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

ANNUAL / FINAL REPORT

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The objectives of this work are to produce hepatitis A virus (HAV) antigens by recombinant DNA technology to identify antigens that may induce a protective immune response, and to construct vectors that can be used to stimulate enteric mucosal immunity against HAV. Viral cDNA sequences encoding capsid proteins or selected parts of capsid proteins VP1 and VP3 have been cloned into several bacterial expression vectors, and have been shown to direct the synthesis of high levels of HAV capsid protein sequences in <i>E. coli</i> and in one case, in <i>Salmonella typhimurium</i>. Large amounts of HAV protein have also been produced in cultured insect cells infected with recombinant baculoviruses. The recombinant HAV proteins have been used to raise antisera in rabbits. In the case of one protein containing VP1 sequence, the antiserum recognized VP1 from purified virus, but it did not neutralize virus infectivity. The immunized rabbit, however, appeared to have been primed to produce a secondary, anamnestic response, which was neutralizing, to challenge with a sub-immunogenic dose of intact virus. Evaluation of the antibodies produced against other recombinant proteins and of the protective nature of the priming reaction are still in progress. An <i>in situ</i> hybridization procedure has been developed, using the plasmids constructed for antigen production as a source of probes to detect viral nucleic acid in infected cells and tissues. The procedure has been used to measure virus growth in cell culture, and for the analysis of liver and other biopsy material from HAV-infected Aotus monkeys. A slot blot hybridization assay as well as a quantitative hybridization focus assay has been developed for virus titration and serum neutralization assay.					
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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 (DHHS Publication No. (NIH) 86-23, Revised 1985).

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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. Production of an inactivated virus vaccine is problematic because of the low yields of virus grown in cell culture, and no candidate attenuated virus vaccine is currently available. In this work, we proposed to identify viral capsid protein antigens that may induce a protective immune response and thus may be used for the development of an effective subunit vaccine. We proposed to produce these antigens by recombinant DNA technology, and to evaluate the effectiveness of presentation via bacterial carriers to stimulate enteric mucosal immunity.

B. Background

A review of the structure and molecular biologic characterization of hepatitis A virus (HAV), as well as a summary of the pathobiology and immune response generated by HAV infection in primates, was presented in the previous annual report. Consequently, only relatively recent data relating to the potential development of a subunit-type vaccine for HAV will be presented here.

Studies of the antigenic relatedness of different strains of HAV have identified only one serotype (1), although nucleic acid hybridization analyses suggest that differences in the regions coding for non-structural proteins exist and may distinguish human from monkey strains of virus. Purified VP1 was capable of eliciting a weak neutralizing anti-HAV response in rats (2), as was a synthetic peptide representing a short sequence from the amino terminus of VP1 in rabbits (3). A protein produced in *E. coli* from recombinant cloned DNA, also representing the amino terminal portion of VP1, was shown to react with rabbit anti-HAV serum, but not with human convalescent serum (4). Surprisingly at least one virus mutant that escapes neutralization by a monoclonal antibody was shown to have an altered amino acid at position 70 of VP3, and no changes in VP1 (5).

Since the start of this project, a great deal of information about the surface structure of several picornaviruses has become available from X-ray crystallographic data (6,7) and from epitope mapping using monoclonal antibodies (8-12). Similar studies are currently in progress for HAV. Using computer alignments of the capsid proteins, as well as the known structures of other picornaviruses, some predictions can now be made as to the topological locations of specific regions of the HAV capsid proteins (13). Assuming that the major external projections on the virus surface are likely to constitute the major immunodominant and neutralization-specific epitopes, attention has been focused on three predicted surface structures: one is composed of a loop extending from a fold in VP1, centered approximately around amino acid residue 100; a second is a conformation-specific epitope composed of part of VP3 and part of VP1; and the third is comprised of part of VP2 and part of VP1. In some, but not all, picornaviruses, the first-mentioned VP1 epitope is immunodominant. Thus, an HAV protein or protein fragment that can elicit a protective neutralizing antibody response in primates has not been identified. It is hoped, but not absolutely clear, that such a protein sequence exists, and that not all HAV neutralization epitopes are composed of conformation-specific contributions from non-contiguous protein sequences. If a linear protein fragment is found to reproducibly stimulate even a weak neutralizing response, several approaches have been demonstrated to enhance such responses. These include production of a polypeptide with multiple tandem repeats of the sequence (14), or inclusion of Tcell stimulating epitopes in the sequence (15,16,17).

An additional consideration is that although the presence of serum anti-HAV correlates with resistance to infection, secretory IgA may also play a role in providing local intestinal immunity (18). 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.

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. Several strains of *Salmonella typhi* have been developed for this purpose; e.g. *S. typhi* Ty 21a (gal E) and *S. typhi* aroA (19). In both cases, avirulence is due to bacterial death of these metabolic mutants under the nutritional conditions encountered in the intestine, 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 *S. typhi* or from *E. coli* mutant strains engineered to increase invasiveness are also under development.

C. Project Aims

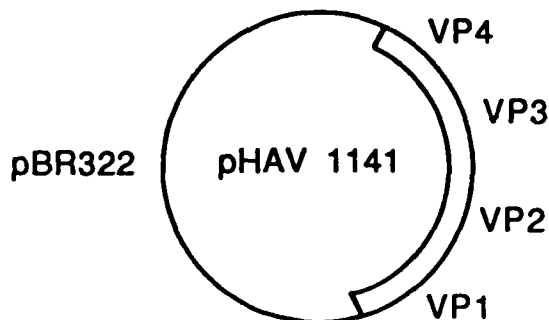
1. 1-1. To construct recombinant bacterial plasmids that express HAV capsid proteins
1-2. To evaluate the immunologic response to the recombinant proteins
1-3. To transform attenuated *Salmonella* strains with HAV recombinant plasmids and evaluate the induction of systemic and mucosal immune response by oral ingestion.
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

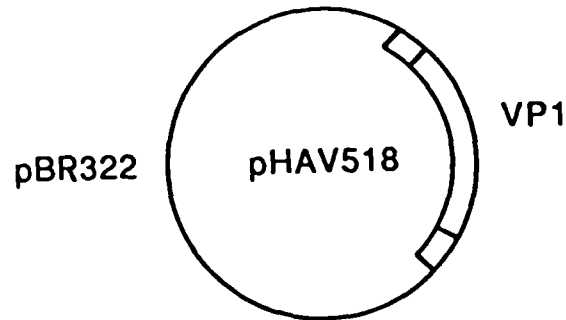
1.1. Construction of recombinant plasmids that express hepatitis A virus capsid protein.

a. Cloning HAV capsid genes.

When this project was initiated, we obtained two clones of *E. coli* that contained partial capsid coding sequences in pBR322 plasmids from John Ticehurst, then at NIH. We grew and prepared DNA from both of these clones, and religated the DNA so as to produce a single plasmid that contained 4.5 kb of contiguous HAV cDNA, including all of the capsid coding sequences. We then subcloned a piece of this cDNA that contained the coding sequences for the P1 protein [VP4,2,3,1]. This plasmid is called pHAV 1141.



In addition, we excised just the VP1 coding region, added EcoRI linkers, and sub-cloned the VP1 region, resulting in plasmid pHAV-518, so that the VP1 sequence could be readily independently manipulated.



b. Bacterial expression vectors.

A number of bacterial expression vectors were examined for their ability to direct the synthesis of either the entire capsid protein sequence or the VP1 sequence in *E. coli*. These vectors all provide a transcriptional promoter, ribosome binding sequence, translational start signal and part of the coding sequence for a bacterial protein, to which the HAV cDNA is fused. These vectors have been described in previous reports. No clones were isolated that synthesized detectable amounts of the entire capsid protein sequence, P1. (PI = VP4, 2, 3, 1). (Expression of the complete P1 protein has also failed in the case of other picornaviruses in other laboratories.) For expression of VP1, the best results were obtained with a pATH vector, which is designed to express a fusion protein containing the amino terminal portion of the *E. coli* TrpE protein, driven by a strong trp promoter. It is inducible, but difficult to shut off completely. Details of the plasmid construction and characterization of the expressed protein have been described (20). Bacteria transformed with this plasmid (pATH-HAV VP1) synthesize large amounts of a fusion protein (M_r 88,000) that reacts with both anti-TrpE serum and rabbit anti-HAV serum that had been raised against intact HAV. This TrpE-HAV fusion protein was 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 pATH-HAV VP1 plasmid has been modified by deletion of the majority of the TrpE sequences so that a smaller fusion protein is produced, consisting of HAV VP1 with about only 17 amino acids of *E. coli* protein. Expression levels are somewhat reduced from this deleted pATH-HAV-VP1 plasmid, but the protein is soluble and is still expressed in significant amounts. One additional vector worth mentioning is a plasmid that creates a fusion protein containing the signal sequence for secretion across the periplasmic membrane. The signal sequence is derived from the *E. coli* alkaline phosphatase gene. Good expression of HAV VP1 sequences was obtained with this vector, but the hybrid protein was rapidly degraded in the bacteria. The success of the pATH vectors to produce TrpE-HAV fusion proteins encouraged us to clone other HAV capsid sequences in this vector. These will be described below in section d.

c. Baculovirus vectors.

Although not in our original proposal, we undertook a side project to attempt to prepare large amounts of HAV VP1 protein from a recombinant baculovirus,

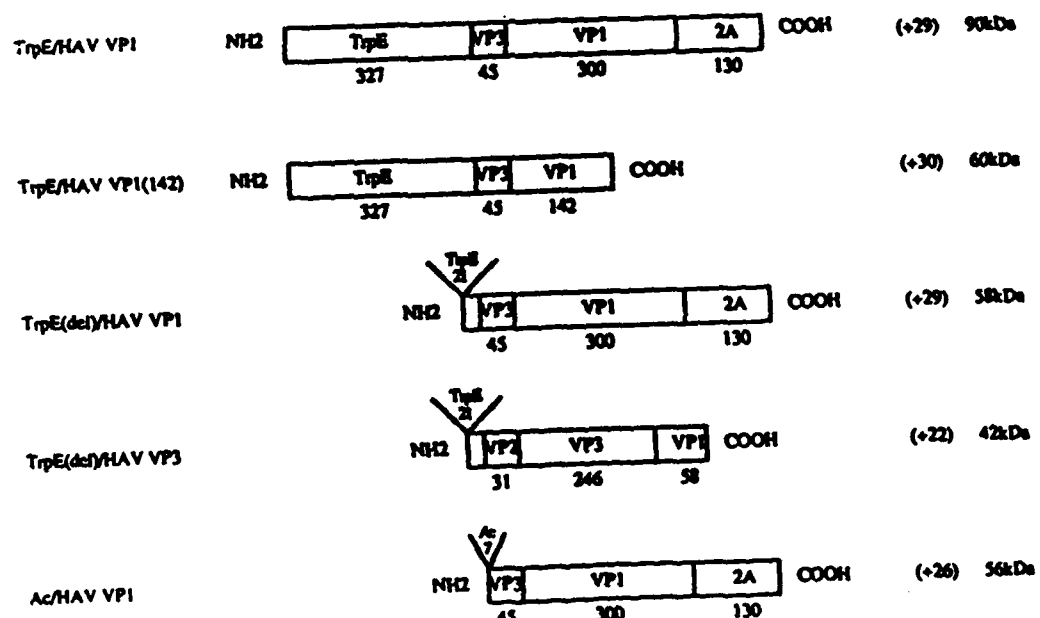
Autographa californica nuclear polyhedrosis virus, in an insect Spodoptera frugiperda (SF9) cell line. This expression system (obtained from Dr. Max Summers) often produces extremely high yields of recombinant proteins. The first HAV protein that we tried to produce in this system was the same VP1-containing protein that we used in the bacterial expression vectors, described above. The results of these studies have been described (21). Recombinant baculovirus was isolated that produced HAV-VP1, fused to only a few amino acids, from the viral polyhedron promoter. Synthesis of large amounts of VP1 can be visualized in the cytoplasm of infected SF9 cells by immunofluorescence, using antiserum to the bacterial TrpE-HAV VP1 fusion protein. The protein produced in insect cells has also been purified and used to immunize rabbits, and the resulting antiserum will be described below.

d. HAV antigens

The initial HAV protein sequence that we produced in either E. coli or baculovirus-infected insect cells contained all of VP1, with a small amount of VP3 sequence to the amino terminal side, and a small amount of 2A sequence to the carboxy terminal side. Previous studies with FMDV peptides had shown that smaller fragments of FMDV VP1 sometimes were more effective as protective immunogens than was the entire FMDV VP1 protein, presumably because the protein fragment presented the appropriate epitope to the immune system in a fashion more similar to what was seen in the intact virion.

We therefore have constructed clones that express the following proteins:

COMPOSITION OF HAV RECOMBINANT PROTEINS



All of the proteins indicated are in various stages of being evaluated for their immunologic activity in rabbits, as indicated in section 1-2.

e. Recognition of recombinant proteins by human convalescent sera.

One anti-HAV serum was provided by Walter Reed Army Institute of Research and about 10 sera were obtained from the Diagnostic Virology clinic in Madrid, Spain, from persons infected with HAV. All of the recombinant proteins are detected on Western blots by at least some of the human sera, suggesting that antigens present on native virus may be present in the recombinant proteins.

1-2. Immunologic response to recombinant proteins.

In general, recombinant proteins were purified by SDS-polyacrylamide gel electrophoresis and used to immunize rabbits, using either Freund's adjuvant or polyacrylamide as adjuvant. In some cases, proteins have been purified by other procedures, in the absence of denaturing agents such as SDS. In one case, an insoluble protein preparation was solubilized with guanidine-HCl prior to immunization. The most complete study was performed with the TrpE/HAV VP1 fusion protein, produced in *E. coli*. Rabbit antiserum raised against this protein specifically identified VP1 from purified HAV. Despite this, the antiserum failed to immunoprecipitate whole virus and failed to neutralize infectivity for cultured BSC-1 cells. However, when a rabbit immunized with the TrpE-HAV VP1 fusion protein was challenged with a sub-immunogenic dose of formalin-inactivated HAV, neutralizing antibody appeared that reacted with intact virus. This antibody developed rapidly (5 days) and was of higher titer and longer lasting than that stimulated by primary vaccination of control rabbits. These data suggest the animal immunized with the HAV VP1 fusion protein from *E. coli* was primed for a secondary, virus neutralizing antibody response after a single sub-immunogenic inoculation of intact virus.

The VP1 protein produced in baculovirus also induced antibody that detects HAV VP1, but does not neutralize infectivity of whole virus. Priming studies are in progress. The TrpE fusion protein containing the amino terminal half of the VP1 has raised antibody, which has not yet been tested for neutralizing activity or for priming ability.

Other proteins are in rabbits but antisera are not yet available for testing.

1-3. Transformation of Salmonella strains with plasmids encoding HAV capsid proteins.

The plasmid encoding the TrpE/HAV VP1 fusion protein has been transferred to restriction-minus and modification-minus strains of *E. coli* and finally used to transform an attenuated *aroA* mutant of Salmonella typhimurium. The HAV fusion protein is expressed constitutively in Salmonella. This approach will allow direct presentation of the HAV capsid antigen to the mucosal immune system of the intestine, where infection by HAV is initiated. We are continuing to evaluate data about Salmonella typhi strain Ty 21a (gal E minus) as well as from invasive *E. coli* strains for possible use as vectors for antigen presentation in the gut.

2. Detection of HAV RNA and antigen in cells and tissues.

a. In situ.

The plasmids constructed for the studies described in section above, were used to prepare probes for the development of an in situ hybridization technique. The probe we usually use is pHAV 1141, containing a 2.8 kb cDNA representing the HAV capsid protein sequences. The plasmid is nick translated in the presence of [³⁵S] dATP, and the product used to probe BSC-1 cells infected for various times with HAV and then fixed with paraformaldehyde. HAV sequences are readily detected in 10-20% of cells infected for 2 days. With time, the proportion of cells positive for HAV sequences increased, as did the intensity of labeling. 100% of cells infected for 7 days were clearly positive. Control, uninfected cells had no reactivity with the HAV probe, or with a control pBR322 probe. For comparison, an immunofluorescence assay was performed on the same infected cell preparations. The fixed cells were permeabilized with Triton X-100 and then incubated with anti-TrpE-HAV VPI antiserum, followed by fluorescein-conjugated goat anti-rabbit IgG as secondary antibody. In our laboratory, using the above-mentioned reagents, the in situ hybridization procedure was significantly more sensitive than immunofluorescence. Viral RNA could be detected 2-3 days earlier than viral antigen, and 100% of cells in the culture scored as positive for HAV by hybridization after only one week, whereas immunofluorescence took almost two weeks to appear in every cell.

The in situ hybridization methodology has also been applied to a variety of fixed tissue sections from liver and other organs of infected Aotus monkeys. HAV sequences were readily detected in the liver parenchyma (in hepatocytes or Kupffer cells) and in perivascular cells. Further efforts to use this technique to evaluate the possibility of extra-hepatic replication of HAV have been thwarted because of the apparent limited availability of monkeys and monkey material for these purposes at Walter Reed.

b. Development of methods for virus titrations, serum neutralization and measuring virus growth in cell cultures

We use a rapid slot blot hybridization procedure to quantitate viral RNA in virus preparations of unknown concentration. Dilutions of the virus preparation are blotted onto nitrocellulose membranes using a commercial slot blot apparatus, fixed and baked to disrupt virions, and the membrane is then hybridized to a HAV probe. Nick-translated plasmid similar to that utilized for the in situ hybridization is an acceptable probe, but sensitivity is increased by an order of magnitude with a riboprobe. To obtain this probe, we inserted the HAV cDNA insert from pHAV 1141 into a pGem vector that contains a T7 RNA polymerase promoter so as to allow transcription in vitro of an HAV RNA of minus strand polarity. [³²P]-UTP is provided as substrate for T7 RNA polymerase, and the specific activity of the resulting probe is very high. In addition, the single stranded RNA-RNA hybridization is extremely efficient. The number of genomes in the virus preparation is obtained by comparing the hybridization signal from a known dilution of virus to that yielded by a standard amount of plasmid DNA, blotted on the same filter.

An adaptation of this method has been used to follow virus replication in infected cells and to perform serum neutralization assays. Approximately 10,000 cells infected with HAV for various times are collected and blotted directly onto membranes, fixed

with glutaraldehyde and hybridized with ^{32}P -riboprobe as above. Preincubation of a standard amount of virus with neutralizing antiserum prior to infection reduces the hybridization signal measured at four days.

Quantitative assays for infectivity and neutralization are being developed that produce hybridization foci, similar to the previously developed radioimmunofocus assay. BSC-1 cells are grown on nitrocellulose membranes in culture dishes or wells. The monolayers are infected with HAV and overlaid with medium in 1% agarose. At a fixed time interval, the agarose is removed and the cells fixed with glutaraldehyde as above and hybridized with ^{32}P -riboprobe. Exposure of X-ray film by the hybridized membrane reveals countable foci of cells replicating HAV.

3. Analysis of human antibody response to HAV infection.

We have solicited a small number of human convalescent sera from patients infected with HAV, primarily for the purposes described in section I, above. We have only small amounts of these sera, and have not made the effort to obtain a larger set. All of the sera in our collection recognize intact virions with much greater sensitivity than they react with individual denatured virus capsid proteins. All sera, however, recognize isolated VP1, whereas VP2 and VP3 are variably detected. We have not examined any sera for the presence of antibodies against non-structural proteins.

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