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A Common Neutralizing Epitope Conserved between the Hemagglutinins of Influenza A Virus H1 and H2 Strains

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When mice were immunized with the A/Okuda/57 (H2N2) strain of influenza virus, a unique monoclonal antibody designated C179 was obtained. Although C179 was confirmed to recognize the hemagglutinin (HA) glycoprotein by immunoprecipitation assays, it did not show hemagglutination inhibition activity to any of the strains of the three subtypes of influenza A virus. However, it neutralized all of the H1 and H2 strains but not the H3 strains. Moreover, it inhibited polykaryon formation induced by the H1 and H2 strains but not by the H3 strains. Two antigenic variants against C179 were obtained, and nucleotide sequence analysis revealed that amino acid sequences, from 318 to 322 of HA_1 and from 47 to 58 of HA_2, conserved among H1 and H2 strains were responsible for the recognition of C179. Since the two sites were located close to each other at the middle of the stem region of the HA molecule, C179 seemed to recognize these sites conformationally. These data indicated that binding of C179 to the stem region of HA inhibits the fusion activity of HA and thus results in virus neutralization and inhibition of cell-cell fusion. This is the first report which describes the presence of conserved antigenic sites on HA not only in a specific subtype but also in two subtypes of influenza A virus.

Although influenza is a mild febrile illness in general, it causes severe symptoms and sometimes death in those who have underlying diseases or in the aged. Moreover, even at the present time, large influenza epidemics become serious public health problems all over the world. However, the effect of currently available vaccines against influenza is extremely variable (17). One of the reasons for such variability comes from the fact that a high frequency of antigenic drift has been observed in the influenza virus hemagglutinin (HA) glycoprotein, which is the major protein capable of inducing neutralizing antibodies (23).

Antigenic variation of HA has been studied in naturally occurring or monoclonally selected variants by conventional serological tests and more recently by nucleotide sequence analyses (43). These studies showed that the amino acid substitutions were clustered in several sites, predominantly in the HA_1 globular domain (44). On the other hand, conserved amino acid sequences were observed in HA, mainly in HA_2, not only within a specific subtype but also among the subtypes (20, 29, 37, 46). Moreover, Graves et al. (9) showed the existence of cross-reactive antigenic determinants in HA_2 by a radioimmunoprecipitation assay. Therefore, it is expected that HA contains conserved antigenic sites which might induce cross-protective antibodies In this study, we have attempted to select monoclonal antibodies (MAbs) which can neutralize the viruses broadly.

By use of our recently developed method whereby infected cells in 96-well plates are stained by the peroxidase-antiperoxidase staining technique (27), we could successfully select a MAb which showed unique characteristics not previously reported. In this study, this MAb was analyzed by molecular techniques together with biological and serological methods. Its importance in the study of influenza virus will be discussed.

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MATERIALS AND METHODS

Viruses. The influenza viruses used were A/PR/8/34 (H1N1), A/Bangkok/10/83 (H1N1), A/Yamagata/120/86 (H1N1), A/Osaka/930/88 (H1N1), A/Suita/1/89 (H1N1), A/Okuda/57 (H2N2), A/Adachi/2/57 (H2N2), A/Kumamoto/1/65 (H2N2), A/Kaizuka/2/65 (H2N2), A/Izumi/5/65 (H2N2), A/Aichi/2/68 (H3N2), A/Fukuoka/C29/85 (H3N2), A/Sichuan/2/87 (H3N2), A/Ibaraki/1/90 (H3N2), A/Suita/1/90 (H3N2), and B/Nagasaki/1/87. These viruses were grown in the allantoic cavities of 11-day-old embryonated hen eggs, harvested, and stored as infectious allantoic fluid at −80°C until further use.

Cells. MDCK (Madin-Darby canine kidney) cells were grown in Eagle minimal essential medium supplemented with 10% fetal bovine serum. CV-1 (monkey kidney) cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum.

HI test. The hemagglutination inhibition (HI) test was performed with receptor-destroying enzyme-treated mouse ascitic fluids by a standard microtiter assay (6).

Neutralization test. The newly developed rapid focus reduction neutralization test for influenza viruses was carried out (27). Mouse ascitic fluids were treated with receptor-destroying enzyme before the test.

Staining test. The ability of MAbs in mouse ascitic fluid to stain cells infected with influenza viruses was studied by the method described for the neutralization test (27), with slight modifications. MDCK cells infected with influenza viruses in 96-well flat-bottom plates (Falcon 3072; Becton Dickinson Labware, Oxnard, Calif.) were rinsed with phosphate-buffered saline (PBS; pH 7.4), fixed with absolute ethanol at room temperature for 10 min, and then treated with four antisera: MAbs serially diluted from 1:100 to the desired dilutions, rabbit anti-mouse immunoglobulin G (IgG) serum (Organon Teknika, Malvern, Pa.) diluted 1:1,000, goat anti-rabbit IgG serum (Organon Teknika) diluted 1:500, and peroxidase-rabbit antiperoxidase complex (Organon Teknika) diluted 1:1,000. Each treatment was 40 min long and was followed by a washing with PBS. Finally, a peroxidase reaction was allowed to develop for about 5 min by the

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method of Graham and Karnovsky (8), in which 0.01% H_2O_2 and 0.3 mg of 3,3′-diaminobenzidine tetrahydrochloride (Wako Chemical Industries, Osaka, Japan) per ml in PBS were used. The cells were then rinsed with tap water and dried. The stained cells were observed under an ordinary light microscope. The staining titers were expressed as the reciprocal of the highest dilution that stained the cells definitely.

Fusion assays. Cell-cell fusion was accomplished by the method of White et al. (41), with a few slight modifications. Monolayers of CV-1 cells were infected with the influenza virus strains. After incubation for 24 h, the cells were washed twice with DMEM and then incubated for 15 min at 37°C in this medium supplemented with trypsin (10 μg/ml). Then the cells were washed twice with DMEM and incubated for 30 min with DMEM or with DMEM plus appropriately diluted C179. Thereafter, the cells were treated for 2 min at 37°C with a fusion medium (RPMI without bicarbonate, 0.2% bovine serum albumin, 10 mM morpholineethanesulfonic acid [MES], 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]) adjusted to pH 5.0. After the medium was completely removed by two washes with DMEM, the cells were incubated for 3 h in DMEM containing 2% fetal bovine serum. At this time, they were fixed with absolute methanol, stained with Giemsa, and observed under a light microscope.

Production of MAbs. For the production of MAbs, BALB/c mice were immunized intraperitoneally with two doses (320 hemagglutinating units per mouse) of A/Okuda/57 (H2N2) (25) 1 month apart. The antigen was emulsified in Freund's complete adjuvant before use. The mice were boosted 2 to 5 months after the second immunization with a similar intraperitoneal injection. Three days later, the spleen cells from these mice were fused with mouse myeloma cells (P3X63Ag8.653) by using polyethylene glycol (19). The culture fluids from the fused cells were screened by the method used in the staining test described above. To detect MAbs which cross-react among subtypes of influenza A virus, MDCK cells infected with each of the three subtypes were stained at the same time with undiluted culture fluids as the first antibody. Antibody-positive cells were cloned by limiting dilution at least three times and were injected intraperitoneally into pristane-treated mice. The resulting ascitic fluids were collected and stored at −20°C before use.

Radiolabeling and immunoprecipitation. Radioimmunoprecipitation assays were carried out as previously described (5). For the preparation of labeled virus antigens, MDCK cells infected with A/Okuda/57 were used.

Selection of variants which are resistant to C179. IgG from ascitic fluid of MAb C179 (IgG2a subclass), which can neutralize H1 and H2 strains of influenza A virus, was purified by affinity chromatography using a IgG purification kit (Pierce, Rockford, Ill.). Neutralization-resistant variants were obtained by growing the H1 and H2 strains in MDCK cells in the presence of purified C179.

Amplification of the HA genes and nucleic acid sequencing. For nucleotide sequencing of the HA genes, viral RNAs in MDCK cells infected with each of the strains were extracted by using guanidinium isothiocyanate (3). The oligonucleotide primer 5′CATACGAAGCAAAAGCAGGGG3′, which is a combination of the 5′ upstream region of the SP6 promoter (CATACGA) and the region complementary to the 3′ terminus of the genomic strand RNA, was used to synthesize cDNAs. To amplify the cDNAs, the polymerase chain reaction (PCR) was applied by using two primers, the above-mentioned primer and 5′AGTAGAAACAAGGGTGTTTTT3′, complementary to the 3′ terminus of the positive-strand RNA. Double-stranded cDNAs were separated by electrophoresis through agarose gels, and a cDNA band corresponding to the HA gene was eluted. After the cDNA was further amplified by PCR, it was directly sequenced by a previously described method (11, 12). Briefly, the PCR-amplified DNA fragment was purified by using 20% (wt/vol) polyethylene glycol 6000 in 2.5 M NaCl. The purified DNA was annealed with sequencing primers which were labeled with [γ-^{32}P]ATP. These primers had been constructed by selecting nucleotide positions conserved in each subtype. Then the DNA was incubated for 10 min at 72°C and elongated by 10 cycles of 1 min at 90°C, 2 min at 55°C, and 3 min at 72°C. Before being loaded onto sequencing gels, the DNA was incubated for 3 min at 95°C and then cooled on ice.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession numbers D13570 to D13584.

TABLE 1. Antibody titers and fusion inhibition activity of C179 against influenza A and B viruses

Virus	Antibody titer of C179 measured by[a]:		Fusion inhibition[b]
	Staining test	Neutralization test	
H1N1			
A/PR/8/34	1,638,400	512	+
A/Bangkok/10/83	1,638,400	512	+
A/Yamagata/120/86	409,600	1,024	+
A/Osaka/930/88	409,600	512	+
A/Suita/1/89	409,600	1,024	+
H2N2			
A/Okuda/57	1,638,400	1,024	+
A/Adachi/2/57	1,638,400	1,024	+
A/Kumamoto/1/65	409,600	1,024	+
A/Kaizuka/2/65	409,600	2,048	+
A/Izumi/5/65	409,600	1,024	+
H3N2			
A/Aichi/2/68	<100	<16	−
A/Fukuoka/C29/85	<100	<16	−
A/Sichuan/2/87	<100	<16	−
A/Ibaraki/1/90	<100	<16	−
A/Suita/1/90	<100	<16	−
B			
B/Nagasaki/1/87	<100	<16	−

[a] In all cases, titers measured by the HI test were <32.
[b] +, cell-cell fusion was inhibited completely by C179 at a dilution of 1:1,000; −, cell-cell fusion was not inhibited at all by C179 at a dilution of 1:10.

RESULTS

Isolation and serological characterization of MAb C179. Since we intended to select MAbs which cross-react broadly, culture fluids from the hybridoma cells were screened by a staining test in which not only homologous A/Okuda/57 (H2N2) but also A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2) were used. Of the many MAbs obtained, five which recognized the NP or M protein reacted to all three subtypes and one, designated C179, reacted to both A/PR/8/34 and A/Okuda/57. As C179 showed intriguing reactivity, we analyzed this MAb in detail serologically.

Antibody titers of C179 were measured by three serological tests against each of the five strains of the three subtypes and one type B strain (Table 1). In the staining and neutral-

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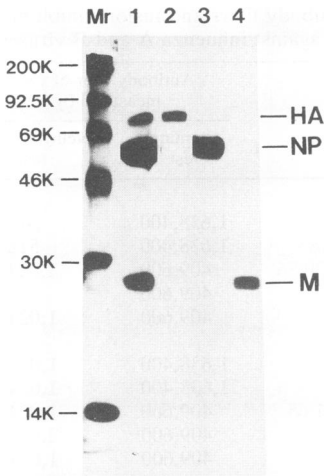

FIG. 1. Demonstration that C179 reacts with the HA glycoprotein by immunoprecipitation assays. For comparison, a polyclonal rabbit serum and MAbs against the NP and M proteins of influenza virus were used. Lanes: Mr, molecular weight markers; 1, anti-A/Okuda/57 rabbit serum; 2, C179; 3, the MAb (C43) which recognizes the NP protein; 4, the MAb (C111) which recognizes the M protein.

ization tests, C179 reacted with all strains of the H1 and H2 subtypes to almost the same degree but not with any of the strains of the H3 subtype and B type, indicating that C179 reacted with H1 and H2 strains specifically. However, it did not react with any of the strains in the HI test.

To confirm the identity of the viral protein which is recognized by C179, immunoprecipitation assays were carried out (Fig. 1). The hyperimmune anti-A/Okuda/57 polyclonal rabbit serum reacted with three major proteins of the virus (Fig. 1, lane 1). C179 and two other MAbs (C43 and C111) that were used as reference antibodies immunoprecipitated one of the three proteins specifically (Fig. 1, lanes 2 to 4). The results clearly demonstrated that C179 recognized the HA protein.

Inhibition of cell-cell fusion by C179. The results mentioned above indicate that C179 neutralized the viruses by mechanisms other than inhibition of virus attachment to a target cell. We then examined the ability of C179 to inhibit cell-cell fusion (Table 1). Figure 2 shows typical results of a fusion assay in which representative strains in each subtype, A/PR/8/34 (H1N1) (A and B), A/Okuda/57 (H2N2) (C and D), and A/Aichi/2/68 (H3N2) (E and F), were used. When monolayers of CV-1 cell infected with each of the strains were treated with trypsin and briefly exposed to pH 5.0, formation of giant polykaryons was observed (Fig. 2A, C, and E). However, in the presence of C179 even at a high dilution (1:1,000), cell-cell fusion by the H1 and H2 strains was completely inhibited (Table 1; Fig. 2B and D). In contrast, the formation of polykaryons by the H3 strains was not inhibited at all, even in the presence of a low dilution of C179 (1:10) (Table 1; Fig. 2F). These results agreed well with those obtained by the serological tests and suggested that C179 neutralized the H1 and H2 strains by inhibiting the fusion activity of HA.

Identification of the antigenic site recognized by C179. When all of the H1 and H2 strains used for the serological tests were grown in the presence of C179, two antigenic variants, A/Suita/1/89(R) and A/Izumi/5/65(R), derived from the parent strains A/Suita/1/89 and A/Izumi/5/65, were selected. The variants did not react at all with C179 in the staining and neutralization tests (Table 2). We then subjected the HA-coding gene of the variants and the parent strains to nucleotide sequencing, and the deduced amino acid sequences were compared. In A/Suita/1/89(R), there were three nucleotide changes, and they were all accompanied by amino acid substitutions at positions 189, 225, and 318 (H3 numbering system [45]) in HA_1. The substitutions at residues 189 and 225 were not responsible for resistance to C179 because these positions were highly variable when amino acid sequences of HA reported so far were compared. Therefore, the amino acid change at residue 318 (Thr→Lys) must be responsible for the resistance to C179. In A/Izumi/5/65(R), only one nucleotide change with an amino acid substitution at residue 52 (Val→Glu) was observed in HA_2.

Since these results suggested that residues 318 in HA_1 and 52 in HA_2 affect the antibody recognition of C179, nucleotide sequences in the vicinity of the residues in all of the influenza A strains used were compared (Fig. 3). It was found that the amino acid sequences from 318 to 322 in HA_1 (designated the A region) and from 47 to 58 in HA_2 (designated the B region) were identical in all H1 and H2 strains except A/Suita/1/89(R) and A/Izumi/5/65(R), in which amino acid changes from T to K at position 318 (HA_1) and from V to E at position 52 (HA_2), respectively, were observed. Residues in the A region of the H3 strains showed a common sequence with only one amino acid difference from the H1 and H2 strains at residue 320. In the B region, five or six amino acid differences were observed between strains in the H3 and other two subtypes. Therefore, these amino acid differences might cause the inability of C179 to react with H3 strains in the serological and fusion inhibition tests.

The residues at 318 (HA_1) and 52 (HA_2) were located close to each other at the middle of the stem region of the HA molecule (Fig. 4). As described in the Discussion, C179 must bind at both antigenic sites in the A and B regions conformationally.

DISCUSSION

Of the several unique biological characteristics of C179, the most important was the ability to neutralize all of the H1 and H2 strains (Table 1). Although C179 did not have HI activity (Table 1), it was confirmed to recognize the HA by immunoprecipitation assays (Fig. 1). Recently, it has been demonstrated that certain sera, and particularly MAbs, may possess virus neutralization activity in the absence of HI activity (14–16, 50). These observations, together with our data, suggested the existence of epitopes on the HA to which the binding antibodies cause virus neutralization by mechanisms other than the inhibition of virus attachment to the target cells. Since the HA initiates infection by mediating both the binding of the virions to receptors on the cell surface and the subsequent fusion of viral and endosomal membranes (40), we can surmise that virus neutralization by C179 was due to the inhibition of fusion activity of the HA. This supposition was clarified by the results of fusion assays; in the presence of C179, polykaryon formation by the H1 and H2 strains, but not by the H3 strains, was inhibited (Table 1; Fig. 2).

To identify the recognition sites of C179, selection of antigenic variants against C179 was attempted, and two antigenic variants resulted. By nucleotide sequencing and determination of the deduced amino acid sequences, it was demonstrated that amino acid substitutions at positions 318 (HA_1) and 52 (HA_2) were responsible for the resistance to

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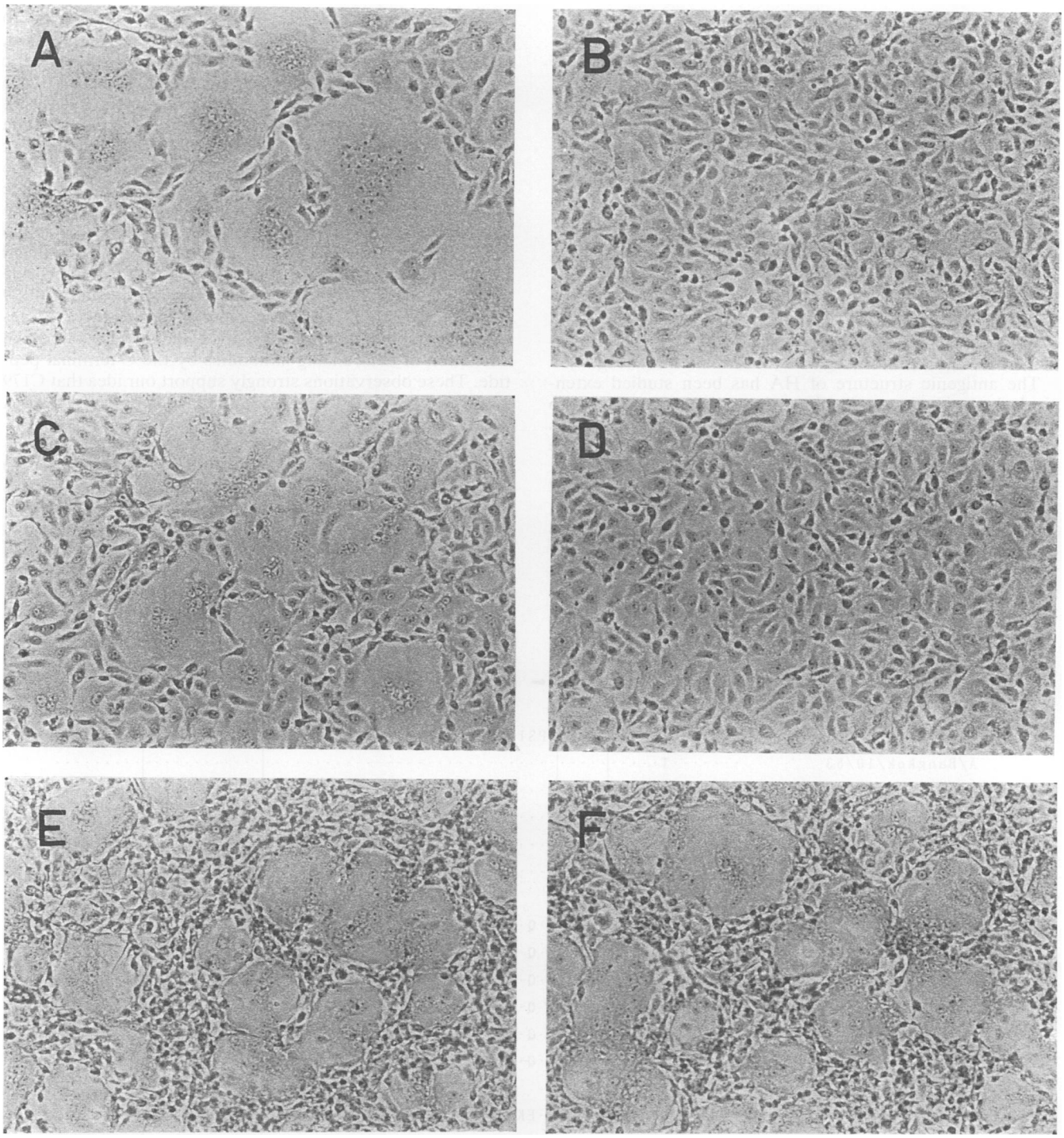

FIG. 2. Cell fusion of CV-1 cells infected with A/PR/8/34 (H1N1) (A and B), A/Okuda/57 (H2N2) (C and D), and A/Aichi/2/68 (H3N2) (E and F) in the absence (A, C, and E) or presence of C179 at a high dilution (1:1,000) (B and D) and a low dilution (1:10) (F).

C179 (Table 2). Moreover, recognition sites of C179 could be restricted to positions 318 to 322 in HA_1 (A region) and 47 to 58 in HA_2 (B region) (Fig. 3). Although we could not determine the precise binding sites of C179 within the two regions, they will become evident in experiments in which oligonucleotide-directed site-specific mutagenesis is used. It has been reported that MAbs directed to HA must bind directly to the immediate vicinity of the amino acid substitutions (18, 43), and most of the antigenic determinants were discontinuous conformational determinants in that amino acids from distant parts of the linear sequence contributed to each antigenic domain (28). These observations, together

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TABLE 2. Characterization of antigenic variants by the serological tests and amino acid sequencing

Virus	Antibody titers of C179 measured by:		Amino acid change(s)
	Staining test	Neutralization test	
A/Suita/1/89	409,600	1,024	
A/Suita/1/89(R)	<100	<16	HA_1 G→R(189) G→D(225) T→K(318)
A/Izumi/5/65	409,600	1,024	
A/Izumi/5/65(R)	<100	<16	HA_2 V→E(52)

with the fact that C179 could not be detected by Western immunoblotting (data not shown), indicate that C179 recognized both the A and B regions conformationally (Fig. 4).

The antigenic structure of HA has been studied extensively by analyzing antigenic variants that are resistant to anti-HA MAbs (4, 7, 21, 24, 36, 44, 47, 49). These studies and cross-competition experiments using MAbs revealed the existence of at least four antigenic domains on the HA molecule (1, 2, 35, 39, 48). Thus, it was shown that almost all of the MAbs reported so far recognized the antigenic sites on the globular head of the HA molecule. In contrast, C179 was demonstrated to recognize the middle of the stem region, where antigenic determinants have not previously been identified. Therefore, this study shows for the first time that the stem region is immunologically important.

Synthetic peptides have been used as immunogens to provide an immune response against influenza viruses (10, 22, 31). Green et al. (10) reported that 18 of 20 synthetic peptides corresponding to each amino acid sequence of the HA_1 molecule could induce antibodies against the intact HA. In particular, antibodies raised against the C terminus of the HA_1 peptide (positions 306 to 329) induced the highest reactivity. Thereafter, the C terminus of the HA_1 molecule was examined and found to have antigenic and immunogenic activity (13, 26, 30). When antibodies raised against the synthetic peptide corresponding to the C-terminal 24 amino acids (305 to 328) of HA_1 were analyzed by enzyme-linked immunosorbent assay, three antigenic sites in this peptide were identified (30). However, the recognition site of C179 (positions 318 to 322) was not included among them. Moreover, the above-mentioned reports did not describe the elicitation of neutralizing antibodies by the C-terminal peptide. These observations strongly support our idea that C179 can neutralize the viruses by simultaneous binding of the A and B regions.

The fusion activity of HA is induced by trypsin or trypsin-like enzymes which cleave the HA precursor, HA_0, into two disulfide-bonded subunits, HA_1 and HA_2 (17, 23). This cleavage generates the fusion peptide at the N terminus of the HA_2 subunit. In the process of membrane fusion, the requirement of several steps, such as a conformational change in HA at low pH and insertion of the fusion peptide into the target membrane, has been proposed (32, 33, 38, 42). C179 seemed to affect any of these steps and inhibit the

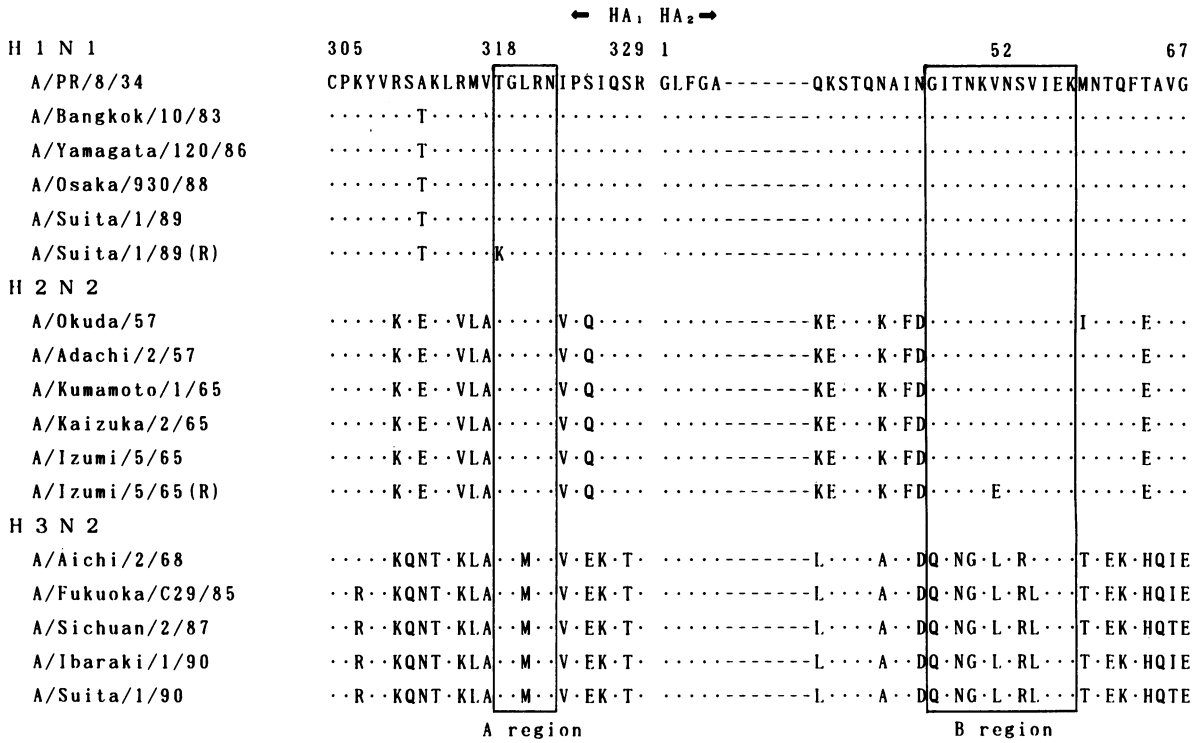

FIG. 3. Comparison of the amino acid sequence of H1N1, H2N2, and H3N2 influenza virus HAs. Sequences are shown from amino acid residues 305 to 329 in HA_1 and 1 to 67 in HA_2. The numbering of amino acid residues of all strains is followed by that of the A/Aichi/2/68 strain. The sites where changes were identified compared with the sequence of the A/PR/8/34 strain are shown. Amino acid sequences from 318 to 322 in HA_1 (A region) and from 47 to 58 in HA_2 (B region) are boxed. In these regions, amino acid residues in the H1 and H2 strains are conserved except in the A/Suita/1/89(R) and A/Izumi/5/65(R) strains, in which amino acid changes from T to K at position 318 (HA_1) and from V to E at position 52 (HA_2), respectively, were observed.

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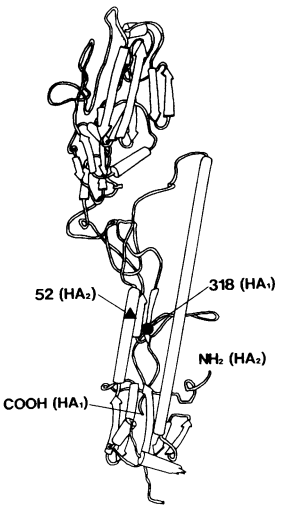

FIG. 4. Locations of altered amino acid residues in the antigenic variants shown in a schematic diagram of the influenza virus HA monomer (44). The carboxyl and amino termini of HA_1 and HA_2 are also shown.

fusion activity of HA. Recently it has been revealed that in the initial step of the conformational change in HA, structural modification, mainly in the stem, is important (34, 42). Therefore, it is most probable that C179 prevents the first event of conformational change in HA by binding the middle of the stem region, and consequently, membrane fusion by HA is inhibited.

Although C179 recognized the epitope near the cleavage site, it was preserved irrespective of the cleavage, as demonstrated by the evidence described below. (i) The virus stocks used for the neutralization tests were the cleaved type, and they were neutralized by C179. (ii) The HA antigens in MDCK cells were the uncleaved type, and they were stained by C179. (iii) After treatment with or without trypsin, the HA antigens on the surface of infected cells were stained by C179. (iv) Inhibition of cell-cell fusion was observed whether the infected cells reacted with C179 either before trypsin treatment or after.

At present, both the H1 and H3 subtypes of influenza A virus have caused large epidemics. Therefore, we are trying to produce a MAb which can neutralize all H3 subtype viruses by the same method in which C179 was selected. If they are obtained, these MAbs may be used for the prevention and treatment of influenza after they are converted to chimeric mouse-human antibodies.

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