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Induction of Hepatitis A Virus-Neutralizing Antibody by a Virus-Specific Synthetic Peptide

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Comparative surface feature analyses of the VP1 sequences of hepatitis A virus (HAV) and poliovirus type 1 allowed an alignment of the two sequences and an identification of probable HAV neutralization antigenic sites. A synthetic peptide containing the HAV-specific amino acid sequence of one of these sites induced anti-HAV-neutralizing antibodies. It is concluded that a structural homology exists between the two viruses, despite minimal primary sequence conservation.

Hepatitis A virus (HAV) has recently been classified on the basis of morphology, epidemiology, and biophysical characteristics as enterovirus type 72, a member of the enterovirus genus of the *Picornaviridae* family (8, 13, 16, 17, 19). As is the case for poliovirus, the prototype member of the enterovirus genus, the hepatitis A virion is a naked icosahedron whose surface is composed of three structural proteins, i.e., VP1, VP2, and VP3 (3, 19). VP1 is the predominantly exposed protein (10). Studies with neutralizing monoclonal antibodies have shown that this structural protein contains at least some of the virion neutralization antigenic sites (10).

Emini et al. (5, 6) identified several poliovirus type 1-specific neutralization antigenic sites on the poliovirion VP1 protein. Synthetic peptides containing the amino acid sequences of these sites are capable of either directly inducing a virus-neutralizing antibody response or priming such an immune response upon inoculation of a subimmunogenic dose of intact virion (6).

cDNA of the genomic HAV RNA has been cloned recently, and partial sequences of the nonstructural (20) and structural (14) gene regions have been obtained. The availability of this information prompted an examination of the structural relatedness between HAV and poliovirus. It was hoped that the detailed knowledge of the latter's antigenic structure would provide insights into the antigenic structure of HAV.

VP1 amino acid sequence comparisons. The VP1 amino acid sequences of HAV and poliovirus type 1 (Mahoney strain) (12, 18) were compared for primary sequence homology by the ALIGN program of Dayhoff (4) (data not shown). The homology was slight, giving an alignment score (4) of 1.93, indicating a probability of 2.5% that the alignment with randomly permuted sequences would give a better score. This degree of homology is generally considered insufficient to establish an evolutionary relationship between proteins on the basis of primary sequence alone. Alignments with different break penalties gave similar alignment scores. Nonetheless, a provisional alignment of the HAV and poliovirus proteins was made.

Comparisons of the predicted secondary structures of the two VP1 proteins by the methods of Chou and Fasman (2) and Garnier et al. (7) showed no predicted structural similarities (data not shown). For example, by the method of Garnier et al. (7), the predicted alpha helical potentials of poliovirus VP1 (1 to 302) and HAV VP1 (5 to 306) aligned poorly, with the alignment having a correlation coefficient (r) of −0.22 (R^2 = 0.049). The alignment of helical potentials in the first 110 residues was actually significantly negative, with r = 0.51 (r^2 = 0.26) indicating a possible anticorrelation. Other possible alignments were similarly poor with helical, extended structure (sheet), turn, or coil potentials of Garnier et al. (7) or comparable potentials described by Chou and Fasman (2). The overall predicted structures, by either method, of the sequences for VP1 of poliovirus and HAV showed no apparent similarities and could not be aligned on the basis of predicted secondary structure.

Hence, in an attempt to increase confidence in the provisional alignment made by the Dayhoff program, a method was developed for comparing the two sequences by predicted surface features, based upon indices of surface probability. This method assumes the absence of significant internal deletions or insertions. For a given amino acid sequence, a point for sequence number n is a normalized product of the surface probabilities of amino acids in positions $n - 2$ to $n + 3$, using the empirical amino acid accessible surface probabilities of Janin et al. (11) which are fractional probabilities (0.26 to 0.97) determined for an amino acid found on the surface of a protein, based upon structural data from 28 proteins. A surface residue is defined as one with >20 Å^2 (2.0 nm^2) of water-accessible surface. With these fractional surface probabilities for amino acids, a surface probability (S) at sequence position n can be defined as follows:

$$S_n = [\prod_{i=1}^{6} \delta_{n+4-i}] * (0.37)^{-6}$$

δ_x is the fractional surface probability for the amino acid at position x. The S_n for a random hexapeptide sequence = 1.0, with probabilities greater than 1.0 indicating an increased probability for being found on the surface.

When the two sequences were compared by this surface probability method, striking similarities were noted (Fig. 1). These similarities, in conjunction with the results of the Dayhoff program analysis, allowed an alignment of the two VP1 sequences and an identification of three HAV sites which correspond to the three previously identified (6)

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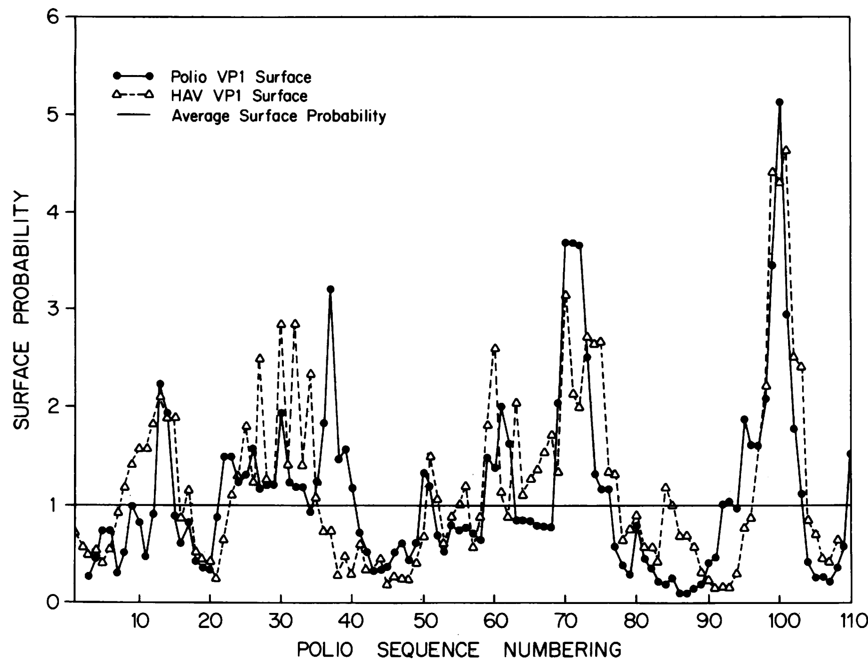

FIG. 1. Surface probability profiles comparing HAV VP1 (5 to 114) with poliovirus type 1 (Mahoney strain) VP1 (1 to 110) (12, 17). The correlation coefficient (r) for the comparison shown is 0.68 (r^2 = 0.46). Comparisons between the C-terminal halves of poliovirus VP1 and HAV VP1 show a marked decrease in surface profile homology. Randomly related sequences show surface probability profiles with r close to zero. Comparisons of surface profiles of poliovirus and HAV VP1 generated by the amino acid hydrophilicity-based method of Hopp and Woods (9) give results similar to those obtained with the surface probability method, though visually not as striking and with a significantly lower correlation coefficient.

poliovirus neutralization antigenic sites (Fig. 2). Once aligned, a degree of amino acid sequence and content similarity was evident between the poliovirus and HAV sites.

Induction of an anti-HAV response by an HAV-specific synthetic peptide. A synthetic peptide was prepared, by the solid-phase method (1) with a Beckman model 990B peptide synthesizer, which contained the amino acid sequence of one of the three targeted HAV sites (Fig. 3). Antibodies in hyperimmune serum raised against either intact HAV or sodium dodecyl sulfate-disrupted HAV were found, by immunosorbent assay, to bind to the free peptide (data not shown). Hence, the peptide may express a conformation or set of conformations equivalent to those found on the intact virion, although the nature of the cross-reactivity is not entirely clear.

The peptide was then linked covalently, independently via either end, to a bovine serum albumin carrier. New Zealand White rabbits and Hartley guinea pigs were each inoculated with 0.7 mg of the respective conjugate per inoculation. Each animal received three injections with Freund adjuvant over a 2-month period. The resultant sera all contained antipeptide immunoglobulin G antibodies. In addition, each serum also contained immunoglobulin G antibody capable of (i) binding directly to purified hepatitis A virions and (ii) neutralizing the infectivity of the virus in cell culture (Table

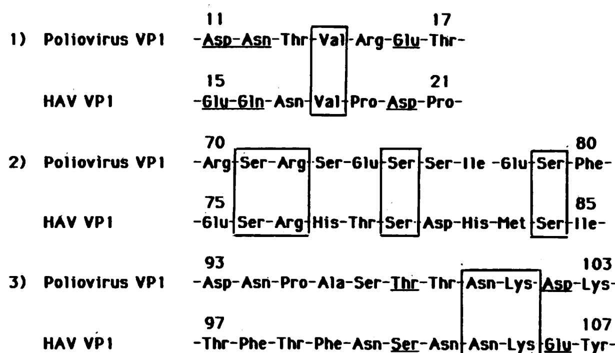

FIG. 2. Alignment of the poliovirus VP1 and HAV VP1 sequences at the three poliovirus-specific neutralization sites. The alignment is based on the alignment of surface probability profiles (Fig. 1). Identical amino acid residues between poliovirus and HAV revealed by the alignment are enclosed in boxes. The alignment of the second sequence, poliovirus VP1 (70 to 80) to HAV VP1 (75 to 85), was shifted by one amino acid from that previously implied (Fig. 1) to maximize the homology. A number of additional similarities in the aligned amino acid sequences are underlined. For example, Asp aligns with Glu, Asn aligns with Gln, and Thr aligns with Ser. Amino acid residues numbering is from the amino terminus of each protein.

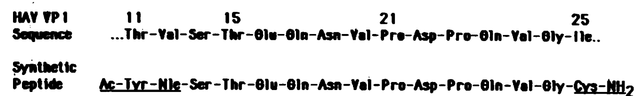

FIG. 3. Synthetic HAV VP1 (11 to 25) analog peptide sequence. The sequence contains the HAV VP1 (15 to 21) segment which was aligned by the surface probability profile analysis with the antigenic poliovirus VP1 (11 to 17) determinant. The underlined Tyr and Cys residues were added for coupling to carriers, and the Nle residue facilitated the quantitation of coupling efficiency. Coupling via the Cys residues was by the method of Liu et al. (14), and the conjugate contained 10 μg of peptide per mg of bovine serum albumin conjugate. Coupling via Tyr was as described by Walter et al. (21) with 40 mol of peptide per mol of bovine serum albumin.

TABLE 1. Antibody responses after immunization with the HAV-specific synthetic peptide[a]

Animal[b]	Reactions with:				HAV neutralization[e]
	Peptide[c]		HAV virion[d]		
	Preserum	Postserum	Preserum	Postserum	
Rabbit					
N-1	0.195	0.307	0.120	0.893	10.0
N-2	0.173	0.451	0.192	1.078	10.0
C-1	0.250	0.421	0.207	0.271	10.0
C-2	0.212	0.289	0.195	1.191	10.0
Control	0.187	0.199	0.155	0.166	<1.0
Guinea pig					
N-1	0.944	1.267	0.817	0.952	10.0
N-2	0.889	2.200	0.987	1.452	10.0
C-1	0.838	1.648	0.660	1.179	10.0
C-2	0.739	1.812	0.920	1.163	10.0
Control	0.791	0.817	0.771	0.780	<1.0

[a] Rabbits and guinea pigs were inoculated as described. Postsera were prepared 2 weeks after the final inoculation.

[b] Animals N-1 and N-2 were inoculated with peptide conjugated to bovine serum albumin via the peptide amino terminus. Animals C-1 and C-2 received the peptide carboxy-terminal conjugate. The control animals were inoculated with unconjugated bovine serum albumin.

[c] Antibody-peptide immunoglobulin G reactions were measured by enzyme-linked immunosorbent assay (6). The values represent optical density readings at 405 nm after the final enzyme-linked immunosorbent assay step and are representative of several experiments.

[d] Antibody-virion immunoglobulin G reactions were measured by enzyme-linked immunosorbent assay. The substrate was purified hepatitis A virions bound to the microtiter plate wells by an HAV-specific monoclonal antibody. The antibody had been determined not to react with the synthetic peptide under study. The values represent optical density readings at 405 nm after the final enzyme-linked immunosorbent assay step and are representative of several experiments.

[e] Neutralization of HAV growth in cultures of newborn Cynomolgus monkey kidney cells was determined as described previously (10). The numbers represent the reciprocal of the highest postserum dilution capable of reducing virus growth by 50%. In each case, preserum exhibited no effect. Hyperimmune anti-HAV serum yields values in this assay of ca. 1,000.

1). The anti-HAV antibody could be removed from the serum by exposure to free peptide (Fig. 4).

It was of interest to determine whether the peptide-induced antivirion antibody was also capable of competing with antibody found in human HAV immune serum. For this purpose, each serum was tested in a modified commerical HAVAB assay which measures antihepatitis A virion immunoglobulin G by competition with a standard human immune serum. The results (Table 2) showed that all of the guinea pig and 50% of the rabbit sera contained such competitive antibodies. Hence, some of the cross-reactive, peptide-specific virion epitopes are also recognizable by the human immune system. It is noteworthy, however, that at least one epitope which is recognized by the rabbit immune system is not recognized by the human.

Finally, the antipeptide antibodies were found to react, by Western immunoblotting, with purified denatured HAV VP1 (data not shown). This result emphasized the potentially sequential nature of the determinant represented by the peptide sequence on the virion surface.

TABLE 2. Competition between human anti-HAV antibodies and anti-HAV peptide antibodies[a]

Animal[b]	CPM of labeled human antibodies	
	Preserum	Postserum
Rabbit		
N-1	3,566	2,695
N-2	3,353	3,259
C-1	3,556	2,038
C-2	2,878	2,759
Control	ND[c]	5,132
Guinea pig		
N-1	4,343	1,430
N-2	5,119	1,147
C-1	3,346	1,622
C-2	4,052	1,773
Control	ND	4,538

[a] Each serum was tested for its ability to compete with antibodies found in human anti-HAV immune serum by a modified commercial HAVAB assay. The assay measures the presence of anti-HAV antibody by the degree to which this antibody competes with radioactively labeled human anti-HAV for binding to intact hepatitis A virions. The assay was performed as suggested by the supplier (Abbott Laboratories), except that equal volumes of test serum and diluent were used. The reported values represent the counts per minute (CPM) of labeled human antibodies bound to virion. With no competition, the assay yielded a value of 5,279 CPM. Competition with unlabeled homologous human immune serum lowered this value to 113 CPM.

[b] See footnote b to Table 1 for animal identification.

[c] ND, Not done.

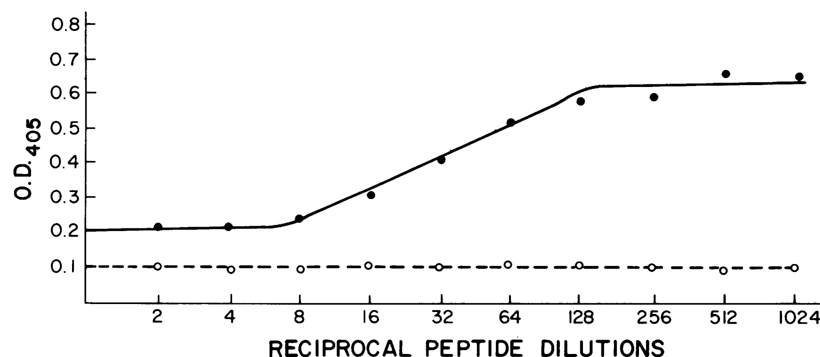

FIG. 4. Competition by free peptide of anti-hepatitis A virion activity in antipeptide serum. Serial 1:2 dilutions of a 1.0 mg/ml peptide solution were mixed 1:1 with a 1:10 dilution of test serum. The mixtures were allowed to incubate overnight at 4°C. Each was then assayed for antivirion activity by enzyme-linked immunosorbent assay (see footnotes to Table 1). The values recorded in the graph represent optical density readings at 405 nm after the final enzyme-linked immunosorbent assay step. The *x* axis is logarithmic ($\log_{10}$) in scale. The results reported here are representative and were obtained with sera from rabbit N-1 (Table 1). Symbols: ●, postserum; ○, preserum. The small degree of positive reactivity seen after complete peptide absorption of the antivirion activity is probably due to binding between antibodies to the carrier protein and residual bovine serum albumin associated with the purified virus.

It is of interest that, despite apparently minimal primary sequence and secondary structure conservation, the surface structure homology between poliovirus and HAV may be significant. This structural similarity may involve the antigenic site presentations of the two viruses. It was clearly possible to identify an HAV-specific neutralization antigenic site solely by comparison to the known locations of such antigenic sites on the poliovirus-specific VP1. The synthetic peptide reported in this communication has considerable potential as a component of a synthetic HAV vaccine, especially since it was shown that antipeptide antibodies could compete with antibodies in human HAV immune serum. Synthetic peptides containing amino acid sequences of the remaining two probable HAV neutralization antigenic sites are currently under study.

Also, in conclusion, it should be noted that the alignment of the HAV and poliovirus VP1 sequences by surface probability analysis confirms the current mapping of the HAV VP1 on the HAV genomic RNA sequence. The mapping has been established by comparison of the RNA sequence with VP1 amino acid sequence data (14).

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