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Immunology

Immune response to synthetic peptide analogues of hepatitis B surface antigen specific for the *a* determinant

(hepatitis B vaccine/antigenic determinant/amino acid sequence)

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ABSTRACT **Recovery from a natural infection with hepatitis B virus or vaccination with purified envelope protein leads to production of antibodies against the hepatitis B surface antigen (HBsAg). Such physiologic response in man is generally directed against the *a* determinant of HBsAg common to all serotypes of the virus. To define the immunochemical specificity of this determinant, the secondary structure of HBsAg was derived from its sequence of 226 amino acids. Hydrophilic stretches expected to contain the antigenic determinants were located between residues 32 and 76 and between residues 110 and 156. Loss of the antigenic activity after chemical modification of lysine residues of HBsAg indicated their critical importance in antigenicity. Because all lysines are located between residues 121 and 160, we selected this region for localization of HBsAg determinants. Solid-phase synthesis was used to prepare seven peptide analogues of HBsAg (PsAs): 122–137, 128–134, 139–147, 139–158, 140–158, 145–158, and 150–158. For experimental immunization of rabbits the synthetic peptides were coupled to keyhole limpet hemocyanin. We studied the antigenicity of each peptide analogue by serologic neutralization of human antibodies specific for the *a* determinant of HBsAg. Analogues 139–147, 139–158, and 140–158 showed antigenicity as well as induction of anti-HBsAg. The rabbit antibodies were inhibited with each of the three peptide analogues and all serotypes of natural HBsAg, having only the *a* determinant in common. These results indicate that the nonapeptide sequence 139–147 represents the total or an essential part of the *a* determinant of HBsAg.**

Hepatitis B surface antigen (HBsAg, originally termed Australia antigen) is localized in the envelope of the hepatitis B virus (HBV). In the plasma of patients with hepatitis B, it is commonly found in three morphologically distinct forms: spherical particles 22 nm in diameter, tubular forms 22 nm in diameter and up to several hundred nanometers long, and more complex structures 42 nm in diameter, which are considered as the complete HBV (1–3). Serologically, HBsAg has one group-specific determinant *a* and two sets of mutually exclusive determinants *d* or *y* and *w* or *r*, giving four major serotypes: *adw*, *ayw*, *adr*, and *ayr* (4). The antibodies against HBsAg (anti-HBs) produced in response to natural infection are protective against reinfection with HBV and the purified envelope protein has been used successfully in vaccination against HBV infection (5). The physiologic anti-HBs response in man is generally directed against the common determinant *a* encountered in all serotypes of HBV. Therefore, immunization with one serotype of HBsAg is protective against all serotypes of HBV (5). The envelope protein is made up of major structural proteins of 25,000 and 32,000 daltons (6). Both proteins have identical amino-terminal and carboxy-terminal sequences and both proteins produce immune responses to the group-specific determinant *a* as well as to their respective subtype-specific determinant *d* or *y* (7). Therefore, it was concluded that HBV coded for a single structural coat protein that exists in a nonglycosylated form (25,000 daltons) and a glycosylated form (32,000 daltons). Cloning of HBV DNA and determination of the nucleotide sequence confirmed this conclusion and provided the complete sequence of 226 amino acids constituting the HBsAg gene product (8).

Characterization of the antigenic structure of several proteins—namely, myoglobin, lysozyme, cytochrome *c*, and tobacco mosaic virus protein—has shown that sequence-dependent antigenic determinants are always localized in hydrophilic regions of the proteins (9). In an attempt to develop an immunogenic synthetic peptide, potentially useful as a vaccine against HBV, we applied this principle in a structural analysis of the amino acid sequence of HBsAg and used the observation of a loss of HBsAg activity on modification of the lysine residues (10). Thus, we expected the major *a* determinant of HBsAg to lie in the amino acid sequence between residues 121 and 160. A synthetic peptide analogue of residues 134–146 with serologic activity specific for the *a* determinant of HBsAg has been reported by us (10, ¶). More recently, antigenicity or immunogenicity (or both) of a number of synthetic peptide analogues of HBsAg (PsAs) have been reported by other investigators (11–14). We report here definitive analyses of the predicted secondary structure of the HBsAg protein, serologic activity of seven PsAs covering the sequence 122–158, immunogenicity of the peptides after coupling to a carrier protein, and serologic specificity of the rabbit antisera. Our results indicate that the sequence 139–147 represents the *a* determinant of HBsAg or an essential part of it.

MATERIALS AND METHODS

Purification of HBsAg. Plasma from a single HBsAg carrier of serotype *adw* was obtained by plasmapheresis. The HBsAg was isolated by a combination of isopycnic banding and differential rate sedimentation through cesium chloride gradients (6). Structural polypeptides of HBsAg were isolated by preparative acrylamide gel electrophoresis (15).

Serological Assays. Using standard solid-phase sandwich radioimmunoassay kits (Abbott Laboratories), we detected sero-

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; anti-HBs, antibody to HBsAg; anti-HBc, antibody to hepatitis B core antigen; anti-HBe, antibody to hepatitis B 'e' antigen (3); PsA, peptide analogue of HBsAg; KLH, keyhole limpet hemocyanin.

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  1  Met Glu Asn Ile Thr Ser Gly Phe Leu Gly Prc Leu Leu Val Leu Gln Ala Gly Phe Phe
 21  Leu Leu Thr Arg Ile Leu Thr Ile Pro Gln Ser Leu Asp Ser Trp Trp Thr Ser Leu Asn
 41  Phe Leu Gly Gly Ser Pro Val Cys Leu Gly Gln Asn Ser Gln Ser Pro Thr Ser Asn His
 61  Ser Pro Thr Ser Cys Pro Pro Ile Cys Pro Gly Tyr Arg Trp Met Cys Leu Arg Arg Phe
 81  Ile Ile Phe Leu Phe Ile Leu Leu Leu Cys Leu Ile Phe Leu Leu Val Leu Leu Asp Tyr
101  Gln Gly Met Leu Pro Val Cys Pro Leu Ile Pro Gly Ser Thr Thr Thr Ser Thr Gly Pro
121  Cys Lys Thr Cys Thr Thr Pro Ala Gln Gly Asn Ser Met Phe Pro Ser Cys Cys Cys Thr
141  Lys Pro Thr Asp Gly Asn Cys Thr Cys Ile Pro Ile Pro Ser Ser Trp Ala Phe Ala Lys
161  Tyr Leu Trp Glu Trp Ala Ser Val Arg Phe Ser Trp Leu Ser Leu Leu Val Pro Phe Val
181  Gln Trp Phe Val Gly Leu Ser Pro Thr Val Trp Leu Ser Ala Ile Trp Met Met Trp Tyr
201  Trp Gly Pro Ser Leu Tyr Ser Ile Val Ser Pro Phe Ile Pro Leu Leu Pro Ile Phe Phe
221  Cys Leu Trp Val Tyr Ile
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FIG. 1. Complete amino acid sequence of HBsAg/*adw* as derived from the nucleotide sequence of the HBsAg gene (8). Hydrophilic regions are indicated by italic type. The hydrophilic subregion selected for synthesis of peptide analogues is underlined.

logic activity for anti-HBs by the AUSAB system, HBsAg by the AUSRIA II-125 system, core antibodies (anti-HBc) by the CORAB system, and the 'e' antibodies (anti-HBe) by the ABBOTT-HBe system. All procedures were carried out according to manufacturer's instructions. For titration, the serum specimens were serially diluted with negative control serum and each dilution was tested in duplicate. The highest dilution giving a positive reaction was considered an end point. For neutralization reactions, a serum dilution one-fourth that of the end point of titration was used. A single human serum containing anti-HBs, specific for only the *a* determinant (10), was used in all neutralization tests. The minimum quantities of purified HBsAg, isolated structural glycopeptide of HBsAg (32,000 daltons), and PsA required to inhibit human anti-HBs were compared. Rabbit antisera raised against carrier peptide conjugates were tested for reactivity with the natural HBsAg in the AUSAB test. The serologic specificity of rabbit antibodies to synthetic peptides was determined by neutralization with HBsAg-positive sera of known *adw* and *ayw* serotypes. In addition, we used Paris Workshop Panel standard sera with different serotypes of HBsAg (P_1–P_9; provided by A. M. Couroucé of the National Blood Transfusion Center, Paris) comprising *adw*, *ayr*, *adyw*, *adywr*, and *ayw* (16). For specificity testing, the HBsAg concentration in the serum of different serotypes was determined by comparative titration using purified HBsAg/*adw* as a standard and adjusting the amount of HBsAg in each neutralizing serum by appropriate dilution with normal human serum.

Structural Analysis. The amino acid sequence of HBsAg (see Fig. 1) was analyzed by a computer program based on the predictive scheme of Chou and Fasman (17). The program performed the necessary calculations, scanned for α-helix and β-sheet nucleation and propagation, and identified the β-turns. A preliminary secondary structure was then manually refined.

PsAs. Based on the lysine-containing hydrophilic region 122–160 and preliminary findings of serologic activity of HBsAg in PsA 134–146 (10), we selected for synthesis PsAs corresponding to residues 122–137, 128–134, 139–147, 139–158, 140–158, 145–158, and 150–158 (see Fig. 1). The peptides were synthesized by Merrifield's method using a modified polystyrene resin (18, 19) and amino acid derivatives commercially obtained from Bachem Fine Chemicals, Torrance, CA. The synthesis was monitored by determination of the amino acid composition. To simultaneously remove the protecting groups and the peptide from the resin, we treated 1.0 g of dried resin with ≈10 ml of anhydrous HF and 2 ml of anisole for 30 min at 0°C and then evaporated the HF and partitioned the peptide/resin mixture three times each between water/ether and 1.0 M ammonia/ether. The water and ammonia phases were separately pooled and lyophilized. The lyophilized products were mixed and purified on a Sephadex G-15 column equilibrated with 0.1 M NH_4HCO_3. The PsAs were analyzed by using a Durrum amino acid analyzer (model D 500) and by high-voltage paper electrophoresis. The peptides showed the expected amino acid composition (Table 1). In high-voltage electrophoretic analysis, PsAs 128–134 and 150–158 showed a single spot with respective electrophoretic mobility (E_M) values of 0.31 and 0.26 (relative to lysine), confirming their homogeneity, while the other peptides showed smeared spots, probably due to random polymerization and oxidation of cysteine residues. The yields of crude peptides were ≈60% and those of the purified products were 30–40% of the calculated values.

Peptide-Carrier Coupling. For efficient coupling of keyhole limpet hemocyanin (KLH, Calbiochem) to peptides, both through the carboxyl terminus and through the amino terminus, two methods of conjugation were developed (20). The methods for attaching a carrier through the carboxyl terminus essentially involved the use of a water-soluble active ester based on 4-hydroxy-3-nitrobenzenesulfonic acid. The amino terminus of the PsA was blocked by acetylation with [^{3}H]acetic anhydride, and coupling of the peptide to KLH was 8–10% (wt/wt). For attaching a peptide to the carrier through the amino terminus, the peptides were activated while on the resin by using the following scheme:

H_2N-peptide-CO-resin

↓ Succinic anhydride

HOOC-CH_2-CH_2-CO-NH-peptide-CO-resin

↓ Pentachlorophenol, carbodiimide

C_6Cl_5O-CO-CH_2-CH_2-CO-NH-peptide-CO-resin

↓ Cleavage

C_6Cl_5O-CO-CH_2-CH_2-CO-NH-peptide-COOH.

Table 1. Amino acid analyses of PsAs

	128–134	139–147	139–158	140–158	145–158	150–158
Ala	0.96		0.93	1.06	0.97	1.00
Asx	0.86	1.80	1.90	1.86	0.92	
Glx	1.10					
Gly	1.00	1.00	1.00	1.00	1.00	
Ile			1.89	1.96	1.80	1.92
Lys		1.08	1.01	0.93		
Met	0.84					
Ser	0.82		1.68	1.57	1.60	1.64
Thr		1.76	2.80	2.60	0.83	
Phe			0.98	0.92	1.09	0.98

Amino acids were analyzed with a Durrum D-500 analyzer equipped with a single-wave-length detector. Proline was not detectable. Cysteine and tryptophan were present in the profile but could not be quantitated. All estimates are based on the value of glycine or alanine (in the absence of glycine).

The activated peptide was cleaved by mild HF treatment (20). One gram of resin containing 0.2 mmol of activated peptide was suspended in 2 ml of anisole and treated with anhydrous HF for 30 sec at −70°C. The mixture was purged with N_2 at 0°C for 2 hr, and then 5 ml of ether and a solution of 20 mg of KLH in 5 ml of 0.1 M borate (pH 8.5) was added. The ether was decanted, and the mixture was stirred overnight at room temperature and filtered to remove resin particles. Unbound peptide was removed by extensive dialysis against phosphate-buffered saline (pH 7.2). The coupling of PsA to KLH was estimated by using [^{14}C]succinic anhydride and was 8–10% (wt/wt).

Production of Antisera. Three-month-old male rabbits were immunized with 100 μg of KLH-peptide conjugate (equivalent to 8–10 μg of PsA) in complete Freund's adjuvant. Three injections were given subcutaneously at intervals of 3 wk. An immunoglobulin fraction was prepared from rabbit serum (anti-PsA 139–147) by $(NH_4)_2SO_4$ precipitation (40%).

RESULTS

Predicted Secondary Structure of HBsAg. Computer analysis of the amino acid sequence of HBsAg (Fig. 1) predicted three large hydrophobic regions as β-pleated sheets (residues 1–31, 75–109, and 157–226) separated by two hydrophilic regions (residues 32–74 and 110–156). Several β-turns were predicted within these hydrophilic regions, whereas no α-helix was revealed. The tetrapeptide 142–145 yielded one of the highest probabilities for β-turn in the computer-derived prediction.

Synthesis and Purification of PsAs. Based on the predicted secondary structure of HBsAg and the importance of lysine residues for antigenicity, we tested seven PsAs localized in the most hydrophilic region: PsA 122–137 (commercially obtained from Bachem Fine Chemicals) and PsAs 128–134, 139–147, 139–158, 140–158, 145–158, and 150–158 (synthesized in our laboratory). Amino acid analyses of the purified PsAs are given in Table 1.

Antigenic Properties of PsAs. HBsAg reactivity of the synthetic peptides was tested as follows: a constant dilution of human anti-HBs (100 μl) adjusted to be one-fourth the predetermined titer was incubated for 20–24 hr at 4°C with various amounts of peptide (0–4 mg) in 100 μl of normal human serum, and the mixture was assayed in the standard system for anti-HBs. Reduction in cpm was computed as percent inhibition; >30% inhibition was considered positive. The variation in the cpm was ±5%. Three of the seven peptides were found to neutralize anti-HBs reactivity: 139–147, 139–158, and 140–158. The minimum concentration of peptide inhibiting anti-HBs reactivity was 1.25 mg/ml for all three peptides (Table 2). At 20–40 mg/ml, these PsAs inhibited reactivity to ≈80%. By comparison, about 1 ng of purified HBsAg or 1 μg of the 32,000-dalton structural glycoprotein of HBsAg inhibited anti-HBs. However, anti-HBs was not inhibited by oxytocin (Cys-Tyr-Ile-Glu-Asp-Cys-Pro-Leu-Gly) or vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly), which are unrelated nonapeptides containing amino acid residues similar to PsA 139–147. None of the PsAs neutralized anti-HBc or anti-HBe activity.

Immunogenic Properties of PsAs. For immunization, the synthetic peptides were coupled to KLH through either the carboxy terminus or the amino terminus of the peptide. The mode of attachment of each peptide is shown in Table 2. By using preimmunization sera as a negative control, the sera of immunized rabbits were tested for anti-HBs activity in the standard anti-HBs assay system. PsAs 139–147, 139–158, and 140–158 induced an anti-HBs response (Table 2). These peptides also inhibited human anti-HBs (see above). All of the rabbit antisera were nonreactive in assays for anti-HBc and anti-HBe activities. Crude immunoglobulins isolated from a rabbit antiserum (anti-PsA 139–147) by 40% ammonium sulfate precipitation showed anti-HBs activity at 12 mg/ml with a positive/

Table 2. Antigenic activity and immunogenicity of HBsAg and PsAs

PsA or protein	Minimum concentration inhibiting anti-HBs (30%), mg/ml	Peptide terminus linked to KLH	Rabbits immunized,* no.	Responders, no.	Positive/negative ratio
122–137	NI	ND	ND	—	—
128–134	NI	COOH	3	0	—
139–147	1.25	COOH	3	1	16
139–147†	1.25	COOH	4	1	5
139–158	1.25	NH_2	3	2	8,3
140–158	1.25	NH_2	3	1	9
145–158	NI	NH_2	3	0	—
150–158	NI	NH_2	3	0	—
Bovine serum albumin	NI	ND	ND	—	—
KLH	NI	ND	ND	—	—
HBsAg	0.000001	ND	ND	—	—
HBsAg glycoprotein	0.001	ND	ND	—	—

ND, not done; NI, not inhibitory.
* All immunized rabbits showed anti-KLH activity in gel diffusion analyses.
† Independent repeat experiment.

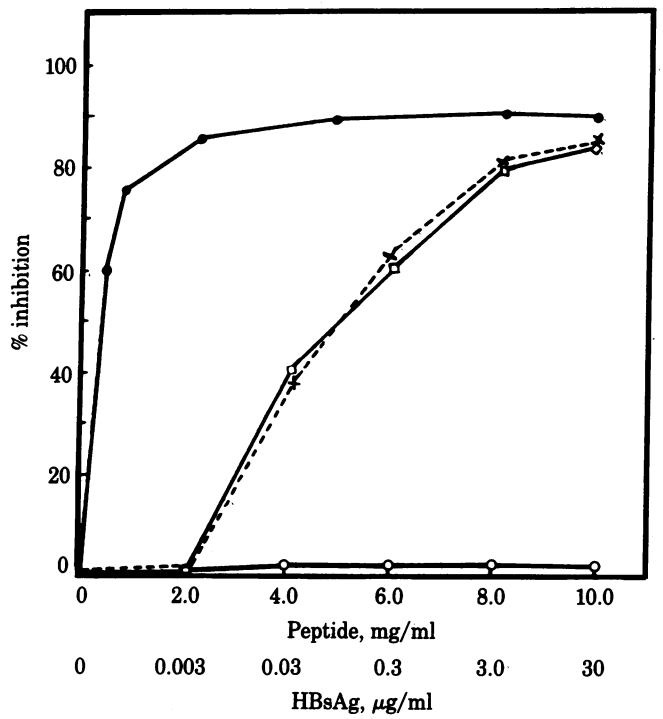

FIG. 2. Inhibition of rabbit antibodies against PsA 139–147 by the autologous peptide (●), natural HBsAg/*adw* (□), natural HBsAg/*ayw* (×), and PsA 150–158 (○).

negative ratio of 8. However, the precise immunoglobulin class of the activity could not be established.

The specificity of the anti-HBs response in rabbits immunized with KLH-PsA conjugates was demonstrated by inhibition with the PsAs and various serotypes of HBsAg. The native antiserum to PsA 139–147, giving a positive/negative ratio of 16, had a titer of 1:8 and was used in inhibition studies. As shown in Fig. 2, the anti-HBs activity in rabbit antiserum to PsA 139–147 was neutralized by the autologous nonapeptide as well as by both serotypes of HBsAg (*adw* and *ayw*). This antibody was also inhibited by each of the nine HBsAg-positive sera from the standard Paris panel. On the other hand, PsA 150–158 was noninhibitory (Fig. 2). Similarly, PsA 1–9 (commercially obtained from Bachem Fine Chemicals), KLH, and bovine serum albumin also failed to inhibit anti-PsA 139–147. Rabbit antisera to PsAs 139–158 and 140–158 were also inhibited by 139–147, whereas PsAs 150–158 and 1–9 were noninhibitory. These results clearly establish that rabbit antibodies to the nonapeptide PsA 139–147 are specific for the common *a* determinant of HBsAg.

DISCUSSION

Current understanding of the molecular immunology of HBsAg stems largely from immunochemical investigations carried out with the 22-nm particles of the HBV envelope protein and the serologic characterization of its various antigenic determinants (10, 16, 21). Identifying the antigenic epitopes of HBsAg became possible when complete amino acid sequence of HBsAg became known from the HBV DNA sequence (8).

Considerable attention has been devoted to identification of the antigenic epitopes of proteins for elucidation of the antigen–antibody binding phenomenon at the molecular level (9). By using a computer program, we predicted the secondary structure of HBsAg and deduced hydrophilic regions between residues 32 and 74 and between residues 110 and 156 (10, ¶) and that the tetrapeptide 142–145 displayed the highest β-turn potential. Hopp and Woods (22) recently described another method of predicting antigenic epitopes of proteins in which the amino acid sequence is analyzed for the hydrophilicity index and the points of highest local average hydrophilicity are either near or located in an antigenic determinant. In agreement with our findings, their analysis of HBsAg predicted the sequence 141–146 to be an antigenic determinant. Chemical modifications of natural HBsAg showed the importance of lysine residues for antigenicity (23, 24). The three lysine residues in HBsAg are located between residues 121 and 160 (Fig. 1). Lysine 160 is in a very hydrophobic environment and may not be accessible for the charge interaction normally involved in antigen–antibody binding. This contention is supported by the recent finding that trypsin does not cleave the Lys-Pro bond between residues 141 and 142 or the Lys-Tyr bond between residues 160 and 161 (25). The Lys-Pro bond is known to be trypsin resistant while the Lys-Tyr bond may not be cleaved due to its inaccessibility. This indicated that one of the antigenic epitopes of HBsAg resides downstream from residue 122. This view has been further substantiated by the expression of the *a* determinant of HBsAg with recombinant DNA techniques using a gene fragment corresponding to the amino acid sequence 121–226 (26). The data shown in Table 2 indicate that there are several peptide sequences in HBsAg that react with human anti-HBs and elicit antibodies that are inhibited by all serotypes of HBsAg and have only the *a* determinant in common. The low anti-HBs response in rabbits immunized with the PsAs is commensurate with the relatively low and variable responses observed when animals were inoculated with recombinant DNA products (26) or with structural polypeptides of HBsAg (27, 28). The fact that the antibodies to PsA 139–147 react equally well with all serotypes in the standard HBsAg panel indicates that the peptide expresses the specificity for the *a* determinant of HBsAg.

Using a different approach, Lerner *et al.* (11) synthesized 13 PsAs covering most of the sequence of HBsAg and demonstrated in rabbits the immunogenicity of at least four different PsAs of the sequence—namely, 2–16, 22–35, 48–81, and 95–109. Because the serologic specificity of the antisera to peptides was not demonstrated with respect to different antigenic determinants of HBsAg, the significance of their findings in relationship to ours is difficult to assess. Dreesman *et al.* (13) recently reported immunogenic activity of disulfide cylized PsAs 117–137 and 122–137 in mice with a single injection of 50 μg of the peptides without coupling them to a carrier protein. Since they expected the subtype-specific determinants in this region (13), testing for the antigenic activity of the PsAs or demonstrating the serologic specificity of the antisera is crucial. Our negative results for antigenic activity of PsA 122–137 may, indeed, be due to the fact that we used only human antibodies specific for the group-specific *a* determinant. The demonstration by Hopp (12) that anti-HBs binds to PsA 138–149 while on the resin is in agreement with the present findings as well as the earlier reports (10, ¶). More recently, Prince *et al.* (14) have used hemagglutination assays to conclude that both the *a* and the *d* determinants are carried by PsA 138–149. However, MacKay *et al.* (26) have tested the immune response to the products of cloned HBV DNA fragments expressed in the *Escherichia coli* system and shown that the *d* determinant is localized between residues 4 and 121 whereas the *a* determinant is localized between residues 121 and 226. The above results are generally in agreement regarding the molecular localization of at least an essential part of the *a* determinant. However, the controversy regarding the localization of the subtype-specific determinants *d* and *y* in the report of Prince *et al.* (14) cannot

be resolved until the specificity of the immune response has been demonstrated. Similarly, the serologic specificity of the immune response needs to be established by Dreesman *et al.* (13) to settle the controversy regarding the localization of the subtype-specific determinants of HBsAg.

In all the studies with PsAs, the antigenicity and immunogenicity of the PsAs are very low in comparison with natural HBsAg. The possibility remains open that the *a* determinant of HBsAg is represented only in part by PsA 139–147. Further work is necessary to establish whether the *a* epitope is made up of a continuous sequence or of a discontinuous sequence with spatially adjacent residues that are not in direct peptide linkage (9, 10) and dependent on a specific conformation possibly involving lysine and cysteine residues (23, 24, 29).

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