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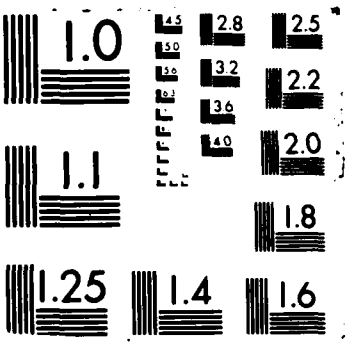
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STUDIES ON THE PATHOGENESIS OF HEPATITIS A AND FEASIBILITY STUDIES ON A HEPATITIS A VACCINE

ANNUAL REPORT

Elvera Ehrenfeld, Oliver C. Richards and
Donald F. Summers

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The objectives of this work are to further our knowledge of the pathogenesis of hepatitis A virus (HAV) infection in man, and to develop recombinant expression vectors for HAV antigens that can be used to stimulate mucosal immunity. Viral cDNA sequences encoding the viral capsid protein, VP1, have been cloned into several bacterial expression vector plasmids, and in one case, efficient production of a fusion protein containing VP1 sequences has been obtained. This fusion protein has been partially purified and used to induce antiserum in a rabbit. The resulting antibodies react with VP1 from purified virions, but do not immunoprecipitate or neutralize intact virus. The immunized rabbit, however, appeared to produce a secondary, anamnestic response to challenge with a sub-immunogenic dose of intact virus. Additional plasmid constructions have been designed to express other forms of VP1 protein and are currently under development. An in situ hybridization procedure has been developed, using probes prepared for our cloning studies. This procedure detects HAV-infected cultured cells with high sensitivity, and has been successfully applied to the analysis of liver biopsy material from HAV-infected Aotus monkeys, provided by Walter Reed. A set of acute and convalescent sera have been obtained for analysis of the anti-HAV antibody specificity. We have developed a "dot blot" hybridization test to evaluate the course of infection in cultured cells.

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Summary

The objectives of this work are to further our knowledge of the pathogenesis of hepatitis A virus (HAV) infection in man, and to develop recombinant expression vectors for HAV antigens that can be used to stimulate mucosal immunity. Viral cDNA sequences encoding the viral capsid protein, VP1, have been cloned into several bacterial expression vector plasmids, and in one case, efficient production of a fusion protein containing VP1 sequences has been obtained. This fusion protein has been partially purified and used to induce antiserum in a rabbit. The resulting antibodies react with VP1 from purified virions, but do not immunoprecipitate or neutralize intact virus. The immunized rabbit, however, appeared to produce a secondary, anamnestic response to challenge with a sub-immunogenic dose of intact virus. Additional plasmid constructions have been designed to express other forms of VP1 protein and are currently under development. An *in situ* hybridization procedure has been developed, using probes prepared for our cloning studies. This procedure detects HAV-infected cultured cells with high sensitivity, and has been successfully applied to the analysis of liver biopsy material from HAV-infected *Aotus* monkeys, provided by Walter Reed. A set of acute and convalescent sera have been obtained for analysis of the anti-HAV antibody specificity. We have developed a "dot blot" hybridization test to evaluate the course of infection in cultured cells.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

A. Statement of the problem.

Hepatitis A virus (HAV) infection, one of the most prevalent infections of man, causes significant morbidity and remains a worldwide public health problem. In this work, we propose to further our knowledge of the pathogenesis of hepatitis A virus infection of man, and to develop recombinant expression vectors for HAV antigens that can be used to stimulate mucosal immunity.

B. Background.

Hepatitis A virus (HAV) is the most common cause of viral hepatitis, accounting for approximately 30,000 reported cases each year during the 1980s (CDC, 1985). It is a particular problem in certain population groups such as day-care center attendees and employees, shellfish eaters, food handlers, patients and staff in institutions for the mentally retarded, travelers, and homosexuals. Although the mortality is very low, HAV infection can cause considerable morbidity and expense to the individual and family (Storch et al., 1979). Transmission occurs predominantly via the fecal-oral route by both common vehicle and person-to-person modes and peaks prior to onset of clinical illness in the source-case making prevention of infection a particularly important public health objective.

Research on the biology of HAV and the pathogenesis of the infection it produces became possible only recently as a result of the successful infection of marmosets (Holmes et al., 1969), visualization of viral particles in stool specimens (Feinstone et al., 1973), and the successful propagation of HAV in tissue culture cells (Provost and Hilleman, 1979). The virus can now be propagated in vitro in a variety of primary, passaged, and transformed cell cultures of human and non-human primate origin. Although the infection in cell cultures is not lytic and, therefore, traditional plaque assays to quantify virus are not possible, viral antigen can be detected in cultured cells by a variety of immunologic methods such as radioimmunoassay, enzyme linked immunosorbent assay, and immunofluorescence and an accurate determination of virus titers can be made using a newly described radioimmunofocus assay (Lemon et al., 1983). Present characterization of HAV reveals it to be a member of the enterovirus genus of the picornavirus family (Coulepis et al., 1982; Melnick, 1982). It is a 27-nm spherical nonenveloped virus with icosahedral symmetry and bands at 1.34 gm/cm^3 in cesium chloride (Siegl and Frosner, 1978). The genome is a single-stranded plus-sense monocistronic RNA of 2.8×10^6 daltons with a 3' poly(A) tract, encodes at least 11 virus-specified polypeptides VP1, VP2, VP3, and VP4, and is extremely stable to chemical and physical agents (Locarnini et al., 1981). Studies of the antigenic relatedness of different strains of HAV have identified only one serotype (Lemon and Binn, 1983). The entire HAV genome has been cloned as cDNA into pBR322 and sequenced revealing little homology with other picornaviruses (von der Helm et al., 1981; Ticehurst et al., 1983; Linemeyer et al., 1985; Baroudy et al., 1985, Najarian et al., 1985).

Following the development of immunologic assays in the early 1970s to detect virus antigen and antibody, studies of the pathobiology and immune response generated by HAV infection in primates became possible. It was observed that as early as one week following oral inoculation of marmosets HAV can be detected in the liver (Provost et al., 1978); in fact, to date replication of virus has been demonstrated only in the liver lobule (Popper et al., 1980) suggesting that virus is transported to the liver from the intestine via the portal veins and that viremia occurs only after replication of virus in the liver. HAV can also be identified by immunoflourescent staining in bile and finally, only after appearance in the liver and concomitant with the onset of prodromal symptoms, fecal excretion of HAV antigen occurs (Mathiesen et al., 1978). Whether HAV infection of hepatocytes is in itself cytolytic and responsible for disease or whether liver cell necrosis results from the host's immunologic response is not clear. With the onset of aminotransferase elevation and jaundice, excretion of HAV in stool has usually ceased and infected hosts are no longer infectious. Despite exhaustive attempts to demonstrate HAV in intestinal epithelial cells of percutaneously and orally inoculated animals by immunocytochemical methods, the presence of HAV antigen in the intestinal mucosa has not been demonstrated. Similar studies using nucleic acid probes have not been reported.

Recent data indicate that the capsid protein VP1 is the important target for HAV neutralizing antibodies (Hughes et al., 1984). Immunization of rats with VP1 purified from cell cultures results in the development of a strong antibody response to the isolated VP1 polypeptide as determined by Western blot analysis and immune precipitation, a good antibody response to the whole virus as demonstrated by competitive radioimmunoassay (RIA) and precipitation of HAV, and low titer neutralizing antibody to HAV as demonstrated by cell culture assay (Hughes and Stanton, 1985).

Although the presence of serum anti-HAV correlates with resistance to reinfection but may be a epiphenomenon as secretory IgA can be detected in stools of patients with acute hepatitis A and, therefore, may play a role in limiting the duration of infectivity and providing local intestinal immunity to prevent reinfection in cases of reexposure (Yoshizawa et al., 1980; Locarini et al., 1980). Resistance to many infections acquired at the mucosal surface is better correlated with the local synthesis, transport, and secretion of specific secretory IgA antibodies than with systemic antibody (Allardyce and Bienenstock, 1984).

A useful test for this hypothesis would be to develop an avirulent bacterial vector, genetically engineered to present viral antigen at the surface mucosa of the gut. Two such vectors have been developed for this purpose: Salmonella typhi Ty 21a (Germanier and Furer, 1975) and Salmonella typhi aro C ( ). In both cases, avirulence is due to bacterial lysis of these metabolic mutants that occurs under the nutritional conditions encountered in the infection, whereas their vaccine protective capacity is likely attributable to induction of local immunity that occurs

during the organisms short-term colonization of the intestine. Additional vectors derived from E. coli mutant strains engineered to manifest increased invasiveness and also under development (S. Formal, personal communication).

The studies being conducted under the auspices of this contract are directed towards the following aims:

C. Project aims

1. 1-1. To construct recombinant bacterial plasmids that express HAV capsid proteins.
   1-2. To evaluate the immunologic response to the recombinant proteins.
   1-3. To transform Salmonella typhi Ty21a oral vaccine strain with HAV recombinant plasmids and evaluate the modified strain's ability to induce a systemic and mucosal immune response.

2. To analyze extrahepatic tissue from infected animals for HAV replication.

3. To determine the specificity of the human immune response to HAV infection.

D. Results

1.1 Construction of recombinant plasmids that express hepatitis A virus capsid protein in bacteria.

When this project was initiated we obtained two clones of E. coli that contained partial capsid-coding sequences in pBR322 vectors from John Ticehurst at NIH. We prepared DNA from both of these clones, cut and religated the DNA so as to produce a single plasmid that contained all of the HAV capsid coding sequences on a single continuous 4.5 kb insert in pBR322. This plasmid is called pHAV113. In addition, since VP1 is believed to represent the major external surface protein and to contain the major immunologically dominant epitope of the virion we excised the VP1-coding region from pHAV113, added EcoRI linkers, and sub-cloned the VP1 region, resulting in plasmid pHAV-518, so that the VP1 sequence could be readily independently manipulated.

Several expression vectors were utilized to attempt to obtain expression of either VP1 alone, or the entire, uncleaved capsid protein precursor, P1, in E. coli. The results obtained with these various vectors are summarized below.

(a) pIN-III expression vectors

This vector contains the E. coli lipoprotein gene promoter plus the sequences coding for the lipoprotein signal peptide and eight amino acids, followed by a cloning

site. This vector was attractive because cloned material should be secreted across the cytoplasmic membrane as a hybrid protein. In addition, a lac UV5 promoter-operator has been inserted upstream of the ribosome binding site so that transcription should require a lac inducer. Both VP1 sequences and the entire capsid coding sequences were cloned into these vectors. Approximately 50 clones were isolated and analyzed. Almost all were in the wrong orientation or had undergone DNA rearrangements or deletions; none expressed HAV protein upon induction.

(b) pATH vectors

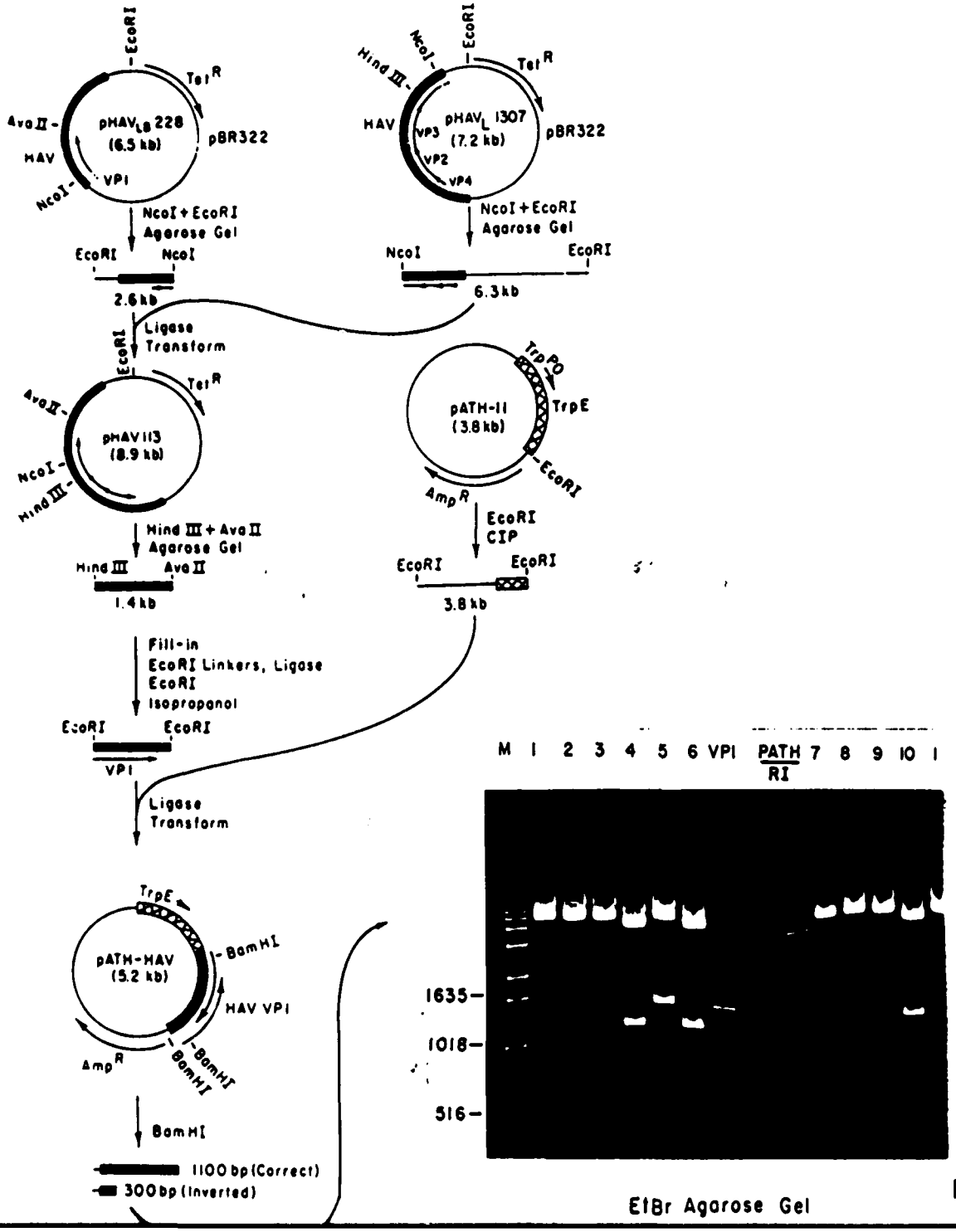
This vector (gift of C. Dieckmann) is designed to express a fusion protein containing the amino terminus of the E. coli Trp E protein, expressed from a strong trp promoter. It is inducible, but difficult to shut off completely.

Fig. 1 shows the scheme used for construction of the plasmid containing VP1 coding sequences. Bacteria transformed with this chimeric plasmid (pATH-HAV-VP1) synthesize large amounts of a fusion protein (M_r 88,000) that reacts with both rabbit anti-TrpE serum and rabbit anti-HAV serum that had been raised against intact HAV. This TrpE-HAV VP1 fusion protein has been partially purified, resolved on a preparative SDS-polyacrylamide gel, eluted from the gel, and used to immunize a rabbit. The resulting antiserum identified the fusion protein and VP1 from purified HAV, and will be described further below.

The success of VP1 expression in pATH vectors encouraged us to attempt expression of the complete capsid coding region in the same vector. No evidence of expression was obtained, however. At the present time, we are constructing additional inserts that contain VP3 and VP1 sequences, but lack VP4 and 2. In addition, we are in the process of modifying the pATH vector so as to delete the major portion of the TrpE coding sequences. This will markedly reduce the size of the fusion protein and permit synthesis of a VP1 molecule with only about 17 amino acids of E. coli protein.

(c) *Other bacterial vector systems*

We have cloned VP1 cDNA sequences into a pAS vector (from Dr. M. Rosenberg, Smith, Kline and French), which expressed from the lambda right promoter and fuses only a single methionine codon to the inserted sequence. Inserted sequences are totally repressed until manipulated by temperature or drug treatment. VP1 protein was expressed in bacteria transformed with this plasmid, but only at low


EcoRI
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AvaII
pHAV LB 228
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pBR322
HAV
VP1
NcoI
NcoI + EcoRI
Agarose Gel
EcoRI
NcoI
2.6 kb
HindIII
pHAV L 1307
(7.2 kb)
VP3
VP2
VP4
6.3 kb
Ligase
Transform
pHAV113
(8.9 kb)
HindIII + AvaII
1.4 kb
pATH-11
(3.8 kb)
TrpPQ
TrpE
AmpR
EcoRI
CIP
3.8 kb
Fill-in
EcoRI Linkers, Ligase
EcoRI
Isopropanol
pATH-HAV
(5.2 kb)
BamHI
HAV VP1
1100 bp (Correct)
300 bp (Inverted)
M 1 2 3 4 5 6 VP1 PATH RI 7 8 9 10 1
1635
1018
516
EtBr Agarose Gel

Figure 1

levels. The complete capsid protein coding sequences again failed to express in this vector.

We are currently working on several additional constructs, using vectors that produce no fusion, as well as vectors that secrete the final protein product. We are optimistic about expression of VP1 in all systems. Expression of the complete P1 capsid coding region has thus far failed in every vector we have tried, and has also failed in the case of foot-and-mouth-disease virus (FMDV) P1 protein, in vectors where FMDV VP1 expresses well. Extensive efforts with the entire P1 region will not be continued.

(d) Baculovirus vectors

Although not in our original proposal, we have undertaken a side project to attempt to prepare large amounts of HAV VP1 by expression from a baculovirus, Autographa californica nuclear polyhedrosis virus, in an insect Spodoptera frugiperda (SF9) cell line. This expression system (obtained from Dr. Max Summers) often produces extremely high yields of recombinant proteins, in a soluble, active form. We hope to utilize the VP1 to induce antibodies to provide a needed reagent for our expression studies, and, in addition, we will test VP1 as an immunogen, in collaboration with WRAIR. We have successfully cloned the VP1 gene into the baculovirus plasmid vector, and have obtained recombinant viruses by co-transformation of viral DNA and recombinant plasmid DNA. Expression studies are currently in progress.

1.2 Immunologic response to TrpE-HAV VP1 fusion protein in a rabbit.

As stated above, the fusion protein was purified by SDS-PAGE and used to immunize a rabbit. The resulting antiserum specifically detects VP1 from purified HAV as well as fusion protein, by Western blot analysis. Antibody to the fusion protein, however, failed to react with intact HAV by three criteria: (i) It did not immunoprecipitate intact HAV, under conditions where polyclonal anti-HAV did; (ii) It did not compete with control anti-HAV serum to react with HAV-coated beads in the commercial (Abbott HAVAB) enzyme immunoassay, using either a normal 1:20 or a modified 1:1 ratio to test serum to labeled anti-HAV serum; (iii) The antibody did not neutralize HAV infectivity of tissue culture cells, as assayed by Dr. L. Binn at WRAIR (see Table 1).

Table 1

ANTIBODY RESPONSES TO WHOLE HEPATITIS A VIRUS AFTER IMMUNIZATION WITH TrpE/HAV VP1 FUSION PROTEIN

SERUM SAMPLE	NORMAL HAVAB	MODIFIED HAVAB	IMMUNO PRECIPITATION	NEUTRALIZATION
Rabbit-1	-	-	ND	-
Rabbit-2	-	-	-	-
ADS R2*	-	-	ND	-
NORMAL RABBIT	-	-	-	-
VAC RABBIT+	+	+	+	+

*ADS R2 = Serum from rabbit 2 adsorbed with E. coli lysate to remove antibodies to TrpE.

+Vac Rabbit = Serum from rabbit vaccinated with immunogenic (0.1 mg) dose of WRAIR killed HAV vaccine.

It has been demonstrated previously ( ) that synthetic peptides representing poliovirus VP1 sequence which did not elicit a poliovirus neutralizing antibody response were able to "prime" the immune system of rabbits for a long-lasting, virus neutralizing antibody response following a single inoculation of intact virus. The ability of the TrpE-HAV VP1 fusion protein to prime was investigated, and these results are summarized in Table 2. A single injection of 0.1 ml of killed whole HAV (ca. 50 ng of WRAIR HAV vaccine) failed to induce an anti-HAV response in 3 rabbits, whereas 0.25 ml and 0.5 ml stimulated antibody to whole virus detectable by modified HAVAB at 2 weeks post immunization. The 0.1 ml sub-immunogenic dose given to a rabbit that had previously received the fusion protein yielded antibody to intact HAV. This antibody developed rapidly (i.e., 5 days after immunization) and was of higher titer than that detected in the control rabbit (1:16 vs 1:4) suggesting that a true secondary immunoresponse had occurred in the animal that had been primed with fusion protein. Preliminary analysis of the immunoglobulin class of anti-HAV by sucrose gradient centrifugation followed by HAVAB assay of the fractions containing IgM and IgG indicate that the predominant antibody response in both animals was of the IgM class. HAVAB assay of the IgG fraction of these rabbit sera after purification on a Staph Protein A column is now underway.

Table 2

ANTIBODY RESPONSE (MODIFIED HAVAB) TO SUBIMMUNOGENIC HAV DOES AFTER PRIMING WITH TrpE/HAV VP1 FUSION PROTEIN

DAY POST-IMMUNIZATION	CONTROL RABBITS 0.1 ml DOSE	CONTROL RABBITS 0.5 ml DOSE	PRIMED RABBIT 0.1 ml DOSE
0	-	-	-
5	-	-	+
8	-	-	+
12	-	+	+
15	-	+	+
21	-	+	+
30	-	+	+

1.3 Transformation of Salmonella typhi Ty 21a (gal E minus) or other potential oral vaccine bacterial strains to evaluate the induction of systemic and mucosal immune response.

All work to date has been in laboratory strains of E. coli in order to characterize the HAV antigens produced and the antibodies induced from these recombinant products. We are continuing to evaluate the data being collected from S. typhi Ty 21a as well as from other Salmonella mutants and invasive E. coli strains.

2. Analysis of tissue for hepatitis A virus RNA.

We have perfected our in situ hybridization technique as a modification of the method of Laurence and Singer ( ). This technique is simple and less time-consuming than that of Haase et al., and the results are reproducible and are quite spectacular. BSC-1 cells were infected with HAV (HM175), and after different times post-infection, samples were fixed with paraformaldehyde and probed with a 2.8 kb HAV cDNA which had been gel-purified and nick-translated with [^{35}S]dATP. HAV sequences were readily detected in about 10% of cells infected for two days. With time, the proportion of cells positive for HAV sequences increased, as did the intensity of labeling. Cells infected for seven days showed 100% infections. Control, uninfected cells had no reactivity with the HAV probe.

Dr. Ludmila Asher of WRAIR has provided liver biopsy sections from pre- and post-infected Aotus monkeys. These sections were processed for in situ hybridization. Pre-infected liver sections and post-infected sections probed with nick-translated pBR322 DNA showed no hybridization. Post-infected liver sections probed with HAV capsid cDNA were clearly positive. HAV sequences were detected in the parenchyma and in perivascular cells. It is unclear at this time whether the infected cells are heptocytes on Kupffer cells. We are currently analyzing additional liver sections from orally infected Aotus monkey, infected (or not) for different periods of time. We are hoping to receive extra-hepatic tissue samples for analysis from WRAIR; however, these studies are apparently limited by the availability of monkeys.

3. Analysis of human immune response to hepatitis A virus infection.

We are now growing HAV (strain HM175) in BSC-1 cells in sufficient quantity to produce partially purified virions for our own analyses. We developed a dot blot hybridization assay to evaluate virus replication and to quantitate viral RNA in infected cells. The resulting virus preparations have been used in dot blot and Western blot assays to analyze the specificity of the human immune response to viral capsid proteins. Both rabbit and human sera react with intact virus with much greater sensitivity than they react with SDS-denatured virus. All convalescent human sera in our collection (obtained from Diagnostic Virology clinic in Madrid, Spain) recognize VP1, whereas VP2 and VP3 and variably recognized. We are currently developing reagents to examine the reactivity of human sera against HAV non-structural proteins.

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