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Immunology

Localization, synthesis, and activity of an antigenic site on influenza virus hemagglutinin

(virus types A and B/HA2 fusion region/synthetic peptides/antibody binding)

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ABSTRACT **This paper reports the antigenicity of the fusion region of the influenza virus hemagglutinin (HA). Two peptides, comprising the fusion region (residues 1–11 of the HA2 part of HA) of strain A and strain B influenza virus, were synthesized and their abilities to bind rabbit, goat, and human anti-influenza antibodies were determined. In addition, 30 anti-HA monoclonal antibodies were examined for their ability to bind the synthetic peptides. In quantitative immunoadsorbent titrations, the two peptides bound considerable amounts of antibodies in rabbit and goat antisera against virus or HA of the A or B strain as well as in several human sera from patients recovering from influenza A. Of the 30 anti-HA monoclonal antibodies, 5 bound completely and 4 bound partially to the peptides. Antibodies were raised in rabbits against the peptides by immunizing with peptide–bovine serum albumin conjugates or with the free peptides. Anti-peptide antibodies were bound by HA and by the intact virus of the respective strain. However, these antisera failed to exhibit significant virus neutralizing activity. In contrast, the monoclonal antibodies that reacted with these peptides inhibited viral infectivity. The results clearly show that residues 1–11 of HA2 represent an important antigenic site on influenza virus.**

The antigenicity of influenza virus coat proteins, hemagglutinin (HA) and neuraminidase, has been the subject of great interest [for recent review see Webster *et al.* (1)]. Attention has been focused by the determination of the covalent structure of several influenza HA variants, through sequence analysis of both the protein (2) and its cloned gene (for review, see ref. 3) and, more recently, by the determination of the three-dimensional structure of the HA of influenza A virus by x-ray crystallography at 3-Å resolution (4). The availability of this structural information permits attempts at elucidation of the immune responses to influenza virus at the molecular level. Localization and synthesis of the major antigenic and immunogenic sites of HA will afford peptides that may be used in an effective vaccination strategy. Also they should lead to the characterization of the type of antibody activity (i.e., whether neutralizing or non-neutralizing) elicited by each site and should permit an understanding of the effect of mutations on antigenic alterations.

HA is a glycoprotein synthesized as a single polypeptide chain (molecular weight, about 77,000, corresponding to 550 residues). After synthesis, it undergoes proteolytic cleavage to give two polypeptide chains, HA1 and HA2, which are held together by disulfide bonds. The NH_2-terminal region of HA2 (the fusion region) is believed to be involved in infection by the virus (5), and, from the x-ray structure (4), the first 10 residues of this region appear to be accessible in the HA monomer. Because of its involvement in viral infection, antibodies may arise against this region in the course of defense against viral infection. The present studies with synthetic peptides comprising the HA2 region (residues 1–11) of influenza A virus (FR/A) and influenza B virus (FR/B) were carried out to investigate the antigenicity of this region.

EXPERIMENTAL

Synthesis and Purification of the Peptides. The covalent structures of the synthetic peptides corresponding to the fusion region (residues 1–11 of HA2) of strain A (FR/A peptide) and strain B (FR/B peptide) influenza virus are: FR/A-peptide, Gly-*Leu*-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu; and FR/B-peptide, Gly-*Phe*-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu.

The peptides were synthesized by solid-phase procedures as described (6, 7). The protecting group for glutamic acid side chain was γ-benzyl ester. The peptides were purified by chromatography on columns (2.0 × 55 cm) of LH-20 in dimethylformamide and their purity was confirmed by peptide mapping and by amino acid analysis of acid hydrolysates.

Coupling of Peptides to Protein Carrier. Synthetic peptides were coupled to succinylated bovine serum albumin (Suc-albumin), succinylated hen lysozyme, or native lysozyme after activation of the carrier carboxyl groups by conversion to *p*-nitrophenyl esters (8). The extent of coupling was determined by amino acid analysis of acid hydrolysates of the peptide–protein conjugates. The coupling levels for Suc-albumin conjugates (in mol of peptide per mol of Suc-albumin) were: FR/A peptide, 31.1; FR/B peptide, 28.3. The levels for lysozyme conjugates were (in mol of peptide per mol of lysozyme): FR/A peptide, 4.80; FR/B peptide, 7.00.

Preparation of Virus and HA. The influenza viruses were from the repository at St. Jude Children's Research Hospital. The viruses were grown in the allantoic sac of 11-day chicken embryos, and the virus particles were purified by adsorption induction on erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10–40% sucrose in 0.15 M NaCl) as described by Laver (9). The HA was isolated from purified virus by the procedure of Laver and Webster (10).

Antisera. Human sera were obtained from individuals within 1–2 weeks after an influenza attack. The virus that infected these individuals was identified as type A.

To prepare rabbit anti-HA antisera, HA (200 μg) was emulsified in Freund's complete adjuvant and injected into the footpad and intramuscularly. The rabbits were given a second intramuscular injection of HA in adjuvant and an intravenous injection in saline 40 days later. Bleedings were performed before vaccination and 7 days after the second injection.

Antibodies against the synthetic peptides were raised in rab-

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Abbreviations: HA, hemagglutinin; Suc-albumin, succinylated bovine serum albumin; FR/A, fusion region of type A virus; FR/B, fusion region of type B virus; HI, hemagglutinin inhibition.

bits by two methods. (*i*) For immunization by peptide–protein conjugates, two rabbits were given initial and booster injections of the peptide–Suc-albumin conjugate (1.0 mg) as an emulsion in complete Freund's adjuvant in the footpads (0.5 ml each) and subcutaneously in the neck (0.5 ml). Serial blood samples from 10 days prior to up to 230 days after the first injection were studied separately. (*ii*) In view of the recent finding (11) that immunization with antigenic site peptides in their free form in complete Freund's adjuvant will stimulate an *in vivo* antibody response, the free peptides were also injected (i.e., without coupling to carrier) into two rabbits. Each animal received 0.50 mg of peptide in 0.5 ml of emulsion in complete Freund's adjuvant, distributed into three sites as above. The immunization schedule is shown in Fig. 1. Serial blood samples from 10 days before up to 200 days after the initial injection were studied separately.

Preparation of Monoclonal Antibodies. Monoclonal hybridoma antibodies were made according to the method of Köhler and Milstein (12) and were specific for the HA or neuraminidase of the virus under study (13, 14).

Preparation of Sepharose Immunoadsorbents. Coupling of proteins to CNBr-activated (15) Sepharose CL-4B was carried out under the optimal conditions for the preparation of active adsorbents (16). The synthetic peptides, which were insoluble in aqueous solution, were coupled to activated Sepharose in dimethylformamide. After coupling, peptide-adsorbents could be handled in aqueous solvents in the usual manner. Adsorbents of unrelated peptides [peptides 1–6 and antigenic site 1 of myoglobin (17)] and of bovine serum albumin, sperm whale myoglobin, and human hemoglobin A were used as controls to determine nonspecific background binding. Adsorbents contained 1–1.5 mg of protein and 0.4–0.7 mg of peptide per ml of packed volume.

Antibody Binding Studies. The IgG fraction was isolated from antisera by ammonium sulfate precipitation followed by DEAE-cellulose chromatography (18). Radioiodination with ^{125}I (carrier-free, New England Nuclear) was carried out by the chloramine-T method (19). Labeled proteins had specific activities of 18–24 μCi/μg (1 Ci = 3.7×10^{10} Bq). Binding of antibodies from mouse hybridomas was also determined by reaction with ^{125}I-labeled protein A or with ^{125}I-labeled rabbit anti-mouse IgG. Immunoadsorbent titration studies using fixed amounts of ^{125}I-labeled immune IgG and varying amounts of peptide or protein adsorbents were done as described (16). Antisera against the peptides were also screened for the presence of antibodies by binding to HA or disrupted virus particles adsorbed on round-bottomed wells of polyvinyl chloride plates as described (20).

Serological Assays. Hemagglutination inhibition (HI) tests were done on the antisera as described (10). Neutralization tests were done by titrating virus infectivity in the presence of pre-infection and post-infection sera. Briefly, serial 1:10 dilutions of virus were incubated (40°C, 30 min) with equal volumes of a 1:10 dilution of heat-inactivated (56°C, 30 min) serum; then the serum/virus mixtures were inoculated into five embryonated eggs per dilution. The eggs were tested for HA activity after 48-hr incubation at 37°C to determine the ID_{50}. Neutralizations were also done in Madin–Darby canine kidney cells (21).

Peptide Protection Against Viral Infection. Mice (BALB/c) were immunized intramuscularly with 5, 10, or 50 μg of the synthetic peptide in complete Freund's adjuvant. Each group of mice was given three more similar doses as boosters at biweekly intervals. The mice were then infected with approximately 10 infectious doses of influenza viruses intranasally. Control animals were infected but not immunized. The lungs were removed 3 days after infection and the virus was titrated in chicken embryos.

RESULTS

Purification and Characterization of the Synthetic Peptides. The purified synthetic peptides were homogeneous by peptide mapping and had the following compositions (in mol of amino acid per mol of peptide): FR/A peptide, $Glu_{0.93}Gly_{2.81}Ala_{2.09}Ile_{1.95}Leu_{0.98}Phe_{2.10}$; FR/B peptide, $Glu_{1.10}Gly_{3.24}Ala_{1.91}Ile_{1.87}Phe_{3.14}$. These compositions are in excellent agreement with those expected from the primary structures.

Binding of Rabbit and Goat Anti-HA Antibodies to Peptides. The binding of anti-HA antibodies to peptides was determined by quantitative immunoadsorbent titration (16) of a fixed amount of ^{125}I-labeled immune IgG with increasing amounts of peptide- or HA-adsorbents. This procedure allows the determination of the maximum amount of antibody (plateau value) that can bind with a given antigen-adsorbent (22). The values were corrected for nonspecific binding (corresponding to 3–5%) by concomitant titration of equal amounts of antibody with similar adsorbent volumes of unrelated proteins (albumin, hen lysozyme, and human hemoglobin A). Considerable amounts of each antibody were bound by the peptide-adsorbents (Table 1). Except for one antiserum (rabbit 476), rabbit and goat antibodies against HA of a given strain also crossreacted with the fusion peptide of the other strain. In some cases the crossreaction was comparable to (goat 123) or higher than (rabbit 75-72 and 78-5 and goat 7) the reaction of the homologous peptide.

Binding of Human Anti-Influenza Virus Antibodies to Peptides. Antibody binding to peptides was determined by immunoadsorbent titrations using fixed amounts of human antibodies and increasing amounts of peptide- or protein-adsorbent. The antibodies bound were quantitated by the double-antibody procedure used in the linear range for the first antibody (23) with ^{125}I-labeled rabbit anti-human IgG. All six human sera had considerable amounts of antibodies that bound to the peptides (Table 2). Except for serum MT, more antibodies were bound to the FR/B peptide than to the FR/A peptide.

Table 1. Binding of rabbit and goat anti-HA antibodies to peptides

Antiserum	Immunizing antigen	Antibody bound,* cpm × 10^{-3} FR/A peptide	FR/B peptide	HA (PC/1/73)
Rabbit:				
464	B/Lee/1940	5.10	12.51	23.66
473	B/Maryland/1/59	1.43	14.10	119.54
476	B/Taiwan/2/62	0	12.95	104.46
479	B/England/13/62	1.08	11.14	64.36
75–72	HI A/NWS-A/Tern/SA/61	8.07	17.65	94.77
78–5	HI A/USSR/90/77	1.30	5.43	88.03
79–6	HI HSWINI (X-53A)	1.53	0.27	38.86
Goat:				
7	HI A/Bel/42	11.72	19.94	23.64
40	HI A/FM/1/47	4.54	2.44	20.48
50	HI A/duck/Urk/63	4.31	1.76	19.89
123	B/Lee/1940	14.00	13.48	29.90
126	B/Hong Kong/1/72	0.96	4.36	10.29

* Binding values in the plateau obtained by quantitative immunoadsorbent titrations using ^{125}I-labeled IgG (2×10^5 cpm). Values are means of six replicate analyses which varied ±1.4% or less and have been corrected for nonspecific binding (3% or less).

Table 2. Binding of human antivirus antibodies to peptides

Human serum	HI titers To A/Br/11/78 (HINI)	HI titers To A/BK/1/79 (H3N2)	Antibody bound,* cpm × 10^{-3} FR/A peptide	FR/B peptide	HA (PC/1/73)
BA	<	<	10.81	20.25	70.78
PA	160	320	15.72	21.89	65.48
ZA	320	320	16.93	22.51	66.19
GB	160	<	6.80	16.56	86.21
NB	<	160	13.73	17.99	55.22
MT	<	160	17.60	13.00	63.04

* As in Table 1.

Binding of Monoclonal Antibodies to Peptides. To examine the clonal incidence of antibodies against this region of HA, 32 monoclonal antiviral antibodies were investigated for their ability to bind to the peptides. Binding was determined by a plate assay using peptide–Suc-albumin conjugates as the binding antigen. Bovine serum albumin, Suc-albumin, lysozyme, and hemoglobin were used as control unrelated antigens to correct for nonspecific binding. The specificity of 30 of the monoclonal antibodies was directed against HA. Of these, nine (30% of the total) bound to the synthetic peptides (Table 3); five of the nine (17%) exhibited comparable binding to the peptides and to intact HA and four (13%) bound only partially to the peptides relative to their binding to intact HA. Antibodies against type A or type B virus bound best with the HA and the corresponding synthetic peptide of that virus type, although considerable crossreactions were observed with the other HA type and its peptide.

Reaction of Anti-Peptide Antibodies with HA and with Intact Virus. When peptide–Suc-albumin conjugates were the immunizing antigens, antibody responses were analyzed by peptide–lysozyme (not succinyl–lysozyme) conjugates because lysozyme and Suc-albumin do not crossreact immunochemically. Correction for nonspecific binding was derived from binding to a lysozyme control. When the free peptides were the antigens, antibodies were analyzed on peptide–Suc-albumin conjugates and lysozyme and Suc-albumin were used as controls.

Antibodies against peptides conjugated to carrier. The peptide-specific antibody response elicited in rabbits by peptide–Suc-albumin conjugates was weak even with monthly immunizations up to 9 months. Table 4 summarizes the binding results with some representative samples from three rabbits. A second rabbit that was immunized with the FR/B peptide conjugate gave no anti-peptide antibodies even after 9 months of immunization. Antibodies to the FR/A or FR/B peptide bound to the corresponding HA (A/Port Chalmers or B/Hong Kong) and, even more significantly, bound in comparable amounts to the corresponding virus strain. Antibodies to the FR/A peptide showed considerable crossreaction with the FR/B peptide and vice versa (see Table 5).

Table 3. Binding of monoclonal mouse antibodies to HA and to synthetic fusion region peptides

Monoclonal antibody	Antibody bound, cpm × 10^{-3} FR/A peptide	FR/B peptide	HA (Texas)	HA (B/HK)
Antibodies that bind completely to peptides				
BHK 174/3	13.60	19.97	7.54	24.92
BHK 82/1	0.10	22.95	14.51	18.80
VIC 21/3	35.96	18.56	39.19	21.55
Texas 185/1	5.39	2.06	5.58	2.12
Texas 10/2	1.99	1.44	1.99	1.42
Antibodies that bind partially to peptides				
BHK 150/3	2.29	3.26	0	9.43
BHK 419/2	4.46	8.52	3.20	32.61
BHK 430/1	17.46	18.19	0.39	56.99
Texas 192/1	9.81	1.34	16.86	1.30

Antibody binding to HA or peptide–Suc-albumin conjugate was determined by reaction with ^{125}I-labeled protein A on polyvinyl chloride plate adsorbents. The values, which have been corrected for nonspecific binding (4% or less) to albumin, Suc-albumin, lysozyme, and hemoglobin, represent the average of triplicate analyses which varied ±1.3% or less.

Antibodies obtained by immunization with free peptides. An example of the antibody response with time in one rabbit that was immunized with the FR/A peptide is shown in Fig. 1. Table 5 summarizes the binding to peptides and to virus strains A and B of antibodies in representative anti-peptide antisera. After immunization with the free peptides, all rabbits mounted a good peptide-specific antibody response, and the titer could be main-

Table 4. Binding of antibodies against peptide–carrier conjugates to HA and to virus*

Antigen	Harvest day	Antibody bound, cpm × 10^{-3} FR/A peptide	FR/B peptide	HA (A/Port Chalmers)	Virus (A/Port Chalmers)
		Rabbit FRA-1			
FR/A	63	5.77	2.76	3.40	3.86
peptide–Suc-albumin	137	8.07	2.66	4.44	6.14
	180	10.35	5.04	6.62	6.72
		Rabbit FRA-2			
FR/A	86	3.86	3.96	3.64	2.75
peptide–Suc-albumin	137	2.69	0.91	2.39	2.13
	180	4.73	3.12	4.58	4.70
		Rabbit FRB-2†			
FR/B	104	2.09	0.92	1.53	1.58
peptide–Suc-albumin	180	5.51	4.26	4.30	3.67

Antibody binding to HA and to peptide–lysozyme conjugate was done as in Table 3. Correction for nonspecific binding (3% or less) was obtained from binding to lysozyme control. Values represent the average of triplicate analyses which varied ±1.2% or less.

* The studies were carried out with antiserum dilutions of 1:100, except for the 63-day serum of rabbit FRA-1 which was at 1:10.

† In this case, HA and virus were B/Hong Kong.

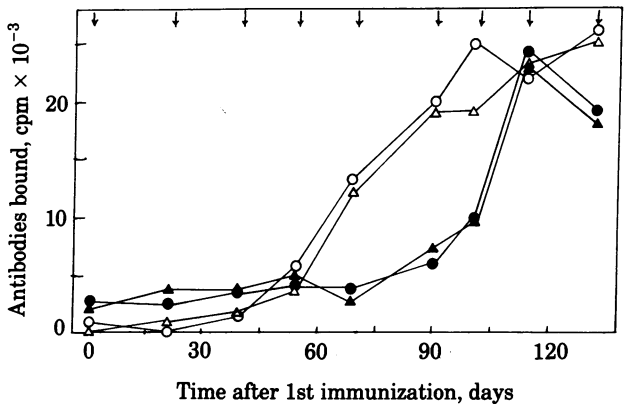

FIG. 1. Time-course of the binding of rabbit anti-FR/A peptide (rabbit FR/A-P2) with: FR/A peptide–Suc-albumin (△); FR/B peptide–Suc-albumin (○); virus A/Port Chalmers (●); virus X-31 (▲). Antisera were obtained from rabbit FRA-P2 which was immunized with free (i.e., without coupling to carrier) FR/A peptide. Binding of antibodies in serial blood samples (1:1,000 dilution) was determined by reaction with ^{125}I-labeled protein A (2×10^5 cpm) on polyvinyl chloride plate adsorbents. For details, see text.

tained by monthly booster injections up to 200 days after the initial immunization. Later blood samples were not tested. Most significantly, almost all the anti-peptide antibodies in the antisera were bound by the corresponding type A or type B virus.

Viral Neutralizing Activities of the Antisera and of the Monoclonal Antibodies. The synthetic peptides failed to protect mice from infection with low doses of either B/Lee/40 or A/Aichi/2/68 (H3N2) influenza viruses (10 ID_{50}) (results not shown). Rabbit anti-peptide antisera at the peak of the antibody response failed to neutralize virus infectivity in plaque assays and in end-point virus titrations or to inhibit hemagglutination or hemolysis of erythrocytes by influenza viruses at pH 5.0 (24). In contrast, the monoclonal antibodies (of the IgG1 isotype) neutralized the homologous virus to very high titers (in excess of 1:10,000) and were effective in inhibiting hemagglutination and for selection of antigenic variants (13, 14).

DISCUSSION

Our selection of the fusion region of HA for synthesis and immunochemical investigation was based on a report (5) implicating its involvement in infection of the cell by the virus. We reasoned that, if this were correct, then in the course of viral infection, antibodies against this region could be produced which may afford protection against viral infection. This would imply that the fusion region is a natural antigenic site on HA, which was confirmed here.

The recognition of this region as an antigenic site under artificial immunization conditions (i.e., injection in complete Freund's adjuvant) would not in itself imply that it is antigenic in humans under conditions of natural infection. However, the finding that sera from several individuals after viral infection had large amounts of antibodies directed against the fusion region unequivocally established it to be an important antigenic site on virus and on HA in humans under conditions of natural influenza infection.

The screening of a panel of anti-influenza monoclonal antibodies afforded information about the clonal incidence of the antibody response to the region. These studies revealed that

Table 5. Binding of antibodies elicited by immunization with free peptides to HA and to virus*

Antigen	Harvest day	Antibody bound, cpm × 10^{-3} FR/A peptide	FR/B peptide	Virus (A/Port Chalmers)	Virus (X-31)
		Rabbit FRA-P1			
FR/A peptide	51	10.00	8.95	9.72	9.60
	80	58.18	50.80	40.64	39.71
	108	62.89	49.87	43.29	46.59
	125	89.65	73.68	64.68	61.32
		Rabbit FRA-P2			
FR/A peptide	51	3.75	5.61	4.13	4.89
	80	19.51	20.02	6.03	7.30
	108	23.12	22.22	24.21	23.15
	125	24.09	26.14	19.27	18.09
		Rabbit FR/B-P1			
				HA (B/Hong Kong)	Virus (B/Hong Kong)
FR/B peptide	66	4.63	10.57	11.99	11.19
	108	31.29	43.43	16.32	17.30
	125	39.73	50.80	24.41	25.86
		Rabbit FR/B-P2			
FR/B peptide	66	11.15	28.98	15.24	14.67
	108	31.24	43.75	15.89	10.26
	125	14.60	35.71	13.90	14.08
	160	26.15	31.64	15.57	16.10

Antibody binding to HA or to peptide–Suc-albumin conjugates was determined as in Table 3. Lysozyme and Suc-albumin were used to correct for nonspecific binding (3% or less). Values are means of triplicate analyses which varied ±1.4% or less.

* Binding studies were carried out with antiserum dilutions of 1:1,000.

about a third of the clones can bind to this region. However, only about 17% of anti-HA clones bound completely to the peptides. The remaining 13% bound only partially, suggesting that they recognize an antigenic site that may encompass additional residues either to the right of glutamate-11 or, less likely in this case, residues that are spatially adjacent from parts that are otherwise distant in sequence. Such discontinuous antigenic sites (25, 26) were first recognized and synthesized in lysozyme (18, 25, 27–30). Minor shifts in antigenic site boundaries were first reported for myoglobin antigenic sites (6, 7, 17). One of the monoclonal antibodies, Vic/21/3, had previously been shown (31) to inhibit neuraminidase activity of homologous virus. Its reaction with the fusion peptide was unexpected and may be due to some unusual crossreactivity of the antibody.

The high antigenicity of the HA fusion region led us to investigate whether anti-peptide antibodies will bind to this region on HA or the virus. Indeed, anti-peptide antibodies, especially those elicited by the free peptides, bound almost completely to HA or to the respective virus. However, it was surprising that these antibodies afforded no significant virus-neutralizing activity. Nevertheless, each of the nine monoclonal antibodies that bound to the fusion peptides had good neutralizing activity. All the monoclonal antibodies were of the IgG1 isotype. We have no satisfactory explanation for the different behavior of the sera and the monoclonal antibodies. It could be related to differences in the immunoglobulin isotype or to differences in affinity or to the possibility that the binding sites for the monoclonal antibodies encompasses additional residues not present on the peptides.

The strong immunogenic activity of this location may be viewed in better perspective when compared with other studies on HA. Slight antigenicity has been reported in the region of residues 91–108 (32) and in some of the carbohydrate side chains (33) of HA1. Recently, 20 peptides comprising almost 75% of the HA molecule were synthesized (34) and, even though most were antigenic when coupled to protein carrier, none of the peptides reacted with anti-HA antibodies, indicating that they did not contain any antigenic sites of HA. It should be noted that the antigenic site described here is not one of the four regions suggested (35) from examination of the x-ray structure (4). However, it is accessible and is involved in infectivity. Also, this site is not predictable from the empirical approach suggested by Hopp and Woods (36) on the basis of hydrophilicity index as derived by analysis of the sequence by the Chou and Fasman (37) method. In fact, the fusion region is so hydrophobic that our synthetic peptides were insoluble in aqueous solvents. Only by coupling to soluble carriers were we able to study their binding activity. Clearly, caution should be exercised in applying such empirical methods to a highly complex phenomenon such as the immune recognition of protein antigens. As originally pointed out when the antigenic structure of myoglobin was first determined (17), antigenic sites should be expected to reside in accessible surface regions, but then not every surface region constitutes an antigenic site. Thus, exposure is not a sufficient criterion for immunogenicity (17, 38). Furthermore, antigenic sites are not necessarily highly hydrophilic regions, and hydrophobic interactions frequently provide major contributions to the binding energy (17). Also, as seen here and reported for hemoglobin α-chain (39), major antigenic sites could reside in surface regions that have very low hydrophilicity or are mostly hydrophobic. Such regions are rendered accessible by the three-dimensional and oligomeric characteristics of a particular protein (39).

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