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SYNTHETIC PEPTIDE VACCINES FOR THE CONTROL OF ARENAVIRUS INFECTONS

ANNUAL REPORT

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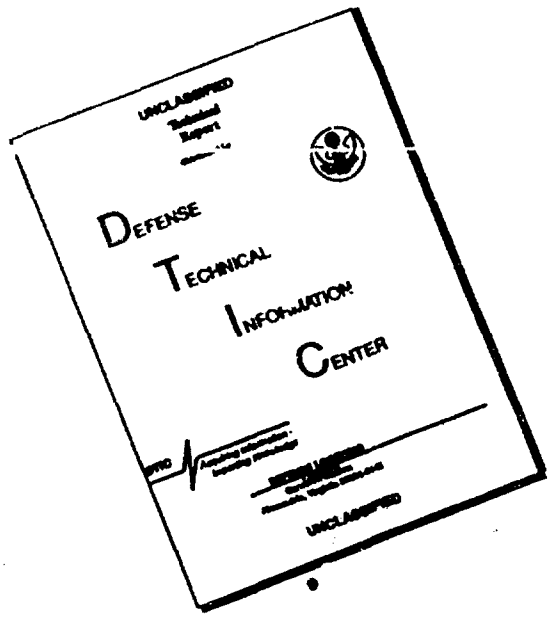

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Arenaviruses are endemic on both the African and South American continents and represent significant public health hazards. Prophylactic immunization, precise diagnostic methods, and effective treatment protocols are not currently available. We are 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 (OVER)

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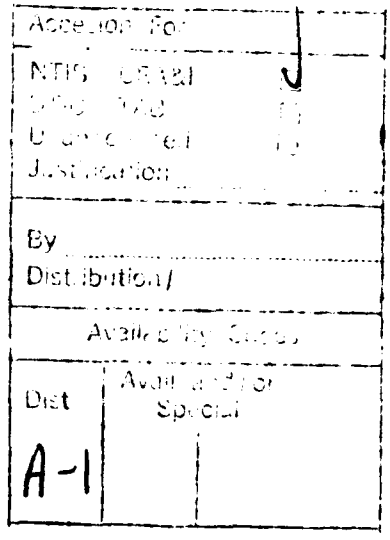
Abstract

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. Synthetic peptides corresponding to immunogenic regions of the viral structural proteins are being synthesized and will be evaluated for the ability to induce immune responses in experimental animals. Experimental approaches to immunization based upon synthetic peptides and polypeptides, vaccinia virus vectors containing LCMV genes, and anti-idiotypic antibodies will be explored. Experimental approaches to immunotherapy for acute arenavirus infections will also be investigated using cloned cytotoxic T-lymphocytes and neutralizing monoclonal antibodies in attempts to modify the course of acute disease. Finally, monoclonal antibodies and cDNA probes against defined type specific and common determinants and sequences will be made in order to facilitate precise diagnosis of arenaviral diseases.



Foreword

In conducting the research described in this report, the investigators 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 (DHEW Publication No. (NIH) 78-23, Revised 1978).

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

Table of Contents

A. Introduction and Overview

During the contract year 1 Aug 1989 - 31 Jul 1990 we made substantial progress in several areas. Most notable was the advancement in our nearest neighbor analysis of the glycoproteins which make up the LCMV spike, the prototypical arenavirus glycoprotein spike. For the first time we are able to propose a cogent theoretical model of the macromolecular organization of the spike. Furthermore we have advanced our studies of posttranslational protein processing to include data on other arenaviruses and have mapped by in situ hybridization the distribution of viral nucleic acid in persistently infected mice. Major areas of focus include:

1. Protein-protein interactions in the glycoprotein spike.

2. Development of a theretical model for the spike structure.

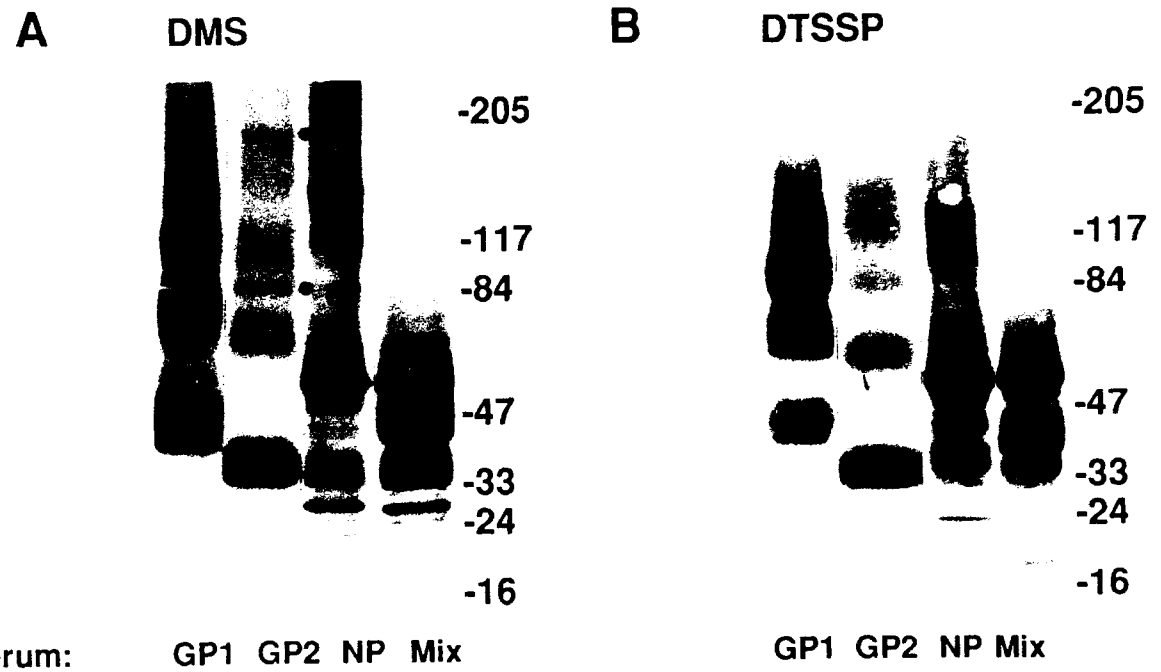
3. N-terminal sequences of the GP-1 and GP-2 glycoproteins of LCM and other arenaviruses.

4. In situ hybridization studies of LCMV RNA distribution in infection.

B. 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. 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 $(GP\text{-}2:NP)_2$. Additional possible complexes of GP-2 with the 12kd Z protein were also occasionally observed (Fig. 1A).

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. 1B). 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 1. 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 hydrophobic



'ig. 1. 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.

TABLE 1. 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).

interactions or ionic bonding.

C. Theoretical Model of the Spike Structure

Based on the cross linking experiments and on the observation of native tetramers of GP-1, we can propose the following working model of the spike structure of LCMV and by extension all similar Old World arenaviruses (Fig. 2). In this model GP-1 forms the tetrameric head of the spike attached by non-covalent interactions to an extended stalk composed of a tetramer of GP-2 molecules in an extended alpha helical configuration. GP-2 spans the membrane interacting internally with NP, the major protein component of the ribonucleoprotein complex. Cryoelectron microscopic examination of highly purified virions appears to support this model. Globular structures are evident at the tips of fibrous stalks which in turn appear to span the virion envelope. It is suggested, based on our previous studies and those of others (Bruns et al., Virology 130:241), that the GP-1 head carries the sites active in viral binding to cells and virus neutralization, therefore we will work to refine our methodology for purifying GP-1 and eliciting an anti GP-1 humoral immune response by active immunization.

D. N-terminal Sequences of GP-1 and GP-2 of Other Arenaviruses

Building upon the results reported in the previous project year, 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. 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, J. Virol. 61:982). Similar results were obtained with the New World arenaviruses Pichinde and Tacaribe (Fig. 3), unequivocally locating the N termini of GP-2 in both viruses and of GP-1 in Pichinde. To date we have been unable to isolate a sufficient quantity of Tacaribe GP-1 to enable sequencing, probably because it comigrates or nearly comigrates with NP of that virus.

MODEL OF THE LCMV GLYCOPROTEIN SPIKE

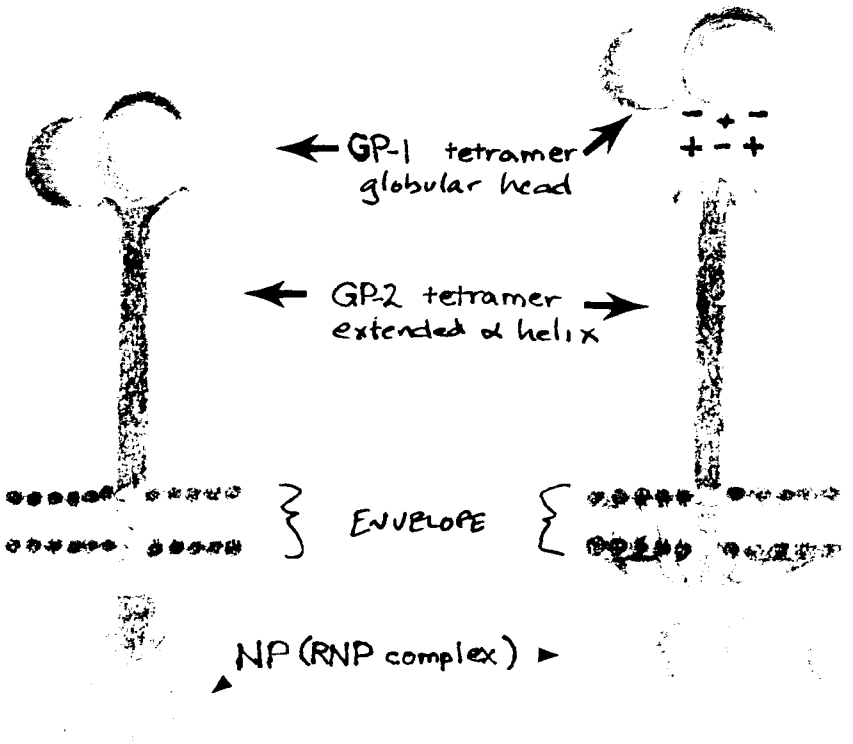


Fig. 2. Our working model for the LCMV glycoprotein spike identifies GP-1 (red) as a disulfide linked tetramer that is non-covalently linked to GP-2 (blue) which also exists as a tetramer. We envision that GP-2 spans the lipid bilayer of the viral envelope (purple) and interacts with NP (orange), part of the RNP complex, via the cytoplasmic tail of GP-2.

ARENAVIRUS GPC CLEAVAGE SITES

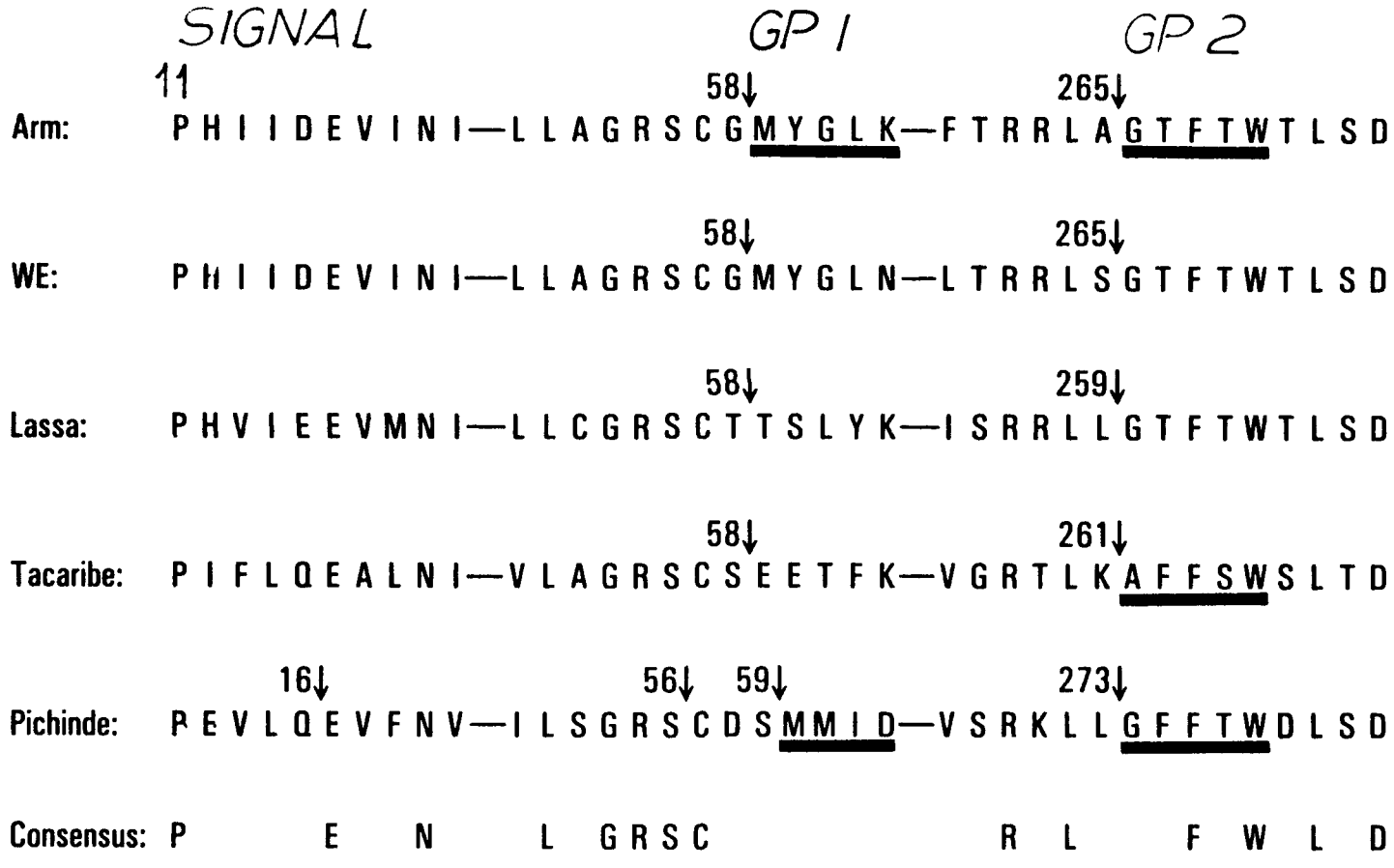


Fig. 3. Underlined sequences have been confirmed by N-terminal amino acid sequencing of virion structural proteins.

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 in vitro expression of the arenavirus glycoproteins from cDNA and to rationally design GP-C expression vectors.

E. 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. 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 results are described in detail in a manuscript entitled "High resolution in situ hybridization to determine the cellular distribution of

lymphocytic choriomeningitis virus RNA in the tissues of persistently infected mice" which has been submitted for publication.

F. Plans for the Coming Year

In the next contract year we will focus on refining our cross linking and nearest neighbor analysis of the glycoprotein spike in order to facilitate expression from cDNA and purification of an immunogenic GP-1 subunit for vaccination. We have recently requested Vaccinia vectors expressing Lassa GP-C and NP genes from Dr. David Auperin, and once these have been obtained and we are comfortable with them we will begin to work toward expression and purification of Lassa GP-C for experimental studies. The observation of a long cleavable signal sequence on LCMV and Pichinde GP-Cs suggests that attempts at expression must accommodate signal cleavage to achieve transport. Furthermore, it may be necessary for more refined structural studies such as X-ray crystallography, to modify or delete this long hydrophobic sequence to obtain soluble glycoprotein. It will also be necessary to define the interaction of GP-2 with the membrane and its transmembrane interaction with the nucleocapsid complex, since this recognition event is highly likely to be an important event in viral maturation. Finally, we will return to our studies of the mechanism of passive protection by antibody in order to look at several biologically important parameters, including requirements for complement, IgG subclass dependence, effect on helper and cytotoxic T-cell activity and potential for trans-placental passage of protection. We will also attempt to duplicate these results by active immunization of mice with purified GP-1 isolated by stripping from virions following by isolation by sucrose gradient sedimentation.

In anticipation of Lassa, we have begun to prepare synthetic peptides and antibodies against Lassa GP-C sequences. The first five of these are indicated by boxes on Fig. 4, and were chosen because of their representation of topographically interesting regions of the Lassa GP-C sequence. These antisera and peptides were also sent to Dr. Peter Jahrling, COTR on this project, for his evaluation in reactivity with native Lassa virus antigens.

Fig. 4 .

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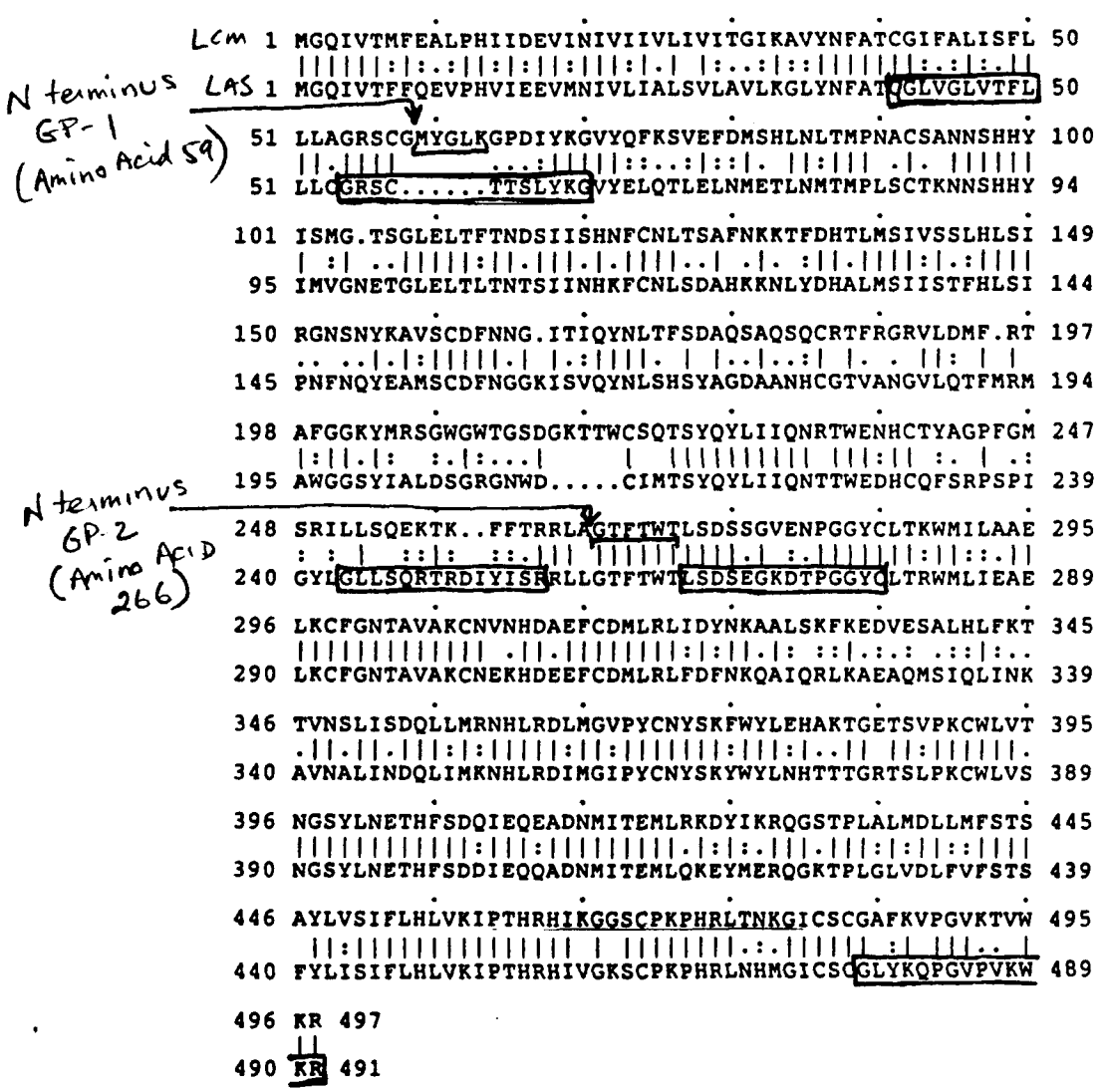

Appendix 1: Publications supported by this document, 1989-90

Published Papers

*Wright, K. E., R. C. Spiro, J. W. Burns and M. J. Buchmeier. 1990. Post-translational processing of the glycoproteins of lymphocytic choriomeningitis virus. Virology 177:175-183.

Fuller-Pace, F. V. and P. J. Southern. 1989. 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. J. Virol. 63:1938-1944.

*previously listed as papers in press.

Papers in Press

Wright, K. E. and M. J. Buchmeier. Antiviral antibodies attenuate T-cell mediated immunopathology following acute lymphocytic choriomeningitis virus infection. J. Virol., 1990, in press.

Wright, K. E., C. Schmaljohn, A. Schmaljohn and M. J. Buchmeier. Arenaviridae and bunyaviridae. In Immunochemistry of Viruses, Vol. 2, von Regenmortel and Neurath, eds., Elsevier, Amsterdam, 1990, in press.

Papers Submitted

Fazakerley, J. K., P. Southern, F. Bloom and M. J. Buchmeier. High resolution in situ hybridisation 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. J. Gen. Virol., 1990, submitted.