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An analysis of the properties of monoclonal antibodies directed to epitopes on influenza virus hemagglutinin

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Summary. Monoclonal antibodies (MAbs) specific for the hemagglutinin (HA) of the H3 subtype of influenza A virus were grouped according to their inability to bind to particular MAb-selected neutralization escape mutants of the virus having an amino acid substitution in one of the five postulated antigenic sites on the molecule. Additional residues critical to the binding of the MAbs were deduced from their patterns of reactivity with a panel of field strains and receptor mutants of the H3 subtype. The relationship of these residues to the actual epitopes recognized by the MAbs was inferred from their location on the three-dimensional structure of the HA molecule. In this way it was generally possible to identify a number of residues that are critical to the integrity of the epitope recognized by each of the MAbs examined. It was found that: (1) Several of these epitopes appear to be discontinuous and some may depend on residues contributed by more than one monomer. For example, residue 205, in the interface between monomers of the HA, was found to affect the integrity of the epitopes for several MAbs, possibly by stabilizing the conformation of residues around the receptor-binding pocket and/or in site B on the adjacent monomer. The activity of these particular MAbs was greatly decreased if the virus was exposed to pH 5. (2) All the MAbs tested neutralized viral infectivity and inhibited hemagglutination, although the single MAb directed to site C, which is the most distant from the receptor-binding site, was the least efficient. (3) Hemagglutination inhibition, and particularly neutralization tests, were more discriminating than ELISA in discerning subtle differences between the corresponding epitopes recognized by MAbs on different field strains. (4) Efficiency of neutralization of infectivity did not correlate consistently with hemagglutination inhibiting efficiency; MAbs postulated to bind to epitopes close to the receptor-binding pocket were very efficient at inhibiting hemagglutination,

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whereas neutralization efficiency tended to be more influenced by the affinity of binding of the MAb. (5) A MAb binding to any particular epitope could affect the binding of a second MAb directed to an epitope within the same or even a different antigenic site. The observed effect was most commonly inhibition of binding, which was not always reciprocal; enhancement of binding was also observed with certain combinations of MAbs. The relative affinity of the MAbs, in addition to steric constraints, were shown to be important factors in the ability to compete for interaction with HA.

Introduction

The nature, number, and location of antigenic sites on the hemagglutinin (HA) of influenza virus have been investigated using a variety of strategies. Comparison of the amino acid sequence of the HA of field strains that have arisen by antigenic drift [3, 47], and of monoclonal antibody (MAb)-selected neutralization escape mutants [8, 30, 35, 49], indicates that the amino acid changes tend to cluster into distinct regions on the HA molecule. On the basis of these findings five antigenic sites on the HA of the H3 subtype have been identified: sites A, B, C, D [54], and E [8, 54]. Similar studies with viruses of the H1 subtype have also identified five antigenic regions on the HA, some of which correspond to those of the H3 HA [7, 15, 33, 36, 37].

The number of antigenic sites on the HA molecule has also been investigated by testing the ability of MAbs to bind to a panel of escape mutants each containing a single amino acid substitution. By grouping together MAbs that demonstrate similar patterns of binding, operational antigenic maps of the HA can be constructed. Such maps of the HA from a strain of the H3 subtype of influenza virus indicate that it possesses at least three antigenic sites, each defined by a group of related MAbs [49]. In a slightly different type of study, Underwood [44, 45] examined the ability of MAbs raised to six field strains of the H3 subtype to bind to a panel of H3 viruses, resulting in the delineation of 6–10 overlapping groups. Operational antigenic maps have also been produced for viruses of the H1 [7, 15], H2 [55], H4 [19], and H7 subtypes [26].

Competitive radioimmunoassays, which examine the ability of an unlabeled MAb to inhibit the binding of a different radiolabeled MAb, allow construction of a topographical antigenic map of the HA. Failure of the unlabeled MAb to reduce the binding of the labeled MAb indicates that the MAbs are binding to distinct sites on the HA molecule, while inhibition of binding indicates that the MAbs recognize the same epitope, overlapping epitopes, or epitopes located in close proximity to each other. Using such an approach, four topographical sites have been described for viruses of the H1 [31] and H3 subtypes [4, 24].

In recent years, the initial concept of discrete antigenic sites on HA has been tempered by the realization that binding of certain MAbs can be affected by changes in more than one site and can be blocked by the binding of MAbs directed to a different site. These findings gave rise to the idea that the available

surface of the distal end of the HA in fact forms a continuum of epitopes. Residues postulated to be critical to the binding of the various groups of MAbs defined by Underwood [44] formed a continuous ring surrounding the receptor-binding pocket. The inability of anti-viral antibodies to bind to reduced and alkylated HA or to small fragments of the molecule [22] suggests that most of the relevant epitopes are discontinuous, i.e., "assembled" from different parts of the amino acid sequence that are in close proximity in the folded structure [2]. It is therefore not possible, in this instance, to answer questions about location and interrelationship of epitopes using synthetic peptides that represent parts of the linear sequence of HA.

Furthermore, even though much of the accessible surface of the head of the molecule may consist of a series of overlapping epitopes capable of binding antibodies in vitro, only certain of these epitopes may be immunodominant or even immunogenic in a given individual. Studies of Haaheim [17], Natali et al. [34], and Wang et al. [46] show that most humans, especially children, mount a limited antibody response to influenza virus which, in the case of HA, is restricted to one or two antigenic sites. Moreover, the spectrum of antibodies produced differs between individuals. These findings provide an explanation for how new epidemiologically important field strains, which are thought to require at least one mutation in each of the major antigenic sites [54], can arise. In view of the fact that the calculated probability of a mutant arising with a substitution in every antigenic site is too low to allow the production of a viable new strain within a single immune individual [15, 49, 58] it is likely these viruses evolve in a step-wise manner by sequential infection of individuals possessing different and limited antibody repertoires. In addition, not all antibodies have the same capacity to neutralize viral infectivity; those that do not neutralize are presumably not relevant to the selection process. MAbs binding to distinct antigenic regions of the H1 subtype HA molecule were found to differ in their neutralization ability [15], and certain MAbs directed to H3 HA inhibited hemagglutination but not infectivity [4].

In this paper, residues in the HA that are critical for the integrity of epitopes recognized by different MAbs have been defined and their relationship to the actual binding site of the MAbs discussed. The efficiency of neutralization of infectivity and inhibition of hemagglutination, and the affinity of binding to HA of MAbs elicited by epitopes in different antigenic sites is compared and the ability of particular MAbs to influence the binding of others is determined.

Materials and methods

Virus strains

The following strains of influenza virus of the H3N2 subtype were used: A/Port Chalmers/1/73 (PC73); A/Victoria/3/75 (Vic75); A/Texas/1/77 (Tex77); A/Bangkok/1/79 (Bang79); A/Philippines/2/82 (Phil82). Reassortant viruses bearing the H3 subtype HA and the N1 neuraminidase of A/Bellamy/42 were also used: A/Memphis/1/71 (Mem71); A/Memphis/102/72 (Mem72); A/duck/Ukraine/1/63 (Duck63).

Neutralization escape mutants Jane, Ken, Meg, Ian, Doug, Don, and Ted were a gift from Dr. W. G. Laver, John Curtin School of Medical Research, Australian National University, Canberra, A.C.T. The mutant Jacinta was selected in this laboratory [6]. These viruses were prepared by growth of Mem71 virus in hens' eggs in the presence of a neutralizing MAbs, and were cloned twice at limiting dilution in eggs as described by Webster and Laver [49]. The mutants differ from the parent virus by a single amino acid substitution in the HA (Table 1).

Horse-serum sensitive (HS^S) and horse-serum resistant (HS^R) viruses, isolated by cloning at limiting dilution in hens' eggs from a laboratory stock of Mem71 virus, were prepared by A. A. Scalzo in this laboratory.

Growth and purification of virus

Virus was grown in the allantoic cavity of 10-day embryonated hens' eggs for 2 days at 35 °C. The eggs were chilled at 4 °C overnight and the allantoic fluid collected, centrifuged (2,000 **g**, 15 min, 4 °C) and aliquots of infectious virus stored at −70 °C. This virus was used for immunization of mice and neutralization experiments.

Virus preparations were purified from allantoic fluid by adsorption to and elution from chicken erythrocytes according to the method of Laver [29]. Some preparations of Mem71 virus, purified from allantoic fluid by rate-zonal centrifugation on sucrose gradients, were obtained from A. Hampson, Commonwealth Serum Laboratories, Parkville, Vic.

Production of MAbs

MAbs 36, 40, 203, 207, 244, 261, 508, and 514 were produced in this laboratory by fusion of Mem71-primed BALB/c spleen cells with the non-secretory myeloma Sp2/O-Ag14 [42] and hybrid cells isolated using the 'HAT' selection procedure [28]. Antibody-positive hybridomas were cloned by limit dilution and the clones grown as ascites in pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, WI, U.S.A.)-primed BALB/c mice. Ascitic fluid was collected from the peritoneal cavity 5–10 days later and stored at −20 °C.

MAbs H14-A2, H14-A21, and H14-B17 (designated here as A2, A21, and B17 respectively), raised against Mem71 virus, were provided by Dr. W. Gerhard, Wistar Institute, Philadelphia, PA, U.S.A. MAbs A95, A106, A170, and A221 (designated 95, 106, 170, and 221 respectively), raised against A/Northern Territory/60/68 (NT68), were a gift from Dr. P. A. Underwood, CSIRO Division of Molecular Biology, North Ryde, N.S.W. [44].

Purification of immunoglobulin G (IgG)

IgG was purified from mouse ascitic fluid by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) according to the method of Ey et al. [12]. Prior to purification, ascitic fluid was treated with freon (30 min, 0 °C) to remove lipid material and adjusted to pH 8.2.

Protein estimation

The protein concentration of IgG preparations was determined either by using the method of Hartree [18], or by determining the absorbance at 280 nm, assuming an extinction coefficient of 1.34 for IgG (1 mg/ml solution, 1 cm path length).

Enzyme-linked immunosorbent assays (ELISA)

The standard ELISA was performed as described by Kida et al. [26] using a coating solution of 50 HAU purified virus/well. The antibody titre was expressed as the reciprocal

of the antibody dilution giving an absorbance of 0.1, which represents at least 5 times the background level of binding.

In some assays the virus was exposed to low pH; virus-coated wells were treated with 100 µl of 0.1 M sodium acetate or sodium citrate buffer pH 4.9 for 1 h at room temperature then returned to pH 7.4 by washing 4 times with phosphate buffered saline containing 0.05% v/v Tween 20 (PBST) prior to the addition of antibody.

The heavy-chain isotype of MAbs was determined by ELISA using 50 µl of a 1/30 dilution of rabbit-(anti-mouse IgG) subclass-specific IgG1, IgG2a, IgG2b, and IgG3 antisera (Miles Scientific, Mulgrave, Vic.) to probe MAbs titrated on virus-coated wells. The isotyping sera were detected using 50 µl of a 1/400 dilution of horseradish peroxidase-conjugated swine Ig directed to rabbit Ig (Dako Immunoglobulins, Denmark).

Hemagglutination and hemagglutination inhibition assays

Hemagglutination and hemagglutination inhibition (HI) assays were performed as described by Fazekas de St. Groth and Webster [14]. The HI titer of antibody was taken as the reciprocal of the mean antibody dilution inhibiting 3 out of 4 hemagglutinating units (HAU) of virus. In microassays, 25 µl of 1% v/v chicken erythrocytes were used and the HI titer was taken as the reciprocal of the highest mean antibody dilution inhibiting 4 HAU of virus.

In some HI assays the virus was exposed to low pH; virus in phosphate buffered saline containing 0.1% sodium azide (PBSA) was held at pH 4.9 for 5 min and then returned to pH 7.4.

Neutralization assays

Assays for neutralization of infectivity utilized a micro allantois-on-shell (AOS) culture system. The medium was RPMI supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 30 µg gentamicin/ml and 10% v/v allantoic fluid. Dilutions of antibody and 16-64 50% AOS-infective doses of virus in 100 µl of medium were incubated for 1 h at room temperature in duplicate 96-well flat-bottomed tissue culture plates (Nunc, Denmark). Egg shell from 11–12 day embryonated hens' eggs, with the chorioallantoic membrane attached, was cut into 3 mm^2 fragments and a fragment added to each well. After incubation (37 °C, 5% CO_2) for 2 days, a 25 µl sample of each well was tested in a micro-HA assay for the presence of virus. The neutralization titer was taken as the reciprocal of the highest mean antibody dilution that inhibited virus growth (< 1 HAU/25 µl).

Radioimmunoassays (RIA)

A solid-phase assay was performed according to the method of Brown et al. [5] using wells coated with 50 HAU purified virus. IgG was radioiodinated using a modification [20] of the method of Greenwood and Hunter [16]. The antibody titer was expressed as the reciprocal of that antibody dilution which gave levels of radioactivity 20 times that of background.

Competition assays

The solid-phase RIA was used to test the ability of different MAbs to bind simultaneously to HA. Dilutions of unlabeled MAb in 25 µl were added to virus-coated wells and, after 1 h, a constant amount of radioiodinated homologous or heterologous IgG which gave less than maximal binding to antigen in the absence of inhibitor, was added in 25 µl. The assay was then completed as described above.

Measurement of affinity

The affinity of binding of IgG to HA was determined using a solid-phase RIA. Dilutions of radioiodinated IgG were added to virus-coated wells and the assay completed as described above. The amount of radiolabeled antibody added and the amount bound to antigen at each concentration was then determined and the data plotted according to Scatchard [41]. The affinity constant was derived from the slope of the line of best fit determined using linear regression analysis. The affinity was measured in two separate assays and expressed as the mean of the values obtained.

Results

Binding of MAbs to escape mutants

HA-specific MAbs raised to two closely related H3 subtype influenza viruses, Mem71 and NT68, were tested by ELISA for binding to a panel of MAb-selected escape mutants of Mem71 virus. These mutants have all been demonstrated to differ from the parent virus by a single amino acid in one of the major antigenic sites of HA (Table 1). To illustrate the nature of the experimental data obtained, the results with a single MAb are presented in Fig. 1. It can be seen that MAb 244 bound to most of the mutants to approximately the same titer as to the parent virus Mem71, but not at all to the mutant Meg, and only with greatly reduced efficiency to Ken. A summary of the data obtained with 15 of the MAbs whose binding was influenced by the changes in one or more of the mutants is shown in Fig. 2. Reactivity of most of these MAbs was affected by an amino acid change in more than one of the mutants, usually at different positions within the same antigenic site although several MAbs were affected by amino acid changes in both sites B and D.

Table 1. Amino acid changes in escape mutant of Mem71 virus[a]

Mutant	Residue	Amino acid change	Antigenic site[b]
Jane	53	N→K	C
Jacinta	60	N→V	E
Ken	143	P→T	A
Meg	144	G→D	A
Ian	188	N→D	B
Doug	189	Q→H	B
Don	205	S→Y	D
Ted	218	G→W	D

[a] Data obtained from [6, 30, 35, 49]

[b] Antigenic sites as defined by Wiley et al. [54] and Daniels et al. [8]

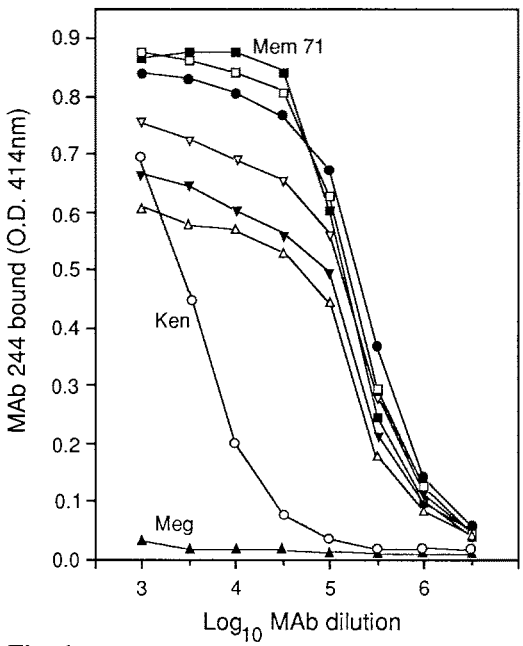


Fig. 1

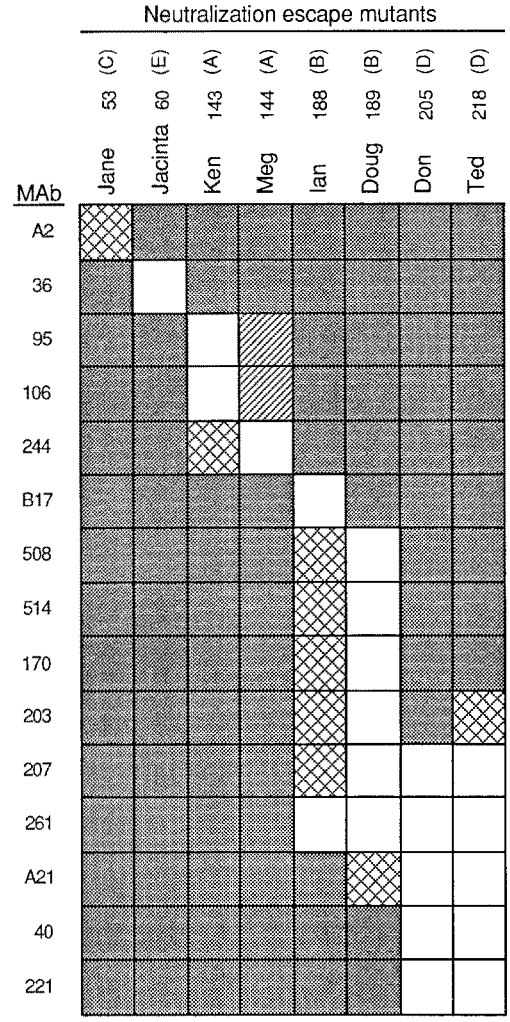


Fig. 2

Fig. 1. Binding of MAb244 to escape mutants of Mem71. MAb 244 was tested for its ability to bind in an ELISA to Mem71 virus (■) and to the escape mutants Jane (●), Ken (○), Meg (▲), Ian (□), Doug (▽), Don (△), and Ted (▼)

Fig. 2. Summary of binding of MAbs to escape mutants of Mem71 in ELISA. The mutants are identified by name, the changed amino acid residue, and the antigenic site within which the residue is located [53]. The ELISA titer for each mutant was compared with that against Mem71. □ No significant binding: titer less than $10^{3.5}$; ▩ reduced binding: titer to mutant $10^{1.5}$–$10^{3.0}$ fold lower than to Mem71; ▩ comparable binding: titer to mutant within ± $10^{0.5}$ titer to Mem71; ▩ enhanced binding: titer to mutant more than $10^{1.5}$ fold higher than to Mem71

Binding of MAbs to field strains

The extent of cross-reaction of each MAb with strains of virus of the H3 subtype that arose by antigenic drift during the period 1971–1982, and also for the avian virus Duck63, the putative progenitor of the H3 subtype [13, 48], was determined by ELISA. A summary of the data obtained is presented in Fig. 3. Two

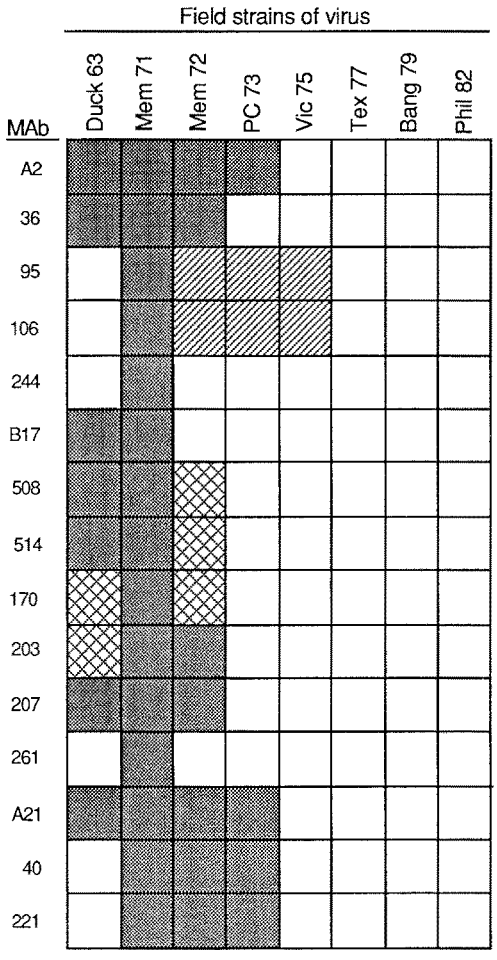


Fig. 3

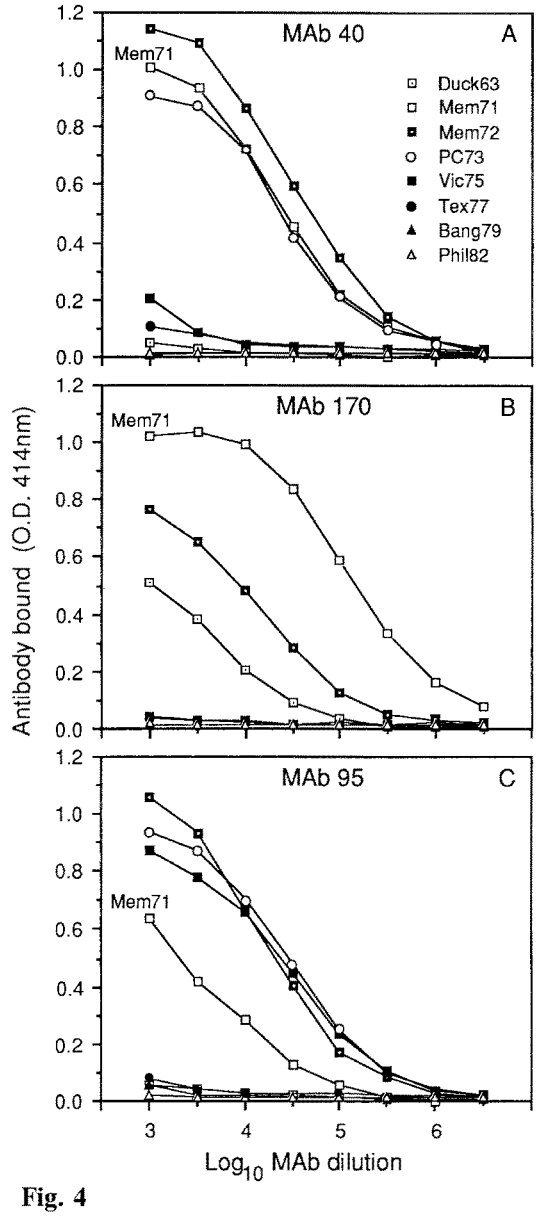


Fig. 4

Fig. 3. Summary of binding of MAbs to field strains of the H3 subtype in ELISA. The titer to field strains was compared with the titer to Mem71 virus. □ No significant binding: titer less than $10^{3.5}$; ▩ reduced binding: titer $10^{1.2}$–$10^{2.0}$ fold lower than titer to Mem71; ▩ comparable binding: titer 10 fold lower to $10^{0.3}$ fold higher than titer to Mem71; ▩ enhanced binding: titer more than $10^{0.5}$ fold higher than titer to Mem71

Fig. 4. Binding of MAbs to field strains. MAbs 40 (**A**), 170 (**B**), and 95 (**C**) were titrated in an ELISA against the field strains shown in the key

MAbs were found to be strain-specific, while the others were cross-reactive to different degrees. The cross-reactive MAbs displayed various reactivity patterns illustrated by the examples shown in Fig. 4. Some bound equally well to two or more strains of virus (Fig. 4A), some site B-specific MAbs showed reduced

binding with certain strains (Fig. 4B), while two site A-specific MAbs actually showed enhanced binding to strains isolated in some later years (Fig. 4C). None of the MAbs showed significant binding to Tex77, Bang79, or Phil82. Those MAbs that displayed reduced levels of binding to particular field strains recognized amino acid changes in sites B and D. Neither the specificity nor the extent of cross-reactivity of binding of the MAbs to field strains was related to any particular antigenic site, as defined by the location of amino acid substitutions in escape mutants that affected the binding of the MAbs.

Influence of HA receptor specificity on binding of MAbs

Gamma inhibitor-resistant (HS^R) mutants of viruses of the H3 subtype, selected by growth in the presence of horse serum, differ from the horse serum-sensitive (HS^S) parents by the amino acid change L→Q at residue 226 within the HA receptor binding site [38]. When the MAbs were tested for HI activity against HS^S Mem71 virus and its corresponding HS^R mutant, only MAbs 40 and 221 were able to discriminate between the two viruses: MAb 40 had an HI titer of 20,480 against HS^S virus and 60 against HS^R virus, while MAb 221 had a titer of 30,720 against HS^S virus and 160 against HS^R virus. This significant decrease in HI activity against the HS^R mutant indicated that substitution of residue 226 results either in a loss in the integrity of the epitope recognized by these two MAbs, or that the MAbs are unable to inhibit the interaction of the HS^R receptor with the different substrate that it recognizes [10]. In view of the fact that both MAbs also displayed substantially reduced (approximately 100 fold less) binding to the HS^R mutant in ELISA (data not shown), it can be concluded that the integrity of the epitopes has been modified by the change at residue 226.

Identification of amino acid residues essential to the epitope recognized by each MAb

Figure 5 shows the amino acid sequence of the HA_1 of the various field strains used in this study. By comparing the sequences it is possible to identify residues whose substitution can account for the pattern of reactivity of each MAb with the different strains. The rationale for identifying these residues is described for two MAbs.

MAb A2

This MAb selected the mutant Jane with the change N^{53}→K [30, 49] and binds to Duck63, Mem71, Mem72, and PC73, but not Vic75, Tex77, Bang79, and Phil82. Residue N^{53} is present in the four strains of virus to which the MAb binds, but also in Vic75 to which the MAb does not bind. Therefore, the change at residue 53 alone cannot account for the pattern of binding of the MAb to the different field strains. An additional residue present in Duck63, Mem71,

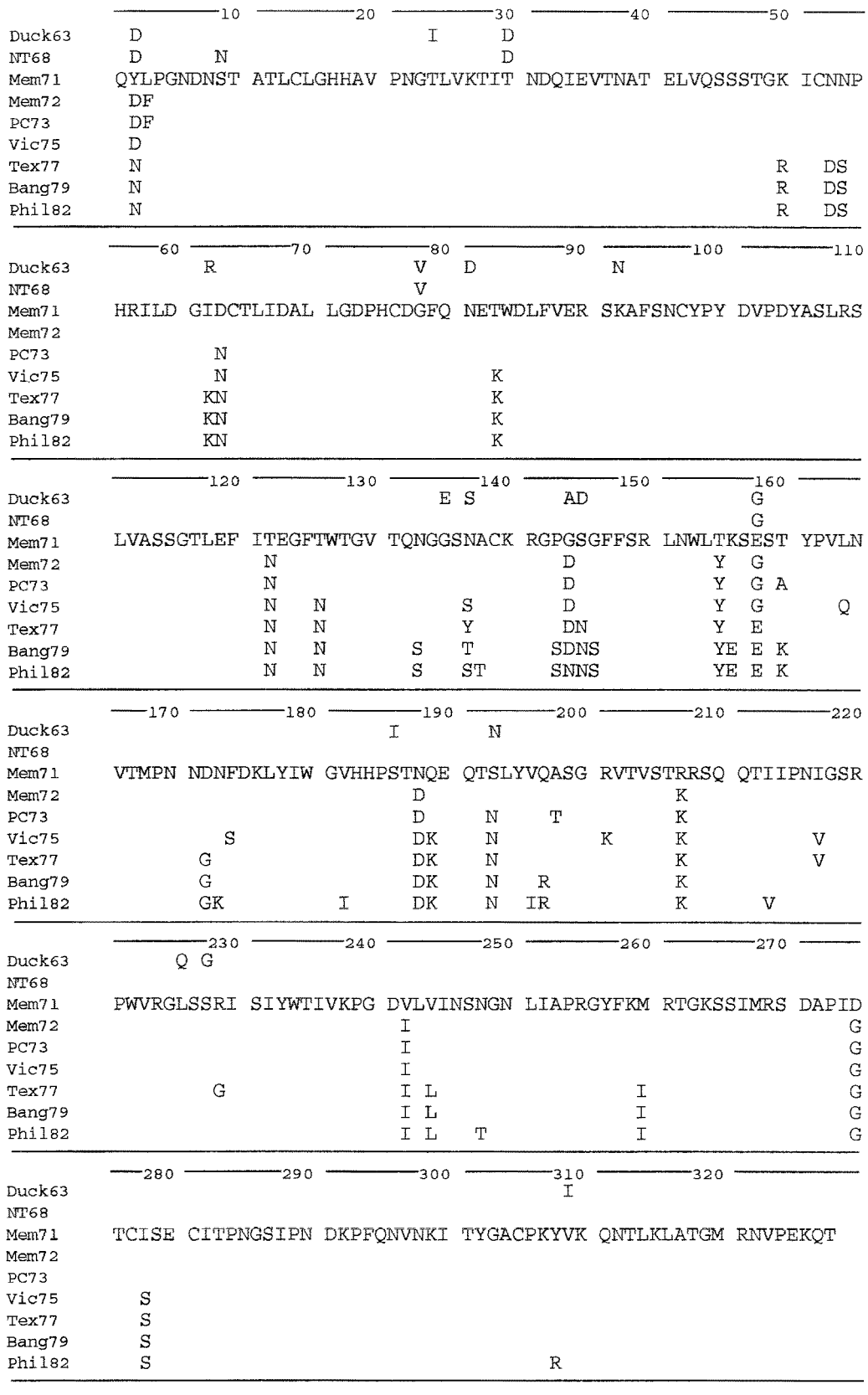


Fig. 5. Amino acid sequence of the HA_1 chain of field strains. The amino acid sequence of Mem71 is shown. For all other strains of virus, only those residues that differ from Mem71 are indicated. Data from Ward and Dopheide [48]; Underwood [44]; Both et al. [3]; Newton et al. [35]; and R. S. Daniels (pers. comm.)

Mem72, and PC73 but altered in Vic75, Tex77, Bang79, and Phil82 must also influence binding of this monoclonal antibody. Throughout the entire HA_1 molecule, only residues 83, 126, 189, and 278 meet these criteria. Although residue 53 is distant in amino acid sequence from these residues, it is in direct contact with residue 278 on an adjacent chain of the HA molecule [54]. Therefore, we predict that MAb A2 binds to strains of virus which possess both N^{53} and I^{278}, but not to N^{53} and S^{278} in Vic^{75}, or D^{53}, and S^{278} in Tex77, Bang79, and Phil82.

MAb 261

This MAb binds only to Mem71 so that one or more residues in the epitope recognized by this MAb must be changed in the other strains of virus. Residues Y^2 and G^{144} are unique to Mem71, but both of these are remote from residues 188, 189, 205, and 218, whose substitution in escape mutants has been shown (Fig. 2) to affect binding of the MAb. Two possible combinations of amino acids explain the reactivity pattern observed: (*i*) both residues 186 and 188 are critical such that this MAb binds to S^{186} and N^{188} in Mem71, but not to I^{186} and N^{188} in Duck63, nor to S^{186} and D^{188} in Mem72, PC73, Vic75, Tex77, Bang79, and Phil82; and/or (*ii*) residues 188 and 193 are critical such that the MAb binds to N^{188} and S^{193} in Mem71, but not to N^{188} and N^{193} in Duck63, D^{188}, and S^{193} in Mem72, nor to D^{188}, and N^{193} in PC73, Vic75, Tex77, Bang79, and Phil82.

Table 2 is a summary of residues within HA whose substitution results in a change in the binding of each of the MAbs. These comprise (*i*) the single amino acid residues that are substituted in the HA of escape mutants and which result in increased or decreased binding to the MAbs, and (*ii*) those residues whose pattern of change in field strains can account for the reactivity of these viruses with the MAbs. In the latter case, the location of the altered residues in relation to those identified with certainty in the escape mutants was considered in compiling the fourth column, namely the residues postulated to be critical to the integrity of the epitope. While it is probable that most of these residues are directly implicated in the epitope itself, i.e., may actually make contact with the paratope of the MAb, some may be situated outside the boundaries of the epitope but nevertheless affect its conformational integrity.

The MAbs identify a number of overlapping epitopes within sites A, B, and D, as indicated by the slightly different combinations of essential residues. Most MAbs may recognize discontinuous epitopes composed of residues remote in amino acid sequence but brought into proximity within the folded structure of the molecule [2]. In this study MAb A2 is a good example. In addition, there is evidence for the existence of either a *quarternary* determinant, i.e., an epitope composed of amino acids contributed by different monomers, or an epitope on one monomer whose integrity is affected by a change in an adjacent monomer. This is illustrated in Fig. 6, in which residues critical for the binding of MAbs 207, 261, and 40 are indicated on separate monomers of the HA trimer. In

Table 2. Summary of amino acid residues critical to the epitope recognized by the MAbs

MAb	Amino acid substitutions in escape mutants that affect binding[a]	Amino acid substitutions in field strains that could theoretically affect binding[c]	Amino acid residues postulated to be critical to the epitope[e]	Antigenic site[f]
A2	53	83, 126, 189, 278	53, 278	C
36	60	63	60, 63	E
95	143, 144	62, 145	143, 144, 145	A
106	143, 144	62, 145	143, 144, 145	A
244	143, 144	2, 144	143, 144	A
B17	188	122, 155, 188, 207, 242, 275	188	B
508	188, 189	63, (188 & 189 & 198)[d]	188, 189, 198	B
514	188, 189	63, (188 & 189 & 198)[d]	188, 189, 198	B
170	188, 189	63, (188 & 193)[d]	188, 189, 193	B
203	188, 189, 218	63, (188 & 193)[d]	188, 189, 193, 218	B/D
207	188, 189, 205, 218	63, (188 & 189 & 198)[d]	188, 189, 198, 205, 218	B/D
261	188, 198, 205, 218	2, 144, (186 & 188)[d], (188 & 193)[d]	(186 &/or 193), 188, 189, 205, 218	B/D
A21	189, 205, 218	83, 126, 189, 278	189, 205, 218	B/D
40	205, 218, 226[b]	137	137, 205, 218, 226	D
221	205, 218, 226[b]	137	137, 205, 218, 226	D

[a] Residues whose substitution in a particular escape mutant resulted in a decrease or increase in the binding of the MAb in an ELISA

[b] Residue that is substituted in the HS^R mutant

[c] Residues deduced from the amino acid sequence of the HA1 of field strains (shown in Fig. 5), whose substitution could account for the pattern of binding of a particular MAb with this panel of viruses. All residues or combinations of residues that could theoretically be implicated are shown, whatever their location on the molecule

[d] Substitutions are required in all of the bracketed residues for abrogation of binding. See example of MAb 261 in the text

[e] Residues implicated as being critical to the binding of a particular MAb (possibly within the epitope recognized by that MAb), comprising those residues whose substitution in escape mutants affected binding of the MAb, plus those of the residues affecting binding to field strains which are located nearby in the folded HA molecule

[f] Antigenic sites as defined by Wiley et al. [54] and Daniels et al. [8]

each case it is apparent that a change at residue 205 in the interface (site D) would exert a greater influence on the critical residues on the neighbouring monomer than those on the same monomer.

Binding of MAbs to pH 5-treated virus

At pH 5 an irreversible conformational change occurs in HA [11, 39, 40, 43] which alters the antigenicity of the molecule [9, 21, 50, 52, 57]. The effect of this conformational change on the binding activity of the MAbs was determined

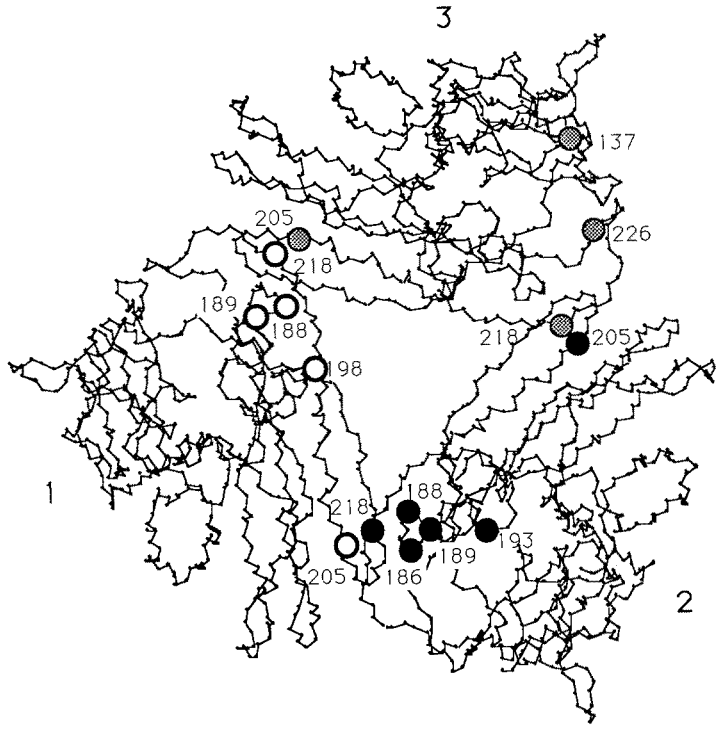


Fig. 6. HA trimer showing amino acid residues postulated to be involved in the epitopes defined by MAbs 207, 261, 40, and 221. The view is from above looking down the axis of symmetry. Residues critical to the binding of particular MAbs are shown on different monomers: MAb 207 in white on monomer (*1*); MAb 261 in black on monomer (*2*); MAbs 40 and 221 in grey on monomer (*3*)

using an HI assay (Fig. 7). MAbs binding to epitopes in sites C, E, and, in some cases, B were found to display enhanced HI activity to pH 5-treated virus, whereas most MAbs binding to sites B/D and D had decreased HI activity.

Neutralization and HI activity of MAbs

In order to determine whether the location of the epitope recognized by each MAb determines its functional significance, MAbs were examined for their neutralizing and HI activity against those field strains to which they demonstrated significant binding titer as measured by ELISA. As shown in Tables 3 and 4, all MAbs had the ability to neutralize viral infectivity and to inhibit hemagglutination of Mem71 and, to differing extents, certain other field strains. In an attempt to compare the neutralization and HI efficiency of the MAbs relative to their binding activity, neutralization and HI titers were expressed as a percentage of the ELISA titers.

All the MAbs in the panel neutralized the infectivity and inhibited the hemagglutination of Mem71 virus, regardless of the antigenic site against which they were directed. The order of sensitivity of the assays employed here was

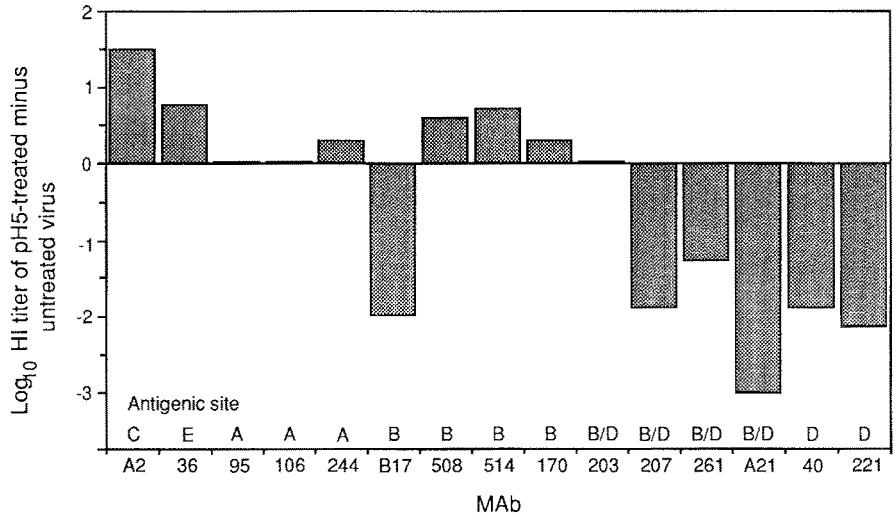


Fig. 7. Effect of pH 5 treatment of virus on HI titer of the MAbs. The MAbs were tested in an HI assay at pH 7 with untreated or pH 5-pretreated Mem71 virus and the ratio of the HI titers was determined

Table 3. Neutralization of viral infectivity by MAbs

MAb	Neutralization titer[a] against				
	Duck63	Mem71	Mem72	PC73	Vic75
A2	2.0 (0.03%)	3.0 (0.06%)	3.5 (0.4%)	2.5 (0.16%)	–
36	2.2 (0.06%)	5.2 (7.9%)	4.7 (1.6%)	–	–
95	–	2.7 (1.0%)	3.5 (1.3%)	4.2 (5.0%)	3.5 (1.0%)
106	–	3.2 (0.5%)	4.2 (0.6%)	5.5 (13%)	4.5 (1.3%)
244	–	6.0 (320%)	–	–	–
B17	3.7 (0.8%)	5.7 (16%)	–	–	–
508	3.0 (0.04%)	6.0 (40%)	4.5 (40%)	–	–
514	3.7 (0.3%)	6.0 (40%)	3.7 (6.3%)	–	–
170	2.7 (1.6%)	5.7 (20%)	3.5 (2.0%)	–	–
203	3.2 (1.3%)	6.0 (40%)	2.5 (0.03%)	–	–
207	4.2 (2.0%)	5.2 (7.9%)	2.2 (0.06%)	–	–
261	–	4.5 (2.5%)	–	–	–
A21	4.5 (1.0%)	5.7 (16%)	5.0 (2.5%)	5.2 (5.0%)	–
40	–	4.5 (10%)	5.2 (32%)	4.0 (2.5%)	–
221	–	5.7 (100%)	5.2 (16%)	4.2 (3.2%)	–

[a] Neutralization titers are expressed as the reciprocal of the highest mean antibody dilution ($\log_{10}$) inhibiting replication of 30 ID_{50} of virus in an AOS assay. In parentheses, efficiency of neutralization: neutralization titer expressed as a percentage of the ELISA binding titer

– Not tested, as previously demonstrated not to bind in ELISA

Table 4. HI titer of MAbs against field strains

MAb	HI titer[a] against				
	Duck63	Mem71	Mem72	PC73	Vic75
A2	<	3.1 (0.08%)	2.5 (0.04%)	<	–
36	2.7 (0.2%)	4.3 (1.0%)	3.7 (0.16%)	–	–
95	–	3.0 (2.0%)	3.5 (1.3%)	3.1 (0.4%)	3.7 (1.6%)
106	–	3.9 (2.5%)	4.0 (0.4%)	3.7 (0.2%)	4.3 (0.8%)
244	–	4.2 (5.0%)	–	–	–
B17	2.5 (0.05%)	4.8 (2.0%)	–	–	–
508	<	4.2 (0.6%)	<	–	–
514	<	4.9 (3.2%)	<	–	–
170	2.2 (2.0%)	4.6 (1.6%)	2.5 (0.2%)	–	–
203	<	5.4 (10%)	<	–	–
207	3.1 (0.16%)	4.6 (2.0%)	<	–	–
261	–	4.3 (1.6%)	–	–	–
A21	4.0 (0.32%)	5.1 (4.0%)	4.6 (1.0%)	3.4 (0.08%)	–
40	–	4.3 (6.3%)	3.8 (1.3%)	3.1 (0.3%)	–
221	–	4.5 (6.3%)	4.4 (2.5%)	3.6 (0.8%)	–

[a] HI titers are expressed as the reciprocal of the highest mean antibody dilution ($\log_{10}$) inhibiting 4 HAU of virus. In parentheses, efficiency of inhibition of hemagglutination: HI titer expressed as a percentage of the ELISA titer

< Titer of less than 2.0

– Not tested

ELISA > neutralization > HI. In general, neutralization titers of the MAbs were approximately 10–100% of their ELISA titers, whereas the HI titers for the majority of the MAbs were in the range 0.5–5% of the ELISA titers. Some interesting exceptions were observed however. The single site C-specific MAb in the panel, A2, was notably inefficient at both neutralization and HI. Moreover, comparison of the three site A-specific MAbs reveals a striking difference between MAb 244, which was the most efficient neutralizing antibody in the panel, and MAbs 95 and 106, which were 300–600 fold less efficient, even though they are evidently directed to epitopes sharing residues with the epitope recognized by MAb 244. The extremely high efficiency of MAb 244 in neutralization tests was not paralleled by an exceptional HI activity. Conversely, MAbs 203, 40, and 221 were relatively efficient at HI.

Examination of the data for other field strains of virus revealed that most, but not all, of the MAbs demonstrated progressively diminishing neutralization and HI titers against strains isolated in the years immediately following 1971.

This analysis of the data obtained with heterologous strains clearly illustrates the fact that neutralization, and also HI, are more susceptible to subtle changes in the epitope than is ELISA. For example, although the site B/D-specific

MAbs 203 and 207 bound equally well to Mem71 and Mem72, they neutralized the later strain 100–1,000 fold less efficiently.

Affinity of binding of MAbs

Affinity constants were derived from modified Scatchard plots constructed from binding data obtained by RIA. Figure 8 shows the data obtained for a representative antibody, MAb 40. In this example, an affinity constant of 6×10^7 M^{-1} was obtained from the slope of the line. A summary of the data obtained for all MAbs is shown in Table 5. The affinity of most MAbs fell within the range 5×10^7 to 5×10^8 M^{-1}. MAb 244 had the greatest affinity for Mem71, whereas MAbs 95 and 106, which are also directed to site A, demonstrated the lowest affinities. This pattern parallels that found in neutralization rather than HI tests. Likewise, the very efficient HI activity of MAbs 40 and 221 with Mem71 which decreased with subsequent isolates, was not correlated with affinity, just as it showed no correlation with neutralization efficiency.

Topography of epitopes recognized by the MAbs

The topography of the epitopes defined by the various MAbs was examined in a competitive RIA. Each MAb was tested for its ability to inhibit the binding to Mem71 virus of other MAbs in the form of radioiodinated IgG prepared

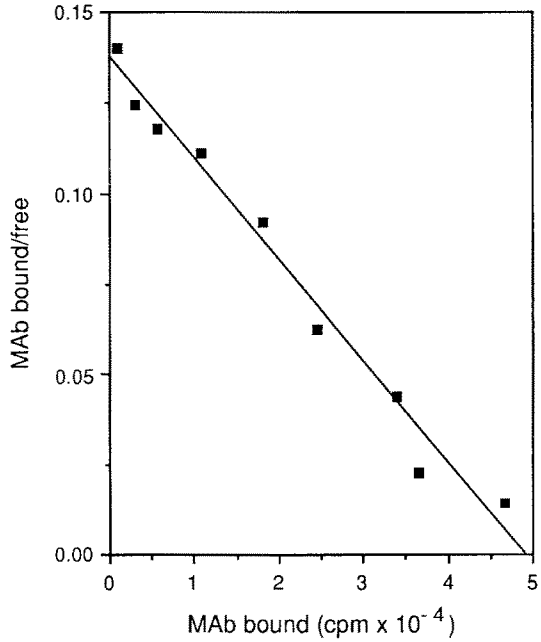


Fig. 8. Scatchard analysis of the binding of MAb 40. Dilutions of radioiodinated MAb 40 IgG were added to Mem71 virus-coated wells of a microtiter plate. After overnight incubation the amount of IgG bound was determined and the results presented as a Scatchard plot [41]. The affinity constant was derived from the slope of the line, which has a correlation coefficient of 0.99

Table 5. Affinity of binding of MAbs to field strains

MAb	IgG subclass	Affinity of binding[a] to				
		Duck63	Mem71	Mem72	PC73	Vic75
36	2a	7.4	8.5	9.0	–	–
95	2b	–	6.8	7.9	8.3	7.9
106	2b	–	7.5	7.9	7.8	7.6
244	2a	–	9.5	–	–	–
508	1	8.8	8.3	6.8	–	–
514	1	8.0	8.4	7.1	–	–
170	2a	7.7	8.7	7.7	–	–
203	1	7.8	8.5	7.8	–	–
207	1	8.2	8.4	7.2	–	–
261	2a	–	8.5	–	–	–
40	2a	–	7.8	7.7	7.4	–
221	2b	–	7.9	8.3	8.2	–

The affinity of binding of MAbs A2, A21, and B17 was not measured as there was insufficient ascitic fluid to isolate IgG

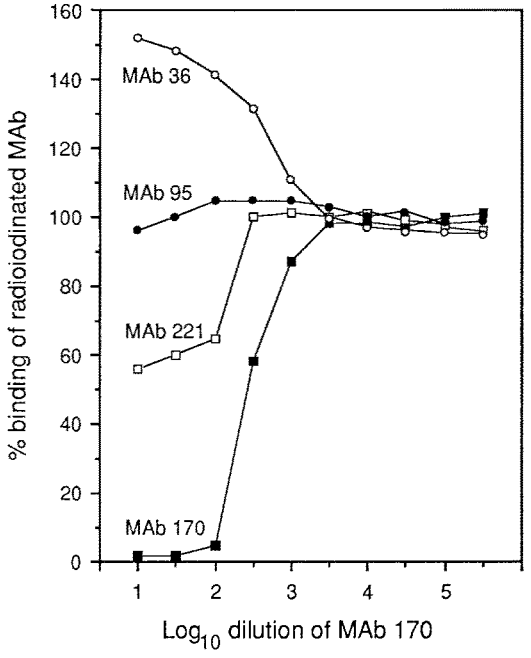
[a] Affinity of binding is expressed as $\log_{10} M^{-1}$ and is the mean of two estimations. The difference in the two values was usually less than $10^{0.5}$

– Not tested, as previously shown not to bind in ELISA

from ascitic fluid. A selection of results in which MAb 170 is the competing antibody is shown in Fig. 9. In this case, the binding of MAb 221 was partially inhibited by MAb 170, no competition occurred with MAb 95, and enhanced binding of MAb 36 to HA was observed. A summary of the results for different MAb combinations is shown in Fig. 10. Many pairs of MAbs showed reciprocal inhibition of binding. In most cases this reciprocal blocking was with MAbs recognizing identical or overlapping epitopes (e.g., MAbs 203, 207, 261, and 170; 40 and 221; 95 and 106), but it was also observed with MAbs binding to epitopes within distinct, though neighbouring antigenic sites (e.g., site A-specific MAb 244 and site E-specific MAb 36). Non-reciprocal inhibition of binding was observed between certain pairs of MAbs, some having overlapping epitopes (e.g., MAbs 207 and 508; 261 and 221; 95 and 244) and some quite separate epitopes (e.g., MAbs 170 and 40; 221 and 244; 106 and 36). In addition, particular MAbs from one topographical region could enhance the binding of MAbs recognizing epitopes in a different site (e.g., MAbs 203 and 36). This enhancement was not always reciprocal (e.g., MAbs 508 and 221).

Discussion

In this study residues critical to the binding of MAbs specific for the HA of the H3 subtype of influenza virus have been identified. Many of these probably form part of the epitope with which the paratope of the antibody combines.



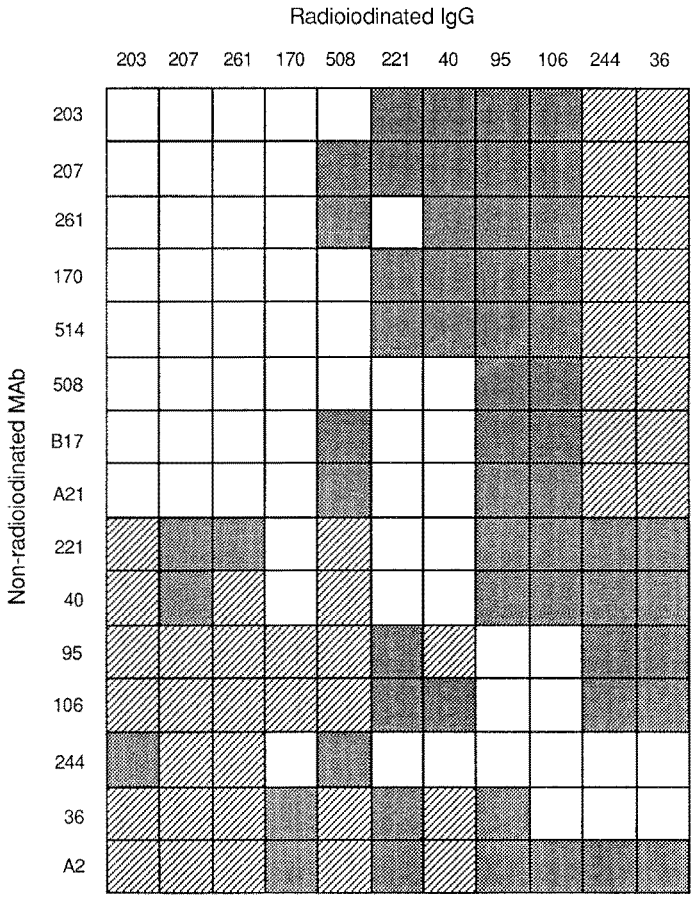


Fig. 9 **Fig. 10**

Fig. 9. Competitive RIA using MAb 170 as the inhibitor. Dilutions of unlabeled MAb 170 were added to Mem71 virus-coated wells of a microtiter plate. After 1 h, a constant amount of radioiodinated IgG of MAbs 36 (○), 95 (●), 170 (■), or 221 (□) was added and allowed to incubate overnight. The amount of bound radioactivity was measured and expressed as a percentage of that bound in the absence of unlabeled MAb 170

Fig. 10. Summary of competitive RIA data. Dilutions of unlabeled MAbs were added to Mem71 virus-coated wells of microtiter plates. After 1 h a constant amount of radioiodinated IgG was added and allowed to incubate overnight. The amount of bound radioactivity was measured and expressed as a percentage of that bound in the absence of unlabeled MAb: □ 0–50%; ▩ 51–120%; ▩ >120%

X-ray crystallographic analysis of the HA molecule from two escape mutants of the H3 subtype, one with a substitution at residue 146 in site A [27] and the other at residue 188 in site B [53], revealed no major structural changes, indicating that the substituted residue is not causing indirect conformational change in an epitope elsewhere but itself constitutes a critical contact residue within the epitope for that MAb.

Nevertheless, residues whose substitution abrogates binding of a MAb need not always lie within the epitope recognized by that MAb. In this study,

MAbs 207, 261, A21, 40, and 221 were all found to be affected by a change at residue 205 and yet this residue is buried about 10 Å below the surface of the HA trimer. Unless gross conformational changes occur during binding, it is difficult to envisage how residue 205 can contact the paratope of these antibodies. As shown in Fig. 6, residue 205 and residue 218, whose substitution also affects these MAbs, are both situated at the interface between monomers in the HA trimer. Mutants with a change at residue 218 that have altered receptor-binding properties have been isolated by Daniels et al. [10]. The substitution in these mutants of glycine to a charged residue was thought to disturb the interface and cause a conformational change in one side of the receptor-binding site. Similar receptor-binding mutants of the H1 subtype that have a change in the interface have also been described [56]. The changes $S^{205} \rightarrow Y$ and $G^{218} \rightarrow W$ in the mutants Don and Ted respectively, which affect the binding of MAbs 207, 261, A21, 40, and 221 in the present study, introduce large hydrophobic residues that may also perturb the interface and cause distant conformational changes.

The binding of MAbs 40 and 221 was also affected by substitution of residue 226 in the HS^R mutant and residue 137 in field strains. These residues lie on different chains that form the perimeter of the receptor-binding pocket [51]. Therefore, although the epitopes recognized by these two MAbs have been assigned to site D because of the involvement of residues 205 and 218, it is more likely that they are close to the receptor pocket and that substitution of the interface residues affects them indirectly. This is consistent with the reactivity of these MAbs with a second class of mutants, recently characterized by Anders et al. [1], which are resistant to beta inhibitors in mouse and bovine serum. MAbs 40 [1] and 221 (C. A. Hartley, pers. comm.) fail to bind the H3 subtype mutant in which the loss of a carbohydrate side-chain associated with the interface is thought to perturb the receptor binding site, resulting in altered receptor specificity. The proposal that these MAbs bind to epitopes close to the receptor-binding pocket is also consistant with their relatively high efficiency in inhibiting hemagglutination.

The binding of MAbs 207, 261, and A21 was also affected by amino acid changes in residues 205 and 218. None of these MAbs distinguished between the HS^S and HS^R viruses, but their binding was susceptible to changes in residues within site B. This suggests that changes in the interface can affect not only those epitopes intimately associated with the receptor binding-site but also other site B-associated epitopes.

Comparison of the HI reactivity pattern of the panel of MAbs with pH 5-treated and untreated virus indicated that conformational changes accompanying this treatment affected the antigenicity of sites B, C, D, and E, but not site A. These data are in accord with previous findings of Webster et al. [50], Daniels et al. [9], Jackson and Nestorowicz [21], and White and Wilson [52]. MAbs A2 and 36, which bind to sites C and E respectively, showed enhanced HI activity to pH 5-treated virus. Such enhanced activity was also observed

with MAbs 508 and 514 which bind to site B. In cotrast, of two other site B-specific MAbs, one displayed reduced HI activity to pH 5 virus and the other was relatively unaffected. This indicates that in the acid-induced form of HA, epitopes in site A remain unchanged, those in sites E and C become more accessible, while the effect upon epitopes in site B depends upon their exact location. If residue 205 is not involved, then those site B epitopes whose integrity is dependent on residue 198 appear to become more accessible, whereas those dependent on residue 193 are unaffected at pH 5. All MAbs whose binding is affected by substitution at residue 205 showed decreased HI activity towards pH 5 treated virus. Jackson et al. [23] have previously proposed that at pH 5, the heads of the individual monomers separate and fold back from the central axis of the molecule. The decrease in HI activity at pH 5 of MAbs dependent upon residue 205 in the interface is therefore consistent wiht the postulate that these MAbs recognize quarternary determinants that span the trimer interface, or that residue 205 serves to stabilize epitopes on an adjacent monomer.

Data in this paper also show that amino acid changes in field strains isolated after Mem71 affect the binding properties of MAbs directed to site A. MAb 244, which was raised to Mem71, is incapable of binding to field strains isolated from 1972 onward. MAbs 95 and 106, in contrast, failed to bind to strains isolated after 1975 but actually showed enhanced binding to Mem72, PC73, and Vic75. These three viruses, together with the escape mutant Meg to which MAbs 95 and 106 also show enhanced binding, possess aspartic acid at position 144 while Mem71 has glycine at this position. These two MAbs were raised against a particular preparation of the H3 strain NT68 known to contain a mutant with the change G→D at 144 [44]. The apparent heteroclitic response of these particular MAbs can be explained if they happened to be elicited by the mutant rather than the parent virus.

MAbs directed to site B showed either no binding or reduced binding to Mem72 and no binding to later strains, indicating that the epitopes of these MAbs underwent antigenic drift early in the evolution of this subtype. Antigenic drift in sites C, D, and E was detected by changes in either PC73 or Vic75. These results agree with those of Webster and Laver [49] and Underwood [44], which indicated that amino acid changes occurring initially in site A and later in site B were important in the evolution of the H3 subtype.

In the present study, designation of residues as being critical to the integrity of a particular epitope was made on the basis of the ability of MAbs to bind to escape mutants and field strains in ELISA. However, the use of functional assays, such as neutralization and HI, indicate that with certain MAbs, such as 203 and 207, amino acid substitutions in a field strain can result in a drastic decrease in the ability to neutralize infectivity and inhibit hemagglutination even though the binding of the MAb as measured by ELISA is relatively unaffected. The finding demonstrates that the neutralization test and to a lesser extent HI, are more discriminating than ELISA in discerning subtle changes in an epitope. This could not be consistently explained on the basis of a reduction

in affinity of binding of the MAb to that field strain. It may mean that the substitution alters the angle of attachment of the antibody molecule and thereby decreases its influence on the receptor-binding pocket. Whatever the explanation, amino acid substitutions abrogating the biological function of a neutralizing antibody may be overlooked if direct binding assays are used exclusively.

Comparison of the biological properties of the various MAbs revealed that MAb A2 was by far the least efficient in both neutralization and HI. Because MAb A2 was the only one directed to an epitope within site C, it remains unclear whether the low activity is unique to this MAb or is a general property of antibodies directed to this antigenic site. It is plausible that the inefficiency of such MAbs is a result of the relatively large separation of site C from the receptor-binding site. With this exception, there was no absolute correlation between the antigenic site recognized by a MAb and its neutralizing or HI activity; MAbs directed to all sites had satisfactory neutralization and HI titers. Furthermore, although there have been examples in other systems of MAbs which neutralize infectivity but do not inhibit hemagglutination [26], these two properties were not divorced in the panel of MAbs in this study. It was observed, however, that the efficiency of the MAbs in neutralizing virus did not always correlate with their efficiency in HI, supporting the idea that inhibition of infection and of hemagglutination can occur by quite distinct mechanisms. MAbs thought to bind to epitopes close to the receptor-binding pocket were very efficient at HI, whereas the efficiency of MAbs in neutralizing infectivity tended to be correlated with their affinity of binding.

The MAbs displayed affinities of binding to Mem71 ranging from $10^{6.8}$ to $10^{9.5}$ M^{-1}, but the majority differed from one another by less than ten-fold. This is comparable with previous studies showing up to a five-fold difference in the affinity of MAbs binding to different antigenic sites on the HA of H3 viruses X-31 and Tex77 [4, 46] and to A/seal/Massachusetts/1/80 (H7) [26], and up to a 35 fold difference in affinity for MAbs binding to distinct epitopes within sites Sa and Sb of the H1 subtype virus A/USSR/90/77 [25]. It is apparent that there can be wide variation in the affinity of binding of different MAbs to the same HA, reflecting the 'goodness-of-fit' of the antibody for antigen.

In competitive binding assays, reciprocal blocking of MAbs recognizing apparently identical epitopes (e.g., MAbs 40 and 221), overlapping epitopes (e.g., MAbs 203, 207, 261, and 170), and epitopes located in close proximity to each other (e.g., MAbs 244 and 36) was observed. Non-reciprocal inhibition of binding to HA was also detected, as illustrated by the results obtained with MAb 244. This site A-specific MAb completely inhibited the binding of MAbs 95 and 106, which are directed to overlapping epitopes also within site A, and yet the binding of MAb 244 to HA was not inhibited by these two MAbs. In this case the non-reciprocal inhibition of binding is probably attributable to the larger difference in affinity of the MAbs involved; MAb 244 has a 100 fold greater affinity than that of MAb 106 and 500 fold greater affinity than MAb 95. MAb 244 also inhibited, non-reciprocally, the binding of MAbs 170, 221, and

40 which recognize epitopes in sites B and D (or around the receptor-binding site) of the molecule. The difference in affinity may still be a factor here, but in the case of MAbs recognizing distinct antigenic sites, non-reciprocal inhibition of binding may indicate a difference in the angle of attachment of the MAbs, such that steric hindrance occurs only in one direction. In addition, the binding of MAb 244 to HA actually enhanced the binding of the site B/D MAbs 207 and 261, and vice versa. Enhanced binding of MAbs to HA in competition assays has also been reported by ourselves [22] and others [32] where it was postulated that binding of the first MAb induced a conformational change in the epitope of the second MAb, resulting in an increased affinity of binding of the latter.

Although a number of operationally distinct antigenic sites have been defined on the HA molecule [54], this type of competition study illustrates the influence that MAbs binding to epitopes within a particular site will have on those binding elsewhere on the molecule, and therefore the practical significance of the sites. In this study, on the basis of inhibition of binding, sites C and E are topographically distinct from sites B and D, but these two separate regions are linked together by site A. In a similar way, the five antigenic sites of A/PR/8/34 (H1) correspond to four topographical sites [7]. If enhancement is also considered, then the influence of MAbs binding at certain sites on those binding elsewhere is considerably broader.

This study serves to highlight the complexity of the repertoire of antibodies elicited in response to a single molecule, both in terms of the range of different epitopes recognized, which includes those that have only very subtle yet significant differences in the critical residues, and in terms of their functional properties and the factors that can influence these. It also illustrates the different interactions that would occur in vivo where antigen encounters a spectrum of different antibodies.

Acknowledgements

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