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Immunologic Priming with Recombinant Hepatitis A Virus Capsid Proteins Produced in *Escherichia coli*

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Hepatitis A virus capsid proteins (VP0, VP3, and VP1) have been synthesized in *Escherichia coli* for use in antigenic and immunogenic analyses. Rabbits immunized with each of these individual recombinant capsid proteins developed a rapid neutralizing antibody response when subsequently challenged with a subimmunogenic dose of whole virus.

Hepatitis A virus (HAV) is provisionally classified as an enterovirus within the picornavirus family with a host range restricted to certain primates (9). HAV differs from other picornaviruses in that there appears to be only one serotype and significant antigenic variation does not occur (10, 12). For this reason and from the observation that low levels of serum-neutralizing antibody confer protection in humans (18), it may be feasible to design successful HAV subunit vaccines.

As the immunological recognition of the surface of the HAV virion is no doubt necessary (although possibly not sufficient) for immune-mediated clearance of the virus, it is important in subunit vaccine development to determine where (or if) immunogenic B- and/or T-cell epitopes reside on the HAV capsid proteins. The HAV virion is composed of three major capsid proteins (VP1, VP2, and VP3) and possibly a fourth (VP4) (6, 20). Based on analogy with other picornaviruses, the latter 17- to 23-amino-acid (aa) peptide presumably derives from proteolytic processing of the amino terminus of the capsid protein precursor, VP0, into components VP4 and VP2 (2, 8). A number of approaches have been utilized to try to elucidate the antigenic nature of HAV (reviewed in reference 14). The results to date suggest that the B-cell immunogenic epitopes of HAV are conformational in nature, dependent on the assembly of the capsid proteins into the virion structure, and difficult or impossible to mimic by isolated synthetic proteins. However, two studies have suggested that immunization with recombinant capsid proteins can prime the immune system for a neutralizing response upon challenge with whole virus (5, 7). In this report, we demonstrate that immunization with recombinant fusion or nonfusion proteins synthesized from the expressed cDNA sequences of each of the HAV capsid proteins (VP0, VP3, or VP1) prime the rabbit immune system for a neutralizing antibody response to a subimmunogenic dose of whole inactivated virus.

Cloning and expression of recombinant proteins. To construct a series of expression plasmids designed to synthesize the major structural proteins of HAV, capsid coding sequences were subcloned from pHAV113 (7) into the expression vectors pATH11 and pATHΔ (Fig. 1). In addition to amino-terminal TrpE sequences (330 or 18 aa for pATH11 and pATHΔ, respectively), as well as 3 to 5 aa encoded by sequences from the multiple cloning site and from synthetic linkers, the constructions have the following HAV-specific compositions: (i) VP0 extends from the third amino acid (Met) after the putative translation start site for virion RNA and includes 242 aa of VP0 plus an additional 8 aa of VP3 at its carboxy terminus; (ii) VP3 contains 32 aa of VP0, all 246 aa of VP3, and 55 aa of VP1 at the carboxy terminus; (iii) VP1 contains 46 aa of VP3, all 300 aa of VP1, and 128 aa of the nonstructural protein, 2A. At the 3′ end of viral sequences, VP0 and VP1 constructions contain another 8 aa encoded by vector sequences before reaching a translation stop codon (TAG), while VP3 contains 18 additional aa.

After induction of bacterial cultures harboring expression plasmids with the tryptophan analog indoleacrylic acid (which derepresses the *trp* operator), cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In most cases, expression of a unique polypeptide (approximately 10 mg per liter of culture) of the predicted molecular weight, according to sequence composition, was observed by Coomassie brilliant blue staining (Fig. 2). The nonfusion (pATHΔ) VP1 protein was synthesized at a much lower level than the other recombinant proteins; the reason for the low level of expression is unknown, but could result from mRNA instability, rapid proteolytic breakdown, or toxicity of the expressed protein to the host bacteria. In addition, expression of the pATH11-VP0 plasmid did not lead to the synthesis of a high level of the predicted 66-kDa TrpE/VP0 fusion protein, but produced high levels of two other unique recombinant polypeptides of approximately 39 and 27 kDa (Fig. 2, lane 4). Immunoblot analysis of these proteins (data not shown) suggested that the 66-kDa protein might have been processed in *Escherichia coli* to yield proteins representing TrpE plus VP4 (39 kDa) and VP2 (27 kDa). However, sequencing of the amino terminus of the 27-kDa protein revealed that it began with a methionine, which is seven residues downstream from the published amino-terminal Asp residue of HAV virion VP2 (4). Inspection of the nucleotide sequence (2) upstream of this ATG (Met) codon (nucleotide 822) revealed an almost consensus *E. coli* Shine-Dalgarno ribosome binding site (AGGAAG) with proper spacing in relation to the start (ATG) codon (7 nucleotides upstream). Therefore, it is likely that the recombinant 27-kDa VP2 polypeptide expressed from the pATH11-VP0 plasmid results from an internal initiation of translation rather than from proteolytic processing.

Preparation of recombinant antigens. The bacterially synthesized antigens fractionated predominantly into the insol-

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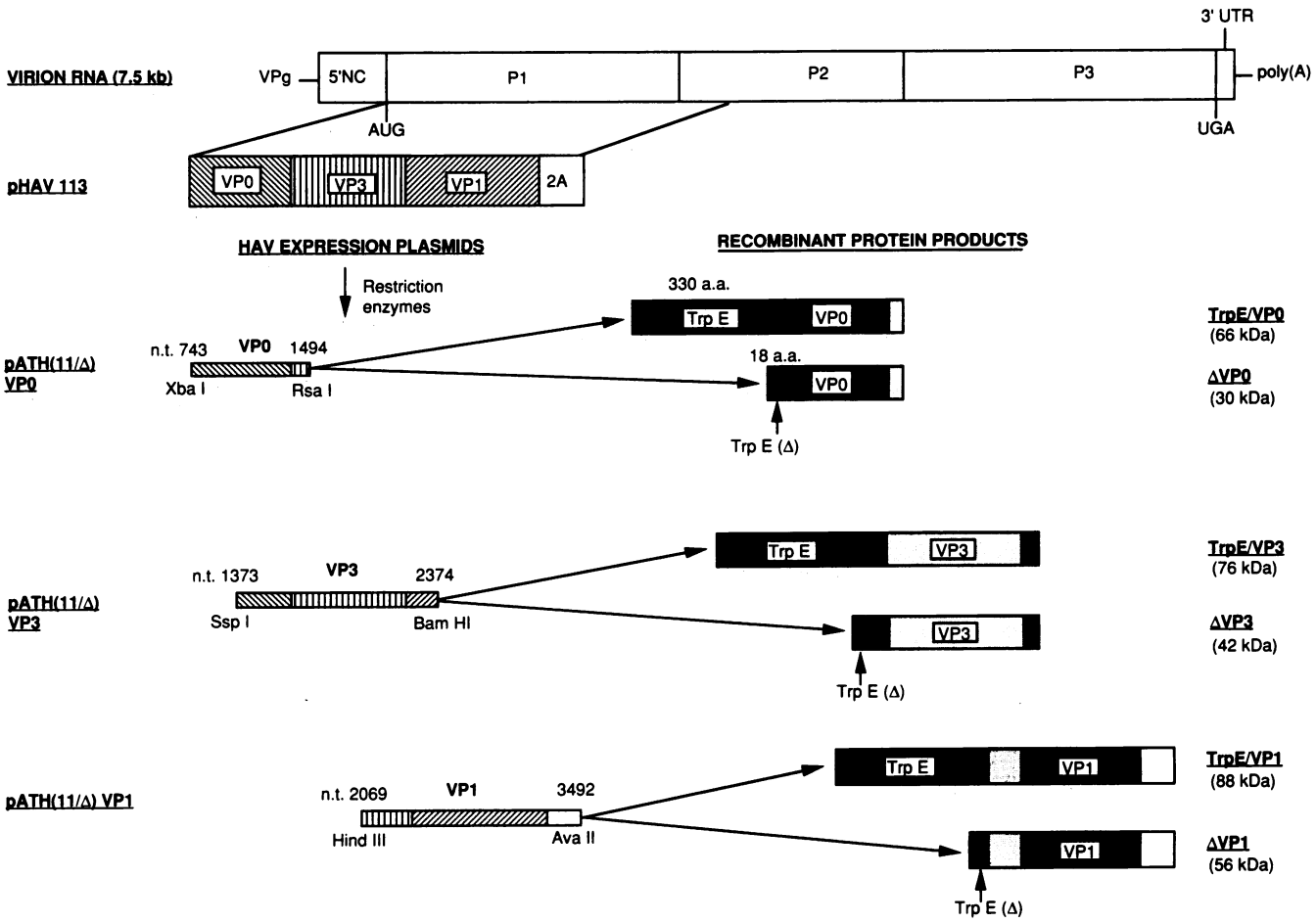

FIG. 1. Schematic representation of the HAV genome with P1 cDNA sequences expanded to indicate the capsid regions cloned into the pATH11 and pATHΔ expression vectors [pATH(11/Δ)]. The terminal HAV nucleotide sequence positions and restriction endonuclease sites utilized for subcloning into the expression vectors are indicated for each cDNA fragment. Arrows to the right of each restriction fragment point to bar representations of each recombinant protein product. These bars illustrate the viral protein subunit composition, and the predicted molecular weight of each recombinant protein is indicated to the right of each bar.

uble pellet after disruption of cells, presumably in the form of intracellular inclusion bodies. Either of the strong chaotropic agents 8 M urea or 7 M guanidine-HCl in conjunction with the reducing agent 2-mercaptoethanol was used to solubilize the recombinant protein-containing fractions. Following solubilization, the urea or guanidine and the 2-mercaptoethanol were removed by dialysis against dilute Tris buffers (10 to 20 mM). This gradual removal of denaturants, followed by incubation with prereduced thioredoxin, which catalyzes the reformation of disulfide bonds, was performed in an attempt to renature and refold the proteins into a more native type of conformation before use as immunogens. Although the presence of disulfide bonds in the HAV capsid proteins is not known and the utility of this protocol in reconstituting immunogenic epitopes dependent on secondary structure has not been investigated, such an approach has been shown to restore activity to denatured enzymes (16, 19). TrpE/VP1, TrpE/VP3, and nonfusion (Δ) VP3 recombinant proteins prepared in this manner were used directly without further purification for rabbit immunizations (Fig. 2, lanes 1 to 3).

Nonfusion VP3 and TrpE/VP0 were further purified with a combination of ion-exchange and gel filtration chromatography (S Sepharose and Sephacryl S-300; Pharmacia LKB, Piscataway, N.J.). Purified ΔVP3 (Fig. 2, lane 5) was used in antigenic analyses and purified TrpE/VP0 moieties were used for both antigenic and immunogenic studies. In particular, gel filtration of the TrpE/VP0 pellet fraction led to the isolation of the three polypeptides (66, 39, and 27 kDa) characteristic of the expression of the pATH11-VP0 plasmid. These three proteins eluted together in a high-molecular-weight fraction and were used for the primary and two booster immunizations of rabbits. The VP2 polypeptide was further purified by elution from an Immobilon membrane (Immobilon-P; Millipore Corp., Bedford, Mass.) (19) (Fig. 2, lane 6) and used as a final booster for rabbits immunized previously with TrpE/VP0 pooled proteins. These recovered proteins were dialyzed, concentrated, renatured, and refolded as above prior to use as immunogens.

Antigenic properties of the recombinant HAV proteins. All of the recombinant antigens were recognized in immunoblot analysis by antisera produced to recombinant proteins which share overlapping HAV sequences or common vector-encoded (TrpE) sequences (data not shown). Although the TrpE/VP1 antigen has been shown previously to react with rabbit anti-HAV virion antisera (7), we could not reproduc-

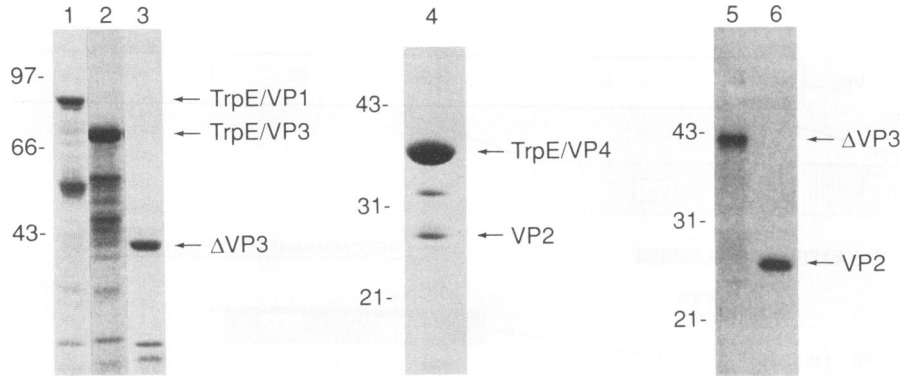

FIG. 2. Coomassie-stained SDS-PAGE gels of the pellet fraction from proteins expressed in derepressed *E. coli* cultures. Expression plasmids used for each preparation were as follows: lane 1, pATH11-VP1; lane 2, pATH11-VP3; lane 3, pATHΔ-VP3; lane 4, pATH11-VP0. Lane 5 shows chromatographically purified ΔVP3 protein from the preparation shown in lane 3; lane 6 shows purified VP2 protein eluted after blotting the VP2 band indicated in lane 4 onto an Immobilon membrane. Molecular mass in kilodaltons is given at the left of each panel.

ibly demonstrate reactivity of any of the recombinant antigens (VP0, VP3, or VP1) with our rabbit antisera raised to Formalin-inactivated HAV. In contrast, each of the recombinant antigens was recognized by anti-HAV containing human convalescent antisera (1:25 dilution) (Fig. 3A), but not by anti-HAV-negative human sera (data not shown). All human sera were exhaustively adsorbed with an *E. coli* lysate containing the expressed product of the pATH11 plasmid (TrpE) to eliminate nonspecific binding to *E. coli* determinants. The observation that the recombinant proteins are recognized by anti-HAV-containing convalescent human sera suggests that these proteins present epitopes which are recognized by at least a portion of the antibodies produced during natural HAV infection.

Immunogenic properties of the recombinant HAV proteins. HAV virions were purified from concentrated infected cell (BS-C-1) supernatants, isopycnically banded in cesium chloride-sucrose gradients (13, 17), and used as antigen in immunoblot analysis. Antisera raised to each of the recombinant HAV capsid proteins reacted with its corresponding denatured HAV virion capsid protein by immunoblot (Fig. 3B). In addition, virion proteins VP1 and VP3 were recognized by both recombinant VP1 and VP3 antisera, presumably as a result of the overlapping capsid regions present in these immunogens (Fig. 1). Likewise, virion protein VP2 appeared to be recognized by recombinant VP3 antisera, although it is difficult to determine with certainty as the gel mobilities of virion proteins VP2 and VP3 are similar. No band corresponding to virion VP4 or VP0 was ever observed by immunoblot analysis of mature (160S) virions.

The ability of the rabbit antibodies to bind to intact HAV particles was determined by commercial enzyme immunoassay (HAVAB; Abbott Laboratories, North Chicago, Ill.). Serum was assayed by a modified HAVAB protocol in which the competitive ratio of test sample to labeled HAV antiserum was reduced from the normal HAVAB ratio of 1:20 to

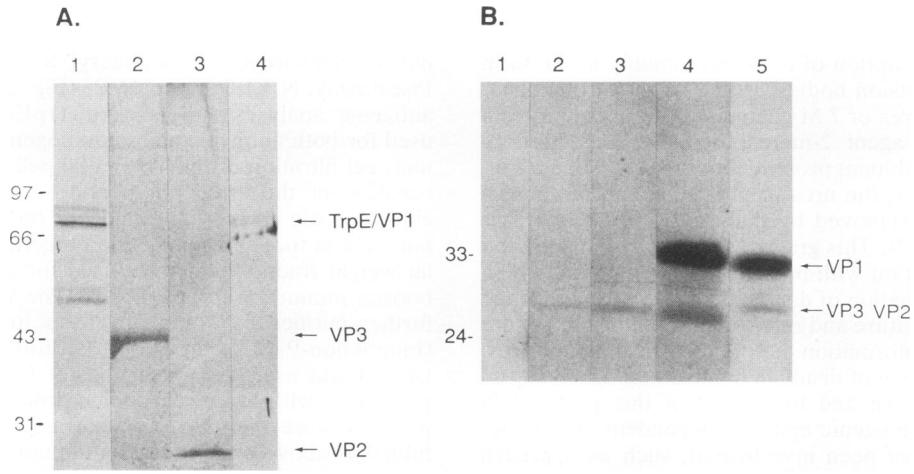

FIG. 3. Immunoblots demonstrating antigenicity and immunogenicity of the HAV recombinant proteins. (A) Immunoblot of proteins synthesized in *E. coli* and probed with a 1:25 dilution of human antisera known to react with HAV virions (HAVAB +). Expression plasmids used for each preparation were as follows: lane 1, pATH11-VP1; lane 2, pATHΔ-VP3; lane 3, pATH11-VP0; lane 4, pATH11. Arrows denote the position of the HAV recombinant protein detected by the human antisera. (B) Immunoblot of dissociated HAV proteins from purified, mature (160S) virions probed with the following rabbit antisera: lane 1, preserum; lane 2, anti-TrpE/VP0; lane 3, anti-VP2 peptide (kindly provided by S. Feinstone); lane 4, anti-ΔVP3; lane 5, anti-TrpE/VP1. Arrows indicate the position of each capsid protein. The arrow labeled VP3/VP2 indicates that the virion proteins VP3 and VP2 have approximately the same gel mobility.

TABLE 1. Antivirion binding (enzyme immunoassay [EIA]) and neutralizing (radioimmunofocus inhibition test [RIFIT]) antibody responses in HAV recombinant protein-primed rabbits given 2 ng of whole HAV

Recombinant antigen	% of preimmune value[a]					
	EIA			RIFIT		
	Day 0	Day 8	Day 26	Day 0	Day 8	Day 26
TrpE/VP0	100	22	40	100	24	24
TrpE/VP3	100	25	31	100	11	5
ΔVP3	100	47	29	100	7	2

[a] Day 0 = 100%. Average values of two rabbits per immunogen are shown.

1:1. A positive response was considered one that produced 50% or greater inhibition of A_{492} given by the same rabbit's preimmune serum. By this criterion, only rabbits immunized with the recombinant nonfusion VP3 protein (ΔVP3) developed a virion-binding antibody response (data not shown).

All rabbit sera were also tested by a sensitive, cell culture-based radioimmunofocus inhibition test (11), analogous to a standard viral plaque assay, except that foci of viral replication are detected autoradiographically after removal of agarose overlays, fixation of cell monolayers with acetone, and incubation with ^{125}I-labeled polyclonal antibody to HAV. Neutralizing antibody was considered present when incubation of serum (1:10 dilution) with a titered amount of virus resulted in a 50% or greater reduction in the observed number of radioimmunofoci given by the same rabbit's preimmune serum. None of the rabbit antisera to recombinant HAV immunogens was found capable of neutralizing infectivity of cultured cells. In contrast, rabbit antisera raised to whole virions, as well as HAVAB-positive human sera, were found to strongly inhibit foci formation (data not shown).

Immunologic priming effect of the expressed HAV proteins. To determine whether the recombinant immunogens were capable of priming an antibody response to intact virus, rabbits immunized previously with the recombinant viral proteins were challenged with a single subimmunogenic dose of Formalin-treated whole HAV (kindly provided by L. Binn) (1) without adjuvant. The dose chosen (~2 ng) had been shown previously not to elicit an antiviral antibody response in control animals, whereas a larger (~10-ng) dose was shown to stimulate an antiviral antibody response in naive rabbits, but only after 2 weeks following inoculation (7). The priming response induced by the TrpE/VP1 fusion protein has been reported previously (7). Both the control and TrpE/VP1 priming experiments have been repeated with reproducible and consistent results. Rabbits immunized with each of the recombinant proteins (TrpE/VP0, TrpE/VP3, or ΔVP3) developed neutralizing antibody within 5 to 13 days after the administration of the subimmunogenic dose of HAV (results of HAVAB and neutralization tests summarized in Table 1). For all antisera tested for priming, radioimmunofocus inhibition test neutralization data were in qualitative accordance with the enzyme immunoassay data.

Although an anamnestic virus-neutralizing antibody response must necessarily contain both T- and B-cell components, we think that the data presented here are most consistent with a helper T (T_H)-cell response to HAV recombinant capsid proteins. A computer analysis, based on the probability of amphipathic helix formation (3, 15) of sequences of each of the HAV virion proteins, revealed that there are possible T_H cell epitopes on each of the capsid proteins. As some of these putative T_H cell epitopes occur in overlapping capsid protein sequences present in the recombinant proteins (Fig. 1), it is impossible to predict which of the HAV virion proteins contain epitopes which may be responsible for the observed priming effect. We suspect that VP0 (or VP2) contains an important priming epitope as there is only an 8-aa overlap with VP3; other epitopes must be present in VP3, VP1, or both. It is not known whether any of the suspected T_H cell epitopes are immunogenic in humans or whether T_H cell immunization with recombinant proteins can protect against HAV infection or disease. Further studies will be needed to answer these questions as well as to address the roles of various components of the immune system not only in immune-mediated clearance of HAV infection, but also in the immunopathology of HAV disease.

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