

## Enumeration of Antigenic Sites of Influenza Virus Hemagglutinin

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The antigenic sites on the hemagglutinin of X-31 (H3) influenza virus have been defined by using a competitive radioimmunoassay with a panel of monoclonal antibodies which includes those known to select variants with substitutions of particular amino acids. The capacity of each monoclonal antibody to block the binding of other radioiodinated monoclonals to purified hemagglutinin permitted classification of the panel into four separate groups, each of which defined a particular antigenic site on the hemagglutinin molecule. Three of these are located on the polypeptide backbone and correspond to the "hinge," the "loop," and the "tip/interface" of the X-ray crystallographic model of Wiley et al. (*Nature* [London] **289**:373-378, 1981). Nonreciprocal blocking of certain anti-interface antibodies by anti-loop antibody suggests that much of the exposed surface of the head of the hemagglutinin molecule extending from the loop to the interface may be a continuum of epitopes. A fourth antigenic site is carbohydrate in nature, presumably situated on the antigenic oligosaccharide side chains. These four domains are in addition to two antigenic sites defined by monoclonal antibodies that inhibit neither hemagglutination nor infectivity (Breschkin et al., *Virology* **113**:130-140, 1981; Yewdell et al., *Nature* [London] **279**:246-248, 1979).

Wiley et al. (23) have recently postulated that the hemagglutinin (HA) molecule of influenza virus A/Hong Kong/1/68 (H3) contains four major antigenic sites. This conclusion was based on the observation that most of the amino acid changes in those antigenically distinct epidemic virus strains of the Hong Kong (H3N2) subtype that have been sequenced, as well as in variants selected in the presence of monoclonal anti-HA antibodies (17, 22) or anti-HA antiserum fractions (5, 19), appeared to cluster into four regions on a three-dimensional model of the HA molecule obtained by X-ray crystallographic analysis (23). Three of these regions, termed by Wiley et al. A, B, and C, were comprised of external residues in what we shall refer to as the "loop," "tip," and "hinge," respectively, whereas the fourth (D) seemed to be located in the "interface" between the three subunits that form the HA spike.

Although there is no doubt that amino acid substitutions in these four regions induce antigenic alterations in the HA molecule, this does not prove that they represent the antigenic sites to which antibodies actually bind; it is quite possible that substitutions in one region could,

by changing the conformation of the molecule, affect an antibody-binding site some distance away. To investigate the binding sites for antibody more directly, we performed competitive binding studies with a panel of monoclonal antibodies which include three that have previously been demonstrated (17) to select laboratory variants with substitutions in regions A, C, and D, respectively. The rationale of this approach is that if two monoclonal antibodies recognize the same epitope, then an excess of one will inhibit the binding of the second. If, however, two antibodies recognize determinants which are distant, then both will bind simultaneously. An intermediate situation may also occur in which steric hindrance occurs between the monoclonal immunoglobulin G (IgG) molecules recognizing epitopes that are distinct but relatively close to one another. The feasibility of this approach has been previously demonstrated in this laboratory (20).

### MATERIALS AND METHODS

**Viruses.** The influenza viruses used in this study were: A/Bel/42(H1N1); the reassortant X-31, bearing the HA of A/Aichi/68(H3), derived by the genetic reassortment between A/Aichi/68(H3N2) and A/PR/8/34(H1N1); and Mem<sub>H</sub>-Bel<sub>N</sub>, bearing the HA of A/Memphis/102/72(H3) and the neuraminidase of

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A/Bel/42(N1), derived by the genetic reassortment between A/Memphis/102/72(H3N2) and A/Bel/42(H1N1). The viruses were grown in the allantoic cavity of 10-day embryonated hen's eggs and purified by rate zonal centrifugation after adsorption-elution from chicken erythrocytes as previously described (15).

**Isolation of HA and its component polypeptide chains.** The HA of each strain of virus was isolated by electrophoresis on cellulose-acetate blocks by the method of Laver (14). The heavy (HA<sub>1</sub>) and light (HA<sub>2</sub>) polypeptide chains of HA were separated by centrifugation on a gradient of guanidine hydrochloride (16).

**Monoclonal antibodies.** Monoclonal antibodies were prepared by using X-31 virus as immunogen in BALB/c mice, as described in detail elsewhere (13). All monoclonal antibodies used in this study were of the IgG class.

**IgG.** IgG directed against mouse IgG was extracted by affinity chromatography on protein A-Sepharose (9) from serum of outbred New Zealand rabbits which had been immunized with murine IgG. IgG from murine ascitic fluid was prepared on protein A-Sepharose by the method of Ey et al. (4).

**Radiolodination.** IgG was iodinated by using a modification (10) of the chloramine T method.

**RIA.** A solid-phase radioimmunoassay (RIA) described previously (3) was employed. Competitive binding assays were carried out by incubating 25 ng of radiolabeled monoclonal IgG with serial dilutions of homologous or heterologous antibody in wells coated with purified HA. After 18 h in a humidified atmosphere, the wells were aspirated and washed, and the radioactivity remaining in individual wells was determined. The results were expressed as percentage inhibition by comparing radioactivity bound in the presence of nonlabeled antibody with radioactivity bound in the absence of nonlabeled antibody.

**Hemagglutination and HI assays.** Hemagglutination and hemagglutination inhibition (HI) assays were performed as previously described (6).

## RESULTS

**Reactivity patterns of monoclonal IgG antibodies against influenza viral HA.** Monoclonal IgG was prepared from ascitic fluid and titrated for HI activity against X-31 and Mem<sub>H</sub>-Bel<sub>N</sub> viruses (Table 1). The monoclonal antibodies can be classified into three groups: those with substantial HI activity (A13, A20, A21, B17, B18), those with low HI activity (A2, A11, B23), and one with no activity (B1). Furthermore, they differ considerably in the ratio of their HI titers against the two viruses. Monoclonal A21, for example, displays a comparably high titer against each virus, whereas monoclonal A20 is specific for the virus used to elicit it.

Failure to inhibit hemagglutination does not necessarily imply failure to bind to HA. It is possible that certain antibodies may bind to HA, even with high avidity, yet have little effect on the biological activity of the molecule because they attach at a location that does not sterically

TABLE 1. Characteristics of monoclonal antibodies

Monoclonal	Code	Immunogen	HI titer against <sup>a</sup> :	
			X-31	Mem <sub>H</sub> -Bel <sub>N</sub>
H14 A2-19	A2	X-31	800	800
H14 A11-5	A11	X-31	80	905
H14 A13-15	A13	X-31	51,200	1,600
H14 A20-14	A20	X-31	512,000	1,131
H14 A21-3	A21	X-31	362,000	256,000
H14 B1-10	B1	X-31	10	10
H14 B17-16	B17	X-31	18,100	400
H14 B18-12	B18	X-31	36,200	1,131
H14 B23-13	B23	X-31	20	905

<sup>a</sup> Reciprocal of the dilution of monoclonal antibody inhibiting 4 HA units of virus.

hinder hemagglutination. This possibility was tested by titrating each monoclonal antibody against a constant amount of purified HA in the solid-phase RIA. Binding of antibody was detected by using radiolabeled rabbit anti-mouse IgG.

The results (Fig. 1) show that even those monoclonal antibodies that displayed little or no HI activity bound well to HA. The cross-reactivity of some of the antibodies evident in Table 1 is also reflected in the binding profiles of Fig. 1. Monoclonal antibody B1 was unique in that it bound comparably to the HA of Bel (H1), Mem<sub>H</sub>-Bel<sub>N</sub> (H3), and X-31 (H3) but had little or no HI activity against any of them (Table 1); proof that it is directed to carbohydrate antigen on the HA is presented below.

Although the use of purified HA may avoid any problems due to accessibility of antibodies to antigenic sites within intact virus, partial denaturation of antigenic determinants may occur during isolation of HA. Although the HI activity of A11 toward Mem<sub>H</sub>-Bel<sub>N</sub> virus is higher than it is toward X-31 virus (Table 1), the binding of A11 to purified HA of Mem<sub>H</sub>-Bel<sub>N</sub> is lower than that to HA of X-31 (Fig. 1). This may indicate that HA from Mem<sub>H</sub>-Bel<sub>N</sub> virus is less stable to purification than is HA from X-31.

**Spatial arrangement of antigenic determinants of the HA molecule.** Fifteen different monoclonal IgG preparations were prepared from ascitic fluid and radioiodinated. Each demonstrated significant binding to HA antigen. Wells of polyvinyl trays were exposed to 100 μl of HA solution at a concentration of 5 μg/ml, which provided an antigen coating insufficient to bind all of the radioactivity in the 25 ng of monoclonal [<sup>125</sup>I]IgG used in the assay. Inhibition of binding of radioiodinated IgG was therefore easily detected. Dilutions of monoclonal antibodies were added to antigen-coated wells in the presence of a constant amount (25 ng) of radiolabeled IgG. In this way, every monoclonal antibody was titrated for its ability to prevent the attachment

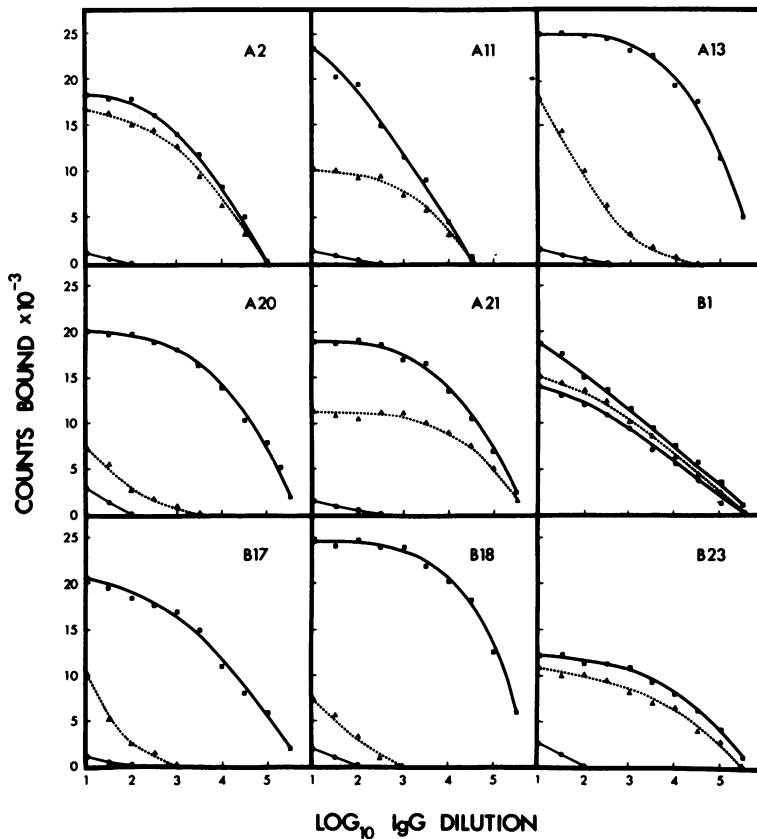


FIG. 1. Binding of monoclonal IgG to HA. Serial dilutions of monoclonal IgG were prepared and introduced to wells of microtiter trays coated with hemagglutinin. After 3 h of incubation, the wells were aspirated and washed, and <sup>125</sup>I-labeled rabbit anti-mouse IgG was added. After an additional 1 h of incubation, wells were washed and excised, and the residual radioactivity was determined. ■, X-31 HA; ▲, Mem 72 HA; ●, Bel HA.

of each radioiodinated member of the panel. In every case, binding of radioiodinated monoclonal IgG was inhibited 100% by nonlabeled homologous antibody. Heterologous antibodies that achieved levels of inhibition similar to those achieved by the homologous antibodies were assigned to the groups described below.

(i) **Group 1.** Radioiodinated antibody B23 was inhibited in its binding to HA only by antibodies A2, A11, and B23 (Fig. 2a). Similarly, antibody A11 was inhibited only by A2, A11, and B23 (Table 2). Antibody A2, on the other hand, was inhibited by A2 and A11 but not by B23 (Fig. 2b). The three monoclonal in this group ap-

TABLE 2. Summary of competitive RIA data

Non-radiolabeled antibody	Inhibition radiolabeled antibody <sup>a</sup> :										Group	Antigenic site
	A2	A11	B23	A20	A13	B18	B17	A21	B1	Anti-host		
A2	+	+	+	-	-	-	-	-	-	-	1	Hinge
A11	+	+	+	-	-	-	-	-	-	-		
B23	-	+	+	-	-	-	-	-	-	-		
A20	-	-	-	+	+	+	-	-	-	-	2	Loop
A13	-	-	-	-	+	+	+	+	-	-		
B18	-	-	-	-	+	+	+	+	-	-	3	Interface/tip
B17	-	-	-	-	+	+	+	+	-	-		
A21	-	-	-	-	+	+	+	+	-	-		
B1	-	-	-	-	-	-	-	-	+	+	4	Carbohydrate
Anti-host	-	-	-	-	-	-	-	-	+	+		

<sup>a</sup> +, 100% Inhibition; -, zero inhibition.

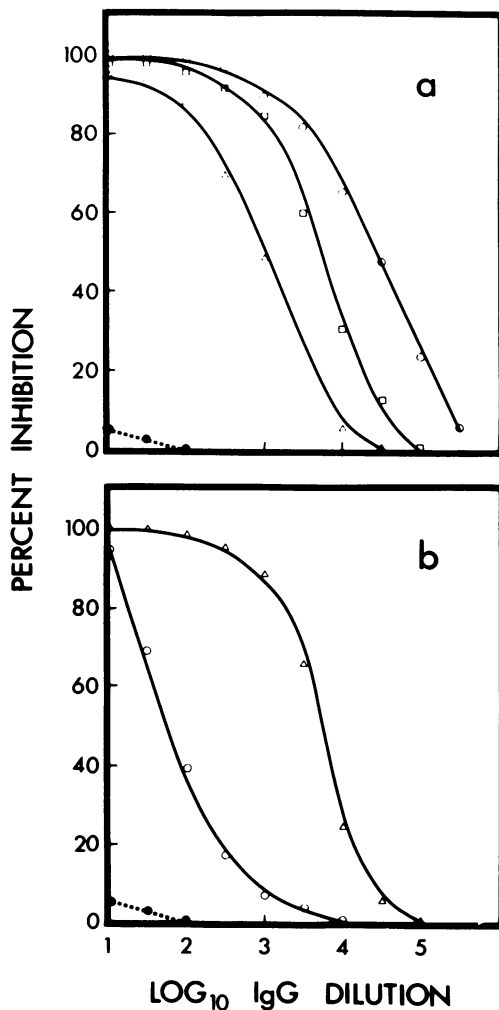


FIG. 2. Inhibition of binding of radioiodinated monoclonal IgG to X-31 HA. With a competitive RIA, the degree of binding of radiolabeled monoclonal IgG to HA was determined in the presence of nonlabeled monoclonal antibodies. (a)  $^{125}\text{I}$ -B23 (25 ng) was examined for its binding properties to HA in the presence of each of the nonlabeled monoclonal antibodies:  $\Delta$ , A2;  $\circ$ , A11;  $\square$ , B23. (b) Binding of 25 ng of  $^{125}\text{I}$ -A2 to HA in the presence of each nonlabeled monoclonal antibody.  $\Delta$ , A2;  $\circ$ , A11. The broken line ( $\bullet$ --- $\bullet$ ) represents the level of inhibition obtained with serial dilutions of each of the remaining monoclonal antibodies of the panel.

peared to bind to a region that is not recognized by any other monoclonal tested.

(ii) **Group 2.** Radioiodinated antibody A20 was inhibited in its binding to HA by the homologous monoclonal (Fig. 3a) and by six monoclonal antibodies provided by R. G. Webster (data not shown). These six monoclonal antibodies were classified by Webster and Laver (22), on the basis of HA reactivity patterns, into a single group which

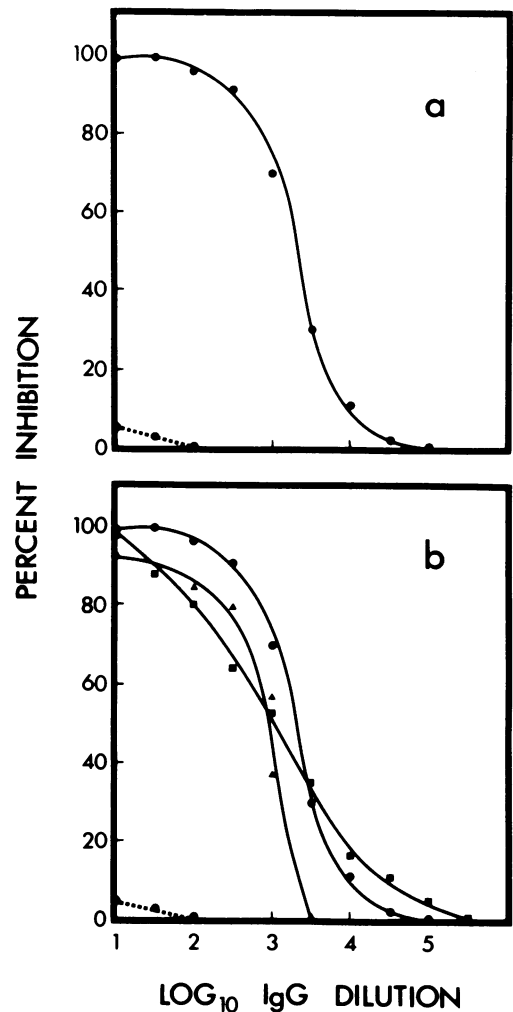


FIG. 3. (a) Binding of 25 ng of  $^{125}\text{I}$ -A20 in the presence of the homologous nonlabeled antibody ( $\bullet$ ). (b) Inhibition of 25 ng of  $^{125}\text{I}$ -A13 ( $\Delta$ ), 25 ng of  $^{125}\text{I}$ -A20 ( $\circ$ ), and 25 ng of  $^{125}\text{I}$ -B18 ( $\blacksquare$ ) in the presence of serial dilutions of nonlabeled A20. The broken line ( $\bullet$ --- $\bullet$ ) represents the level of inhibition obtained with serial dilutions of each of the remaining monoclonal antibodies from the panel.

included A20. Although none of the other members of our panel of antibodies was able to prevent the binding of A20, A20 itself was able to compete with A13 and B18 in binding to HA (Fig. 3b).

(iii) **Group 3.** Monoclonal antibodies A13, A21, B17, and B18 blocked one another completely and reciprocally (Fig. 4) and, with the exception of A13 and B18 (which were inhibited by A20 [Fig. 3b]), were not affected by any of the other antibodies tested.

(iv) **Group 4.** Monoclonal antibody B1 defines an antigenic site which is separate from each of

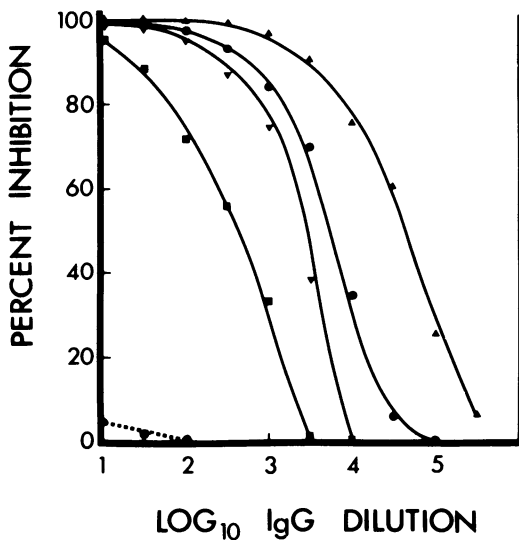


FIG. 4. Degree of inhibition obtained when 25 ng of  $^{125}\text{I}$ -A21 was incubated with serial dilutions of nonlabeled A13 ( $\nabla$ ), A21 ( $\blacktriangle$ ), B17 ( $\bullet$ ), and B18 ( $\blacksquare$ ). None of the other antibodies ( $\bullet$ --- $\bullet$ ) exhibited any significant inhibition.

those defined by the other antibodies. It was not inhibited by any other members of the panel, nor did it inhibit them. However, a rabbit antibody raised against host carbohydrate antigen prepared from uninfected chorioallantoic membrane (11) was able to prevent the binding of B1 to HA; furthermore, B1 was able to prevent the binding of this anti-carbohydrate antibody (Fig. 5). The binding properties of monoclon B1 to different strains of HA (Fig. 1) indicated that it is directed against the carbohydrate moiety of HA.

The results of the competitive RIAs are summarized in Table 2.

#### DISCUSSION

By using a relatively small number of monoclonal antibodies, three antigenic domains have been identified on the polypeptide of influenza viral HA in this study by a competitive RIA. These three domains are defined by the monoclonal antibodies A2, A20, and A21, previously shown by Laver et al. (17) to select variants with amino acid substitutions at positions 54, 133 and 143, and 205, respectively. Inspection of the three-dimensional model of the HA monomer recently presented by Wilson et al. (24) indicates that residue 54 is situated in the hinge region centered around cysteine 52 and cysteine 277 in HA<sub>1</sub> (putative antigenic site C of Wiley et al. [23]), residues 133 and 143 are both in the prominent loop projecting from the side of the head of the molecule (site A of Wiley et al. [23]), and residue 205 lies within the interface (site D of Wiley et al. [23])

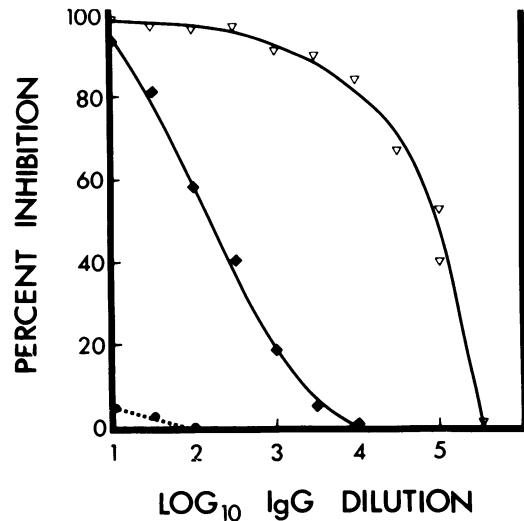


FIG. 5. Inhibition of 25 ng of  $^{125}\text{I}$ -B1 in the presence of nonlabeled B1 ( $\nabla$ ) and a rabbit IgG preparation directed against host antigen ( $\bullet$ ). ( $\bullet$ --- $\bullet$ ) Inhibition level achieved with the remaining monoclonal antibodies.

near the tip of the molecule. Our demonstration that monoclonal antibodies A2, A20, and A21 bind simultaneously and non-competitively to the isolated HA molecule supports the proposal of Wiley et al. (23) that the hinge, the loop, and the interface constitute separate antigenic sites.

As no monoclonal was available which is known to select variants with amino acid substitutions in the region designated as the tip of the molecule, we have no evidence that the antigenic site Wiley et al. (23) postulate to be located between residues 187 and 196 of HA<sub>1</sub> is sufficiently separate from the interface to be distinguishable as a fourth site by competitive RIA. All that can be said here is that whereas three of our monoclonal antibodies (A13, B17, and B18) totally blocked the binding of A21, monoclonal A20, recognizing the loop, blocked two of these antibodies (A13 and B18) but was not itself blocked by any of them. There are a number of possible explanations for such nonreciprocal blocking (2). One is that antibodies to the loop may induce a conformational alteration elsewhere on the molecule, e.g., the tip or interface; such allosteric changes, suggested by the data of Jackson et al. (12), have now been clearly demonstrated in virus PR8 (H1) by Lubeck and Gerhard (18a). A second possibility is that A13 and B18 recognize epitopes located between the interface and the loop, perhaps in or near the tip of the molecule. Such a region, comprising amino acid residues 155 to 160, has recently been described (1). Indeed, it is likely that most of the exposed surface of the head of the HA molecule

is potentially antigenic and comprises a continuum of adjacent (overlapping or nonoverlapping) epitopes. The clustering of these epitopes into apparently separate antigenic domains (loop, tip, and interface) may be fortuitous.

The hinge, however, does seem to represent an antigenic site quite distinct from the others. Monoclonal antibodies A2, A11, and B23 blocked the binding of no other antibodies and were not blocked by any other. Although all three bound efficiently to isolated HA (Fig. 1), each displayed very low HI activity, consistent with the remoteness of the hinge region from the putative erythrocyte-binding cleft at the tip of the molecule (24). This site may correspond to the C<sub>b</sub> region described by Gerhard and colleagues (7, 8, 18).

Two additional antigenic domains have been described in HA of the H3 subtype (Memphis/102/72) by Breschkin et al. (2) and the H1 subtype (A/PR/8/34) by Gerhard and colleagues (7, 8, 18). Antibodies directed against these sites inhibit neither hemagglutination nor infectivity. It has been postulated that they are situated well down the spike (7). Since they are relatively conserved (7, 8, 18), they may not correspond to any of the four sites of high substitution mapped on the model of Wiley et al. (23).

The carbohydrate determinant identified in this paper presumably occurs on those particular oligosaccharide side chains that are antigenic and immunogenic. Earlier studies from this laboratory have indicated that only certain of the *N*-acetylglucosamine (complex or type I) oligosaccharides and none of the oligomannosides (mannose rich, simple, or type II) are antigenic (3a, 21) and that the antigenic determinant they carry is cross-reactive between subtypes of influenza A (11).

The major advantage of competitive binding studies is that they enable one to define discrete antigenic domains to which monoclonal antibodies bind non-competitively. Their limitation is that they tend to underestimate the number of such domains because of steric hindrance between the IgG molecules. Moreover, distinct epitopes within a given domain will not be discriminated unless the angles of attachment of their respective antibodies are such as to avoid steric hindrance. For example, using this approach with PR8 (H1), Lubeck and Gerhard (18) were unable to discriminate between two epitopes previously demonstrated to be distinct by HI reactivity patterns of mutants selected with monoclonal antibodies. More precise definition of the topography of the epitopes within each domain, including such questions as whether a single domain contains several epitopes, whether such epitopes overlap, or whether the antigenic domains themselves overlap, will require

the application of finer probes such as monoclonal Fab fragments.

#### ACKNOWLEDGMENT

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