalin, the other noradrenalin. In the hamster, the noradrenalin-secreting cells are located principally in the outer region of the medulla and have a pale (eosin)-staining cytoplasm; the adrenalin-secreting cells are located more centrally and have a darker staining cytoplasm (Yates *et al.*, 1962). Differential



cytoplasmic (eosin) staining was apparent in the adrenal medulla; although cells with both darker and paler staining cytoplasm were infected, a greater intensity of signal was generally present in darker staining cells, presumably adrenalin-secreting cells (Fig. 6*e*). The levels of adrenal catecholamines in mice persistently infected with LCMV have not been investigated.

Many positive cells were also present in the adrenal cortex, particularly in the zona reticularis and zona fasciculata (Fig. 6). The intensity of labelling seen in the medulla was not present in the positive cells of the cortex and cells in the outer zona glomerulosa of the cortex remained predominantly uninfected (Fig. 6*a*, *d*). Strong signal was seen in the connective tissue capsule. Five groups of steroids are secreted by the adrenal cortex; these are progestogens, corticosteroids, mineralocorticoids, androgens and oestrogens. The levels of these steroids have not been measured in mice persistently infected with LCMV except in the case of cortisol levels, which were measured in BALB/Wehi mice persistently infected with the Armstrong and WE strains of LCMV and found to be normal (Oldstone *et al.*, 1984*b*). The uninfected zona glomerulosa produces mineralocorticoids such as aldosterone (Tait *et al.*, 1970). Given the changes in the level of hormones produced by pancreatic islet, anterior pituitary and thyroid epithelium cells persistently infected with LCMV, it is possible that changes in the levels of corticosteroids, but not mineralocorticoids, also exist in these mice. This awaits further study.

In the pancreas, many of the epithelial cells lining the ducts were positive. The majority of islet cells showed some signal but small foci of cells were strongly positive (Fig. 7*a*, *b*). These strongly positive cells were most numerous in the outer layers and were consistent with the normal distribution of $\beta$ cells; both the Armstrong and the WE strains of LCMV have been shown to persist in the $\beta$ cells of the islets of Langerhans in the pancreas, the infection being associated with hyperglycaemia and abnormal glucose tolerance (Oldstone *et al.*, 1984*b*; Rodriguez *et al.*, 1985; Tishon & Oldstone, 1987). Electron microscopy has demonstrated virions budding from these cells and double antibody labelling studies have identified most of the infected cells in the islets as $\beta$ cells. The present study confirms the previous distribution of the infection and extends these findings by demonstrating viral genetic material in the islet and acinar cells of the pancreas. The focal pattern of infection observed in the islets could result from spread of virus in distinct territories of interconnecting, communicating islet cells (Orci *et al.*, 1984) via intracellular connections.

In the thyroid gland, strong signal was present in the epithelium of the ducts. Some thyroglobulin-producing epithelial cells were positive, as were supporting cells between the follicles; in both cases the infection was focal (Fig. 7*d*). Viral nucleoprotein has been detected previously in thyroid epithelial cells by immunohistochemistry, and virus has been observed budding from these cells by electron microscopy (Klavinskis & Oldstone, 1987*a*). In the parathyroid glands, occasional individual positive cells of both major cell types, the acidophils and principal cells, were positive, with a greater number of acidophils than principal cells being infected (Fig. 7*e*).

In the salivary glands intense signal was present in the ductile epithelium, and the mucus- and serum-secreting tissues, and scattered foci of positive acinar cells were observed throughout (Fig. 7*c*, *f*). Persistence of LCMV within other secretory tissues includes persistence within the lachrymal gland (Blount *et al.*, 1986) and the well documented infection of growth hormone-producing cells in the anterior pituitary which results in reduced levels of growth hormone mRNA (Oldstone *et al.*, 1985*b*; Valsamakis *et al.*, 1987).

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Fig. 4. Following intracerebral infection of adult mice with LCMV, virus replicates in the meninges. (*a*) Dark field microscopy showing bright positive staining for viral RNA in the meninges overlying the cerebellum 2 days post-infection. In persistently infected animals viral nucleic acid can be demonstrated in many tissues. (*b*, *c*) By dark field illumination, the infection of hepatocytes can be clearly seen to be predominantly focal (*c*, arrows), suggesting cell-to-cell spread of the infection. By dark field illumination of this haematoxylin and eosin stained section, the nuclei of the hepatocytes appear orange, the cytoplasm green and the signal white. Some of these foci of hepatic infection are inflammatory with invading mononuclear cells (*b*, arrows). (*d*) Individual cells in all layers of the transitional epithelium of the bladder are positive for viral nucleic acid and may be sites of infectious virus release into the urine. The lumen of the bladder is directly below this large, swollen, positive epithelial cell (arrow). (*e*, *f*) In the testes, viral nucleic acid is found predominantly in the spermatogonia (arrowed). These are the basal stem cells of the germinal epithelium and give rise to the inner series of differentiating cells which are rarely positive for viral nucleic acid. (*e*) Dark field microscopy showing the infection confined to chains of adjacent spermatogonia (arrows) in the basal layers of four seminiferous tubules. (*f*) Higher power, showing a cross-section of the germinal epithelium. Only the basal spermatogonia are positive (arrow). (*g*, *h*, *i*, *j*, *k*) Examples of cell types infected in the CNS detected by (*g*, *i*, *k*) *in situ* hybridization, (*h*, *j*) immunostaining using an antibody to the viral nucleocapsid. (*g*) Positive neurons (e.g. arrows) in the mitral cell layer of the olfactory bulb. (*h*) Large neurons in the thalamus. Staining is cytoplasmic (arrow). (*i*) Positive cells (arrowed) in a white matter tract of the spinal cord; their alignment in chains is characteristic of oligodendrocytes. (*j*) Putative oligodendrocytes in a white matter tract in the brain stem, including cells staining positive for viral antigen (arrows). (*k*) Positive cells (arrowed) in the choroid plexus.

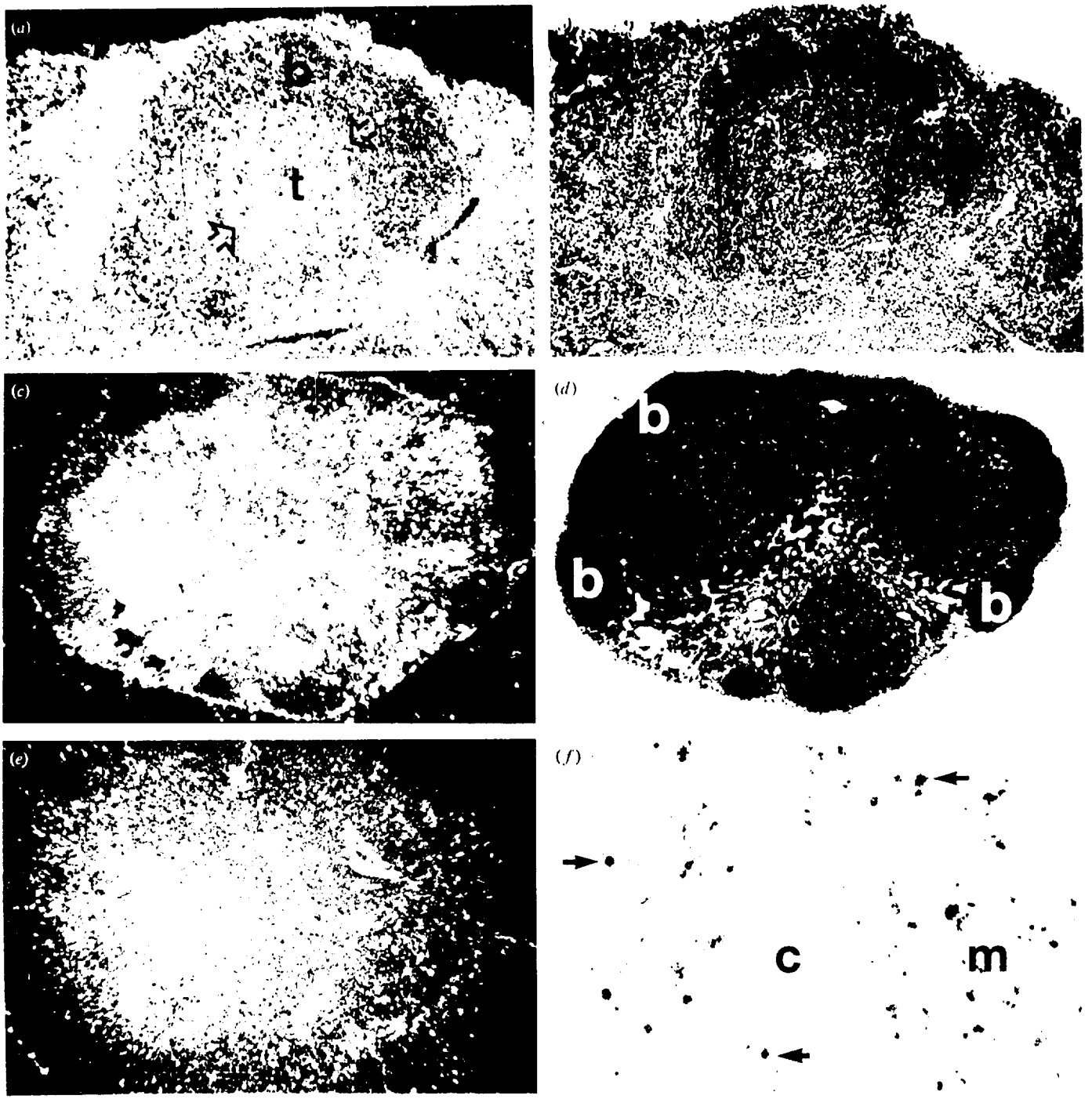



Fig. 5. Lymphoid tissue. (*a*, *b*) Dark and light field illumination of the same section of a spleen, showing cells positive for LCMV nucleic acid. Positive cells are scattered throughout the red pulp, particularly in the marginal zone, but are not as numerous as suggested by dark field microscopy because haemosiderin deposits also appear white under this type of illumination. In the white pulp, many positive cells are present in the central T cell-dependent area (t, between the arrows), but not in the surrounding B cell area (b). Germinal centres (not shown) were also negative. (*c*, *d*) Dark and light field illumination of the same section of a lymph node. Positive cells are distributed throughout the tissue except in the B cell follicles (b) in the outer cortex. These same areas which stain dark under light field microscopy (e.g. b) do not contain positive cells by dark field illumination. (*e*, *f*) Low power, dark field microscopy (*e*), and higher power, light field microscopy (*f*) of the thymus. Scattered positive cells (arrowed) are present throughout the medulla (m) and more rarely in the cortex (c).

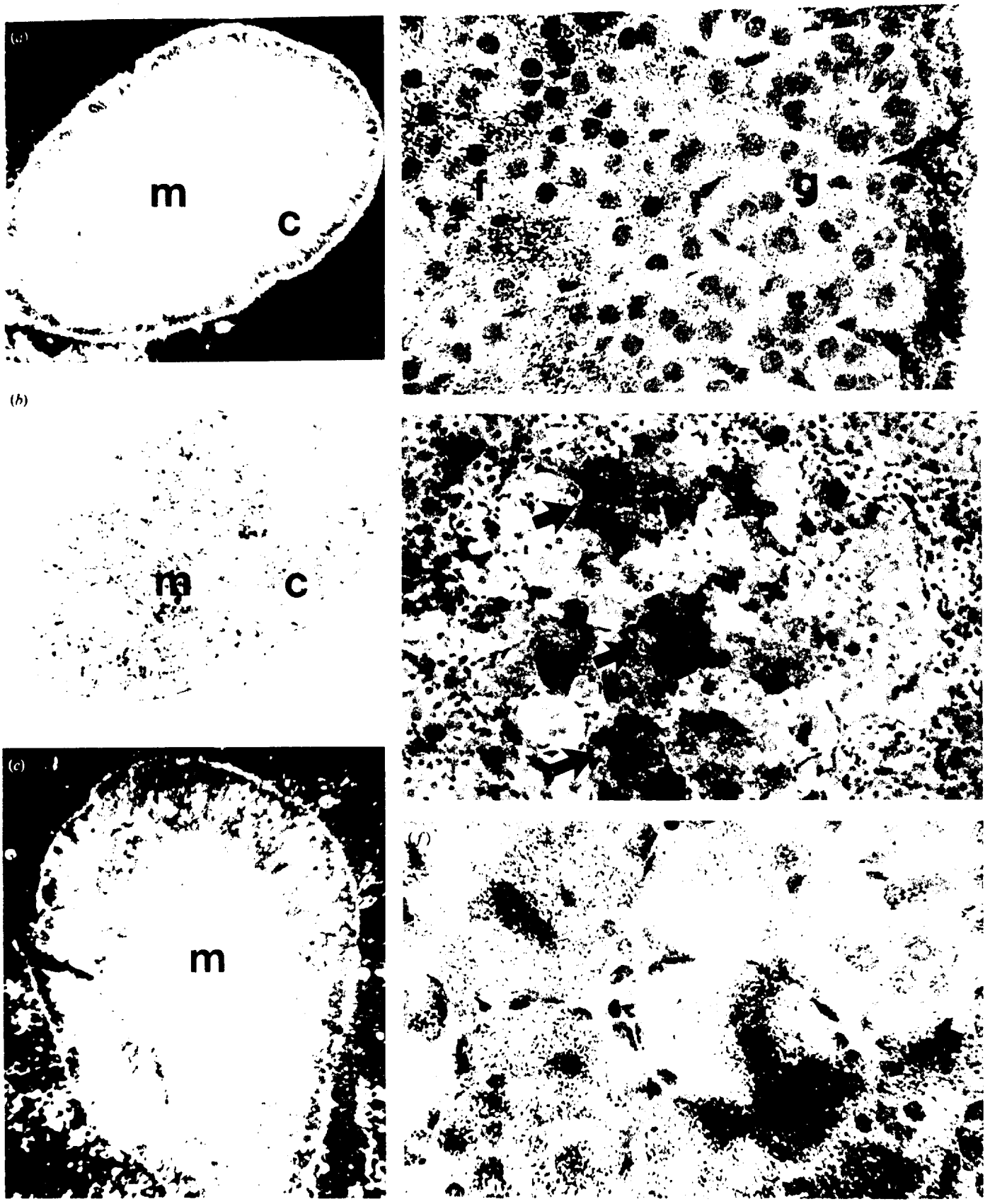



Fig. 6. The adrenal gland. (*a*, *b*) Dark and light field illumination of the same section of an adrenal gland, showing infection of all areas except the outer layer of the cortex (c). The most intense signal is seen in the medulla (m). (*c*) An adrenal gland from another mouse, dark field illumination, with intense staining of the medulla (m). Signal in the cortex is less than that seen in the gland mentioned above. The outer layer of the cortex is again negative. (*d*) High power microscopy of the cells in the outer layer of the cortex, the zona glomerulosa (g). These cells remain uninfected despite positive cells in the underlying zona fasciculata (f) and the overlying connective tissue (c). (*e*) Higher power microscopy of the adrenal gland shown in (*a*) and (*b*) showing intense signal in the darker eosin-staining adrenalin-secreting cells of the medulla (arrows). The surrounding, paler staining, noradrenalin-secreting cells appear negative. (*f*) Cells that are intensely positive and cells that are negative may be adjacent.

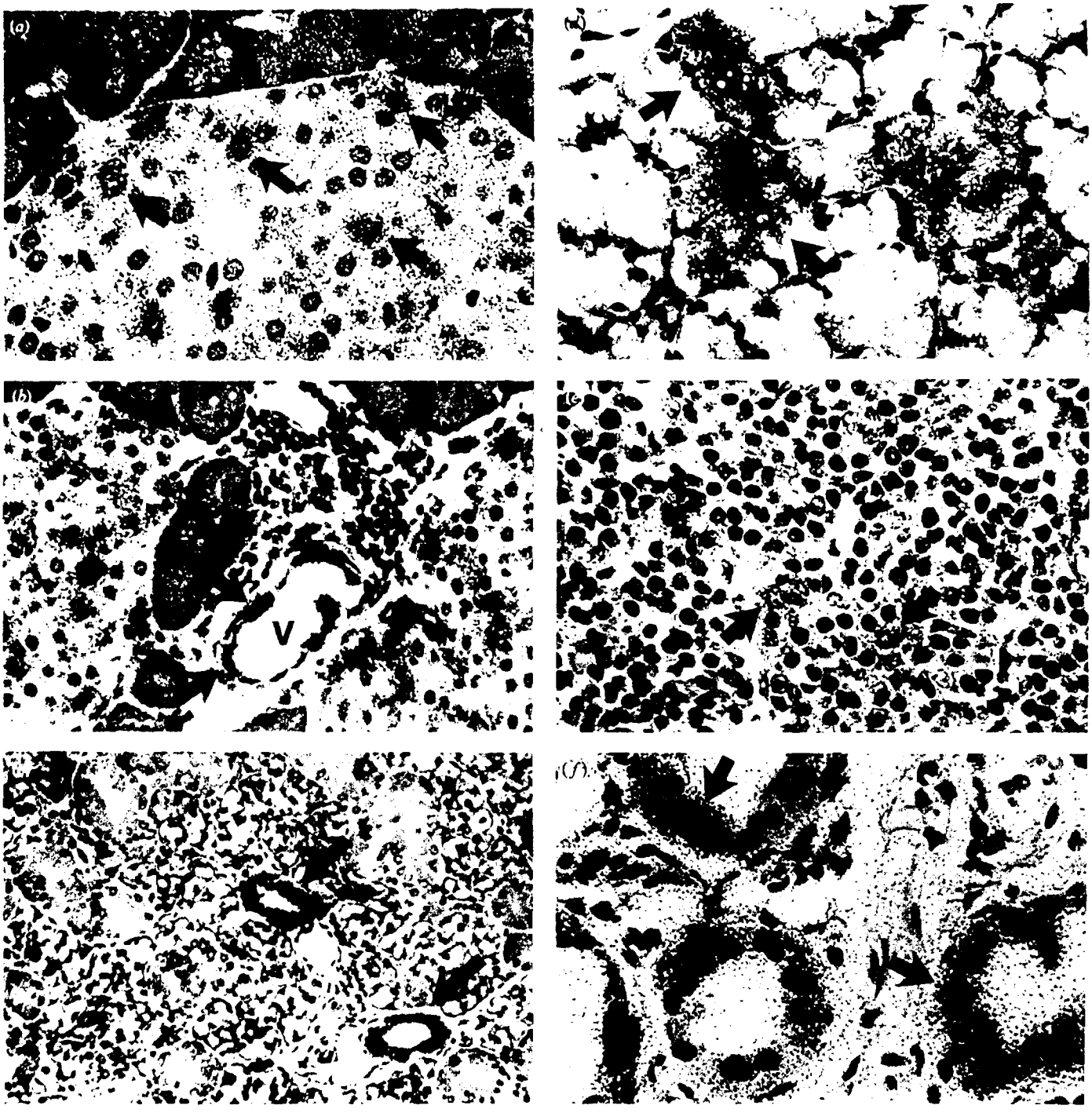



Fig. 7. The endocrine and exocrine systems. (*a*) Positive cells (e.g. arrows) in an islet of Langerhans in the pancreas. (*b*) An inflammatory infiltrate adjacent to a vessel (v) between two islets. Note the positive endothelial cells (arrows). The inflammatory cells do not spread out into the surrounding tissue despite infection of adjacent islet cells. (*c*)Salivary gland showing the positive epithelial cells of two ducts (arrowed), occasional acinar cells are also positive. (*d*) Foci (e.g. arrows) of infected cells are found throughout the thyroid gland. (*e*) Occasional, scattered, positive cells (e.g. arrows) in the parathyroid gland. (*f*) Many epithelial cells of the salivary ducts are infected (e.g. arrows) and probably contribute to the infectious virus found in saliva.

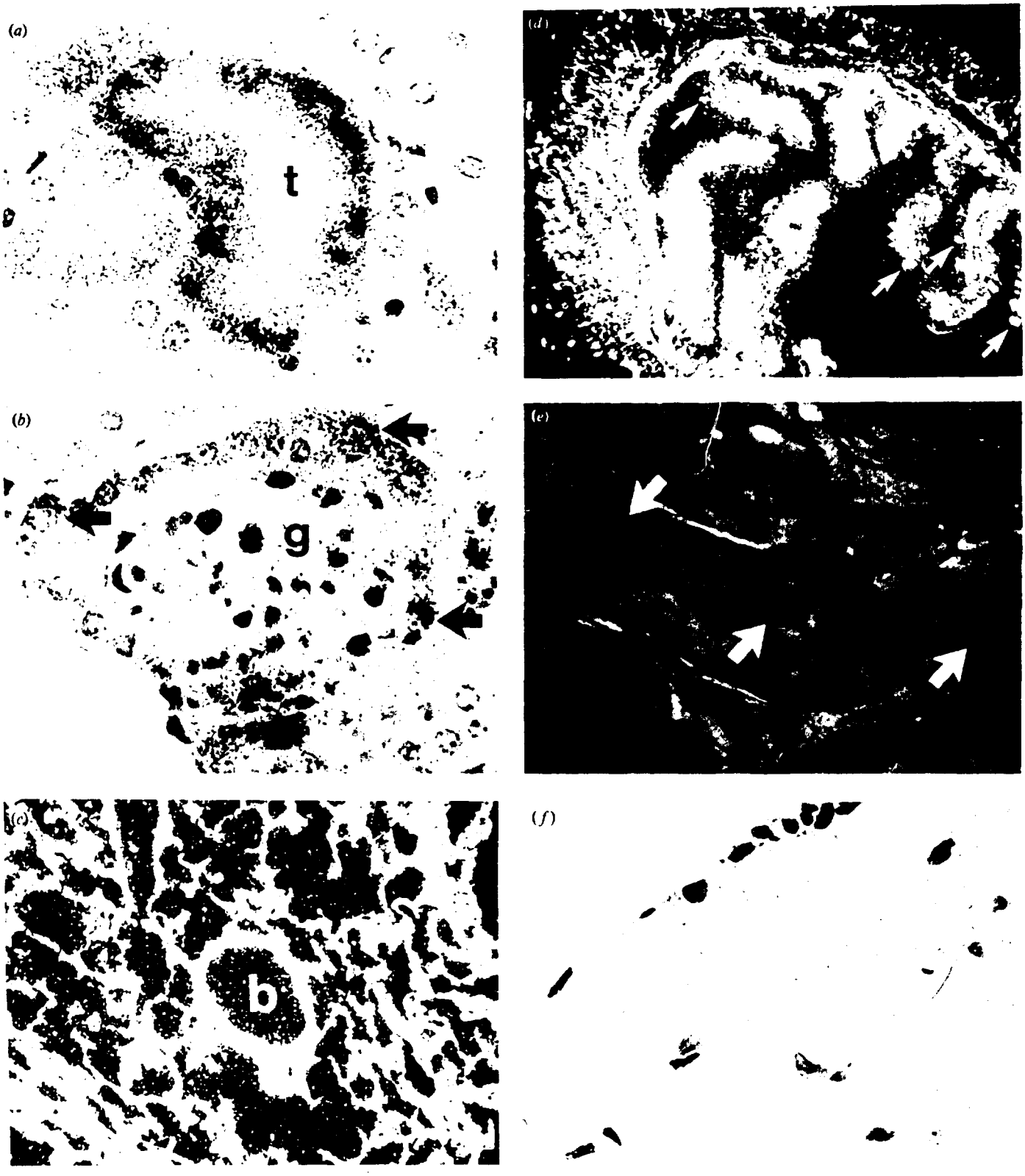



Fig. 8. Kidney, lung, bladder and muscle. (*a*) Infection of the cells of one proximal renal tubule (t). Infected and uninfected tubules are found throughout the kidney. As in this instance, all the cells of any one tubule are usually positive; positive cells are not found scattered throughout different tubules. (*b*) Positive tubule cells surrounding the glomerulus (g). (*c*) In the centre of the field, the columnar epithelial cells of a bronchiole (b) are positive; dark field microscopy. Other positive cells, including alveolar macrophages, are scattered throughout the tissue. (*d*) Low power, dark field microscopy, cross-section of the bladder. Scattered positive cells (e.g. arrows) are present in all layers of the transitional epithelium (see also Fig. 4*d*) and in the muscular bladder wall. (*e*) Isolated, positive cells (arrowed) in the smooth muscle of the bladder wall. (*f*) Striated muscle is notable for a total lack of positive cells.

*Lung, liver and kidney*

In the lung the columnar epithelial cells around the bronchioles were strongly positive (Fig. 8*c*). The alveolar lining cells were rarely positive, with the few infected cells usually found in small foci; several positive alveolar macrophages were observed.

Scattered foci of infected hepatocytes were observed throughout the liver, but the majority of cells remained uninfected (Fig. 4*c*); necrosis and inflammation were observed within or adjacent to some of these foci of infection (Fig. 4*b*). The epithelial cells of the bile duct were labelled, as were some cells, probably Kupffer cells, lining the sinusoids.

Hybridization signal was observed in numerous renal tubules distributed throughout the cortex and medulla and extending into the collecting ducts (Fig. 8*a*, *b*). Within glomeruli, signal was observed predominantly over the cytoplasm of proximal tubular cells, but occasional mesangial cells were also labelled. As in other tissues, vascular endothelial cells were also infected.

*Testes*

In the testes, the presence of viral nucleic acid was dependent upon the differentiation state of the germinal epithelium. The most striking feature was chains of strongly positive spermatogonia in the basal layers of the germinal epithelium in many seminiferous tubules (Fig. 4*e*, *f*). In contrast, signal was rarely evident in spermatocytes and spermatids and when observed was widely dispersed over several infected cells at a much lower intensity than that seen in spermatogonia. Strong signal was also present over individual cells in the interstitial areas, including capillary endothelial cells and Leydig cells; the squamous epithelial cells of the tunica albuginea were positive and occasional positive cells were seen in all layers of the ductus epididymis.

In the mouse testes, spermatogenesis takes place in a cyclic manner and 14 different stages of epithelial development have been recognized (Ewing *et al.*, 1980; De Rooij, 1988). The germ cells can be divided into three groups, spermatogonia which are the stem cells (De Rooij, 1983; Huckins, 1971) and are found adjacent to the basement membrane, spermatocytes which undergo meiotic divisions, and spermatids which become spermatozoa. Viral nucleic acid was observed in chains of spermatogonia but not in single, or only rarely in paired cells. Infection of adjacent spermatogonia is likely to occur via specialized intracellular bridges that exist between these cells (Dym & Fawcett, 1971); these bridges are large enough to allow the passage of organelles and would thus allow ready passage of viral material. Communication via these bridges is considered to be important in the synchronous division of the spermatogonia (Huckins, 1978). Confinement of viral nucleic acid to a distinct subgroup of cells in the germinal epithelium provides an example of a virus infection related to the state of differentiation of the cell and suggests that transmission of infection by infected sperm is unlikely.

The infection of Leydig cells, the main source of testosterone in the adult male mouse, in the interstitium of the tubules is consistent with the infection of endocrine tissue throughout the body. Only small numbers of these cells were infected, perhaps insufficient to affect levels of testosterone, although this has not been investigated.

*Muscle, heart, fat and bladder*

Many viruses such as the alphaviruses replicate to high titres in skeletal muscle, but this tissue was striking for its lack of involvement during persistent LCMV infection (Fig. 8*f*). No viral material was observed in muscle fibres, the only signal in this tissue being over endothelial cells. Occasional smooth muscle-cells of the bladder were infected (Fig. 8*e*) and very rarely signal was observed in the myocardium (not shown). Possibilities for the lack of muscle cell involvement include the absence of a virus receptor in these cells or unfavourable conditions for virus replication within the cell. In contrast, virus was present in both brown and white fat, in which the majority of cells were strongly positive (not shown). All layers of the transitional epithelium of the bladder contained foci of positive cells (Fig. 8*d*, 4*e*).

*Pathology*

Necrosis with infiltrating inflammatory cells was apparent only in the liver, within or adjacent to the foci of infection (Fig. 4*b*). A few tissues, including the thyroid and salivary glands, pancreas and lungs had accumulations of inflammatory cells (Fig. 7*c*), from which cells did not appear to spread into the surrounding tissue. Accumulations of mononuclear cells in the tissues of persistently infected animals have been described previously and shown to be predominantly plasma cells secreting virus-specific immunoglobulins (Moskophidis *et al.*, 1987). We have extended these findings by showing that a few of these inflammatory cells are positive for virus.

*Arenavirus disease*

These results relate to several aspects of arenavirus infection and disease. All known arenaviruses naturally infect rodents which shed virus into the environment in urine, faeces and saliva. Human infection is probably

from infected urine as an aerosol or by direct contamination of human food stocks. Persistence of virus within cells of the salivary gland and its ducts, cells lining the ureter, renal tubule cells and the transitional epithelium of the bladder indicate the likely sites of virus production and release into the saliva and urine.

Despite the infection of numerous cells throughout most tissues of the body, with the exception of a few hepatocytes, infected cells appeared morphologically normal. The effects of LCMV infection are subtle and are manifest as changes in the differentiated function of specialized cells, as has been described in the pituitary and thyroid glands, pancreas, immune system and neurons (Oldstone *et al.*, 1984*a*). Here we have reported that viral nucleic acid is also present in specific cells of the adrenal cortex and medulla, the parathyroid glands, the testes and, in the brain, the brachium of the superior colliculus was strongly positive. It is likely that dysfunctions of homeostasis can also exist in these tissues.

The cell types described as positive for LCMV nucleic acid during the persistent infection were consistently positive in all mice examined and represent the cell types that the virus can both infect and within which viral RNA can be replicated; LCMV clearly has the ability to infect a wide range of cells. The receptor for this virus is as yet undetermined but must be a widely distributed molecule, perhaps present on all cells. The absence of infection in, for example, myocytes does not necessarily imply a lack of a virus receptor on these cells because infection could also be restricted at any point in the virus life-cycle after attachment to a cell surface receptor.

### *Nature of the persistent infection*

Mice persistently infected with LCMV have a persistent plasma viraemia (Hotchin, 1962), and it is not clear whether the detection of viral nucleic acid, proteins or budding virions in a cell indicates that the cell is persistently infected or has just become infected. It is possible that many cells are infected for a short period of time and then eliminate the virus and that what we are observing is just a snapshot in time. If it were possible to observe the same tissue at earlier and later time points in the same living animal, perhaps totally different individual cells in a population would be infected. Alternatively, it may be that once infected, cells remain infected for the life of the animal, or there may be cell types that remain permanently infected whereas others are infected only transiently.

The situation may resemble that found *in vitro*, where cell cultures can be persistently infected by LCMV, but individual cells do not remain so and pass through cycles of infection, referred to by Hotchin (1974) as cyclical transient infection. In an individual cell, infection and virus production are followed by shutdown of virus replication, elimination of viral antigen and refraction to reinfection before the cell becomes infected again; these events are likely to be linked to the cell cycle. If this same phenomenon also exists *in vivo*, different cells of a given cell type may be at different stages in the cell cycle whereas other cell types may not exhibit all stages of the cycle. For example, some cells may remain at a stage that allows continuous production of virus, whereas others are at a stage that allows production of little or no infectious virus. The presence of viral nucleic acid in cells at certain stages of spermatogonial differentiation and the infection of immature lymphocytes but not mature circulating lymphocytes may be examples of this. Furthermore, although persistent infection is non-cytopathic in most tissues, when these tissues are explanted and cultured, infected dividing cells are destroyed by the virus (Lehmann-Grube, 1971).

Strong positive *in situ* hybridization or immunostaining with an anti-nucleoprotein antibody may not necessarily indicate abundant virus production, but could result from the accumulation of viral ribonucleoprotein aggregates with minimal infectious virus production; for example, during the persistent infection of neurons production of viral glycoprotein is reduced (Oldstone & Buchmeier, 1982). Transfer of LCMV-specific cytotoxic lymphocytes to persistently infected mice rapidly clears the extraneural infection, but viral material is cleared only slowly from the CNS (Oldstone *et al.*, 1986). One possibility is that the *in situ* signal detected in neurons results from ribonucleoprotein complexes, and that the slow decay of these complexes is responsible for the observed delay in clearance of viral material from the brain. In this case, infection of neurons would be predominantly unproductive and transient. Different neurons would be continually infected from a productive source, perhaps cerebral endothelial cells, neurons that are in a different state of activation or even the strongly positive cells in the brachium of the superior colliculus. Viral ribonucleocapsid complexes in infected neurons would first accumulate and then decay without viral production and cell destruction.

The exact events of the persistent infection remain to be elucidated, and are likely to be complex and may vary from tissue to tissue. The absence of tissue destruction represents a highly refined virus–host relationship which allows both survival of the host and continual release of infectious virus into the environment, but results in disturbance of homeostasis in immune, endocrine and exocrine tissues.

We are most grateful to Drs J. Sprent, F. Chiasari and C. Wilson of Scripps Clinic for their help and advice, and to Michelle Zandonatti for excellent technical assistance. This is publication number 6514-NP from the Department of Neuropharmacology, Research Institute of

Scripps Clinic, La Jolla, California, U.S.A. This work was supported by a British Multiple Sclerosis Society fellowship to J.K.F. and NIH grant A116102 and US Army Contract C-6234 to M.J.B. The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

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*(Received 25 January 1991; Accepted 14 March 1991)*

JOURNAL OF VIROLOGY, June 1991, p. 3001–3006
0022-538X/91/063001-06$02.00/0
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Vol. 65, No. 6

# Antiviral Antibodies Attenuate T-Cell-Mediated Immunopathology following Acute Lymphocytic Choriomeningitis Virus Infection

KATHRYN E. WRIGHT† AND MICHAEL J. BUCHMEIER*

*Department of Neuropharmacology, Research Institute of Scripps Clinic, La Jolla, California 92037*

Received 5 December 1990/Accepted 4 March 1991

**The role of antiviral antibody in resistance to acute lymphocytic choriomeningitis virus infection has been examined by passive transfer of monoclonal antibodies and intracerebral challenge infection. Protection of mice from lethal T-cell-mediated acute disease was observed following passive administration of antibodies either 1 day before or up to 2 days after infection. Viral replication was suppressed in protected mice, and the cytotoxic T-cell response to virus was also diminished. Virus was cleared from the brain and other tissues of protected mice without development of lethal immunopathology, suggesting that preexisting antibody may play a significant role in modulating potentially destructive effects of T-cell-mediated immune responses to pathogens.**

Infection of mice with lymphocytic choriomeningitis virus (LCMV) can have three outcomes: (i) a transient asymptomatic acute infection when adult immunocompetent mice are infected extraneurally, (ii) life-long persistence after infection of neonates or immunosuppressed adult mice, or (iii) acute, fatal lymphocytic choriomeningitis which develops after intracranial (i.c.) infection of adult mice. It has been established that in all three infections, T-cell-mediated responses are of primary importance in clearing virus, but an auxiliary role for antibody has never been ruled out or examined in depth.

Persistent infections occur when T-cell-mediated responses fail to develop or are selectively abrogated (13, 37), and transfer of virus-specific T cells can clear such infections (24). Specific T lymphocytes are able to mediate a more rapid clearance of virus in transient asymptomatic infections (10, 20, 22, 40), and transfer of virus-specific T cells early after infection can reduce virus replication in the brains of mice infected i.c., thus preventing the development of lethal choriomeningitis (1, 2, 34).

Virus-specific antibodies can be demonstrated in persistently infected carrier mice in the form of immune complexes lodged in the kidney (7, 25) and free antibody in the serum (9, 35). Some carrier mice may even produce low levels of neutralizing antibody (7), but virus is not cleared. It is evident from these results that antibody alone is not adequate to eliminate virus or prevent establishment of persistence. Few experiments have been conducted to examine the role of antibody in either clearing or preventing acute infections. Neutralizing antibodies are generated only late in acute infection, at a time when virus has already been eliminated (21). Others have shown that animals infected with a strain of LCMV eliciting only low titers of neutralizing antibody were still immune to a secondary i.c. challenge with the virus (18), suggesting that neutralizing antibody titer did not correlate directly with protection. Selective abrogation of humoral responses by elimination of T-helper cells in vivo had no effect on viral clearance in acutely infected mice (22), and transfer of immune serum 1 day prior to i.c. challenge did not prevent disease (23). However, evidence has been presented recently supporting an auxiliary role of antibody in clearance. Cerny and colleagues (11, 12) found that clearance of LCMV in adult mice infected intravenously was slowed in mice depleted of B cells by treatment with anti-mu serum from birth. Virus was eventually eliminated, indicating that antibody only played an auxiliary role in clearing the infection. In another study, viral replication in spleens and livers of mice after intraperitoneal (i.p.) infection was significantly reduced when hyperimmune serum was transferred before infection (33).

We have described B-cell epitopes on the glycoproteins of LCMV by using monoclonal antibodies (MAbs). The strain of LCMV in use in our laboratory, Armstrong 4 (Arm 4), possesses a minimum of four B-cell epitopes on the major surface glycoprotein, GP-1, and three epitopes on the second glycoprotein, GP-2 (26). Two of the GP-1 epitopes are recognized by MAbs with neutralizing activity. MAbs directed against all other epitopes on GP-1 and GP-2 are nonneutralizing (26). In this study, we examine the requirements for and mechanisms of protection from challenge infection by passive transfer of these MAbs.

## MATERIALS AND METHODS

**Virus.** The strains of LCMV used in these experiments, Arm-4 and Arm-5, were plaque purified from a stock of Armstrong CA-1371 (26). Working stocks of virus were prepared by infecting BHK-21 cells at a multiplicity of infection of 0.1 and harvesting the supernatants 48 h later. Virus stocks were titrated by plaque assay on Vero cell monolayers (15). Infectious virus in tissues was titrated similarly after brains from individual mice were homogenized. Purified virus was prepared as previously described (8).

**Mice.** Female BALB/c ByJ (*H-2*$^d$) and C57BL/6 (*H-2*$^b$) mice were obtained from the breeding colony at the Research Institute of Scripps Clinic. All mice were used between 6 and 8 weeks of age. Mice were infected by i.c. inoculation with 500 PFU of LCMV stock diluted in phosphate-buffered saline. This dose constituted greater than 500 50% lethal doses for both the Arm-4 and Arm-5 virus strains.

**MAbs.** The generation and characterization of murine MAbs against LCMV are described elsewhere (6, 26).

**ELISA and Western immunoblotting.** An enzyme-linked immunosorbent assay (ELISA) using purified lymphocytic

* Corresponding author.

† Present address: Department of Microbiology, School of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5.

TABLE 1. Characterization of anti-LCMV MAbs

| MAb (ascites fluid) | Epitope specificity[a] | ELISA titer | Neutralization titer (50% plaque reduction) |
|---|---|---|---|
| 2.11.10 | GP-1D | 204,000 | 17,700 |
| 258.2 15 | GP-1A | 12,800 | 562 |
| 67.2 | GP-1C | 16,384 | Nil[b] |
| 1-1.3 | NP | 204,800 | Nil |
| 9-7.9 | GP-2B | 800 | Nil |
| 83.6 | GP-2A | 4,096 | Nil |

[a] As determined previously (26).
[b] Less than 50% plaque reduction at a 1:20 dilution.

choriomeningitis virions as antigen was performed as described previously (39). For Western blotting, 5 μg of purified LCMV per lane was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with polyclonal sera. Bound antibody was detected with $^{125}$I-protein A.

**In vitro cytotoxicity assay.** Control effector cells were prepared by injecting mice with $2 \times 10^5$ PFU of virus i.p. and harvesting splenocytes at day 7. Splenocytes were harvested from mice receiving MAb and 500 PFU i.c. at the times specified for each experiment. After disruption of spleens, cells were washed, cleared of erythrocytes, and counted. Cell lines used as targets in cytotoxicity assays were BALB Cl.7 (*H-2*$^d$) and MC57 (*H-2*$^b$). Mock-infected cells or cells infected 2 days previously with LCMV at a multiplicity of infection of 1.0 were incubated for 1 h with 100 μCi of $^{51}$Cr and washed three times; $5 \times 10^4$ cells were plated per well. Effector cells were added to give a final volume of 200 μl; effector/target ratios were 50:1, 25:1, 12.5:1, and 6.2:1. All samples were run in triplicate wells. Maximum release was calculated in wells with target cells and detergent; spontaneous release was calculated in wells with targets and medium only. Plates were incubated for 5 h, after which time 100 μl of supernatant was removed from each well and counted in a gamma counter. The percent specific release was calculated as (experimental − spontaneous)/(maximum − spontaneous) × 100.

## RESULTS

**Protective effect of antiviral MAbs.** To determine whether the humoral immune response to epitopes defined by various MAbs had any protective role in vivo, we examined the ability of various MAbs to protect adult mice against lymphocytic choriomeningitis disease induced by i.c. challenge with LCMV. Each antibody was titrated by ELISA and neutralization assay prior to use (Table 1). Ascites fluid was given i.p. the day before and the day of i.c. infection. Passive transfer of MAb to GP-1 but not to GP-2 or to the viral nucleoprotein (NP) protected mice (Fig. 1). Control ascites fluid containing MAb directed against murine hepatitis virus also had no effect. Mortality was reduced from over 90% in unprotected mice to 0 to 20% in protected mice. The capacity of specific MAb to protect mice against i.c. challenge with LCMV did not depend on in vitro neutralizing activity (Table 1).

The MAb that appeared to be most efficient in its ability to protect mice, 2.11.10, recognizes an epitope present on Arm-4 but absent on another LCMV strain, Arm-5 (26, 39). To further test the specificity of MAb-mediated protection, we transferred 2.11.10 to mice and then challenged the mice

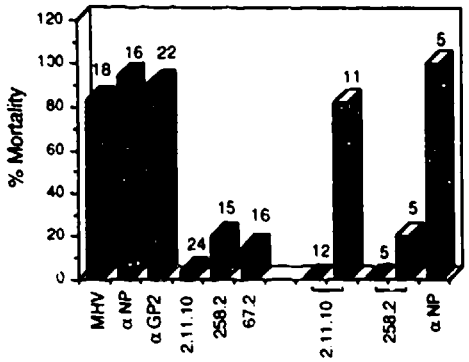



FIG. 1. Mortality following challenge in mice passively protected by MAb. MAbs (indicated at the bottom) to GP-1 but not GP-2 or NP protect mice against lethal challenge with LCMV. Mice were given a single dose of 0.2 ml of ascites fluid and then challenged i.c. with 500 PFU of LCMV Arm-4 (■) or Arm-5 (▨) 1 day later. Cumulative mortality was scored for 21 days after challenge. The number of animals is indicated at the top of each bar. MHV, mouse hepatitis virus.

with either Arm-4 or Arm-5. Another group of mice received an MAb (258.2) directed to an epitope shared by both virus strains. The results of these experiments show that MAb 2.11.10 protects against challenge with Arm-4 but not Arm-5, while MAb 258.2 protects against challenge with either virus (Fig. 1); MAb directed against NP had no protective effect.

We wished to determine whether protection was dependent on the presence of MAb at the time of infection or whether antibody transferred after infection could also mediate protection. The data in Table 2 indicate that transfer of MAb as late as 2 days postinfection (p.i.) significantly reduced mortality, but transfer at day 3 p.i. had little effect. The minimal amount of each ascites fluid that would mediate protection was not determined, but in these experiments as little as 0.2 ml of 2.11.10 was protective.

**Clearance of virus in protected mice.** To determine the mechanism by which antibody was protective, we examined viral titers in brain tissues of infected mice in the days following infection. On the first day, virus was detected above threshold levels in 5 of 11 unprotected mice and in none of the 10 protected mice. By day 4 and continuing on day 6, virus replication was suppressed in the protected mice, as indicated by a 1- to 3-log reduction in central nervous system virus titer (Fig. 2). Surviving mice totally cleared infectious virus. No infectious virus was detected at day 14 or 30 p.i., nor was viral antigen detected by indirect immunofluorescence (data not shown) in the brains of sur-

TABLE 2. Protection from lethal choriomeningitis by transfer of MAb 2.11.10 after infection

| Day of treatment after infection | Treatment | % Mortality (*n*) |
|---|---|---|
| 0[a] | 2.11.10[b] | 0 (10) |
| | Saline | 89 (9) |
| 1 | 2.11.10 | 0 (10) |
| | Saline | 75 (4) |
| 2 | 2.11.10 | 20 (5) |
| | Saline | 100 (5) |
| 3 | 2.11.10 | 80 (5) |

[a] Day of infection with 500 PFU of virus i.c.
[b] 0.2 ml of ascites fluid injected i.p.

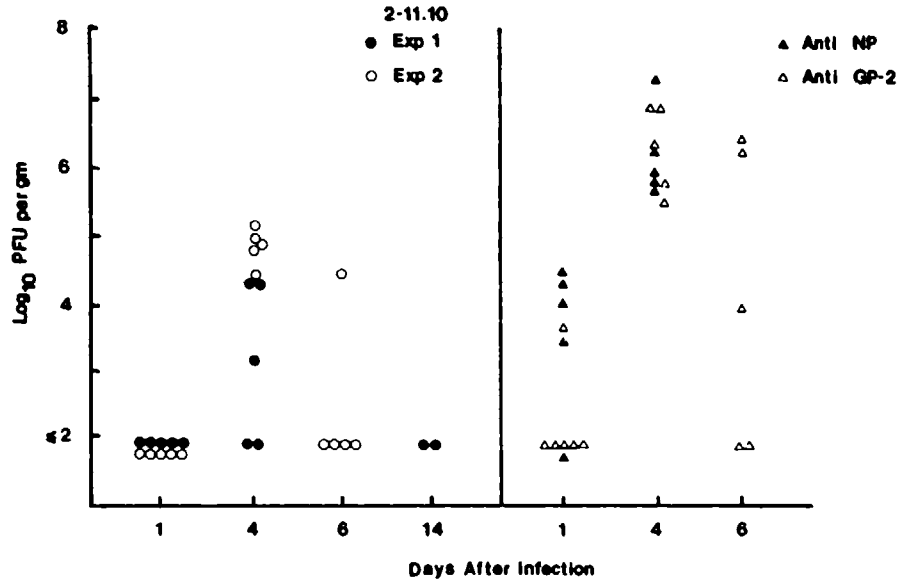



FIG. 2. Virus titers in tissues of protected and unprotected mice following challenge infection. Mice were given 0.2 ml (2.11.10 anti-GP-1) or 0.4 ml (9-7.9 anti-GP-2 and 10.7.5 anti-NP) of ascites on day −1 or day 0 and then challenged i.c. with 500 PFU of virus. Mice were sacrificed at the indicated days, and brain virus content was determined. Results of two experiments are shown. Each point represents one determination.

viving mice at 60 and 100 days. These results suggest that transferred antibody prevented disease by limiting the extent of viral replication in the central nervous system.

**Antiviral immune responses in protected mice.** Fatal lymphocytic choriomeningitis is the result of a T-cell-dependent mononuclear infiltration into the brains of infected mice. In the protected mice, either the quantity of antigen was not adequate to attract sensitized T cells into the brain or virus replication was so limited that the mice did not become immunized. The latter possibility seemed unlikely, as virus replicated in the brains of protected mice, and such mice underwent a crisis between days 3 and 5 p.i. when they showed mild symptoms of disease but recovered by day 7 when their unprotected counterparts were dying. To address this issue, we examined protected mice for viremia and for the presence of LCMV antibodies in their sera at various times after infection. No virus was detected in the sera of

TABLE 3. CTL induction in passively protected mice

| Effector cell source[a] | MAb transferred[b] | Target cells (% $^{51}$Cr release) | | |
|---|---|---|---|---|
| | | $H\text{-}2^d$ LCMV | $H\text{-}2^d$ mock | $H\text{-}2^b$ LCMV |
| Expt 1 | | | | |
| Day 7 | None | 85.5 | 0 | 0 |
| Day 6 | Anti-GP-83.6 | 30.0 | 0 | 0 |
| Day 7 | | 67.2 | 0 | 0.7 |
| Day 6 | Anti-GP-12.11.10 | 8.3 | 0 | 0.1 |
| Day 7 | | 16.9 | 0 | 0 |
| $H\text{-}2^b$ | None | | | 64.4 |
| Expt 2 | | | | |
| Day 7 | None | 68.5 | 0 | 4.0 |
| Day 7 | Anti-GP-83.6 | 52.8 | 1.3 | 0.9 |
| Day 7 | Anti-GP-167.2 | 16.0 | 1.2 | 0 |
| Day 9 | 67.2 | 16.8 | 0 | 0.4 |
| Day 11 | 67.2 | 12.8 | 3.0 | 0 |
| Day 14 | 67.2 | 3.1 | 0.9 | 0 |
| $H\text{-}2^b$ | None | | | 66.2 |

[a] Splenocytes taken on the indicated day after infection (BALB c Byj mice; $H\text{-}2^d$). Effector target ratio is 50:1.

[b] The indicated MAb (0.2 ml) was injected i.p. 1 day prior to infection.

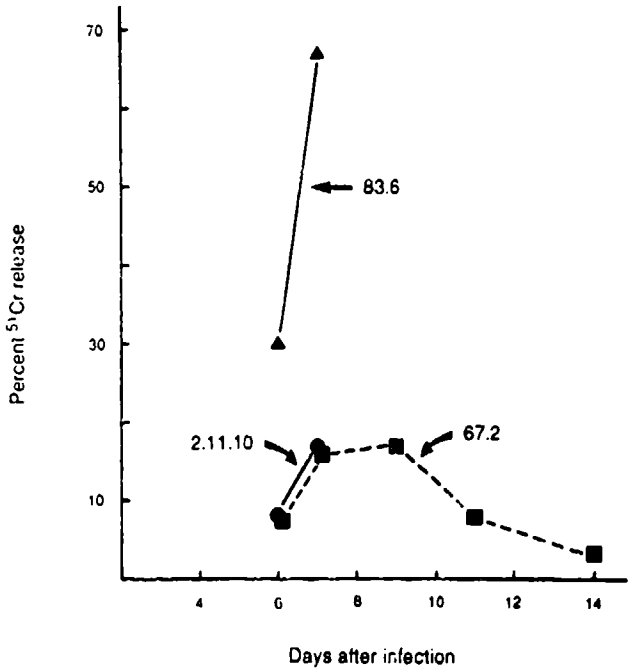



FIG. 3. Attenuated CTL responses in antibody-protected mice. CTL activity in the splenocyte population was determined at an effector:target ratio of 40:1 for protected mice (2.11.10 and 67.5) and unprotected mice (83.6). Note that unprotected mice rapidly developed high levels of CTL activity, while protected mice made relatively poor responses. Unprotected mice typically died by days 7 to 8; hence, activity could not be followed further.




FIG. 4. Immunopathology in the brains of unprotected (A) and protected (B) mice 6 days after i.c. challenge with 500 PFU of LCMV Arm-4. Mice protected with MAb 2.11.10 showed only very mild mononuclear infiltrates in meninges and choroid plexus, while mice receiving a nonprotective antibody (9-7.9) had substantial meningeal infiltrates. Such mice usually died by 7 days after challenge, while protected mice survived indefinitely.

mice at day 4 p.i., but by 7 days low levels were present, and at 30 days p.i. all surviving mice had titers of anti-LCMV antibody ranging from 1/800 to 1/3,200. Passively transferred monoclonal immunoglobulin G has been shown to persist in mice with a half-life of 12.7 days (32). Therefore, to distinguish newly synthesized antibody from the passively transferred antibody, we examined the specificity by Western blotting. Using this method, we found that sera of protected mice contained antibody specific for all of the LCMV structural proteins and hence was newly synthesized (data not shown).

We also examined cytotoxic T-cell (CTL) responses in the spleens of protected and unprotected animals at selected times after infection. Specific antiviral cytotoxic activity could be demonstrated in the spleens of unprotected mice (given MAb 83.6) after i.c. challenge with virus. This activity peaked at days 6 to 7 p.i. and was comparable to CTL activity in the spleens of mice immunized by the i.p. route (Table 3). Cytotoxic activity was also generated in the spleens of mice protected by MAb 2.11.10 or 67.2. However, CTL activity appeared slightly later, 7 to 9 days p.i., and was lower than that in unprotected mice. This cytotoxic activity

tapered off between days 11 and 14 p.i. (Fig. 3). These results suggest that absence of disease in protected mice was linked to a reduced level of cytotoxic activity rather than to a delayed response.

**Pathology in the brains of mice.** To examine this aspect further, unprotected mice, given either anti-GP-2 or anti-NP MAb, and protected mice, given MAb 2.11.10, were sacrificed at selected times after challenge, and their brains were examined histopathologically to assess choriomeningitis. Unprotected mice showed characteristic lesions of lymphocytic choriomeningitis (38), with mononuclear infiltrates in the meninges and choroid plexus (Fig. 4A), while protected mice showed only mild infiltration of these tissues (Fig. 4B).

## DISCUSSION

Infection of mice with LCMV has been the model system of choice for demonstrating the role of T-cell-mediated immunity, particularly CTL, in enveloped viral infections. There is little doubt that clearance of primary infection is impossible without CTL (13, 24, 37). For this reason, the role of antibody responses in either primary or secondary infections with the virus has been largely neglected. Our data clearly demonstrate that antibody to LCMV is effective in attenuating lethal T-cell-dependent choriomeningitis initiated following i.c. infection with the virus. This attenuation appears to be due to the combination of reduced antigen load in the brains of protected mice and decreased levels of CTL activity. This latter finding may indicate prevention of induction or active suppression of CTL by the transferred antibody or simply that adequate virus or viral antigen does not reach the periphery to elicit a strong response. We favor the latter explanation because of the observed rapid clearance of virus from the brain. These results are similar to the reported findings of Thomsen and colleagues in mice infected by the i.p. route after transfer of serum (34). In these animals, virus replication is markedly restricted to the extent that neither cell-mediated nor humoral responses were elicited.

It appears that protection is not dependent on in vitro neutralizing activity; in our hands, both neutralizing (2.11.10 and 258.2) and nonneutralizing (67.2) MAbs were effective. This is not totally unprecedented. Hyperimmune serum that reduced LCMV replication after i.p. infection did not have demonstrable in vitro virus neutralizing activity (38). Furthermore, nonneutralizing antisera or MAbs have been shown to protect against infection with herpes simplex virus (3), Semliki Forest virus (4), Sindbis virus (29), and Newcastle disease virus (36). Although virus neutralizing activity is not necessary for protection against LCMV, the protective epitopes all appear to be present on the major surface glycoprotein, GP-1. There are several postulated mechanisms by which nonneutralizing antibody can be effective in clearing virus in vivo, including lysis of infected cells by complement (28, 30), activation of complement by antibody-virus complexes resulting in increased chemotactic activity for phagocytic cells and opsonization of antibody-virus complexes (17), and antibody-dependent cell-mediated cytotoxicity (27, 30). High titers of complement-fixing antibodies are induced during acute infection of mice with LCMV, but these antibodies are largely directed to NP (16, 31), with only low titers of antibody to GP-1-fixing complement (5, 19). This suggests that one of the other mechanisms may account for the effects of transferred MAb. This view is supported by the observation that protection is effective in complement component C5-deficient mice (3a).

Whatever the mechanisms, our results indicate that even for a virus that is cleared initially by cell-mediated responses, antiviral antibody can limit virus spread and attenuate potentially destructive T-cell-mediated immune responses. Thus, vaccines that elicit strong humoral responses to GP-1 epitopes should be effective against infection by these viruses. The presence of antibody or the rapid induction of memory humoral responses would be generated (14). Antibody alone may not be adequate to completely prevent replication or clear virus after infection, but it may limit the infection at a time when T-cell responses are not strong. Clearly, it is advantageous to define those B-cell epitopes on pathogens like LCMV which will elicit cooperative antibody and to design and present vaccines which produce the desired responses.

## ACKNOWLEDGMENTS

We thank Mary Paine for technical assistance and Jim Johnston for manuscript preparation.

This work was supported by Army contract C-6234 and PHS grant AI 16102. K.E.W. was supported by an Arthritis Society of Canada fellowship.

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