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Chemically synthesized peptides of hepatitis B surface antigen duplicate the *d/y* specificities and induce subtype-specific antibodies in chimpanzees

(vaccine/radioimmunoassay/subtype determinant/chimpanzee animal model/amino acid sequence)

JOHN L. GERIN*, HANNAH ALEXANDER†, J. WAI-KUO SHIH*‡, ROBERT H. PURCELL§, GAIL DAPOLITO*, RONALD ENGLE*, NICOLA GREEN†, J. GREGOR SUTCLIFFE†, THOMAS M. SHINNICK†, AND RICHARD A. LERNER†

*Division of Molecular Virology and Immunology, Georgetown University Medical Center, Rockville, Maryland 20852; †Committee for the Study of Molecular Genetics, Research Institute of the Scripps Clinic, LaJolla, California 92037; and §Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT **Synthetic peptides, predicted from the nucleotide sequence of the S gene of hepatitis B virus were analyzed in terms of the established specificities of the hepatitis B surface antigen. The analysis indicated that the group-specific *a* antigen is composed of at least three nonoverlapping sequences and that a relatively hydrophilic region of the surface antigen protein, spanning amino acid residues 110–137, specifies the major *d* and *y* subtype system. The *d/y* subtype appears to depend on changes in one or more variable amino acids at positions 127, 131, and 134 of the hepatitis B surface antigen protein. Peptide 49 (consisting of amino acid sequences of the *y* subtype for the region 110–137), coupled to a carrier protein and mixed with an adjuvant, stimulated a brisk anti-*y* response in chimpanzees, the relevant model of human response to hepatitis B virus immunization and infection. Experimental challenge with homologous hepatitis B virus resulted in a pattern of partial protection. The results offer promise for the application of chemically synthesized peptides as vaccines in the prophylaxis of hepatitis B virus disease.**

Although a number of studies have suggested that the immunogenicity of a protein molecule depends largely on its conformation (1–3), more recent investigations have shown that reasonably short chemically synthesized peptides elicit antibodies reactive with virtually any region of an exposed surface of a protein (4). Coupled with the rapid determination of amino acid sequences via nucleic acid sequence analysis technology, synthetic peptides offer promise for vaccines that, in molecular terms, can be designed with a precision not heretofore possible.

Hepatitis B virus (HBV) represents an important test model of the synthetic peptide vaccine approach. Current vaccines for HBV (5) consist of subviral components of the virus surface coat (HBsAg) purified from the plasma of chronically HBV-infected donors and inactivated. HBsAg has been the subject of extensive immunochemical characterization and consists of a group-specific (*a*) and two sets of subtype-specific (*d/y*, *w/r*) determinants (6, 7); these specificities are associated with a single polypeptide (8, 9), the entire 226-amino acid sequence of which has been determined from the nucleotide sequence of the S gene (10) of HBV (11–13). Clinical trials have established the safety and efficacy of current HBsAg vaccines but these vaccines are limited in supply and relatively expensive for those world populations with the highest burden of HBV disease; also, certain theoretical risks remain (14). Chemically synthesized peptides, therefore, may offer advantages in terms of cost and safety of HBV vaccination programs if they can be shown to duplicate HBsAg specificities, elicit antibodies of established epidemiologic importance, and protect natural hosts.

It has been shown by radioimmunoprecipitation (15) and commercial solid-phase radioimmunoassays for anti-HBs (16) that antisera to synthetic peptides predicted from the nucleotide sequence of various regions of the S gene of HBV react with native HBsAg. We report here an analysis of the specificities of synthetic peptides in terms of the established HBsAg determinants and the immunogenicity of one such peptide in chimpanzees, the relevant human model of response. The data indicate that the group-specific (*a*) determinant is composed of at least three nonoverlapping sequences and that a relatively hydrophilic region of the HBsAg protein, residues 110–137 (numbered 1–226 from the amino terminus), specifies the major *d/y* subtype system. A peptide of residues 110–137, predicted by the HBV DNA sequence of a HBsAg/*ayw* donor, stimulated a brisk anti-HBs response in chimpanzees that consisted of antibody of the *y* subtype determinant.

These findings indicate that chemically synthesized peptides can duplicate serologically important determinants and elicit in natural hosts immune responses corresponding to those that occur during infection.

MATERIALS AND METHODS

Synthetic Peptides and Rabbit Anti-Peptide Sera. Peptides predicted from the HBV DNA sequence (11) were synthesized by the Merrifield solid-phase methods, coupled to carrier protein (KLH; keyhole limpet hemocyanin), and used to prepare rabbit antisera to individual peptide residues as described (15). The antisera to peptide 3 (residues 2–16), peptide 4 (residues 22–35), peptide 1 (residues 48–81), and peptide 6 (residues 95–109) have been shown to react with native HBsAg (15, 16). The protein region encompassing residues 110–137 contains a high degree of variation among the published nucleotide sequences, and peptides to residues 110–137 (peptide 49) and residues 125–137 (representing the COOH-terminal portion of peptide 49 and designated 49a) were synthesized based on the nucleotide sequence of HBV DNA from a HBsAg/*ayw* donor (12). A peptide comparable with peptide 49 but containing amino acid substitutions predicted from the nucleotide sequence of a HBsAg/

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Abbreviations: HBV, hepatitis B virus; HBsAg, HBV surface antigen; KLH, keyhole limpet hemocyanin.

‡ Present address: Division of Blood and Blood Products, National Center for Drugs and Biologics, U. S. Food and Drug Administration, Bethesda, MD 20205.

adw donor (11) was also prepared and was designated peptide 72. The amino acid sequences of peptides 49, 49a, and 72 are presented in Fig. 1 and rabbit antisera to each of these peptides were prepared as described (15) for the other regions of the HBsAg protein.

Reagents and Serological Assays. The specificities of synthetic peptides representing different regions of the HBsAg protein were analyzed by binding of rabbit antipeptide antibodies to five (*ayw1*, *ayw2*, *ayr*, *adw2*, and *adr*) of the known HBsAg subtypes (P_1–P_9; ref. 17) distributed by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases. The individual HBsAg sera were diluted to equivalent HBsAg concentrations, as determined by Ausria II (Abbott), and bound to anti-HBs-coated beads of the same Ausria II kit. The beads were washed, incubated with the rabbit test antiserum (1:5 in phosphate-buffered saline), washed, and allowed to react with iodinated IgG of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Results are expressed as the ratio of cpm of bound test sample(s) to that of normal rabbit serum (311 ± 22 cpm); values ≥ 1.8 were considered positive based on a bimodal distribution of positive and negative values. Hyperimmune rabbit anti-HBs/*ayw* and anti-HBs/*adr* sera prepared in this laboratory to purified HBsAg (18) were used as positive controls in the assay.

Highly purified HBsAg preparations of *adw* and *ayw* subtype specificities were iodinated and used as ligands in radioimmunoprecipitation assays with selected rabbit antipeptide antibodies, as described (15). Competition radioimmunoprecipitation analyses were carried out by the addition to the reaction mixture of increasing amounts of unlabeled HBsAg of either the *adw* or *ayw* subtype as described in the figures. The reaction of peptide 49 with rabbit antipeptide antibodies and various monospecific antibodies to HBsAg determinants was carried out as described in the legends. Guinea pig antisera to native HBsAg were those distributed by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases and certified by the World Health Organization as *d/y* typing reagents. Monotypic antibodies were prepared from these reagents by affinity chromatography and characterized (19).

Chimpanzee Studies. Three chimpanzees (*Pan troglodytes*) seronegative for markers of HBV infection were inoculated intramuscularly with a preparation of peptide 49–KLH complex (1 mg of peptide 49 per 0.5-ml dose) in adjuvant as indicated below. Animals were housed, maintained, and manipulated in a commercial primate-holding facility as described (20). Sera were obtained weekly and monitored for anti-HBs by a commercial radioimmunoassay (Ausab, Abbott) and antipeptide 49 by radioimmunoprecipitation as described above. The *d/y* subtype specificity of the anti-HBs response in selected sera was determined by the method of Hoofnagle *et al.* (21).

Peptide 49

110 120

Phe-Pro-Gly-Ser-Ser-Thr-Thr-Ser-Thr-Gly-Pro-Cys-Arg-Thr-Cys-

130

Met-Thr-Thr-Ala-Gln-Gly-Thr-Ser-Met-Tyr-Pro-Ser-Cys

⟼ peptide 49a

Peptide 72

110 120

Ile-Pro-Gly-Ser-Thr-Thr-Thr-Ser-Thr-Gly-Pro-Cys-Lys-Thr-Cys-

130

Thr-Thr-Pro-Ala-Gln-Gly-Asn-Ser-Met-Phe-Pro-Ser-Cys

FIG. 1. Amino acid sequences of peptides 49, 49a, and 72. Sequences correspond to residues 110–137 of HBsAg as predicted from the *S* gene nucleotide sequences of HBV DNA from *ayw* (peptide 49; ref. 12) and *adw* (peptide 72; ref. 11) donors, respectively. Amino acids underlined in peptide 72 are positions of variability between the two sequences. Peptide 49a consists of the COOH-terminal 12 amino acids of peptide 49 (residues 125–137).

RESULTS

The specificities represented by synthetic peptides were analyzed by the binding of antipeptide antibodies from individual rabbit antisera to native HBsAg of established subtypes (Table 1). Antibodies to three nonoverlapping peptide regions (peptides 3, 6, and 49) bound to HBsAg in this assay, and the patterns of reactivity, although different from one another, were not consistent with a unique specificity for determinants of the subtype *d/y* and *w/r* systems; rather, the group-specific *a* determinant appears to be a collection of specificities distributed throughout the HBsAg protein. Two of the three peptides (nos. 3 and 6) are located in relatively hydrophobic regions of the HBsAg protein; peptide 3 represents the amino-terminal end and peptide 6 includes the juncture of the central hydrophobic region with a major hydrophilic sequence. Antibodies to peptide 4 (residues 22–35), a hydrophobic region, and peptide 1 (residues 48–81), a long hydrophilic region, failed to bind in this assay (data not shown) although the antisera were positive by Ausab and other assay systems (15, 16).

The antibodies against peptides 49 and 49a reacted with each of the five HBsAg subtypes. The broad reactivity of the peptide 49 and 49a antisera and the sequence variability and hydrophilic nature of the peptide 49 region, as well as its proximity to a glycosylation site (residues 146–148), make this peptide of particular interest. We have further evaluated the specificity of peptide 49 by immunoprecipitation. Peptide 49 (unlinked to KLH) was iodinated by the chloramine-T method through the tyrosine residue at position 134 and allowed to react with animal antisera (Table 2). The iodinated peptide 49 was precipitated by antipeptide 49 and antipeptide 49a sera but not by rabbit antisera to peptides 3, 4, 1, and 6 of the HBsAg protein. Guinea pig sera distributed by the National Institute of Allergy and Infectious Diseases and certified by the World Health Organization as *d/y* subtyping reagents and monospecific antibodies to the *a*, *d*, and *y* determinants derived from these reagents were used to define the specificities represented by peptide 49. ^{125}I-Labeled P49 was precipitated by both guinea pig reference reagents. The antipeptide activity of the anti-HBs/*ad* serum was due to the anti-HBs/*a* component since anti-HBs/*a*, but not anti-HBs/*d*, precipitated the peptide. In contrast, both the anti-HBs/*a* and anti-HBs/*y* fractions of the anti-HBs/*ay* serum precipitated the iodinated ligand, indicating that both

Table 1. Rabbit antisera to synthetic peptides: Binding to HBsAg subtype specificities by solid-phase assay

Peptide	Residues*	Binding to HBsAg subtype, S/N				
		ayw1	*ayw2*	*ayr*	*adw2*	*adr*
3	2–16	1.2	2.8	2.7	3.7	1.8
6	95–109	1.8	2.3	1.2	3.0	2.6
49	110–139	4.3	5.9	2.3	2.4	3.0
49a	125–139	6.1	6.7	4.3	4.1	1.8

Results are expressed as the ratio of cpm of bound test sample (S) to that of normal rabbit serum (N; 311 ± 22 cpm); S/N values ≥ 1.8 are considered positive. Binding of hyperimmune rabbit anti-HBs/*ayw* and anti-HBs/*adr* to the different subtype antigen-coated beads, used here as positive controls, varied from S/N = 5.6 to S/N = 14.2

* Coded by the *S* gene of HBV and numbered 1–226 from the NH_2 terminus.

Table 2. Specificity of synthetic peptide 49 (residues 110–137): Radioimmunoprecipitation with animal antisera

	Residues*	S/N
Antiserum to synthetic peptide		
Rabbit anti-P3	2–16	<1
-P4	22–35	<1
-P1	48–81	<1
-P6	96–109	<1
-P49	110–139	765
-P49a	125–139	614
Antiserum to native HBsAg		
Guinea pig anti-HBs/*ad*		11.8
-HBs/*a*		7.7
-HBs/*d*		1.7
Guinea pig anti-HBs/*ay*		66.3
-HBs/*a*		73.5
-HBs/*y*		33.5
Rabbit anti-HBs/*ay*		92.4
Rabbit anti-HBs/*adr*		53.8

Synthetic peptide 49, residues 110–139, was iodinated by the chloramine-T procedure; free ^{125}I was removed by gel chromatography on a Sephadex G-10 column. The ^{125}I-labeled P49 (1×10^5 cpm) was incubated with the test antiserum in NET/RIP buffer [NET, 150 mM NaCl/5 mM EDTA/50 mM Tris·HCl, pH 7.5/5 mM KI/0.02% NaN_3; RIP, 1% Nonidet P-40/0.05% Triton X-100/0.025% sodium deoxycholate/dextran sulfate (0.25 μg/ml)/20% gamma globulin-free calf serum in NET buffer] at 37°C for 30 min and then at 4°C for 18 hr. A 100-μl volume of *Staphylococcus aureus* (10% suspension in RIP buffer) was added, the incubation was continued for 30 min at room temperature, and the mixture was centrifuged for 2–5 min in a Microfuge tube. The pellet was washed twice in high-salt buffer (2.5 M KCl/0.1 M NaCl/0.5% Nonidet P-40/5 mM Tris·HCl, pH 7.5) and then suspended in low-salt buffer (0.1 M NaCl/0.5% Nonidet P-40/5 mM Tris·HCl, pH 7.5); its radioactivity was determined in a gamma spectrometer. Results are expressed as the ratio of cpm of the test sample(s) (S) to that of normal or preimmunization animal serum (N) (guinea pig, N = 25; rabbit, N = 60); an S/N value of ≥ 2.1 was considered positive. Antisera to synthetic peptides were prepared in rabbits as described (15). Guinea pig antisera to native HBsAg were those distributed by the Research Resources Branch of the National Institute of Allergy and Infectious Diseases; monotypic antibodies were prepared from these reagents by affinity chromatography.

* Amino acid residues of HBsAg, numbered from the NH_2 terminus and corresponding to the synthesized peptide.

group (*a*)- and subtype (*y*)-specific determinants of HBsAg are located within amino acid residues 110–137. This supports the suggestion (15) that this region corresponds to the domain of the HBsAg molecule conferring subtype specificity. Despite comparable antibody concentrations, the antipeptide 49 activity of the anti-HBs/*a* from the guinea pig *ay* reagent was higher than that from the *ad* reagent, which could indicate subtype differences unrelated to the *d/y* system. Indeed, the primary amino acid sequences of HBs/*ay* and HBs/*ad* differ substantially within the peptide 49 region (6 of 28 residues). The fact that rabbit antibody to HBsAg/*adr*, which is heterologous in both the *d/y* and *w/r* systems, also precipitates the ligand confirms the location of an *a* determinant within the peptide 49 structure. Competitive inhibition assays were used to estimate the relative anti-*a* and anti-*y* activities of rabbit antiserum to peptide 49 (Fig. 2). While native HBsAg, subtype *ayw*, fully inhibited the reaction between anti-peptide 49 antibody and iodinated HBsAg/*ayw*, HBsAg of heterologous subtype (*d*) competed at only low levels (15–20%), indicating that the predominant rabbit anti-HBs response to peptide 49 immunization was to the subtype-specific *y* determinant.

Localization of the *d/y* subtype determinants to the protein region spanned by residues 110–137 was further verified by analysis of rabbit antisera to peptide 72, a peptide synthesized

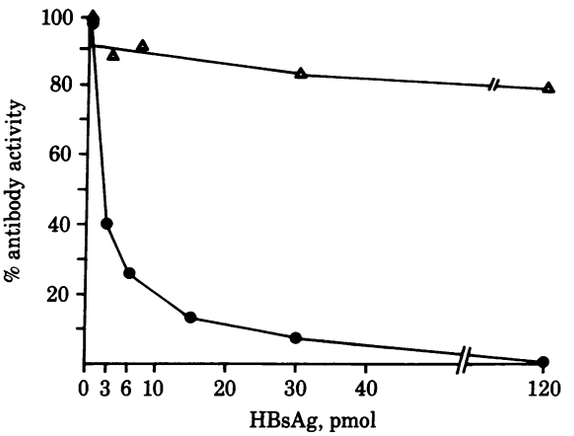

FIG. 2. Anti-HBs subtype analysis of rabbit antiserum to peptide 49 by competitive inhibition assay. The 100% antibody activity value represents the difference between cpm of ^{125}I-labeled HBsAg/*ayw* precipitated by antiserum to peptide 49 and cpm of a negative serum control. Increasing amounts of purified and unlabeled HBsAg/*adw* (△) or HBsAg/*ayw* (●) were added to the reaction mixture and the proportional reduction in precipitated radioactive material was taken as an estimate of antibody activity.

according to the predicted amino acid sequence of subtype *adw* (Fig. 1). As shown in Table 3, antiserum to peptide 49 reacted predominantly with HBsAg/*ayw*, as previously noted. In contrast, antiserum to peptide 72 precipitated only HBsAg, subtype *adw*. These data show that the *d* and *y* determinants are both localized to residues 110–137 and that their specificities hinge on changes in one or a combination of the variable amino acids noted in that region of the HBsAg protein. In an attempt to further identify the critical residues, rabbit antiserum to peptide 49a, representing the COOH-terminal 12 amino acids of peptide 49 (Fig. 1), was similarly analyzed. Antiserum to peptide 49a precipitated only HBsAg, subtype *ayw*, indicating that at least the *y* determinant depends on one or more amino acids at positions 127, 131, and 134 of the *S* gene product.

The use of peptide 49 as an immunogen was evaluated in the chimpanzee model of human response. Three chimpanzees seronegative for all markers of HBV were immunized with the peptide 49–KLH complex (Fig. 3). A brisk primary and secondary response was observed in all three animals as measured by a solid-phase radioimmunoassay (Ausab), although the degree of the response was clearly higher with the use of Freund's incomplete adjuvant in chimpanzee 1 as compared with alum or alum/pertussis in the other two chimpanzees. The serum antipeptide 49 response in chimpanzee 1 (Fig. 3*B*) paralleled the anti-HBs (Ausab) response. The differences between the antipeptide and Ausab titers in the early response are probably due to the fact that Ausab is particularly sensitive to IgM an-

Table 3. HBsAg subtype specificity of rabbit antisera to synthetic peptides

	^{125}I-Labeled HBsAg precipitated	
	ayw	*adw*
Peptide 49	14.7	6.3
Peptide 72	1.4	9.0
Peptide 49a	15.4	1.0

Results are expressed as the ratio of cpm of the precipitated test serum divided by that of a negative control serum. Values are averages of three separate experiments.

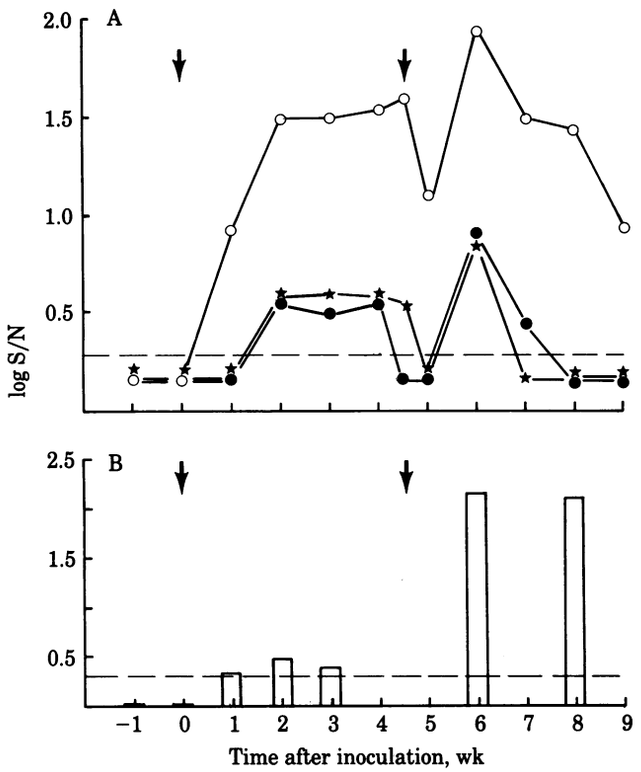

FIG. 3. Kinetics of anti-HBs response in chimpanzees inoculated with synthetic peptide 49–KLH complex. Each inoculum consisted of a 1:1 mixture of the complex with Freund's incomplete adjuvant (chimpanzee 1, ○), alum-adsorbed complex (chimpanzee 2, ●), or alum-adsorbed complex with added pertussis (1×10^{10} cells) (chimpanzee 3, ★); the inocula were administered (↓) intramuscularly and contained 1 mg of P49 per 0.5-ml dose. Sera were obtained weekly and monitored for anti-HBs by Ausab (*A*) and anti-P49 by radioimmunoprecipitation as described in Table 2 (*B*).

tibody while the immunoprecipitation assay selects for IgG. The *d/y* subtype specificity of the anti-HBs response was determined by the method of Hoofnagle *et al.* (21) at wk 2, 3, 4, 6, and 8 for the high-responder chimpanzee 1 and at wk 6 for chimpanzees 2 and 3. The anti-HBs activity of undiluted serum from chimpanzee 1 was totally blocked by HBsAg/*ay* and not blocked by HBsAg/*ad*, establishing that the anti-HBs activity throughout the course of response was entirely anti-HBs/*y*, not anti-HBs/*a* or *d*. An anti-HBs/*y* response was also observed in the wk 6 sera of both chimpanzees 2 and 3, although the low titer prevented the exclusion of anti-HBs/*a* in one of these.

The Ausab and antipeptide 49 titers of all three chimpanzees decayed to baseline values with time and subsequent boosts with the adjuvanted peptide–KLH complex resulted in only low-level transient responses, possibly due to rapid clearance of the complex by high titers of anti-KLH in the three test animals (data not shown).

DISCUSSION

HBsAg has been the subject of extensive immunochemical study over the last decade and, while numerous antigenic specificities have been described, there is clear consensus that HBsAg consists of a group-specific (*a*) and two sets of "allelic" subtype (*d/y*, *w/r*) determinants specified by the HBV genome. The *a* and *d* or *y* determinants have a proven association with the major 226-amino acid protein (8, 9), the primary structure of which is established from the nucleotide sequence of the *S* gene. The ability to construct by chemical synthesis peptides that span selected regions of the protein provided an opportunity to further define the serologically important antigens of HBsAg. In this paper, we report that antibodies to synthetic peptides, representing three nonoverlapping regions of the HBsAg protein, bind to native HBsAg of known subtypes in a pattern of group (*a*) specificity, thereby indicating that the *a* antigen represents a collection of determinants at least some of which depend on only the local conformation of a relatively short amino acid sequence. The recent report by Bhatnagar *et al.* (22) that an additional region (residues 139–147) also represents an *a* determinant further supports this conclusion.

Based on the conserved and variable regions of the protein sequences predicted from the published nucleotide sequences, it has been predicted (15) that the protein region between positions 110 and 137 would encompass subtype-specific determinants of HBsAg; experimental verification of that prediction is provided in this report. Peptide 49, synthesized from the sequence of a *y* subtype, was precipitated by anti-*y* and not anti-*d* monotypic antibodies prepared from certified *d/y* subtyping reagents, and antisera to peptide 49 from rabbits and chimpanzees contained anti-*y* as the predominant anti-HBs component. The fact that antipeptide 49 reacted with native HBsAg and, conversely, that antibody to native HBsAg recognized the peptide implies that the *y* determinants are identical. Substitution into residues 110–137 of amino acids predicted from the nucleotide sequence of a *d* subtype resulted in anti-*d* specificity of rabbit antipeptide serum, establishing that both the *d* and *y* subtype determinants are encompassed within this protein region of HBsAg. Our results with peptide 49a suggest that amino acid substitutions involving one or more residues at positions 127, 131, and 134 are critical to at least *y* subtype specificity. Support for that conclusion comes from structural analysis of *adw* and *ayw* polypeptides (23), in which amino acid differences at positions 131 and 134 were consistent with subtype specificity of the donor protein. Among the various reports on antibodies against synthetic peptides that react with HBsAg, several are of interest with respect to the *d* and *y* specificities studied here. Dreesman *et al.* (24) investigated two cyclic peptides (117–137 and 122–137), both of which are contained within the region we studied. They did not report the subtype specificity of their response but, judging from their sequence, we would expect it to be anti-*y*. They did, however, suspect that cyclization of their peptide was important in generating antibodies reactive with native HBsAg. Although deliberate cyclization of the peptide may generate novel specificities, the present data show that it is clearly not essential for generation of antibodies reactive with HBsAg. The report of Prince *et al.* (25) is more problematic. They synthesized a peptide (residues 138–149) and concluded that it contained the *d* determinant. Possibly, the *d* subtype specificity encompasses a rather large domain or consists of split determinants about the Cys-Cys-Cys sequence (residues 137–139); Bhatnagar *et al.* (22), however, examined the same 139–147 region and concluded it to represent an essential part of the *a* determinant. In partial agreement with the data reported here, MacKay *et al.* (26), using subclones of the *S* gene expressed in *Escherichia coli*, reported localization of the *d* determinant between residues 4 and 121. While we have here localized the *y* determinant to residues 125–137, our data do not exclude the possibility that *d* involves amino acids between residues 110 and 125.

The recent demonstration (27) that synthetic peptides corresponding to regions of the VP1 of foot-and-mouth disease virus-stimulated serotype-specific neutralizing antibody to test animals and protection against experimental virus challenge has provided strong impetus to the use of synthetic peptides in vaccination strategy. As shown here, the fact that chimpanzees, the

model of human HBV infection, responded to synthetic peptide immunization with antibody of established serological importance offers us the possibility that synthetic peptides will provide safe and inexpensive alternatives to current vaccines for the prevention of HBV-associated diseases. The transient nature of the response, however, and the lower response using alum adjuvant points out the need for further work on carrier proteins, adjuvants, and immunization regimens. Repeated immunization of the three chimpanzees described here failed to induce stable anti-HBs responses (data not shown). Nevertheless, intravenous challenge with hepatitis B virus (HBV/*ayw*) late in the immunization course resulted in protection against infection in one chimpanzee, an attenuated infection without disease in another, and a typical pattern of acute hepatitis in the remaining animal (chimpanzee 1). These data, therefore, indicate that immunization with peptide 49 provides at least partial protection against homologous virus despite low or undetectable levels of anti-HBs activity at the time of experimental challenge. Ultimately, similar challenge experiments will be necessary to identify the HBsAg specificities and level of response required for maximum protection. Cross-protection experiments to date (5) indicate that anti-*a* protects against challenge with HBV of either *adw* or *ayw* subtype. The present data, however, establish that the *a* determinant represents a collection of specificities distributed throughout the HBsAg protein; the selection of those sequences that correlate with the stimulation of protective antibody for synthesis will require considerable effort. Alternatively, a mixture of two synthetic peptides (e.g., peptides 49 and 72) containing both of the *d/y* subtype determinants might well provide a broad protection to virus challenge.

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