Hepatitis B virus vaccine: Identification of HBsAg/a and HBsAg/d but not HBsAg/y subtype antigenic determinants on a synthetic immunogenic peptide*

(epitopes/peptide synthesis)

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ABSTRACT On the basis of theoretical considerations, a peptide (H peptide) was synthesized by Hopp and Woods [Hopp, T. P. & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. USA* 78, 3824–3828]. This peptide contains a sequence of six amino acids postulated to represent the major epitope, or antibody-combining site, of hepatitis B virus surface antigen (HBsAg). We have used passive hemagglutination inhibition with monospecific antibodies against the a, d, and y subdeterminants of this antigen and against human serum albumin to investigate the antigenic specificities on this peptide, and we have found it to contain the HBsAg/a and HBsAg/d but not HBsAg/y or human serum albumin subdeterminants. When the peptide was conjugated onto human erythrocytes and injected into mice, it induced the formation of anti-HBsAg with and without the use of Freund's adjuvant. If anti-HBsAg/a confers immunity to infection with hepatitis B virus, as is generally thought, these findings may permit the development of a synthetic vaccine lacking all unnecessary antigenic determinants.

Discovery of hepatitis virus-specific antigens (1–3) and their association with the outer membrane of hepatitis B virus (HBV) (4, 5) has led to rapid advances in the prevention of HBV infection. This has been achieved through detection of HBV carrier blood donors by testing for the HBV surface antigen (HBsAg) (6, 7), and by passive (8–12) and active (13–18) immunization.

The protective effect of hepatitis B immune globulin, prepared from plasma selected for high anti-HBsAg content, as well as the demonstration that protective immunity can be induced, albeit at high cost, by the purified 23,000-dalton HBsAg polypeptide isolated from HBV-associated subviral particles (19), has suggested that anti-HBsAg is a protective antibody. Other surface specificities on the HBV virion could also play a similar role (18, 20).

HBsAg also exhibits generally mutually exclusive strain-specific subtype antigenic determinants—e.g., d/y (21) and w/r (22)—in addition to the determinant a common to all strains. Available evidence suggests that the subtype specificities play no role in providing protective immunity (23, 24), and thus that HBsAg/a may be the critical determinant.

It is thus of obvious interest to learn the amino acid sequence of the antibody-combining site, or epitope, of HBsAg/a. Progress towards this goal has depended on two key advances: isolation and characterization of HBV DNA (25) and determination of its sequence through recombinant DNA methods (26–29). This permitted prediction of the amino acid sequence of the product of the HBsAg gene (the *s* gene) (30, 31). Using a theoretical model, Hopp and Woods (32) were then able to predict a putative dominant HBsAg epitope (termed H epitope): the

sequence Lys-Pro-Thr-Asp-Gly-Asn corresponding to positions 141–146 on the HBsAg protein. They then synthesized a tetradecapeptide (H peptide) containing residues 138–149 of HBsAg plus two glycine residues at its COOH terminus, using classical solid-phase peptide synthetic methodology. The four cysteinyl residues were replaced by α -aminobutyric acid in order to prevent polymerization and other side reactions common to sulfhydryl-containing peptides. Polystyrene beads covalently coated with H peptide bound more 125 I-labeled anti-HBsAg than did uncoated beads (33).

We report herein confirmation of the prediction that the H peptide contains a major epitope for HBsAg, and we further demonstrate that this sequence of amino acids contains the HBsAg/a and HBsAg/d epitopes, but not the epitope of HBsAg/y or human serum albumin. Furthermore, we demonstrate that when bound to a carrier the peptide induces the synthesis of anti-HBsAg *in vivo*.

MATERIALS AND METHODS

H Epitope Polypeptide Preparations. Initial experiments were done with intact polystyrene beads (XE305A resin beads, Rohm and Hass, Philadelphia) on which the H peptide had been synthesized. These beads contained 10 mg of peptide per 25 mg.

For immunization it was necessary to remove the peptide from the polystyrene beads with hydrofluoric acid (34). The isolated polypeptide was then attached to aldehyde-stabilized human erythrocytes (35), or polymerized with 2.5% (vol/vol) glutaraldehyde in 0.01 M sodium phosphate buffer (pH 7.2)/0.15 M NaCl for 1 hr at room temperature and overnight at 4°C. The polymerized preparation was passed through a Sephadex G-200 column and the void volume fraction was recovered.

Passive Hemagglutination and Hemagglutination Inhibition. These tests were carried out as described (36), except that HBsAg/ay cells were obtained from Electronucleonics Laboratories (Bethesda, MD). In addition, some experiments utilized aldehyde-stabilized human group O erythrocytes covalently coated with HBsAg/ad (36) or with human serum albumin. Monospecific antibody reagents were prepared as described (36). Anti-HBsAg/a is abbreviated anti-HBs/a, etc.

Immunization of Mice. Female Swiss ICR strain mice, weighing 20–25 g, were immunized as described.

Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; HBsAg/a, HBsAg subtype determinant a (subtype determinants d and y are indicated similarly); H epitope, dominant HBsAg epitope; anti-HBs/a, antibody to HBsAg/a (antibodies to other subtype determinants are indicated similarly); IU, international unit; TAP buffer, 0.01 M sodium phosphate (pH 7.2)/0.15 M NaCl/0.5% bovine serum albumin/0.0025% polyvinylpyrrolidone/0.005% Tween 80.

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Anti-HBsAg Quantitation by Solid-Phase Radioimmunoassay. Anti-HBsAg was also quantitated with a parallel line assay (18) by comparison with the World Health Organization international hepatitis B immune globulin standard no. 1, which contains 100 international units (IU)/ml.

RESULTS

Determination of Antigenic Specificities on H Peptide Attached to Polystyrene Beads. To determine the antigenic specificities present on the H peptide, we carried out the following experiment: Monospecific antibodies to the a, d, and y specificities of HBsAg were prepared, titrated by passive hemagglutination, and diluted to a titer of 1:2 to 1:4. In addition, anti-human serum albumin was titrated against albumin-coated erythrocytes and similarly diluted. Twenty-five milligrams each of uncoated polystyrene beads, beads coated with normal human serum albumin, and beads with 10 mg of attached H-polypeptide were washed twice with 0.01 M phosphate buffer (pH 7.2)/0.15 M NaCl/0.5% bovine serum albumin/0.0025% polyvinylpyrrolidone/0.005% Tween 80 (TAP buffer) and then immersed in 200 μ l of diluted antibody. After 30 min at 37°C and 1 hr at 4°C, with shaking, the beads were removed by centrifugation (5000 rpm, 10 min in a SS 34 rotor (Sorvall)) and the antibody in the supernate was quantitated by passive hemagglutination against human type O erythrocytes coated with HBsAg/ad by the chromic chloride method, similar cells coated with HBsAg/ay, or human serum albumin.

The results, shown in Table 1, revealed that the peptide-coated beads, but not the two types of control beads, adsorbed anti-a and anti-d antibodies, but not anti-y. Furthermore, none of the beads nonspecifically adsorbed anti-albumin.

We conclude that the polypeptide tested contains HBsAg/a and HBsAg/d but not HBsAg/y or albumin specificities and that the beads can adsorb at least 50–75% of monospecific anti-HBs/a and anti-HBs/d.

The antibody-binding capacity of the H peptide was limited. Ten milligrams of peptide was able to neutralize no more than 1 mIU of anti-HBs/a.

Immunogenicity of H Peptide. To investigate the immunogenicity of the H peptide we coupled the solubilized peptide to aldehyde-treated human erythrocytes and injected mice by subcutaneous and intraperitoneal routes, with 10^8 erythrocytes coupled to 5 or 50 μ g of peptide, with and without emulsification in Freund's complete adjuvant. Eighteen days after injection the mice were bled. The blood from each group was pooled and allowed to clot, and the separated serum was tested for anti-HBsAg by the AUSAB radioimmunoassay (Abbott). As will be seen in Table 2, anti-HBsAg was detected in pooled blood from groups that received subcutaneous injections of 5 μ g of peptide without adjuvant, and 5 and 50 μ g of peptide with adjuvant.

All mice then received a booster injection of 5 μ g of glutaraldehyde polymerized peptide with Freund's incomplete adjuvant when Freund's complete adjuvant had been used in the initial immunization, and without adjuvant in the remaining groups. Two weeks after the booster the mice were again bled and individual sera were prepared and tested for anti-HBsAg as before. This time anti-HBsAg response was seen in 33–50% of mice in each group except for those receiving the larger dose of peptide without adjuvant.

DISCUSSION

Our findings indicate that the H peptide, as predicted, contains HBsAg activity, particularly HBsAg/a and HBsAg/d, but not HBsAg/y or human serum albumin. This peptide can adsorb about 50–75% of monospecific anti-HBs/a and anti-HBs/d antibody activity from solution. These results confirm the immunologic specificity of the H peptide. They further indicate that the a and d specificities are located together on this par-

Table 1. Adsorption of monospecific antibodies by H peptide-coated beads

Antibody*	Adsorbent, polystyrene beads coated with	Titer with coated erythrocytes [†]				Conclusion: Antibody adsorbed
		HBsAg/ad		HBsAg/ay [¶]	Human serum albumin	
		CrCl ₃ [‡]	Aldehyde [§]			
Anti-HBs/a	Uncoated	1:2	1:2	1:2	(-)	-
	Normal human serum	1:4	1:2	1:2	(-)	-
	H peptide	(-)	(-)	(-)	(-)	+
	No beads	1:4	1:2	1:4	(-)	
Anti-HBs/d	Uncoated	1:2	1:4	(-)	(-)	-
	Normal human serum	1:4	1:2	(-)	(-)	-
	H peptide	(-)	(-)	(-)	(-)	+
	No beads	1:4	1:2	(-)	(-)	
Anti-HBs/y	Uncoated	(-)	(-)	1:4	(-)	-
	Normal human serum	(-)	(-)	1:4	(-)	-
	H peptide	(-)	(-)	1:4	(-)	-
	No beads	(-)	(-)	1:4	(-)	
Anti-human serum albumin	Uncoated	(-)	(-)	(-)	1:4	-
	Normal human serum	(-)	(-)	(-)	1:4	-
	H peptide	(-)	(-)	(-)	1:4	-
	No beads	(-)	(-)	(-)	1:4	

* Monospecific antibodies diluted to a titer of 1:2 to 1:4 in TAP buffer.

† (–) = no reaction with undiluted antibody.

‡ Human O erythrocytes coated with purified HBsAg/ad by chromic chloride method (36).

§ Human O erythrocytes coated with purified HBsAg/ad by aldehyde method (35).

¶ Human O erythrocytes coated with purified HBsAg/ay by chromic chloride method (36).

|| Human O erythrocytes coated with purified human serum albumin by aldehyde method (35).

Table 2. Immunogenicity of H peptide conjugated to aldehyde-stabilized human erythrocytes

Route	First injection		P/N at 18 days	Booster injection		Anti-HBsAg 2 weeks after booster			
	H peptide,* μg/dose	Adjuvant		H peptide polymer,† μg/dose	Adjuvant	No. tested	No. positive	% positive	Mean P/N in responders
i.p.	50	None	<1.5	5	None	6	0		—
s.c.	50	None	<1.5	5	None	6	0		—
i.p.	5	None	<1.5	5	None	6	3	50	3.8
s.c.	5	None	2.1	5	None	6	2	33	3.0
i.p.	50	FCA	<1.5	5	FIA	6	2	33	5.6
s.c.	50	FCA	6.6	5	FIA	6	3	50	10.2
i.p.	5	FCA	<1.5	5	FIA	6	2	33	3.2
s.c.	5	FCA	3.0	5	FIA	6	3	50	3.4

i.p., Intraperitoneal; s.c., subcutaneous; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; P/N, ratio of cpm of sample to cpm of negative controls in AUSAB test (Abbott).

* H peptide was coated onto 10⁸ erythrocytes per inoculum.

† H peptide was polymerized by glutaraldehyde.

ticular sequence of amino acids, most probably including the sequence Lys-Pro-Thr-Asp-Gly-Asn.

Recently, Vyas (37) reported that a synthetic peptide, beginning at position 134 in the s protein sequence, had HBsAg activity demonstrable by hemagglutination inhibition against anti-HBs. The subtype composition of the antiserum, or epitope(s) was not reported. Lerner and his colleagues (38) have also synthesized immunogenic peptides based on the sequence of HBsAg (38). Seven of 13 peptides synthesized elicited antibody with the peptides used for immunization, and four of these antisera reacted also with native HBsAg polypeptides. It is unclear why their peptide corresponding to portions 140–148, which includes the H epitope region, was only marginally immunogenic.

The limited adsorptive capacity of H peptide-coated beads—i.e., 1 mIU of anti-HBsAg adsorbed per 10 mg of peptide—suggests that the peptide as presently constituted does not closely mimic the tertiary structure of the native epitope. This is not surprising, because the native sequence contains cysteine groups on either side of the epitope: positions 137, 138, 139, 147, and 149. These probably form disulfide bridges, one of which may circularize the region containing the epitope. Reduction largely destroys HBsAg activity (39), which suggests that a disulfide-bonded loop configuration is vital to full antigenic activity in this region. Synthesis of analogues containing cysteine residues will allow testing of the immunological consequences of loop closure in this peptide.

Effective vaccines for prevention of HBV infections are now available (14–17), and others are contemplated or under development. These include vaccines derived from viral components circulating in the blood of chronic carriers (14–17) as well as the possible use of HBsAg expressed in prokaryotes such as *Escherichia coli* (40), in eukaryotic cells such as mouse (41) or human (42) cells in cell culture, or even in human HBsAg-secreting hepatocellular carcinoma cells (43). Vaccines have been proposed that would contain all membranous components of the virion or its subviral particles (18), isolated 20-nm subviral membrane particles (14–17), isolated polypeptides from the subviral particles (19), micellar aggregates of such isolated polypeptides (43), and isolated polypeptide fragments attached to suitable carriers (44).

Almost all of the above approaches suffer from two possible disadvantages, one theoretical, the other practical. Such vaccines could contain unwanted antigenic components, derived from elsewhere in the virion or its associated particles, or from the host cells in which these were synthesized. It is difficult, if not impossible, to remove all detectable host components—e.g.,

human serum albumin, IgG, and some other serum proteins—from even the most highly purified HBsAg particles (45–48). Indeed, when such particles derive from HBV e antigen-positive donors, they adsorb efficiently to aggregated human serum albumin (48). The possibility that such host components, if present in a vaccine including adjuvant might give rise to autoimmune complications has been raised (49) and sought for but so far not found (50–54). This is then still only a theoretical danger; on the basis of experience with previous far less pure vaccines, it is probably not of major significance.

The second disadvantage of most presently contemplated HBV vaccines is the high cost. Expensive vaccines cannot be afforded in most regions of the world in the foreseeable future.

A small peptide can be inexpensively and rapidly synthesized in large quantities. For example, 500 mg of H peptide was synthesized by one of us in about a week. Thus a practical solution to the problem of world-wide mass immunization against HBV may be at hand. Important model precedents for this approach are available (55, 56).

Erythrocytes were used as a convenient carrier for immunization in this study. We recognize that these would not provide a suitable carrier for use in vaccines, and it is necessary to find a more practical carrier.

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