

AD \_\_\_\_\_

GRANT NO: DAMD17-89-Z-9022

TITLE: NEW APPROACHES TO HEPATITIS A VACCINE DEVELOPMENT

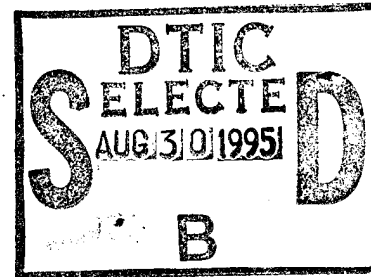
PRINCIPAL INVESTIGATOR: Stanley M. Lemon, M.D.

CONTRACTING

ORGANIZATION: The University of North Carolina at Chapel Hill  
CB# 7030, 547 Burnett-Womack  
Chapel Hill, North Carolina 27599-7030

REPORT DATE: October 1, 1994

TYPE OF REPORT: Final Report



PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick  
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19950829 161

# 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.

<b>1. AGENCY USE ONLY (Leave blank)</b>	<b>2. REPORT DATE</b> October 26, 1994	<b>3. REPORT TYPE AND DATES COVERED</b> Final 3/1/89-9/1/94	
<b>4. TITLE AND SUBTITLE</b> New Approaches to Hepatitis A Vaccine Development		<b>5. FUNDING NUMBERS</b>  Grant No.  DAMD17-89-Z-9022	
<b>6. AUTHOR(S)</b>  Stanley M. Lemon, M.D.		<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>  5-35868	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> The University of North Carolina at Chapel Hill CB# 7030, 547 Burnett Womack Chapel Hill, NC 27599-7030		<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research & Materiel Command Fort Detrick Frederick, MD 21702-5012		<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>			
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for public release; distribution unlimited		<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 words)</b>  Neutralization epitopes on the surface of the hepatitis A virus (HAV) capsid were characterized in detail. A single antigenic site on the virus capsid was found to be reactive with one murine neutralizing monoclonal antibody, but larger peptides representing this region did not elicit anti-HAV neutralizing antibodies in rabbits. Poliovirus antigenic chimeras were engineered which expressed amino acid sequences of HAV epitopes, but these chimeras lacked relevant HAV antigenicity and immunogenicity. The structure and function of the 5' nontranslated region of the genome was characterized. Translational control elements were mapped. Infectious HAV cDNA constructs were engineered which contain large deletion mutations within a pyrimidine rich region located immediately upstream of the internal ribosomal entry site. Some of these mutations result in a marked temperature-sensitive phenotype. The attenuating effect of these mutations remains under study. Significant qualitative differences were demonstrated in anti-HAV antibodies present following administration of immune globulin vs. active immunization.			
<b>14. SUBJECT TERMS</b>  Hepatitis A vaccine, Synthetic oligopeptides, monoclonal antibodies		<b>15. NUMBER OF PAGES</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified		<b>16. PRICE CODE</b>	
<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

**FOREWORD**

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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.

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 (NIH Publication No. 86-23, Revised 1985).

In conducting research utilizing recombinant DNA technology, the investigators adhered to current guidelines promulgated by the by the National Institutes of Health, including the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

*Stan G. Hom*

28 Oct 1994

PI

Date

<b>Accession For</b>	
NIIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By _____	
Distribution/_____	
<b>Availability Codes</b>	
Dist	Avail and/or Special
A-1	

**TABLE OF CONTENTS**

Title Page ----- 1  
Report Documentation Page (DOD1473) ----- 2  
Foreword ----- 3  
Table of Contents ----- 4  
Abstract ----- 5  
Introduction ----- 6  
Narrative Report ----- 8  
Conclusions ----- 20  
References ----- 21

**ABSTRACT**

Neutralization epitopes on the surface of the hepatitis A virus (HAV) capsid were characterized in detail. A single antigenic site on the virus capsid was found to be reactive with one murine neutralizing monoclonal antibody, but larger peptides representing this region did not elicit anti-HAV neutralizing antibodies in rabbits. Poliovirus antigenic chimeras were engineered which expressed amino acid sequences of HAV epitopes, but these chimeras lacked relevant HAV antigenicity and immunogenicity. The structure and function of the 5' nontranslated region of the genome was characterized. Translational control elements were mapped. Infectious HAV cDNA constructs were engineered which contain large deletion mutations within a pyrimidine rich region located immediately upstream of the internal ribosomal entry site. Some of these mutations result in a marked temperature-sensitive phenotype. The attenuating effect of these mutations remains under study. Significant qualitative differences were demonstrated in anti-HAV antibodies present following administration of immune globulin vs. active immunization.

## INTRODUCTION

**Hepatitis A as a military hazard.** Hepatitis A is a potentially debilitating infectious disease which may reach epidemic proportions under poor sanitary conditions. Military history records many instances in which epidemics of this disease have substantially hindered the operational effectiveness of armed forces (Bancroft and Lemon, 1984). Because hepatitis A is less prevalent in the United States than in many other countries, the prevalence of antibody to hepatitis A virus (HAV) is very low among American soldiers. Relatively recent surveys suggest that less than 15% of American soldiers have naturally acquired anti-HAV (Bancroft and Lemon, 1984), indicating that the vast majority of soldiers are susceptible to hepatitis A. In the 1990s and beyond, American forces are most likely to encounter this virus when deployed overseas to developing regions where hepatitis A may be endemic. The risk of exposure to HAV is substantially magnified when previously existing public health facilities and sanitation practices are disrupted by military conflict, such as occurred within Kuwait during Operation Desert Storm. This recent large scale mobilization of American forces reemphasized the risks and special problems posed by HAV to military operations. While short-term protection against hepatitis A was provided by timely administration of immune globulin (IG) prior to deployment, supplies of IG were rapidly exhausted. In addition, readministration of IG is required at a minimum of 6 month intervals in order to maintain continued protection. This was not a problem due to the short duration of Operation Desert Storm, but reimmunization with IG may be difficult to accomplish with troops which remain deployed for periods longer than 6 months. There is thus a critical need for a vaccine that is capable of providing safe, long-term, active immunity against HAV. It is important that such a vaccine be available to the military forces of the United States at reasonable cost.

**Formalin-inactivated hepatitis A vaccines.** Following the successful development of a prototype, formalin-inactivated HAV vaccine produced in cell culture by Army investigators (Binn et al., 1986), formalin-inactivated HAV vaccines have been developed by several commercial vaccine manufacturers (for recent reviews, see Siegl and Lemon, 1990, and Lemon and Shapiro, 1994). A clinical study of an inactivated HAV vaccine produced by Merck & Co. (VAQTA™) was carried out in children in Monroe, NY, during 1991. This study demonstrated a high level of protection against symptomatic hepatitis A following administration of one dose of this vaccine (Werzberger et al., 1992). A similar study carried out in Thailand by U.S. Army investigators demonstrated a similar level of efficacy after two doses of an inactivated HAV vaccine produced by SmithKline Beecham (HAVRIX™) (Innis et al., 1994). While similar efficacy studies have not been carried out in adults, these vaccines appear to offer a level of protection that would be more than adequate for protection of military forces operating in hepatitis A endemic regions (Lemon, 1994).

However, several problems continue to hinder the use of these formalin inactivated HAV vaccines by the U.S. military. Neither of these vaccines is yet licensed by the Food and Drug Administration for use within the United States. In addition, available data suggest that multiple doses of formalin inactivated HAV vaccines will be required in adults in order to elicit even moderately long-lasting protective levels of immunity. Multiple-dose schedules, with late booster doses given at least 6 months after the first immunization, may prove inconvenient for use in military populations. Furthermore, the costs of these vaccines is likely to be high. This is due to the comparatively poor in vitro yields of antigen obtained with current vaccine virus strains, as well as the purification procedures required for production of an acceptable, modern vaccine. The current retail price of HAVRIX™ in Switzerland is approximately US\$ 48.00 per dose (Lemon and Shapiro, 1994). Although competition and government procurement practices are certain to result in lower purchase prices, the high costs of these inactivated vaccines are likely to prohibit the universal use of inactivated vaccines among U.S. military forces for many years. Because of the uncertainties concerning the future use of inactivated HAV vaccines by

the U.S. military, work under this grant has focused on efforts to develop alternative approaches to development of HAV vaccines.

Recent clinical trials of inactivated vaccines (Werzberger et al., 1992; Innis et al., 1994) have offered a unique opportunity to determine serological correlates of protection against hepatitis A. Accordingly, we have taken advantage of the availability of serum samples collected during the Monore efficacy trial of the Merck vaccine (VAQTA™) (Werzberger et al., 1992) to evaluate the protective immunity conferred by this vaccine. This was accomplished using a combination of quantitative, solid-phase radioimmunoassay, viral neutralization assays, and a novel viral radioimmuno-precipitation assay (RIPA) for anti-HAV antibodies developed under support of this contract. These studies will provide useful benchmarks for future HAV vaccine trials.

**Subunit peptide vaccines for protection against hepatitis A.** In preliminary studies, we identified an immunodominant neutralization antigenic site on the surface of the HAV capsid (Stapleton et al., 1987; Ping et al., 1988; Cox et al., 1990). These studies indicated that the  $\beta$ B- $\beta$ C loops of capsid proteins VP3 and VP1 contribute to an immunogenic structure on the virus surface that dominates in the human immune response (Ping et al., 1988). Although, this site appeared to be largely conformationally defined, we reasoned that short oligopeptide sequences representative of the relevant regions of VP3 and VP1 should be both antigenic and potentially immunogenic with respect to HAV neutralizing activity. This has been shown to be the case with peptides representative of antigenic sites in other picornaviruses, including type 1 poliovirus (Chow et al., 1985), human rhinovirus 14 (Francis et al., 1987), and foot-and-mouth disease virus (FMDV) (Bittle et al., 1982). Peptide immunogens are highly stable reagents; they are potentially very inexpensive and extremely safe inasmuch as they are chemically defined. We had previously shown that only low levels of neutralizing antibody are required for protection against hepatitis A (Stapleton et al., 1985), a fact which was recently confirmed during an efficacy trial with the Merck inactivated HAV vaccine (Werzberger et al., 1992). We thus postulated that peptide immunogens might have practical application to the prevention of hepatitis A. A large component of the research supported by this contract thus focused on exploring the potential for development of HAV peptide vaccines. As described below, this work encompassed both a detailed characterization of neutralization epitopes on the surface of the HAV capsid, as well as the evaluation of the antigenicity and immunogenicity of multiple synthetic HAV peptides. In addition, we evaluated poliovirus antigenic chimeras expressing segments of the HAV capsid proteins.

**Live, attenuated HAV vaccines.** Previous attempts to develop an attenuated HAV vaccine have focused on several cell culture-adapted virus strains (for a review, see Lemon, 1985; Siegl and Lemon, 1990). However, such cell culture-adapted viruses appear to replicate poorly in the primate liver, and have been shown to have relatively poor immunogenicity in humans. Less highly passaged cell culture-adapted variants might offer better immunogenicity, but are likely to have unacceptable hepatitis-inducing activity. A better understanding of the molecular basis of attenuation for existing attenuated HAV stains and the mechanisms responsible for the adaptation of HAV to growth in cell culture would be very helpful to the rational development of new and potentially better vaccine candidates. In addition, novel approaches to selecting attenuated HAV vaccine candidate strains should be accorded a high priority. During the course of this contract, studies in our laboratory have focused on the 5' nontranslated region (5'NTR) of the HAV genome and its role in the adaptation of the virus to growth in cell culture (Brown et al., 1991; Day et al., 1992; Brown et al., 1994; Whetter et al., 1994). These studies provided a detailed view of the structure and function of this important region of the viral genome, and led to the construction of several viable HAV mutants with large deletions within the 5'NTR (Shaffer et al., 1994). Similar mutations have since been constructed within the genetic background of virulent HM175 virus. It is likely that one or more of these mutants possess an attenuation phenotype that may serve useful as the basis for development of a

novel attenuated vaccine candidate. The attenuation phenotype of one of these new mutants is currently being evaluated in collaboration with Dr. Suzanne Emerson and Robert Purcell of the National Institute of Allergy and Infectious Diseases.

**Molecular epidemiology and genetic diversity of HAV.** Efforts at vaccine prevention of hepatitis A will be aided by a firm understanding of the extent of genetic diversity among HAV strains circulating worldwide as well as knowledge of the molecular epidemiology of this virus. Thus, under support of this grant, we evaluated the genotypic variation among human and primate HAV strains and the worldwide distribution of specific HAV genotypes. These studies were facilitated by the development of a polymerase chain reaction-based method for direct sequence analysis of virus present in fecal samples (antigen-capture PCR, Jansen et al., 1990). The results of these studies indicate that there are two major genotypes of human HAV which are each distributed in a global fashion, as well as two additional minor genotypes which are represented thus far by only one strain each (Jansen et al., 1990; Robertson et al., 1992). All evidence accumulated to date indicates that these human HAV genotypes are antigenically closely related if not identical. In collaboration with Dr. John Ticehurst at WRAIR, the antigen-capture PCR methodology has been modified for detection and analysis of fecal hepatitis E virus, another health hazard of military significance.

## NARRATIVE SUMMARY OF RESEARCH

The following section contains a narrative summary of research which was accomplished under support of this contract. More complete details for each of the research areas described below may be found in the 5 annual reports which have been submitted previously to the U.S. Army Medical Research and Development Command, and appropriate references to these reports are included herein.

### 1. Evaluation of synthetic peptide immunogens for protection against HAV

We adopted several experimental approaches to this problem:

**Epitope mapping studies.** We extended efforts to map the neutralization epitopes of HAV by the isolation and characterization of additional monoclonal antibody resistant neutralization escape HAV mutants. Studies examining the neutralization resistance phenotypes of these mutants, coupled with identification of the capsid protein mutations responsible for neutralization escape, resulted in the most complete mapping to date of epitopes recognized by murine and human monoclonal antibodies (Ping and Lemon, 1992; Day et al., 1991) (see Annual Report No. 3). Altogether, we characterized a series of 21 murine monoclonal antibody-resistant, neutralization escape mutants derived from the HM175 virus strain. The escape phenotype of each mutant was associated with reduced antibody binding in radioimmunofocus assays. Neutralization escape mutations were identified at Asp-70 and Gln-74 residues of the capsid protein VP3, as well as Ser-102, Val-171, Ala-176, and Lys-221 of VP1. With the exception of the Lys-221 mutants, substantial cross-resistance was evident among escape mutants tested against a panel of 22 neutralizing monoclonal antibodies, suggesting that the involved residues contribute to epitopes comprising a single antigenic site. As mutations at one or more of these residues conferred resistance to 20 of 22 murine antibodies, this site appears to be immunodominant in the mouse. However, multiple mutants selected independently against any one monoclonal antibody had mutations at only one or at the most two amino acid residues within the capsid proteins, confirming that there are multiple epitopes within this antigenic site and suggesting that single amino acid residues contributing to these epitopes may play key roles in the binding of individual antibodies. A second, potentially independent, antigenic site was identified by three escape mutants with different substitutions at Lys-221 of VP1. These

mutants were resistant only to antibody H7-C27, while H7-C27 effectively neutralized all other escape mutants. These data support the existence of an immunodominant neutralization site in the antigenic structure of HAV which involves residues of VP3 and VP1, and a second, potentially independent site involving residue 221 of VP1.

In collaboration with Dr. Leonard Stein of the University of North Carolina, we evaluated the human antibody response to HAV by similar characterization of several neutralizing human monoclonal antibodies. Peripheral blood lymphocytes collected from a patient with recent hepatitis A infection were transformed with Epstein-Barr virus and grown in limiting dilution culture (Day et al., 1991). Three transformed lines secreting anti-HAV antibodies were established in continuous culture. Antibody secreted by two cell lines failed to neutralize an escape mutant with a substitution involving Asp-70 of VP3. One of these antibodies also demonstrated only weak neutralizing activity against escape mutants with substitutions at Ser-102 and Ser-114 of VP1 (Day et al., 1991; Ping et al., 1992; Cox et al., 1990). These resistance patterns are similar to those identified with murine monoclonal antibodies to HAV (Ping et al., 1992), indicating that the human B-cell response is comprised, at least in part, of antibodies directed against epitopes overlapping those recognized by the mouse. In more recent collaborative studies with Dr. Alan Lewis of the Wellcome Research Laboratories, Beckingham, we demonstrated excellent neutralizing activity associated with a recombinant human monoclonal antibody to HAV produced in chinese hamster ovary cells (Lewis et al., 1993).

**Protease sensitivity of the HAV capsid.** We examined the protease sensitivity of the HAV capsid. We found that high concentrations of either trypsin or chymotrypsin caused nearly complete cleavage of capsid protein VP2 of hepatitis A virus, but did not significantly reduce the infectivity, thermostability or antigenicity of the virus (Lemon et al., 1991). Chymotrypsin also had a lesser effect on VP1. These findings indicate the presence of a protease-accessible VP2 surface site which neither contributes significantly to the dominant antigenic site nor plays a role in attachment of virus to putative cell receptors.

**Octapeptide PEPSCAN analysis and immunogenicity of synthetic peptides.** Based on the information provided by the epitope mapping studies, we synthesized on polyethylene pins over 240 nested octapeptides (7 residue overlaps) representing the primary sequence of regions of the HAV capsid proteins VP3 (residues 50-91) and VP1 (residues 80 to 300) which the escape mutant analysis indicated to be involved in the binding of neutralizing antibodies. We probed such peptides with monoclonal and polyclonal anti-HAV antibodies in peptide ELISAs (PEPSCAN) (Geysen et al., 1984, 1987). All but one of the panel of neutralizing monoclonal antibodies described above failed to bind to any of these octapeptides, indicating that the cognate neutralization epitopes are conformationally determined. These results were consistent with other studies carried out in collaboration with Dr. David Sangar of Wellcome Biotech, Beckingham, which failed to find neutralizing antibodies in guinea pigs immunized with peptides representing 30 different segments of the three capsid proteins of HAV (Lemon et al., 1991). However, the PEPSCAN approach resulted in the identification of linear peptide sequences located at or near the carboxyl terminus of VP1 of HAV, which is recognized by the unique H7-C27 antibody which binds outside of the immunodominant antigenic site (Ping and Lemon, 1992). It is interesting that the H7-C27 epitope, as defined by PEPSCAN, is located approximately 35 amino acid residues downstream of the Lys-221 residue at which several different mutations confer resistance to H7-C27 mediated neutralization (Ping et al., 1992). These data thus suggest that mutations at Lys-221 cause resistance against H7-C27 not by altering residues within the antibody-binding site proper, but by a distal effect on the conformation of the carboxyl terminal segment of the VP1 molecule. Several peptides representing the sequence of the H7-C27 reactive region were synthesized in bulk and conjugated to keyhole limpet hemocyanin, but antibodies raised to these peptides in rabbits and guinea pigs failed to neutralize HAV (see Annual Report No. 4).

**Poliovirus-HAV antigenic chimeras.** We characterized the antigenicity and immunogenicity of chimeric picornaviruses in which selected HAV peptide sequences replaced residues within an antigenic loop of capsid protein VP1 of the Sabin type 1 poliovirus (see Progress Report Nos. 1 and 2). This work was carried out in collaboration with Prof. Jeffrey Almond of the University of Reading and Dr. Phillip Minor of the National Institute of Biologic Standardization and Control (NIBSC), Potters Bar. Chimeric picornaviruses were created by inserting peptide sequences from HAV capsid proteins into the  $\beta$ B- $\beta$ C loop of VP1 of Sabin strain type 1 poliovirus (PV-1). Fifteen viable chimeras were generated. Each retained the ability to be neutralized by polyclonal PV-1 antisera (Lemon et al., 1992). Two chimeras (H15 and H2) stimulated production of low levels of HAV neutralizing antibodies in immunized rabbits or mice, although in both cases only a small fraction of immunized animals produced this response. The H15 chimera, which contains residues 13-24 of HAV VP1, elicited HAV neutralizing antibodies in 3 of 9 rabbits and at least 1 of 7 immunized mice. These results indicate that a neutralization domain exists in this region of VP1. However, human sera with high titers of antibodies to HAV failed to immunoprecipitate or neutralize this chimera, suggesting the absence of a significant antibody response to this neutralization domain following natural infection. Sera from rabbits immunized with H15 that did not develop HAV neutralizing antibodies contained antibodies reactive with the HAV peptide segment expressed by the H15 virus, indicating substantial differences in the specificities of antibodies elicited by this peptide segment among individual immunized rabbits. These results suggest that the H15 HAV peptide segment may assume more than one immunogenic conformation within the capsid structure of the chimera, and that the amino terminal neutralization domain present in HAV VP1 has important conformational attributes. Flexibility of the H15 peptide insert was further suggested by a high level of sensitivity of this chimera to neutralization by a related anti-peptide antibody which was itself devoid of HAV neutralizing activity. One of 16 rabbits immunized with the H2 chimera (residues 101-108 of HAV VP1) developed HAV neutralizing antibodies, confirming both the presence and highly conformational nature of a neutralization antigenic site involving these residues of HAV (Lemon et al., 1992).

Two additional poliovirus antigenic chimeras expressing the VP1 H7-C27 linear epitope described in (2) above were constructed during the final year of the contract. These chimeras are not neutralized by the H7-C27 antibody, but their immunogenicity remains to be evaluated (see Annual Report No. 5).

**Summary.** This extensive series of studies provides strong evidence that the major neutralization epitopes of HAV are exquisitely dependent upon the conformation adopted by individual polypeptides within the context of the native capsid structure. It is likely that single epitopes are comprised of residues derived from multiple capsid polypeptides, as both VP1 and VP3 residues are involved in the major immunodominant antigenic site (Ping and Lemon, 1992). Although the reactivity of the H7-C27 monoclonal antibody with linear peptide sequences provides a tantalizing suggestion that peptides representing this region of VP1 might have relevant immunogenicity if properly displayed, work in this direction has thus far been disappointing. In aggregate, these studies indicate that subunit approaches to HAV vaccine development are not likely to be successful unless they involve the expression and assembly of the entire HAV capsid structure.

## **2. Mutations associated with attenuation phenotype of cell culture-adapted HAV**

We continued efforts to better understand the molecular basis of the attenuation of HAV that frequently accompanies the adaptation of the virus to growth in cell culture. This work follows that supported under a previous contract with the U.S. Army Medical Research and Development Command (DAMD17-85-C-5272).

**Attenuation phenotype of the HM175/P16 virus.** In an effort to characterize mutations which are responsible for the cell culture-adaptation and attenuation of HAV, we molecularly cloned and fully sequenced a cell culture-adapted HAV variant (HM175/P16 virus, Jansen et al., 1988) which was initially isolated by Binn and coworkers at the Walter Reed Army Institute of Research (Binn et al., 1984). Although owl monkey challenge experiments with a passage-related virus, HM175/S30 (a neutralization escape variant) suggested that HM175/P16 might be virulent (Lemon et al., 1990), a study carried out in collaboration with COL. James LeDuc, USAMRIID indicated that the plaque-purified HM175/P16 virus is highly attenuated in owl monkeys (Taylor et al., 1993) (see Annual Report No. 4).

In this collaborative study, we assessed the attenuation/virulence phenotype of HM175/P16 virus in 4 anti-HAV negative *Aotus nancymai* monkeys. During the 10 month period of baseline observation, serum ALT values in the four owl monkeys ranged from 49.5 to 68.3 units/L, and AST values from 99.8 to 161.9 units/L (Taylor et al., 1993). Significant elevations of these serum enzymes (greater than 2.5 fold above the baseline mean value) occurred in only one animal (769) following intravenous inoculation with the HM175/P16 virus. This animal had a baseline mean ALT of 68.3 units/L, and developed serum ALT levels of 202 and 221 (reflecting 3.0- and 3.2-fold increases above the baseline mean), 15 and 26 days following virus inoculation. AST levels in animal 769 also peaked 15 days after inoculation, at 3.1-fold above the mean baseline value (see Annual Report No. 4). Liver biopsies were examined by Dr. L. Asher of WRAIR. The initial biopsies from all 4 animals showed normal histology, and normal ultrastructure by electron microscopy. Liver biopsies taken 21 days after inoculation from monkeys 769 and 778 showed minimal non-specific changes: swelling of hepatocytes and increased eosinophilia and granularity of the cytoplasm with focal disruption of the liver cell plates. Biopsies taken 37 days after inoculation from monkeys 1017 and 1022 (day 37) were normal. Inflammation and necrosis were not present in any of the biopsies taken after inoculation. The only abnormalities noted by electron microscopy were in the second biopsy from 769. This biopsy specimen revealed degenerative changes in the cytoplasm of hepatocytes, including swollen mitochondria, dilated endoplasmic reticulum and disrupted cellular membranes. Viral antigen was not detected in tissue sections by immunofluorescence.

Only 3 of the 4 animals developed antibodies to HAV following inoculation with HM175/P16 virus. The earliest and strongest antibody response occurred in animal 769, which developed antibodies to HAV that were detectable in a competitive-inhibition radioimmunoassay by 40 days after inoculation (Taylor et al., 1993). Sera collected from this animal 60 or more days after inoculation competed strongly (generally >85%) with radiolabeled human polyclonal IgG for binding to HAV in the radioimmunoassay. Antibody was not detected in animal 1017 until 68 days after inoculation, and only competed weakly (up to 66%) with labelled antibodies in the radioimmunoassay even at 96 days following inoculation (postinfection chimpanzee serum employed as a control in these assays generally blocked the binding of over 90% of labelled human polyclonal antibody) (data not shown). Antibody was not noted in the third animal (1022) until 307 days following inoculation, when all four animals were rebled and tested by a commercial immunoassay (HAVAB™, Abbott Laboratories). The interval between inoculation of HM175/P16 virus and the first appearance of antibodies to HAV was substantially longer than that we have observed in earlier studies of owl monkeys inoculated with virulent hepatitis A viruses carried out in collaboration with USAMRIID and WRAIR investigators (Taylor et al., 1993).

Because HM175/P16 virus is well adapted to growth in cell culture, it was possible to monitor virologic events in infected monkeys using radioimmunofocus assays which provided a quantitative measure of the titer of virus present. Viremia was demonstrated only in animal 769, in which virus was present continuously in the serum from day 8 to day 33 following inoculation, peaking at  $2.7 \times 10^3$  rfu/ml on day 33. Fecal samples collected from the 2 animals which developed antibodies to HAV within 96 days of inoculation were similarly tested by

radioimmunofocus assay for the presence of infectious virus. Infectious virus was identified only in the feces of animal 769. This animal shed infectious virus from 12 to at least 44 days (the last day tested) following virus inoculation. Peak fecal shedding of virus ( $1.3 \times 10^5$  rfu/gm feces) occurred 30 days after inoculation. Virus was not recovered from fecal samples collected from animal 1017 up to 44 days after inoculation, although this animal also developed anti-HAV antibodies within 68 days of inoculation. None of the fecal samples collected from 769 or 1017 generated a reproducible positive result in a radioimmunoassay for detection of HAV antigen (data not shown). This reflects the low level of fecal shedding even by animal 769. Virus was not recovered in cultures inoculated with throat swabs taken from any animal.

The genome of HM175/P16 virus contains a total of 19 mutations from the reported sequence of the wild-type virus genome (Jansen et al., 1988). Only 8 of these mutations result in amino acid substitutions within the HAV polyprotein, while 6 mutations are located within nontranslated regions of the genome. Although independently adapted to growth in cell culture, the sequence of the attenuated HM175/P35 virus studied at the National Institutes of Health (Cohen et al., 1987) shares a number of mutations from the wild-type genome in common with HM175/P16 virus within the 5'NTR, and the VP2, 2B and 3D<sub>pol</sub> proteins (Jansen et al., 1988). Current evidence suggests that a relatively small number of mutations in the P2 (proteins 2B and 2C) and 5'NTR of the genome are responsible for cell culture-adapted phenotypes of the virus (Emerson et al., 1992; Day et al., 1992). Less is known about mutations in the viral genome which result in attenuation of the hepatovirulence of HAV. Although these two virus phenotypes are related, it is clear that not all cell culture-adapted viruses are attenuated (Lemon et al., 1990; Taylor et al., 1993). Cohen et al. (1989) inoculated marmosets with viruses rescued from chimeric infectious cDNA clones derived from wild-type and attenuated variants of the HM175 strain of HAV. The results of these studies suggested that attenuation of HM175/P35 virus for marmosets could be attributed to mutations within the P2 and P3 regions of the HAV genome. However, further studies will be required in order to learn whether or not the attenuation phenotypes of HM175/P16 and HAV/7 derive from a common set of mutations that are present in both virus variants. It is not clear whether the minimal disease and more active replication of virus observed in animal 769 represented partial reversion of the attenuation phenotype of HM175/P16 virus.

**Role of mutations within the 5'NTR in cell culture adaptation of HAV.** We demonstrated that several mutations present within the 5'NTR of the attenuated HM175/P16 virus, at bases 152 and/or 203-7 and base 687, act to enhance the growth of HAV in BS-C-1 cells (see Report No. 3) (Day et al., 1990; Day et al., 1992). We constructed chimeric infectious cDNA clones in which regions of the 5'NTR of cell culture-adapted HM175/P35 virus were replaced with cDNA from either wild-type virus (HM175/wt) or HM175/P16 which was independently isolated, but is a closely related cell culture-adapted virus (Day et al., 1992). Substitution of the complete 5'NTR of HM175/P35 with the 5'NTR of HM175/wt resulted in virus with very small replication foci in continuous African green monkey kidney (BS-C-1) cells, indicating that 5'NTR mutations in HM175/P35 virus are required for optimal growth in these cells. A chimera with the 5'NTR sequence of HM175/P16 retained the large focus phenotype of HM175/P35 virus, while the growth properties of other viruses having chimeric 5'NTR sequences indicated that mutations at bases 152 and/or 203-7 enhance replication in BS-C-1 cells. These findings were confirmed in one-step growth experiments, which also indicated that radioimmunofocus size is a valid measure of virus replication competence in cell culture. An additional mutation at base 687 of HM175/P16 had only a minor role in enhancing growth. In contrast to their effect in BS-C-1 cells, these 5'NTR mutations did not enhance replication in continuous fetal rhesus kidney (FRhK-4) cells. Thus, mutations at bases 152 and/or 203-7 enhance the replication of HAV in a highly host cell-specific fashion. We suspect that these mutations alter the affinity of the 5'NTR RNA for specific cellular proteins which play a role in viral replication, possibly by supporting or repressing viral translation. These hypothetical alterations in the affinity of the 5'NTR RNA for cellular proteins could be due either to subtle

changes in the secondary or tertiary structure of the RNA, or to changes in the primary structure (RNA sequence) of a protein binding site (see below).

**Mutations associated with cytopathic HAV.** Variants of HAV (pHM175 virus) recovered from persistently infected green monkey kidney (BS-C-1) cells (pHM175 virus) induced a cytopathic effect during serial passage in BS-C-1 or fetal rhesus kidney (FRhK-4) cells (Cromeans et al., 1989). Epitope-specific radioimmunofocus assays showed that this virus comprised two virion populations, one with altered antigenicity including neutralization-resistance to monoclonal antibody K24F2, and the other with normal antigenic characteristics (Lemon et al., 1991). Replication of the antigenic variant was favored over virus with normal antigenic phenotype during persistent infection, while virus with the normal antigenic phenotype was selected during serial passage. Viruses of each type were clonally isolated and designated HM175/18f (normal antigenicity) and HM175/43c (escape mutant). Both viruses were cytopathic in cell cultures and displayed a rapid replication (RR/CPE<sup>+</sup>) phenotype when compared to the noncytopathic HM175/P16 virus which is closely related to the virus used to establish the original persistent infection. The two cytopathic virus clones contained 30 and 33 nucleotide changes from the sequence of HM175/P16 virus. Both shared a common 5' sequence (bases 30-1677), as well as sequence identity in the P2/3 region (bases 3249-5303 and 6462-6781) and 3' terminus (bases 7272-7478). VP3, VP1 and 3CP<sup>10</sup> contained different mutations in the two virus clones, with amino acid substitutions at residues 70 of VP3 and 197 and 276 of VP1 of the antigenic variant. These capsid mutations did not affect virion thermal stability. A comparison of the nearly complete genomic sequences of three clonally isolated cytopathic variants was suggestive of genetic recombination between these viruses during persistent infection, and indicated that mutations in both 5' and 3' nontranslated regions and in the nonstructural proteins 2A, 2B, 2C, 3A, and 3DPol<sup>1</sup> may be related to the RR/CPE<sup>+</sup> phenotype.

The location of mutations in HM175/18f (Lemon et al., 1991) (see Annual Report No. 2) suggested an important role for the 2C protein in controlling the enhanced growth of this virus in cell culture. As reported in Annual Report No. 3, we constructed a series of recombinant full-length infectious cDNA clones in which we replaced the 5'NTR, P2 and 3'NTR regions of the infectious pHAV/7 clone (HM175/P35 virus, Cohen et al., 1989) with cDNA amplified from corresponding regions of HM175/18f virion RNA by polymerase chain reaction. The validity of these constructions was confirmed by DNA sequence analysis. Transfection of FRhK-4 cells with RNA transcribed from these constructs demonstrated that mutations in the P2 region of HM175/18f confer a rapid replication (RR<sup>+</sup>) phenotype on the HM175/P35 virus (generating large foci in 7 days compared with 14 days in transfection/radioimmunofocus assays) (see Report No. 3). These results were subsequently confirmed in BS-C-1 cells using virus rescued from the original transfections. There was no apparent enhancement of the growth of pHAV/7 (HAV/7) recombinants in either cell type with the additional inclusion of the 5'NTR of HM175/18f virus. Because radioimmunofoci generated by HM175/P35 recombinants containing the 5'NTR, P2 and 3'NTR sequences of HM175/18f were significantly smaller than foci generated by HM175/18f virus itself, additional mutations outside of these regions of HM175/18f virus are likely to contribute significantly to its growth properties.

Additional chimeric viruses have been constructed to define in greater detail the role played by individual P2 proteins, as well as other regions of the HM175/18f genome, in determining the RR/CPE<sup>+</sup> phenotype. These more recent studies indicate that mutations in the 2AB and 2C proteins act cooperatively and are both required for rapid growth of the RR/CPE<sup>+</sup> virus. Mutations in the 5'NTR and P3 regions do not appear to play a role in the rapid growth phenotype. These studies are important because they are providing information concerning the genetic determinants of rapid growth of HAV in cell culture. This information may prove useful in designing candidate vaccine viruses which replicate more efficiently in cell culture. In addition, these studies have provided an infectious cDNA clone of a virus with a rapid

replication phenotype (pG3/7-18fP2) which has proven very useful in other molecular genetic studies of HAV (see below, Shaffer et al., 1994).

### 3. Structure and function of the 5'NTR of HAV

As described above, we demonstrated that mutations in the 5' NTR of HAV play an important role in the host-range change accompanying adaptation of the virus to growth in cell culture (Day et al., 1992). The fact that similar 5'NTR mutations have been shown to be critical for the attenuation of the Sabin oral poliovirus vaccine strains (Minor, 1992) suggests that these 5'NTR mutations may play a role in the attenuation of HM175/P16 virus. These findings thus led us to initiate a detailed analysis of structure-function relationships within the 5'NTR of HAV.

**Secondary structure of the 5'NTR RNA.** As described in Annual Report No. 2, we developed a model of the secondary structure of the 5'NTR of HAV, and validated this model by physical means (Brown et al., 1991). We determined the nearly complete nucleotide sequence of the 5'NTR of two genetically divergent HAV strains (PA21 and CF53), and included these data in a comparative phylogenetic analysis of the HAV 5'NTR. We identified covariant nucleotide substitutions predictive of conserved secondary structures, and utilized this information to develop a model of the 5'NTR secondary structure which was further refined by thermodynamic predictions and physical probing of synthetic RNA transcripts with single- and double-strand ribonucleases. According to this model (Brown et al., 1991), the 5'NTR comprises six major structural domains. Domains I and II (bases 1-95) contain a 5' terminal hairpin and two stem-loops followed by a single-stranded and highly variable pyrimidine-rich tract (96-154). The remainder of the 5'NTR (domains III-VI, bases 155-734) contains several complex stem-loops, one of which may form a pseudoknot, and terminates in a highly conserved region containing an oligopyrimidine tract preceding the putative start codon by 13 bases. To determine which structural elements might function as an internal ribosome entry site (IRES), RNA transcripts representing the HAV 5'NTR with progressive 5' deletions were translated in rabbit reticulocyte lysates. The translation product was truncated, unprocessed P1 polyprotein. Removal of the 5'-terminal 354 bases of the 5'NTR had little effect on translation. However, deletion to base 447 slightly decreased translation, while deletion to 533 almost completely abolished it. These data indicate that sequences 3' of base 355 play an important role in the translation mechanism utilized by genomic-length HAV RNA. Significantly, this region shares several conserved structural features with the IRES element of murine encephalomyocarditis virus (EMCV).

**Demonstration and mapping of an IRES element in the HAV 5'NTR.** Based on preliminary studies involving the *in vitro* translation of 5' terminally truncated RNA transcripts representing segments of the 5'NTR (Brown et al., 1991), we considered it likely that the NTR contains an IRES element functioning in the initiation of viral translation (see Annual Report No. 2). To formally test this hypothesis, synthetic bicistronic RNAs, with all or part of the HAV 5'NTR placed in the intercistronic space, were translated in rabbit reticulocyte lysates (Brown et al., 1994). In the presence of an upstream cistron designed to block ribosomal scanning, the HAV 5'NTR was capable of directing the internal initiation of translation, confirming the presence of an IRES. Analysis of various deletion mutants demonstrated that the 5' border of the IRES is located between nucleotides 151 and 257, while the 3' border extends to the 3' end of the 5'NTR, between nucleotide 695 and the first initiation codon at 735. Except for a segment between bases 638 and 694, deletion of stem loop structures between bases 151 and the 3' end of the 5'NTR inhibited or abolished translation. The addition of a 5' cap structure ( $m^7GpppN$ ) to monocistronic or bicistronic transcripts decreased the translation of a reporter gene downstream of the HAV 5'NTR, but enhanced translation of the upstream cistron in bicistronic transcripts. This indicates that a 5' cap structure is inhibitory to HAV IRES-directed translation initiation and that the cap structure and the HAV IRES probably compete for the same limiting translation factors. The efficiency with which monocistronic constructs containing the HAV 5'NTR

directed translation in reticulocyte lysates was compared with monocistronic constructs containing the IRES of the more rapidly growing EMCV. These results indicated that the HAV 5'NTR was more than 25-fold less active than the EMCV IRES in producing translation product. HAV 5'NTR directed translation was inhibited by the presence of a one-fifth molar quantity of RNA containing the EMCV IRES, while a 5-fold molar excess of the HAV 5'NTR did not inhibit EMCV IRES directed translation. The relatively weak activity of the HAV IRES may thus be due to a reduced affinity for cellular translation factors which are present in limiting quantities in rabbit reticulocyte lysate. This low level IRES activity may contribute to the slow and inefficient replication cycle of HAV.

**HAV translational control in vivo.** Since important differences have been found between translation of poliovirus RNA in rabbit reticulocyte lysates and in vivo, we considered it important to gain an understanding of HAV IRES function in a biologically relevant in vivo system (Whetter et al., 1994). Toward this end, we created an HAV-permissive monkey kidney cell line (BT7-H cells) that stably expresses T7 RNA polymerase and which carries out cytoplasmic transcription of uncapped RNA from transfected plasmids containing the T7 promoter. In this cell line, T7-polymerase-transcribed RNAs containing the HAV 5'NTR fused to sequence encoding bacterial chloramphenicol acetyltransferase were translated very poorly compared with transcripts containing either the IRES of EMCV or a short control leader which did not contain a picornaviral IRES. Transcripts with large 5' deletions in the HAV 5'NTR were translated significantly more efficiently than transcripts containing the full length 5'NTR, most likely reflecting a shift from IRES-directed translation to translation initiation by 5' end-dependent scanning. Surprisingly, transcripts containing a large internal deletion in the 5'NTR (nts 158-633), which were predicted to retain substantial secondary structure at the 5' end, were translated approximately 8-fold more efficiently than transcripts containing the complete 5'NTR. Translation of RNAs containing either the EMCV IRES or the control leader was enhanced 2- to 5-fold by coexpression of the poliovirus 2A protease ( $2A^{pro}$ ), which mediates inhibition of 5' cap-dependent translation of cellular mRNAs.  $2A^{pro}$  also enhanced translation of RNAs containing the HAV 5'NTR with deletions between nucleotides 158-633, 1-532 or 1-633. However, coexpression of  $2A^{pro}$  did not enhance translation of RNAs which contained the full length HAV 5'NTR or the 5'NTR with deletions between nucleotides 86-248 or 1-161, which both retain significant IRES activity in reticulocyte lysates. These data indicate that the HAV IRES functions at a minimal level even in cells which are permissive for HAV replication and that HAV IRES-directed translation is not improved by eliminating competition from capped, cellular mRNAs for limiting translation factors. Again, these data suggest that low levels of translation directed by the 5'NTR may contribute substantially to the slow, minimally-productive growth cycle of HAV in cultured cells.

In recent studies with this BT7-H cell system, we have examined the translational efficiency of bicistronic constructs containing wild-type or HM175/P16 virus 5'NTR segments in the intercistronic space. These studies show that the P16 5'NTR initiates translation significantly more efficiently than the wild-type 5'NTR, providing evidence in favor of the hypothesis that the 5'NTR mutations contribute to enhanced growth of the virus in cell culture by facilitating interactions with cellular translation factors.

**Cellular proteins which bind to the 5'NTR of HM175/P16 virus.** Data presented in the previous section indicate that mutations in the 5'NTR which enhance growth in cell culture (Day et al., 1992) are likely to function by altering the affinity of the viral RNA for specific cellular factors which interact with the 5'NTR, and which are capable of influencing viral replication. Cellular translation initiation factors are good candidates for such proteins, although cellular proteins which bind to the 5'NTR also play roles in replication of the viral RNA.

To define potential interactions between the HAV 5'NTR RNA and cellular factors involved in translation, we characterized the binding of ribosome-associated proteins present in

several cell types to synthetic RNAs representing segments of the 5'NTR using a UV cross-linking/label transfer assay. Five major proteins (p30, p30.5, p39, p57 and p110) were identified (Chang et al., 1993). p30 and p39 were present in ribosomal salt washes prepared only from HAV-permissive BS-C-1 and FRhK-4 cells, while p57 was found only in HeLa cells and rabbit reticulocyte lysates. p30.5 and p110 were present in all cell types. Both p30 and p39 bound to multiple sites within the 5'NTR. Efficient transfer of label to p30 occurred with minimal RNA probes representing nts 96-155, 151-354, and to a much lesser extent 634-744, while label transfer to p39 occurred with probes representing nts 96-155 and 634-744. All of these probes represent regions of the 5'NTR which are rich in pyrimidines. Competitive inhibition studies indicated that both p30 and p39 bound with greater affinity to sites in the 5' half of the NTR (a probe representing nts 1-354), than to the more 3' site (probe 634-744). Binding of p39 (but not p30 or p110) to the probe representing nts 96-155 was inhibited in the presence of an equal amount of proteins derived from HeLa cells, suggesting that p39 shares binding site specificity with one or more HeLa cell proteins. These results demonstrate that ribosome-associated proteins which bind to the 5'NTR of HAV vary substantially among different mammalian cell types, possibly accounting for differences in the extent to which individual cell types support growth of the virus. We suspect that mutations in the 5'NTR which enhance the growth of HAV in certain cell types may reflect specific adaptive responses to these or other proteins.

#### 4. HAV variants with large deletion mutations in the 5'NTR: potential novel attenuated vaccine candidates

**Viable 5'NTR deletion mutants.** The 5'NTR of the HM175 strain of HAV contains several pyrimidine rich regions, the largest and most 5' of which (pY1) is an almost pure polypyrimidine tract located between nts 99-138 which includes five tandem repeats of the sequence motif (U)UUCC(C). Previous modeling of the RNA secondary structure suggested that this region was likely to be single-stranded, but repetitive RNase V1 cleavage sites within these (U)UUCC(C) motifs indicated that pY1 possesses an ordered structure. To assess the role of this domain in replication of the virus, a series of large deletion mutations were created which involved the pY1 domain of an infectious cDNA clone. Deletion of 44 nucleotides between nts 96-139, including the entire pY1 domain, did not reduce the capacity of the virus to replicate in BS-C-1 or FRhK-4 cells, as assessed by the size of replication foci in radioimmunofocus assays or by virus yields under one-step growth conditions. In contrast, viable virus could not be recovered from transfected RNAs in which the deletion was extended in a 5' direction by 3 additional nucleotides ( $\Delta$ 93-134), most likely due to the destabilization of a predicted stem-loop structure upstream of pY1. Deletion mutations extending in a 3' fashion to nts 140, 141 or 144 resulted in moderately ( $\Delta$ 96-140,  $\Delta$ 96-141) or strongly ( $\Delta$ 99-144,  $\Delta$ 116-144, and  $\Delta$ 131-144) temperature-sensitive (*ts*) replication phenotypes. Although deletion of the pY1 domain did not by itself affect the replication phenotype of virus, the additional deletion of sequence elements within the pY1 domain (nts 99-130) substantially enhanced the *ts* phenotype of  $\Delta$ 131-144 virus. These data suggest that the (U)UUCC(C) motifs within the pY1 domain are conserved among wild-type viruses in order to serve a function required during infection *in vivo* but not in cell culture. In contrast, the single-stranded region located immediately downstream of pY1 (nts 140-144) is essential for efficient replication in cultured cells at physiological temperature. Viruses with deletion mutations involving nts 140-144, and viruses with large pY1 deletions but normal replication phenotypes in cell culture. Otherwise virulent viruses with similar mutations may have attenuation properties which could be exploited for vaccine development.

**Nature of the defect in *ts* mutants.** We compared the thermostability of the  $\Delta$ 131-144 virus with that of the P16-pY1 parent, in order to determine whether the reduction in titer of this *ts* strain at the nonpermissive temperature might reflect increased thermolability of virions due to altered interactions between capsid proteins and genomic RNA. The infectious titers of the P16-pY1 and  $\Delta$ 131-144 viruses were reduced to a similar extent following brief incubation at temperatures ranging from 50-60 °C (Shaffer et al., 1994). Thus the *ts* phenotype of  $\Delta$ 131-144

virus is not related to reduced thermostability of the virus. Additional studies suggest that the *ts* phenotype is due to a defect in RNA replication (Shaffer and Lemon, manuscript in preparation). This conclusion is based on the fact that insertion of a *ts* mutation into 5'NTR reporter gene constructs did not lead to reductions in 5'NTR-directed translation in transfected cells which were incubated at the nonpermissive temperature. In addition, RNase protection experiments indicate that the *ts* defect is associated with a marked reduction and delay in positive-strand RNA replication which closely parallels the reductions and delay in formation of infectious HAV (Shaffer and Lemon, manuscript in preparation). These latter findings argue strongly against a defect in viral packaging, and are consistent with the fact that the *ts* defect in virus replication is not corrected by brief shifts to the permissive temperature at either the beginning or end of the infection period (Shaffer and Lemon, manuscript in preparation).

**Impact of 5'NTR deletion mutations on the virulence of HAV.** The work described above was carried out with mutations created within the genetic background of a rapidly replicating, cell culture-adapted and almost certainly highly attenuated virus. This has allowed rapid characterization of the impact of these deletion mutations on the replication of the virus, but precludes immediate assessment of their potential attenuating effects. In order to determine the impact of these 5'NTR deletion mutations on the virulence of HAV, identical mutations must be created in the genetic background of a virulent HAV variant. Toward this end, we obtained an infectious cDNA clones of HM175/wt (wild-type) and HM175/8Y viruses from Dr. Suzanne Emerson of Dr. Robert Purcell's laboratory at the National Institute of Allergy and Infectious Diseases. The 8Y clone contains the full-length wild-type sequence of HM175 virus with a single substitution mutation at nt 3889 (2B protein) which facilitates replication in cell culture (Emerson et al., 1992). Virus may be rescued from the 8Y clone by RNA transfection of FRhK-4 cells or by direct RNA injection into primate (marmoset) liver. In contrast, virus can only be rescued reliably from the wt cDNA clone by direct intrahepatic injection of RNA. Thus far, two mutations have been created in the HM175/wt and HM175/8Y background:  $\Delta 99-137$  and  $\Delta 131-144$ . The validity of the plasmid constructions was documented by DNA sequencing. However, virus was rescued only from the 8Y clones following transfection of FRhK-4 cells. Collaborative studies have been initiated with Dr. Emerson to determine whether 8Y $\Delta 99-137$  virus remains capable of causing disease in marmosets following direct intrahepatic injection of synthetic RNA.

## 5. Genetic heterogeneity of HAV strains

In collaboration with Dr. Betty Robertson of the Hepatitis Branch, Centers for Disease Control and Dr. Yasuo Moritsugu of the NIH, Japan, we established an extensive data base containing the partial genomic sequences of over 150 unique HAV strains. Collectively, we have determined 168 bases of sequence from each virus at the putative VP1/2A junction, using the AC/PCR method (Jansen et al., 1990). Development of this method was described in detail in the final annual report of our former contract with the U.S. Army Research and Development Command (DAMD17-85-C-5272) and in Report No. 1 of this grant submitted in 1990. The analysis of partial genomic sequences has confirmed the existence of considerable genetic diversity among human HAV strains (Jansen et al., 1990; Robertson et al., 1992). Results of these studies support the existence of 7 distinct HAV genotypes (defined arbitrarily as strains differing at more than 15% of bases sequenced), four of which are human viruses and three of which appear to be restricted to primates (African green monkey and the cynomolgus monkey). A number of human isolates have been identified which are related closely in sequence to the PA21 strain which was previously isolated from New World owl monkeys held at the Gorgas Memorial Laboratory in Panama by Binn and Lemon at the Walter Reed Army Institute of Research. The current evidence thus suggests that PA21 is representative of genetically distinct human HAV strains (genotype III), and is not in fact a simian agent. Thus far, there is no evidence for significant antigenic variability among human viruses from different genotypes,

although the possibility of such genetic variability remains as detailed studies of antigenicity have thus far only been carried out with genotype I and III strains (Lemon et al., 1992).

## 6. Serologic correlates of protection conferred by inactivated HAV vaccines

**Neutralizing antibody response to inactivated vaccine.** In order to gain a better understanding of the protective antibody response induced by formalin-inactivated hepatitis A vaccine, we analyzed the anti-HAV response in two cohorts of children who were immunized with a highly purified, formalin-inactivated CR326F' strain hepatitis A vaccine (VAQTA™) manufactured by Merck & Co. In a recent controlled clinical trial (Werzberger et al., 1992), this vaccine was shown to induce complete protection against clinically apparent hepatitis A (100% efficacy, 97-100% CI) within 3 weeks of administration of a single 25 antigen unit dose to children aged 1 to 16 years. This study was carried out in collaboration with Merck investigators, and used several different methods for detection of anti-HAV: conventional solid-phase immunoassay (modified HAVAB) in comparison to an international standard, two different assays for measurement of HAV-neutralizing antibodies, the radioimmunofocus inhibition test (RIFIT) and HAV antigen reduction assay (HAVARNA), and a novel radioimmunoprecipitation assay (RIPA) for detection of anti-HAV (see Annual Report Nos. 4 and 5).

Sera collected from 70 children four weeks after administration of a single dose of inactivated HAV vaccine (VAQTA™, Merck & Co.) were tested for HAV-specific neutralizing antibody activity. These children were participants in two separate clinical studies, MSD-020 (a vaccine immunogenicity study) and MSD-023 (the Monroe efficacy study) (Werzberger et al., 1992). The geometric mean titers (gmt) of anti-HAV antibodies detected by modified HAVAB in these study groups were 49.3 and 45.7 mIU/ml, respectively. Thirty-four of 70 (49%) sera contained neutralizing antibodies detectable by RIFIT ( $\geq 80\%$  reduction in focus number at a 1:8 serum dilution), while 56 of 57 (98%) tested sera contained neutralizing antibodies detectable in the HAVARNA assay ( $\geq 50\%$  antigen reduction at a 1:4 serum dilution). Thus, under these conditions, the HAVARNA assay was more sensitive than the RIFIT assay for detection of the early neutralizing antibody response to HAV vaccine. This difference in sensitivity reflected the more stringent criterion for positivity in the RIFIT assay, and was matched by differences in the gmt of neutralizing antibodies detected by these two methods. By RIFIT, the reciprocal gmt for neutralizing antibody was 12.4 and 23.5 for the MSD-020 and MSD-023 vaccine recipients, respectively, compared with 61.0 and 93.9 by HAVARNA (gmts calculated only for positive serum samples). Matched preimmunization sera were tested by RIFIT only. Of these, only 1 of 70 (1.4%) tested positive in this neutralizing assay (RIFIT titer = 1:8).

**Neutralizing antibodies following administration of immune globulin.** Immune globulin administered intramuscularly in a dose of 0.06 ml/kg is known to produce highly effective, short term protection against clinical hepatitis A. This protection is due exclusively to the presence of circulating passively administered antibodies to HAV. Thus, it was of interest to compare the levels of antibodies present following immunization with inactivated vaccine with those present shortly after administration of immune globulin. The level of antibody detectable by modified HAVAB 7 days after administration of immune globulin to a group of 20 adults (MSD-005 study) was similar to that present in both groups of children 4 weeks after immunization with inactivated vaccine (46.8 mIU/ml, vs. 49.3 and 45.7 mIU/ml respectively). However, all 20 (100%) of the immune globulin recipients were positive for neutralizing antibodies by RIFIT, compared with only 34 of 70 (49%) vaccine recipients. These results suggest that there may be qualitative differences in the antibodies present shortly after passive and active immunization, with greater levels of virus neutralizing activity associated with similar antibody levels measured by quantitative modified HAVAB.

**Detection of antibodies to HAV by immunoprecipitation of  $^3\text{H}$ -labelled virus.** A subset of the serum samples collected prior to and 4 weeks after immunization from children enrolled in MSD-020 were tested for anti-HAV activity by RIPA at 1:8 and 1:80 dilutions. In general, a lower percentage precipitation value was obtained at the 1:80 dilution with most pre-immunization serum samples, reflecting less nonspecific precipitation of the labelled virus (see Annual Report No. 4). In contrast, dilution had an opposite effect with serum collected postimmunization. Generally higher precipitation values were obtained at 1:80 compared with 1:8 serum dilutions. Serum samples capable of precipitating >30% of label at either 1:8 or 1:80 serum dilutions were considered positive for HAV-specific precipitating antibodies (see Annual Report No. 5). Using this conservative criterion, each of the preimmunization sera tested negative by RIPA, while 37 of 38 MSD-020 postimmunization sera were positive, 26 of 38 (68%) at 1:8 dilutions, and 37 of 38 (97%) at 1:80.

Sera collected from adult subjects enrolled in the MSD-005 study 7 days after administration of immune globulin were tested for the presence of precipitating antibodies by RIPA at 1:8 and 1:80 dilutions. Surprisingly, only 8 of 20 (40%) sera were positive, and none of these sera had RIPA titers >1:8. Sera collected following administration of immune globulin had much lower RIPA activity than sera collected 4 weeks after administration of inactivated HAV vaccine, even though globulin recipients had comparable titers of antibody measured by modified HAVAB and somewhat higher serum neutralizing antibody titers measured by RIFIT (see Annual Report No. 5).

These results indicate that there are significant qualitative differences in circulating anti-HAV antibodies present following administration of immune globulin and immunization with inactivated HAV vaccine, despite similar quantitative results in solid-phase immunoassays and assays for serum neutralizing antibodies. In addition, these studies have defined protective levels of antibodies following both active and passive forms of immunization. The results should provide useful benchmarks to be applied in future clinical trials of hepatitis A vaccines.

## CONCLUSIONS

An extensive series of studies provided strong evidence that the major neutralization epitopes of HAV are exquisitely dependent upon the conformation adopted by individual polypeptides within the context of the native capsid structure. It is likely that single epitopes are comprised of residues derived from multiple capsid polypeptides, as both VP1 and VP3 residues are involved in the major immunodominant antigenic site. Although the reactivity of the H7-C27 monoclonal antibody with linear peptide sequences near the carboxyl terminus of VP1 provides a tantalizing suggestion that peptides representing this region of VP1 might have relevant immunogenicity if properly displayed, work in this direction has thus far been disappointing. In aggregate, these studies indicate that subunit approaches to HAV vaccine development are not likely to be successful unless they involve the expression and assembly of the entire HAV capsid structure.

The genetic diversity of circulating HAV strains was characterized and 7 distinct genotypes (4 human and 3 simian) were defined. There is no evidence for serotypic diversity among different human HAV genotypes.

The serological response to inactivated HAV vaccines was compared with the antibody response following administration of immune globulin. Qualitative differences were found in the relative activities of these antibodies when assessed in virus neutralization and immunoprecipitation assays. These differences suggest that lower affinity antibodies are induced by active immunization with formalin-inactivated virus. Protective levels of antibodies were defined by solid-phase immunoassay and virus neutralization assays. The results of these studies provide useful benchmarks for future vaccine trials.

The attenuation phenotype of cell culture-adapted HM175/P16 virus was defined, and mutations within the 5'NTR of its genome were shown to contribute to its ability to grow in cultured African green monkey kidney cells. These findings prompted a detailed examination of the structure and function of the 5'NTR. These studies included the detailed mapping of an IRES element present within the 5'NTR which is similar to that of other picornaviruses, but which has very weak activity in directing translation *in vivo*. This weak translational activity is likely to be important in determining the generally slow and noncytopathic replication cycle of HAV. Cellular proteins which bind specifically to the viral 5'NTR RNA were characterized; these proteins may be involved in the host range specificity of cell culture adapted, attenuated viruses. These results offer a better understanding of the basic mechanisms underlying adaptation of existing attenuated HAV vaccine candidates to growth in cell culture. In addition, they may point the way to development of new and novel attenuated vaccine candidates. A series of viable HAV mutants with large deletions in the 5'NTR were constructed based on the detailed understanding of the structure and function of this region of the genome obtained in these studies. The effects of these 5'NTR mutations on the growth and virulence properties of virus in nonhuman primates is presently under investigation and offers fertile ground for future studies.

## REFERENCES

- Bancroft WH, Lemon SM. 1984. Hepatitis A from the military perspective. In: Gerety RJ, ed. Hepatitis A. Orlando, Florida: Academic Press, 81-100.
- Binn LN, Lemon SM, Marchwicki RH, Redfield RR, Gates NL, Bancroft WH. 1984. Primary isolation and serial passage of hepatitis A virus strains in primate cell cultures. *J. Clin. Microbiol.* 20:28-33.
- Binn LN, Bancroft WH, Lemon SM, et al. 1986. Preparation of a prototype inactivated hepatitis A virus vaccine from infected cell cultures. *J Infect Dis* 153:749-756.
- Bittle JL, Houghten RA, Alexander H, Shinnick TM, Sutcliffe JG, Lerner RA, Rowlands DJ, Brown F. 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* 298:30-33.
- Brown EA, Day SP, Jansen RW, Lemon SM. 1991. The 5' nontranslated region of hepatitis A virus: secondary structure and elements required for translation in vitro. *J. Virol.* 65:5828-5838.
- Brown EA, Zajac AJ, Lemon SM. 1994. In vitro characterization of an internal ribosomal entry site (IRES) present within the 5' nontranslated region of hepatitis A virus RNA: comparison with the IRES of encephalomyocarditis virus. *J. Virol.* 68: 1066-1074.
- Chang KH, Brown EA, Lemon SM. 1993. Cell type-specific proteins which interact with the 5' nontranslated region of hepatitis A virus RNA. *J Virol* 67:6716-6725.
- Chow M, Yabrov R, Bittle JL, Hogle JM, Baltimore D. 1985. Synthetic peptides from four separate regions of the poliovirus type 1 capsid protein VP1 induce neutralizing antibodies. *Proc Natl Acad Sci USA* 82:910-914.
- Cohen JI, Ticehurst JR, Feinstone SM, Rosenblum B, Purcell RH. 1987. Hepatitis A virus cDNA and its RNA transcripts are infectious in cell culture. *J Virol* 61:3035-3039.
- Cohen JI, Rosenblum B, Feinstone SM, Ticehurst J, Purcell RH. 1989. Attenuation and cell culture adaptation of hepatitis A virus (HAV): a genetic analysis with HAV cDNA. *J Virol* 63:5364-5370.
- Cox EM, Emerson SU, Lemon SM, Ping L-H, Stapleton JT, Feinstone SM. 1991. Use of oligonucleotide-directed mutagenesis to define the immunodominant neutralization antigenic site of HAV. In Brown F, Chanock RM, Ginsberg HS, Lerner RA eds. "Vaccines 90. Modern Approaches to New Vaccines Including Prevention of AIDS." Cold Spring Harbor. Cold Spring Harbor Laboratory Press, pp. 169-173.
- Cromeans T, Fields HA, Sobsey MD. 1989. Replication kinetics and cytopathic effect of hepatitis A virus. *J. Gen. Virol.* 70:2051-2062.
- Day SP, Lemon SM. 1990. A single base mutation in the 5' nontranslated region of HAV enhances replication in vitro. In: Brown F, Chanock RM, Ginsberg HS, Lerner RA, eds. *Vaccines 90*, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 175-178.
- Day SP, Murphy P, Brown EA, Lemon SM. 1992. Mutations within the 5' nontranslated region of hepatitis A virus RNA which enhance replication in BS-C-1 cells. *J Virol* 66:6533-6540.

- Day SP, Stapleton JT, Cox EM, Feinstone SM, Lemon SM and Stein LD. 1991. Evaluation of the human B lymphocyte response to hepatitis A virus. In: Hollinger, FB, Lemon SM, and Margolis H (eds) *Viral Hepatitis and Liver Disease*, Williams and Wilkins, Baltimore, pp.69-70.
- Emerson SU, Huang YK, McRill C, Lewis M, Purcell RH. 1992. Mutations in both the 2B and 2C genes of hepatitis A virus are involved in adaptation to growth in cell culture. *J Virol* 66:650-654.
- Francis MJ, Hastings GZ, Sangar DV, et al. 1987. A synthetic peptide which elicits neutralizing antibody against human rhinovirus type 2. *J Gen Virol* 68:2687-2691.
- Geysen HM, Meloen RH, Barteling SJ. 1984. Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proc Natl Acad Sci USA* 81:3998-4002.
- Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. 1987. Strategies for epitope analysis using peptide synthesis. *J Immunol Methods* 102:259-274.
- Innis BL, Snitbhan R, Kunasol P, et al. 1994. Protection against hepatitis A by an inactivated vaccine. *JAMA*: 271:1328-1334.
- Jansen RW, Newbold JE, Lemon SM. 1988. Complete nucleotide sequence of a cell culture-adapted variant of hepatitis A virus: comparison with wild-type virus with restricted capacity for *in vitro* replication. *Virology* 163:299-307.
- Jansen RW, Siegl G, Lemon SM. 1990. Molecular epidemiology of hepatitis A virus defined by an antigen-capture/polymerase chain reaction method. *Proc Natl Acad Sci USA* 87:2867-2871.
- Lemon SM, Amphlett E., and Sangar D. 1991. Protease digestion of hepatitis A virus: disparate effects on capsid proteins, antigenicity and infectivity. *J. Virol.* 65:5636-5640.
- Lemon SM, Barclay W, Ferguson M, et al. 1992. Immunogenicity and antigenicity of chimeric picornaviruses which express hepatitis A virus (HAV) peptide sequences: Evidence for a neutralization domain near the amino terminus of VP1 of HAV. *Virology* 188:285-295.
- Lemon SM, Binn L, Marchwicki R., et al. 1990. In vivo replication and reversion to wild-type of a neutralization-resistant variant of hepatitis A virus. *J. Infect. Dis.* 161:7-13.
- Lemon SM, Jansen RW, Brown EA. 1992. Genetic, antigenic and biological differences between strains of hepatitis A virus. Hollinger FB, Andre F (eds). *Proceedings of an International Symposium on Active Immunization Against Hepatitis A*. Vaccine 10 (suppl 1) S40-44.
- Lemon SM, Murphy PC, Shields PA, Ping L-H, Feinstone SM, Cromeans T, Jansen RW. 1991. Antigenic and genetic variation in cytopathic hepatitis A virus variants arising during persistent infection: evidence for genetic recombination. *J. Virol.* 65:2056-2065.
- Lemon SM, Ping L-H, Day SP, Cox E, Jansen, R, Amphlett E., Sangar D. Immunobiology of hepatitis A virus. In: Hollinger, FB, Lemon SM, Margolis H (eds) *Viral Hepatitis and Liver Disease*, Williams and Wilkins, Baltimore, pp. 20-24.
- Lemon SM, Shapiro CN. 1994. The value of immunization against hepatitis A. *Infectious Agents and Disease* 3:38-49.
- Lemon SM. 1985. Type A viral hepatitis: new developments in an old disease. *N. Engl. J. Med.* 313:1059-1067.

- Lemon SM. 1994. Inactivated hepatitis A vaccines. *JAMA* 271:1363-1364.
- Lewis AP, Lemon SM, Barber KA, Murphy P, Parry NR, Peakman TC, Sims MJ, Worden J, and Crowe JS. 1993. Rescue, expression and analysis of neutralizing human anti-hepatitis A virus monoclonal antibody. *J. Immunol.* 151:2829-2838.
- Minor PD. The molecular biology of poliovaccines. 1992. *J. Gen. Virol.*, 73:3065-3077.
- Ping L-H, Jansen RW, Stapleton JT, Cohen JI, Lemon SM. 1988. Identification of an immunodominant antigenic site involving the capsid protein VP3 of hepatitis A virus. *Proc Natl Acad Sci USA* 85:8281-8285.
- Ping L-H, Lemon SM. 1992. Hepatitis A virus neutralization epitopes defined by analysis of neutralization escape mutants selected against murine monoclonal antibodies. *J. Virol.* *J Virol* 66:2208-2216.
- Robertson BH, Jansen RW, Khanna B, et al. 1992. Genetic relatedness of hepatitis A virus strains recovered from different geographic regions. *J Gen Virol* 73:1365-1377.
- Shaffer DR, Brown EA, Lemon SM. 1994 Large deletion mutations involving the first pyrimidine-rich tract of the 5' nontranslated RNA of human hepatitis A virus define two adjacent domains associated with distinct replication phenotypes. *J. Virol.* 68:5568-5578.
- Siegl G, Lemon SM 1990. Recent advances in hepatitis A vaccines. *Virus Res.* 17:75-92.
- Stapleton JT, Jansen RW, Lemon SM. 1985. Neutralizing antibody to hepatitis A virus in immune serum globulin and in the sera of human recipients of immune serum globulin. *Gastroenterology* 89:637-642.
- Stapleton JT, Lemon SM. 1987. Neutralization escape mutants define a dominant immunogenic neutralization site on hepatitis A virus. *J. Virol.* 61:491-498.
- Taylor KL, Murphy PC, Asher LVS, LeDuc JW, Lemon SM. 1993. Attenuation phenotype of a cell culture-adapted variant of hepatitis A virus (HM175/p16) in susceptible new world owl monkeys. *J Infect Dis.* 168:592-601.
- Werzberger A, Mensch B, Kuter B, et al. 1992. A controlled trial of a formalin-inactivated hepatitis A vaccine in healthy children. *N Engl J Med* 327:453-457.
- Whetter LW, Day SP, Elroy-Stein O, Brown EA, Lemon SM. 1994. Low efficiency of the 5' nontranslated region of hepatitis A virus RNA in directing cap-independent translation in permissive monkey kidney cells. *J. Virol.* 68:5253-5263.