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A Fairly Conserved Epitope on the Hemagglutinin of Influenza A (H3N2) Virus with Variable Accessibility to Neutralizing Antibody

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A monoclonal antibody LMBH5 was derived from mice which had been immunized with A/Victoria/3/75 (H3N2)-type recombinant, secreted hemagglutinin (HA), and were subsequently challenged with a potentially lethal dose of X31 [A/Aichi/2/68 (H3N2) × A/PR/8/34 (H1N1)] virus. LMBH5 reacted strongly with the native and low-pH-induced conformations of the HA of A/Aichi (X31 strain) and A/Victoria (X47 strain), but very weakly with the native structure of the HA of A/Philippines/2/82 (X79 strain) and not at all with the HA of A/Guizhou/54/89 H3 (NIB25 strain). However, the acid-induced conformations of the latter two viruses were recognized by LMBH5. The antibody prevented infection of MDCK cells with X31 and X47, whereas X79 virus was partially neutralized by LMBH5. X31 monoclonal escape variants had single amino acid substitutions (Ser 227 → Pro) near the interface. The data obtained suggest that the neutralizing LMBH5 reacts with a fairly conserved epitope of influenza A (H3N2) virus, which as a result of antigenic drift becomes inaccessible in the native state of the HA.

INTRODUCTION

Hemagglutinin (HA), the major surface glycoprotein of influenza virus, is subject to frequent, accumulating mutations known as antigenic drift. Such alterations enable the virus to cause epidemics almost annually and contribute to the failure to control influenza effectively by vaccination. HA is a trimer built up of two structurally distinct domains: a globular head composed entirely of HA1 and an elongated stem structure consisting of HA2 and part of HA1 (Wilson *et al.*, 1981). Five major variable antigenic sites, designated A to E, have been defined on the globular region and have been shown to bind neutralizing antibodies (Wiley *et al.*, 1981; Jackson *et al.*, 1982; Daniels *et al.*, 1983b; Skehel *et al.*, 1984). At a lower pH (5.0–5.5), optimal for membrane fusion, HA undergoes an irreversible conformational change (Skehel *et al.*, 1982; Ruigrok *et al.*, 1988). The distal globular domain loses its trimeric structure, which leads to specific changes in the antigenicity at sites B and D (Yewdell *et al.*, 1983; Daniels *et al.*, 1983a). Usually, binding of antibodies recognizing sites A, C, and E are not affected, although reactivity for some antibodies specific for site C may be enhanced (Jackson and Nestorowicz, 1985).

The other parts of the molecule are fairly conserved. Yet, almost nothing is known about conserved epitopes, their recognition on the neutral and acid-induced HA conformations, or the role of antibodies binding to these epitopes in immune protection. Repeated infections induce antibodies cross-reactive to conserved determinants, but by and large these antibodies do not have neutralizing capability (Francis *et al.*, 1953; Virelizier *et al.*, 1974). Tamura *et al.* (1991) demonstrated that such antibodies augment the uptake of virus into cells via Fc-receptor entry and therefore can affect the T-cell responses to influenza virus. Several attempts have been made to isolate neutralizing, cross-reactive anti-HA antibodies, using synthetic peptides or denatured HA subunits as immunogens (Green *et al.*, 1982; Niman *et al.*, 1983; Sánchez-Fauquier *et al.*, 1987). However, neutralization and protection tests gave unsatisfactory results. Recently, a monoclonal antibody with cross-neutralizing activity among H1 and H2 strains was isolated (Okuno *et al.*, 1993, 1994). Antigenic variants demonstrated that the antibody binds to the stem region of HA and that the epitope contained HA1 and HA2 amino acid sequences.

In this paper we describe experiments with a newly isolated, neutralizing monoclonal antibody (mAb) which recognizes a conformation-dependent, fairly conserved epitope on the HA of subtype H3. The binding characteristics of the antibody to different HA provide new insights into the drift phenomenon of influenza virus.

MATERIALS AND METHODS

Viruses

Recombinant viruses between the WHO reference H3N2 strains A/Aichi/2/68 (X31), A/Victoria/3/75 (X47), A/

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Philippines/2/82 (X79), A/Guizhou/54/89 (NIB25), and A/PR/8/34 (H1N1), as well as the 63.3, 63.D, and V68x X31 variants were kindly provided by Dr. J. J. Skehel and Dr. A. R. Douglas. They all express surface glycoproteins of the H3N2 subtype. Viruses were grown in allantoic fluid of 10-day-old, embryonated chicken eggs and were purified on a Matrex Cellufine sulfate column (Amicon, Danvers, MA). Purified virus was stored in PBS at 4°. Hemagglutination inhibition (HI) tests with recombinant viruses were performed with chicken erythrocytes.

Variant selection

Variants of X31 were obtained by mixing equal volumes of allantoic fluid containing virus and diluted ascitic fluid containing monoclonal antibodies. The mixtures were used to inoculate embryonated eggs (Daniels *et al.*, 1983a). Variants were cloned by limited dilution.

Sequence analysis of viral HA genes

Five overlapping viral HA gene fragments were amplified in a reverse transcriptase–polymerase chain reaction. The primers used contained a conserved HA-specific sequence and the M13 reverse or −21 M13 sequence at the 5′ site. Amplified products were separated on a 1.5% agarose gel, isolated, and sequenced using dye-labeled M13 or −21 M13 primers (Sanger *et al.*, 1977).

Monoclonal antibodies

mAbs HC3 and HC59 MRC11 were kindly provided by Dr. J. J. Skehel and Dr. A. R. Douglas. HC3 was prepared against X31 HA and recognizes antigenic site A (Daniels *et al.*, 1983a). HC59 MRC11 was prepared against A/Port Chalmers/1/73 HA and also recognizes A/Victoria/3/75 HA.

Isolation of mAb LMBH5

For isolation of cross-reactive, neutralizing antibodies we used a previously described immunization and challenge protocol in mice using recombinant, secreted HA (HA0s), derived from A/Victoria/3/75 virus, as immunizing agent. Briefly, Balb/c mice (8–9 weeks old; SCK, Mol, Belgium) received a primary immunization with 2 μg HA0s emulsified in Ribi adjuvant. Three weeks later, the mice were boosted with 2 μg HA0s emulsified in monophosphoryl lipid A and muramyl dipeptide. Another 3 weeks later, the immunized mice were challenged with 20 LD_{50} of the mouse-adapted X47 influenza virus. Immunized mice survived the challenge, whereas control mice succumbed (Vanlandschoot *et al.*, 1993). In the present experiments, we challenged with the mouse-adapted heterologous X31 strain (derived from A/Aichi/2/68). Fifty percent of the immunized mice survived the infection, whereas all control mice died. Splenic lymphocytes from mice which had survived the challenge were fused with SP2/OAg-14 myeloma cells as described (Köhler and Milstein, 1975). Hybridoma cell supernatants were screened in ELISA on X31 and X47 virus. Positive cultures were subcloned twice and retested. Antibodies binding to the X31 and X47 viruses were then tested for binding to the X79 and NIB25 viruses.

ELISA and acid treatment of virus preparations

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with purified virus dissolved in PBS. To obtain acid-induced HA conformation, the plates were incubated with 50 m*M* citrate–100 m*M* NaCl, pH 5, for 20 min at room temperature. Virus required to maintain the native HA conformation was incubated with PBS. After two washings (0.1% Triton X-100 in PBS), the wells were blocked with 0.1% BSA in PBS, followed by two washing steps. Ascitic fluid (1 μg/ml in PBS–0.1% BSA) was added and the plates were incubated for 2 hr at room temperature. After two washings, the wells were incubated for 45 min at 37° with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Substrate solution (1 mg/ml Na *p*-nitrophenol in DEA) was added and the intensity (*A* values) of the color was measured at 405 nm at appropriate times.

Infection and immunofluorescence testing of MDCK cells

MDCK cells were grown on glass coverslips and infected with 100 HAU of virus. After 1 hr the inoculum was removed and the cells were incubated for 10 hr at 37°. Cells were fixed immediately with 3% paraformaldehyde in PBS; to obtain acid-induced HA conformation, cells were first incubated in PBS with 10 μg/ml trypsin (Sigma Chemical Co.), followed by incubation in 50 m*M* citrate–100 m*M* NaCl, pH 5, for 20 min at room temperature. After fixation, cells were washed three times with PBS and incubated for 1 hr at room temperature in ascitic fluid diluted 1:2000 in PBS. After two washings, the cells were incubated in 1 ml anti-mouse Ig-FITC (1/200 in PBS; Boehringer Mannheim, Germany) for 1 hr at room temperature.

ELISA of HA on virus-infected cells

MDCK cells in microtiter plates were infected with virus. After 5 hr, cells were fixed with 3% paraformaldehyde in PBS; to obtain acid-induced HA conformation, cells were first incubated in PBS with 10 μg/ml trypsin (Sigma Chemical Co.), followed by incubation in 50 m*M* citrate–100 m*M* NaCl, pH 5, for 20 min at room temperature. ELISA was performed as described.

Virus neutralization assay

Virus was mixed with ascitic fluid, serially diluted one to two, or with PBS. The mixtures were left at room temperature for 30 min and added to the MDCK cells grown in microtiter plates. After 1 hr the inoculum was removed

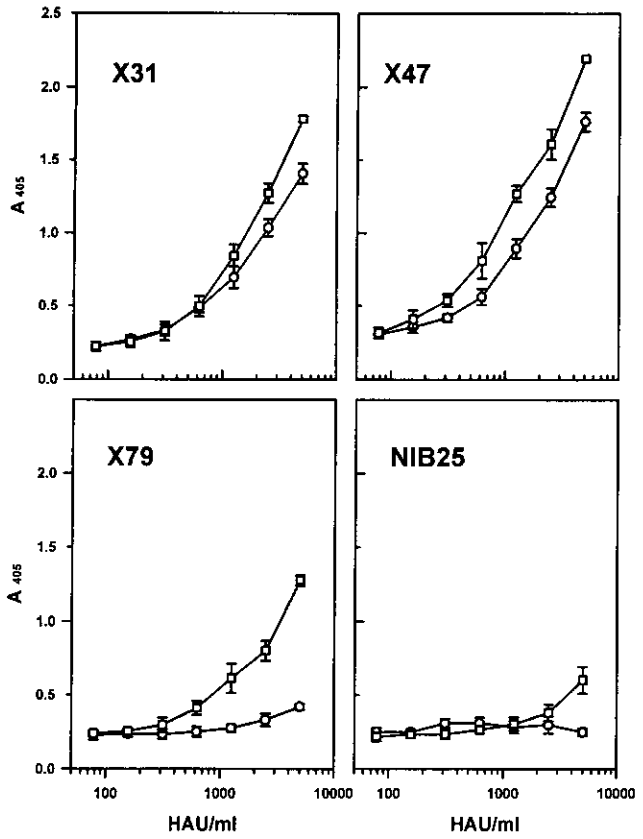

FIG. 1. Effect of low-pH treatment on binding of LMBH5 to X31, X47, X79, and NIB25 viruses stored at 4°. Purified virus, grown in eggs, was serially diluted one to two (5000 to 50 HAU/ml). O, untreated virus; □, low-pH-treated virus. Error bars represent SD (n = 4).

and the cells were incubated for 5 hr at 37°. ELISA was performed as described.

PAGE and Western blotting

Viral proteins were separated on a polyacrylamide gel (Laemmli, 1970) and transferred to nitrocellulose. The filter was blocked with PBS–0.1% Tween 20. HA was visualized with LMBH5 ascitic fluid (1 μg/ml PBS–0.1% Tween 20) followed by alkaline phosphatase-conjugated goat anti-mouse IgG. NBT and BCIP were used as substrates.

RESULTS

As described under Materials and Methods, mice were immunized with HA0s (A/Victoria/3/75) and challenged with mouse-adapted X31. Spleens of two survivors were used for obtaining hybridomas. One newly isolated mAb, LMBH5 (IgG1 isotype), was selected for detailed characterization. It reacted in ELISA with the neutral conformation of X31 and X47, weakly with X79, but not with NIB25 (Fig. 1). Prolongation of the incubation of X79 and NIB25 virus with LMBH5 for 18 hr at 37° did not result in enhanced binding of the antibody to the viruses (data not shown). After treatment at pH 5, higher A values were observed for the X31 and X47 viruses. A more spectacular effect was observed with X79. For this virus, low-pH treatment resulted in binding of LMBH5 to a degree similar to that obtained for X31 and X47 in their native forms. LMBH5 also bound, to a small extent, to pH 5-treated NIB25 virus.

Western blotting tests with LMBH5

To determine whether the LMBH5 antibody recognized a conformation-dependent epitope, 5000 HAU of the four viruses was denatured with or without β-mercaptoethanol, subjected to electrophoresis on a 10% polyacrylamide gel, and transferred to a nitrocellulose filter. LMBH5 produced strong bands with the nonreduced HA from the X31 and X47 viruses and weaker bands with the nonreduced HA from the X79 and NIB25 viruses. There was no reaction with the HA1 or HA2 subunits from the four viruses in Western blotting tests in a reducing environment (Fig. 2).

Immunofluorescence tests with LMBH5

As expected from ELISA and Western blotting experiments, LMBH5 gave a strong immunofluorescence reactivity with X31- and X47-infected MDCK cells (Figs. 3a and 3b). There was a weak staining on X79-infected cells and no signal at all on NIB25-infected cells (Figs. 3c and 3d). When the infected cells were first treated with trypsin and subjected to low pH, there was also a strong immunofluorescence staining of X79-infected cells and a weak staining of NIB25-infected cells (Figs. 3g and 3h). Remarkably, trypsin and low-pH treatment increased the number of fluorescent, X79-infected cells. Such an effect was not observed with X31- or X47-infected cells (Figs. 3e and 3f).

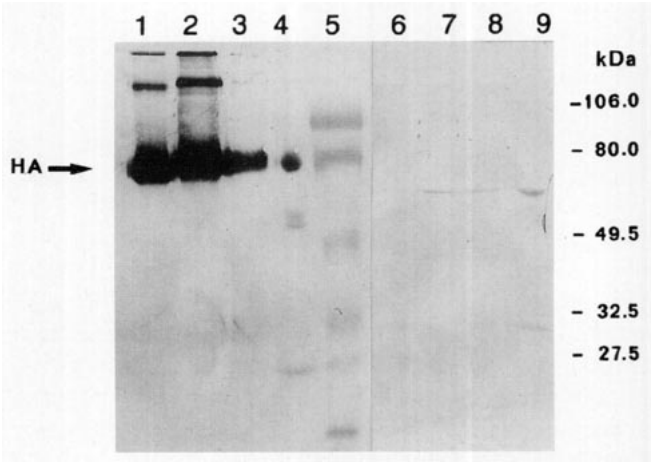

FIG. 2. Ability of LMBH5 to bind to X31, X47, X79, and NIB25 HA separated under nonreducing (lanes 1–4) and reducing (lanes 6–9) conditions (lane 5 represents the prestained M_r markers). Virus proteins were separated by SDS–PAGE on a 10% gel and transferred to a nitrocellulose membrane. The higher bands presumably represent dimeric and trimeric HA molecules.

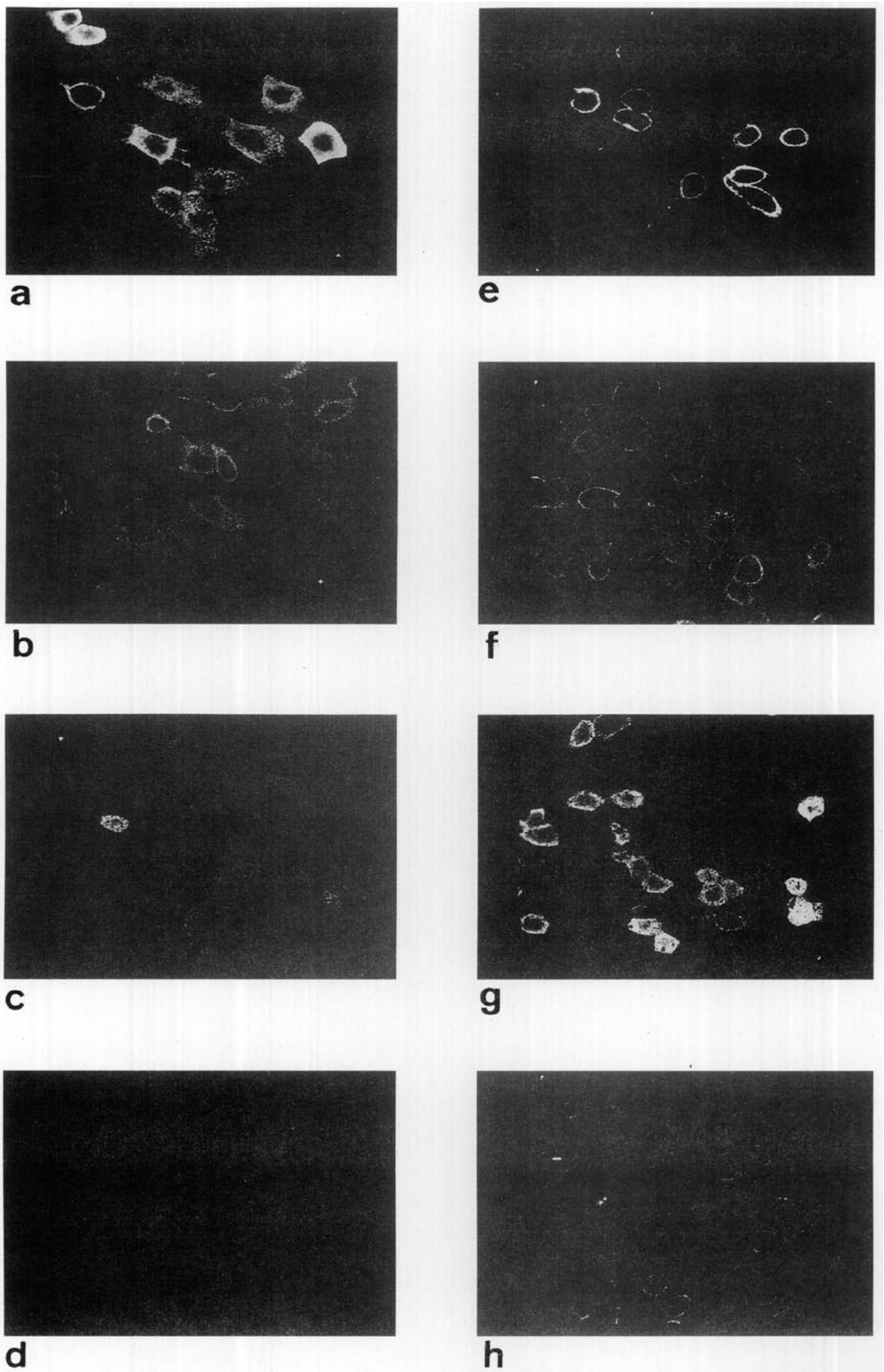

FIG. 3. Immunofluorescence staining of MDCK cells infected with (a, e) X31, (b, f) X47, (c, g) X79, and (d, h) NIB25 viruses. e, f, g, and h represent infected cells subjected to trypsin and low-pH treatment before fixation with paraformaldehyde.

TABLE 1

Comparison of Binding of LMBH5 to Infected Cells and Purified Virus

	Infected MDCK cells				ELISA			
			pH 7				pH 7	
Virus	HAU	pH 5 *A*	*A*	%	HAU	pH 5 *A*	*A*	%
X31	500	1.497	0.994	66	5000	1.541	1.159	75
	250	1.436	0.878	61	2500	1.032	0.789	76
	125	1.305	0.682	52	1250	0.603	0.448	74
X47	500	1.467	0.847	58	5000	1.881	1.459	77
	250	1.290	0.715	55	2500	1.294	0.941	73
	125	1.157	0.474	41	1250	0.955	0.590	62
X79	500	1.314	0.018	1.4	5000	1.028	0.186	18
	250	1.022	0.009	0.9	2500	0.553	0.098	18
	125	0.728	0.006	0.8	1250	0.366	0.042	11

Note. $\%\ \text{pH 7} = \dfrac{100 \times (A - A_{\text{uninfected}})\ \text{pH 7}}{(A - A_{\text{uninfected}})\ \text{pH 5}}$.

ELISA on infected cells

ELISA on infected cells gave results similar to those obtained with immunofluorescence and ELISA tests using purified viruses (Table 1). Binding of LMBH5 to infected cells was enhanced after trypsin and acid treatment, especially for X79-infected cells. This test could not be used to detect weak binding of LMBH5 to NIB25-infected cells. Assuming that maximum binding was achieved after trypsin and acid treatment (100%), this would mean that 0.8 to 1.4% of the native X79 HA molecules expressed on MDCK cells was recognized by LMBH5. For the X31 and X47 HA, this value was 52 to 66% and 41 to 58%, respectively (Table 1). Compared to the ELISA results (74 to 76% for X31, 62 to 77% for X31, 11 to 18% for X79; Table 1) these values are 9 to 22% lower.

Accessibility of the LMBH5 epitope at different pH

HI tests with X31, X47, X79, and NIB25 were performed to determine the narrow pH range at which the LMBH5 epitope became more accessible. Low pH-treated virus was obtained by adding 50 μl of 50 m*M* citrate buffer (pH 5, 5.4, 5.8, and 6.8) or 50 m*M* Tris·HCl (pH 6.8) to 25 μl of virus. After 10 min, 10 μl of 1 *M* Tris·HCl (pH 7.4) was added. The results of the HI tests show that the epitope became more accessible only after pH 5.4 (Table 2). After pH 5 treatment, however, the epitope became fully accessible. The same results were obtained by ELISA (data not shown).

Accessibility of the LMBH5 epitope on uncleaved HA

To determine whether trypsin treatment is necessary to expose the LMBH5 epitope, binding of LMBH5 to trypsin or low-pH-treated, or trypsin and low-pH-treated (pH 5) HA was determined in ELISA on infected cells. Trypsin treatment alone did not result in an enhanced binding of LMBH5 (Fig. 4). However, acid treatment of uncleaved HA already resulted in a stronger binding of LMBH5, especially to X79-infected cells, indicating that irreversible conformational changes had occurred in the uncleaved HA. Trypsin treatment is, however, needed to completely expose the epitope (Fig. 4).

Neutralization by LMBH5

The neutralization assay demonstrated that LMBH5 can completely block X31 and X47 infection of MDCK cells, whereas X79 infection was only partially inhibited, even at very high doses of antibody (Fig. 5). Comparing the 50% inhibition values (IV_{50}) for the HC3 and HC59 mAbs, neutralization by LMBH5 was slightly less efficient. The IV_{50} values further demonstrate the specificity of HC3 for X31 and of HC59 for X47.

X31 monoclonal variants

LMBH5 was used to select X31 escape mutants. The amino acid sequences were deduced from the complete sequence of the HA genes. X31 mutants (X31-L) had

TABLE 2

Determination of the Accessibility of the LMBH5 Epitope at Different pH

	HI titers			
pH treatment	X31	X47	X79	NIB25
5.0	4608	9216	4608	288
5.4	576	576	288	576
5.8	576	288	<18	<18
6.2	576	288	<18	<18
6.8	576	288	<18	<18
7.4	576	288	<18	<18

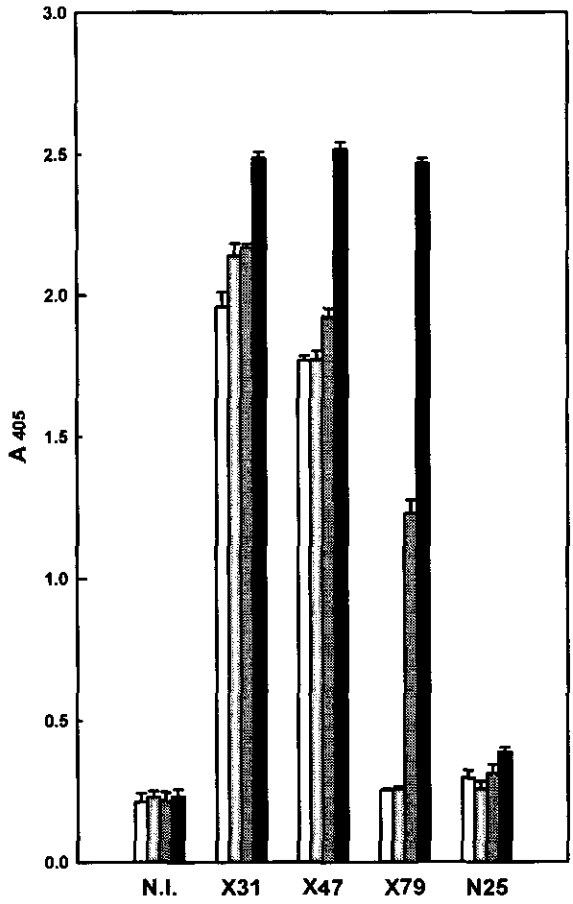

FIG. 4. Effect of trypsin treatment or low-pH treatment on binding of LMBH5 to MDCK-expressed HA. Cells infected with X31, X47, X79, and NIB25 were treated only with trypsin (light gray bars) or with citrate pH 5 (dark gray bars) or with trypsin and citrate pH 5 (black bars). White bars represent untreated cells. Error bars represent SD (n = 4).

a single nucleotide change, resulting in an amino acid substitution of Ser 227 → Pro. LMBH5 could not inhibit hemagglutination of erythrocytes by these variants (Table 3A). ELISA on infected cells showed that LMBH5 did not bind to X31-L HA, neither to the native nor to the acid-induced conformation (Table 3B). LMBH5 did not inhibit hemagglutination of X31 variants 63.D (deletion of amino acids 224 to 230), 63.E (Ser 193 → Asn; Leu 226 → Pro), or V68x (Ser 193 → Arg). ELISA on cells infected with these variants demonstrated that LMBH5 did not bind 63.D and 63.E HA, whereas binding to V68x HA was observed (Tables 3A and 3B). The HC3 mAb recognized all X31 variants tested, showing that possible differences in infection efficiency could not be responsible for the results obtained.

DISCUSSION

Using the neutralizing mAb LMBH5 we identified an epitope on the HA of influenza A (H3N2) viruses X31 and X47. This conformation-dependent epitope, as demonstrated by Western blot analysis (Kapaklis-Deliyannis *et al.*, 1993), proved to be present in influenza A (H3N2) virus strains isolated between 1968 and 1989. Sometimes the virus preparation had to be incubated at pH 5 before reactivity with the mAb could be demonstrated. This indicates that the epitope was not fully accessible in the native structure or that it was slightly changed and fitted better on the antibody-binding site after incubation at pH 5. HI tests showed that the epitope only became fully accessible around the pH of fusion. We obtained X31 LMBH5 escape mutants which differ from the X31 wild-type virus at Ser 227, a conserved residue in the human H3 subtype. The HA of the A/Duck/Ukraine/1/63 (H3N8) virus, which is believed to be a possible ancestor of the Hong Kong virus, also has Pro in position 227 (Fang *et al.*, 1981). Residue 227 is part of the conserved "left side" of the receptor-binding pocket (residues 224 → 230; Weis *et al.*, 1988), and is located in the proximity of the monomer interfaces. The specific behavior of the LMBH5 epitope and the fact that LMBH5 could no longer bind to this mutant HA suggest that Ser 227 can be part of the epitope. This is further supported by HI and ELISA results with 63.D and 63.E viruses. These variants, selected using a monoclonal antibody (HC63) which also recognizes a fairly conserved epitope, were shown to be adsorption

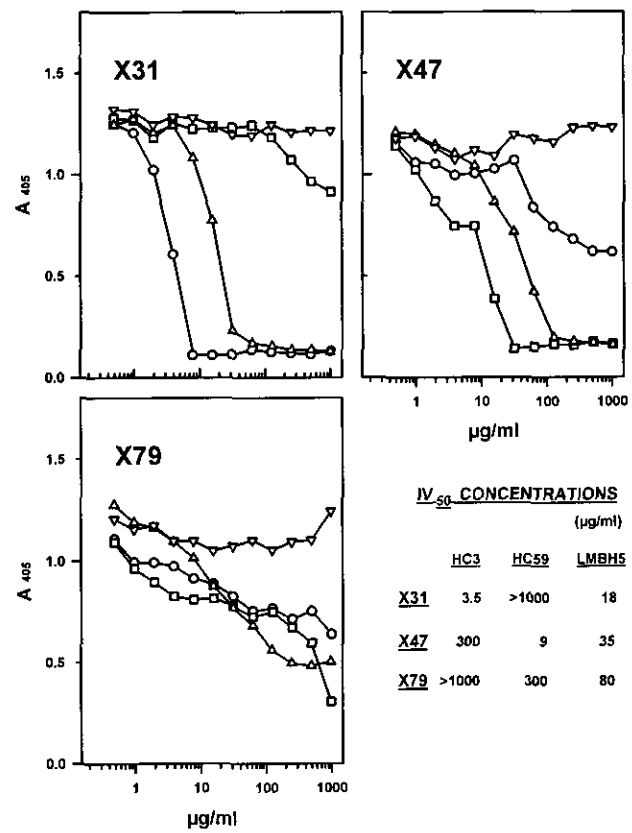

FIG. 5. Neutralization of X31, X47, and X79 by HC3 (○), HC59 (□), LMBH5 (△), or PBS (▽). Ascitic fluid was serially diluted one to two (1000 to 0.4872 µg/ml). Twenty-five microliters of each dilution was mixed with 25 µl purified virus (8000 HAU/ml). Infection was performed as described. Cells were subjected to trypsin and low-pH treatment before fixation with paraformaldehyde. ELISA was performed as described using mAb LMBH5.

TABLE 3

(A) HI Titers of mAbs LMBH5 and HC3 against X31 Variants

	LMBH5	HC3
X31	576	>9000
X31-L	<18	>9000
63.D	<18	>9000
63.E	<18	>9000
V68x	<18	>9000

(B) Binding of LMBH5 to Cells Infected with X31 Variants

		LMBH5			HC3		
			pH 7			pH 7	
	HAU	pH 5 *A*	*A*	%	pH 5 *A*	*A*	%
X31	500	0.702	0.350	50	0.898	0.711	80
X31-L	500	0.054	0.009		0.834	0.720	86
63.D	500	0.034	0.021		0.880	0.746	85
63.E	500	0.041	0.002		1.143	0.842	74
V68x	500	0.517	0.351	68	0.751	0.719	96

Note. $\%\ \text{pH 7} = \dfrac{100 \times (A - A_{\text{uninfected}})\ \text{pH 7}}{(A - A_{\text{uninfected}})\ \text{pH 5}}$.

variants (Daniels *et al.*, 1987). The inability of LMBH5 to bind to 63.D HA (deletion of amino acids 224 to 230) and the difference in binding of LMBH5 to 63.E (Ser 193 → Asn; Leu 226 → Pro) and V68x (Ser 193 → Arg) suggest that Leu 226, and perhaps other residues, are part of the epitope in addition to Ser 227. However, due to its structure, Pro can have such an effect on the folding of the protein chain that the correct conformation of the epitope, necessary for binding of LMBH5, is destroyed. Ser 193 → Arg substitution in V68x HA abrogated inhibition by LMBH5 but not binding of LMBH5. V68x HA has acquired the ability to agglutinate erythrocytes containing the SAα2,3 Gal linkage, compared to the X31 HA (Daniels *et al.*, 1987). So the V68x mutant qualifies as a receptor binding mutant. Such mutations have been shown to facilitate variation at immunodominant sites (Fazekas de St. Groth, 1977; Temoltzin-Palacios and Thomas, 1994). As inhibition by LMBH5 is abrogated, it is possible that this mutation allows the V68x virus to escape neutralization by LMBH5.

The immunofluorescence experiments showed that only a small percentage of the X79-infected MDCK cells expressed HA in which the LMBH5 epitope was accessible at neutral pH. Immunofluorescence was greatly enhanced by treatment of the cells with trypsin and low pH. Similarly, binding of LMBH5 to X79 was detectable in ELISA on infected cells, but was weak with the native form of the HA, whereas low-pH treatment of the virus induced full reactivity. These phenomena may be explained in the light of recent studies on influenza virus HA synthesis in the endoplasmic reticulum and subsequent processing. In the past few years it has become clear that the M2 protein functions as a proton pump (Pinto *et al.*, 1992) to maintain correct folding of the HA in post-Golgi compartments during transport to the cell surface (Hay *et al.*, 1985; Hay, 1989; Ciampor *et al.*, 1992a,b; Steinhauer *et al.*, 1991). Recently, it was demonstrated that a HA2 monomer-specific monoclonal antibody directed against an epitope inaccessible in the native HA still recognized approximately 7% of the molecules at neutral pH. After acid treatment, the hidden epitope became fully accessible (Varečková *et al.*, 1993). Our results support the hypothesis that the HA spikes on the cell surface and on the virions do not form a homogeneous population and that probably a portion of incorrectly folded HA molecules can escape quality control in the endoplasmic reticulum (Sugrue *et al.*, 1990; Varečková *et al.*, 1993). It is also possible that minor conformational changes, probably at the trimer interface, are much more likely to occur when HA molecules are cleaved. We observed 9 to 22% less binding of LMBH5 to native MDCK-expressed HA molecules compared to the binding on purified virus. MDCK-expressed HA is not cleaved, resulting in a more stable trimer during transport. However, the enhanced binding of LMBH5 to low pH-treated, uncleaved HA clearly demonstrated that conformational changes are possible in the globular domain. After trypsin treatment, no differences in binding were observed; this indicates that other factors might be responsible for the differences observed in binding to native, egg-derived, purified virus HA and MDCK-expressed HA (such as the cell type, transport of cleaved HA through the trans-Golgi network, or conformational changes due to the purification procedure).

Neutralization tests demonstrated that LMBH5 could completely inhibit infection of MDCK cells by X31 and X47. Complete inhibition of the X79 virus, even at high doses of LMBH5, was not achieved, although the IV_{50} value was only 2- to 4-fold higher than that obtained for the X31 and X47 virus, respectively. The ELISA results suggest that LMBH5 can bind 11 to 18% of the native X79 HA molecules (purified egg-derived virus was used for infection). This would mean that LMBH5 binds, on average, only to 165 to 270 HA molecules per virion. At low HIU:HAU ratios, IgG-mediated aggregation considerably neutralizes virus (Outlaw *et al.*, 1990). Taylor *et al.* (1987) also found an initial steep rate of neutralization, viz. a 10-fold loss of infectivity for 200 mAbs. It has also been demonstrated that only one IgG molecule is bound per HA trimer on virions in solution (Taylor and Dimmock, 1994). These results might explain why neutralization by LMBH5 is only slightly less efficient than neutralization by HC3 or HC59.

Which amino acid change(s) is (are) exactly involved in the phenomenon of inaccessibility of the epitope is difficult to determine at present. We can only conclude that one of the drift mutations or a combination of several changed amino acids is responsible for the results observed. Such mutations might have occurred at or near the epitope or elsewhere (allosteric effect). Yewdell *et al.* (1993) reported such an allosteric effect in the HA complex of influenza A/PR/8/34 (H1N1) virus. Another explanation of the possibly lower accessibility of the epitope of LMBH5 is the appearance of a carbohydrate side chain on the HA molecule in the neighborhood of the epitope. Such an event can indeed mask antigenic sites (*Caton et al., 1982*; *Daniels et al., 1983a,b*; *Skehel et al.*, 1984). Compared to the X31 and X47 HA sequence (Verhoeyen *et al.*, 1980; Min Jou *et al.*, 1980), two new potential glycosylation sites are present on the X79 HA (Asn 144 and Asn 246; *Nakajima et al., 1988*), while only the new glycosylation site on Asn 246 is present on the NIB25 HA (L. X. Shan *et al.*, unpublished results). It remains to be investigated if these new potential sites are really glycosylated and if so, whether they are responsible for the inaccessibility of the LMBH5 epitope.

Our results suggest that during the antigenic drift of influenza A (H3N2) virus, the LMBH5 epitope, although conserved, became less accessible to the antibody in the native state of the X79 HA. Due to "misfolding" or small conformational changes of some HA molecules, virus is still partially neutralized and LMBH5 binding to X79-infected cells can be achieved. ELISA, immunofluorescence, and HI tests with NIB25 suggest that the LMBH5 epitope became virtually inaccessible, even on the acid-induced conformation of the NIB25 HA. Thus influenza viruses can evade neutralization by mAbs, not only by changing amino acids of the corresponding epitopes, but also by changing amino acids outside these epitopes. Evidence is also presented that receptor-binding mutations in the variable antigenic sites might be responsible for the functional inactivation of neutralizing antibodies directed against conserved parts of the HA.

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