JOURNAL OF VIROLOGY, Jan. 1994, p. 517–520
0022-538X/94/$04.00+0

Vol. 68, No. 1

Protection against the Mouse-Adapted A/FM/1/47 Strain of Influenza A Virus in Mice by a Monoclonal Antibody with Cross-Neutralizing Activity among H1 and H2 Strains

YOSHINOBU OKUNO,* KEN-ICHIRO MATSUMOTO, YUJI ISEGAWA, AND SHIGEHARU UEDA

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan

Received 8 July 1993/Accepted 8 October 1993

The monoclonal antibody designated C179 was found to neutralize all of the H1 and H2 strains of influenza A virus studied (Y. Okuno, Y. Isegawa, F. Sasao, and S. Ueda, J. Virol. 67:2552–2558, 1993). In the present study, the ability of C179 to protect mice from the lethal effect of the A/FM/1/47 (H1N1) strain was examined. When the mice were injected intraperitoneally with 100 μg of C179 per mouse a day before the virus challenge (2.0×10^3 focus-forming units per mouse), all of the mice survived. Moreover, significantly higher survival rates were observed in mice receiving 1,000 μg of C179 per mouse 2 days after the virus challenge than in those receiving phosphate-buffered saline alone. These results indicate that C179 is effective not only for prevention but also for treatment of mice infected with H1 and H2 strains. The possibility that C179 can be used for passive immunization in humans is discussed.

We have demonstrated in an earlier article (15) that a monoclonal antibody, termed C179, has unique characteristics not previously reported. Although C179 was selected from the spleens of mice immunized with the A/Okuda/57 (H2N2) strain (12) of influenza A virus, it neutralized all of the H1 and H2 strains studied. By the hemagglutination inhibition and fusion inhibition tests, it was found that C179 neutralized the H1 and H2 strains by inhibiting the fusion activity but not the binding activity of the hemagglutinin (HA) glycoprotein. Analysis of the antigenic variants that escaped from the neutralizing activity of C179 by nucleotide sequencing revealed that C179 recognized the conformational epitope in the middle of the stem region of HA in which amino acid sequences were conserved in the H1 and H2 strains. Since the antigenic variability of the HA has hampered the control of influenza, the antibody has a possibility of being used for the prevention of influenza. In the present study, we conducted animal experiments to discover whether C179 can protect mice from lethal influenza virus infection.

To characterize the biological activities of C179 in more detail, we analyzed it quantitatively in in vitro experiments by using affinity-purified immunoglobulin G of C179. Two kinds of assay systems, neutralization and fusion inhibition tests, were applied. The strains used for these tests were A/FM/1/47 (H1N1), A/Okuda/57 (H2N2), and A/Aichi/2/68 (H3N2), which represent each of the three subtypes of influenza A virus. Stock viruses of these strains were prepared in the allantoic cavities of 11-day-old embryonated hen eggs.

Figure 1A shows the results of the neutralization test. The test was performed by a previously described method (16). As previously reported (15), the H1 and H2 strains but not the H3 strain were neutralized by C179. The concentrations of C179 required to completely neutralize the viruses were 200 μg for A/FM/1/47 and 50 μg for A/Okuda/57. Although A/Okuda/57 was neutralized more efficiently than A/FM/1/47, it is not clear whether the difference was due to the ability to respond to C179 or was merely an aberration within the test system.

Fusion inhibition assays were performed by our method (15), with slight modifications. This time, infected CV-1 cells in 96-well tissue culture plates were incubated overnight in a medium containing 5 μg of trypsin per ml instead of a trypsin treatment for 30 min 1 day after the infection. The fusion index was obtained by the following formula: fusion index = 1 − (number of cells/number of nuclei). As shown in Fig. 1B, C179 inhibited the fusion activity of A/FM/1/47 and A/Okuda/57 but not of A/Aichi/2/68. The kinetics of fusion inhibition of the two strains were almost identical, showing a complete inhibition at a concentration of 25 μg/ml.

To investigate the protective effect of C179 against influenza A virus in mice, the A/FM/1/47 strain, which caused a high mortality rate in mice (13), was used as the challenge virus. It was made clear that C179 recognized two consecutive amino acid sequences (designated regions A and B) of HA which were conserved among all of the H1 and H2 strains studied (15). We then conducted nucleotide sequencing of A/FM/1/47 and confirmed that the amino acid sequences of the A (TGLRN) and B (GITNKVNSVIEK) regions were the same as those of other H1 and H2 strains. In the present study, 4-week-old female BALB/c mice were administered intraperitoneally with 1 ml of phosphate-buffered saline (PBS; pH 7.4) or PBS containing appropriate amounts of C179 before or after the intranasal inoculation with 25 μl containing 2.0×10^3 FFU (focus-forming units) of A/FM/1/47 per mouse under light ether anesthesia. This dose of A/FM/1/47, which resulted in the highest mortality rate in mice, was determined by the preliminary experiments in which the mice were inoculated with 10-fold serially diluted virus. The higher dose (2.0×10^4 FFU per mouse) did not necessarily result in a higher mortality rate. Figure 2A shows the results of the mouse survival rates when the mice were given C179 a day before the virus challenge. Ten mice each in the three groups received 1,000, 100, or 10 μg of C179, respectively. As the control, 12 mice received PBS alone. All of the mice receiving 1,000 or 100 μg of C179 remained alive, whereas those receiving 10 μg of C179 or PBS showed low survival rates. These data indicate that 100

* Corresponding author. Mailing address: Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565, Japan. Phone/Fax: 81-6-875-1170.

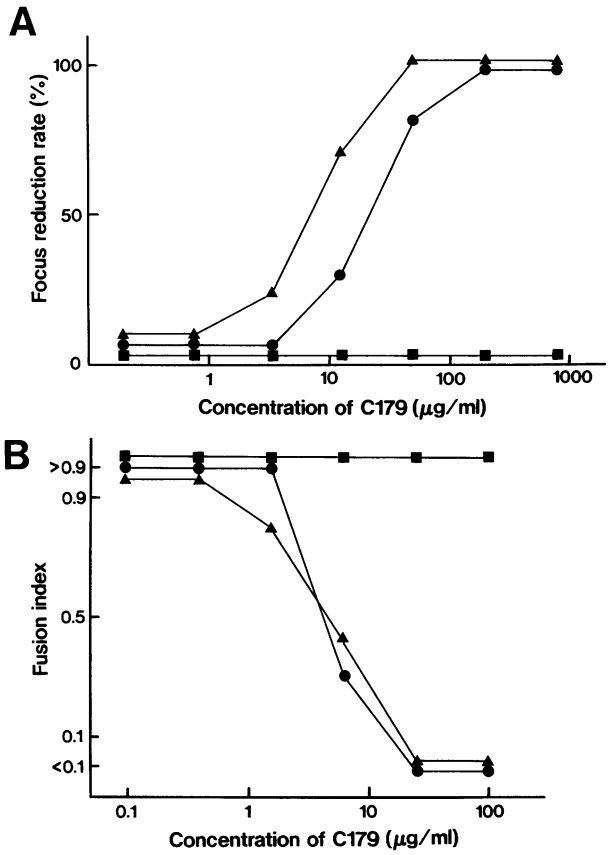

FIG. 1. (A) Kinetics of neutralizing activity of C179 against A/FM/1/47 (H1N1) (●), A/Okuda/57 (H2N2) (▲), and A/Aichi/2/68 (H3N2) (■). The neutralization test was performed by a previously described method (16). Suspensions of MDCK cells were distributed to each well of 96-well flat-bottom plates and incubated in a CO_2 incubator at 37°C to make monolayer sheets. On the following day, C179 was serially diluted in fourfold steps in 96-well round-bottom plates. Then, diluted C179 and control diluent were combined with an equal volume (25 μl) of influenza virus adjusted to give a final control count of about 50 FFU per well. After incubation for 1 h at 37°C, 25 μl of each mixture was transferred to wells of 96-well flat-bottom plates in which MDCK cells had been seeded on the previous day and adsorbed for 30 min at 37°C. After the virus inocula were removed and washed with PBS, the cells were covered with Eagle minimal essential medium containing 0.5% tragacanth gum and 5 μg of trypsin per ml. At 20 to 24 h after inoculation, the medium was removed, and the cells were fixed with absolute ethanol at room temperature for 10 min. Then, the cells were dried, and focus staining was done by successive treatment of the cells with rabbit anti-A/Okuda/57 serum (1:1,000), goat anti-rabbit immunoglobulin G serum (1:500) (Organon Teknika, Malvern, Pa.), and peroxidase-rabbit antiperoxidase complex (1:1,000) (Organon Teknika) for 40 min each. Finally, a peroxidase reaction was allowed for about 5 min according to the method described by Graham and Karnovsky (5), in which 0.01% H_2O_2 and 0.3 mg of 3,3'-diaminobenzidine tetrahydrochloride per ml in PBS were used. The cells were then rinsed with tap water and dried. The stained foci were counted under an ordinary light microscope by turning the plates upside down. (B) Kinetics of fusion inhibition activity of C179 against A/FM/1/47 (H1N1) (●), A/Okuda/57 (H2N2) (▲), and A/Aichi/2/68 (H3N2) (■). Fusion inhibition was assayed by a method previously reported (15). Monolayers of CV-1 cells in 96-well tissue culture plates were infected with each strain at a multiplicity of infection of 0.01 and incubated for 24 h at 37°C in the presence of trypsin (5 μg/ml). Then, the cells were incubated for 30 min with a medium containing serially diluted C179. Thereafter, they were treated for 2 min at 37°C with a fusion medium (pH 5.0) and were further incubated for 3 h in the culture medium. At this time, they were stained with a Giemsa stain and were examined under a light microscope. The fusion index was obtained by the method described by Arikawa et al. (1), in which the following formula was applied: fusion index = 1 − (number of cells/number of nuclei).

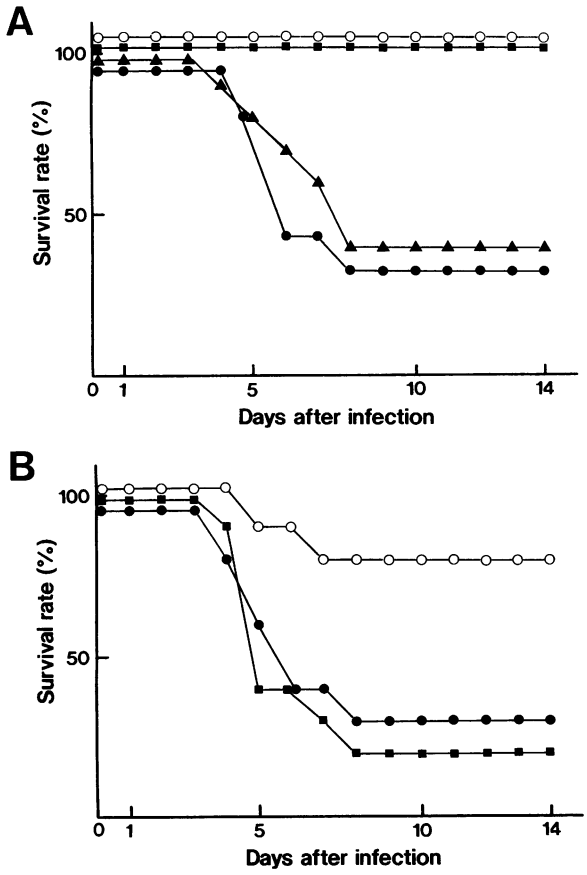

FIG. 2. Effect of C179 against infection with A/FM/1/47 (H1N1) in mice. (A) Four-week-old female BALB/c mice received 1,000 (○), 100 (■), or 10 (▲) μg of C179 per mouse or PBS (●) 24 h before the virus challenge. (B) Four-week-old female BALB/c mice received 1,000 (○) or 100 (■) μg of C179 per mouse or PBS (●) 2 days after the virus challenge.

μg of C179 per mouse is enough to protect mice from the lethal effect of A/FM/1/47.

Figure 2B shows the results of mouse survival rates when mice were given C179 2 days after the virus challenge. Ten mice in each group, which received 1,000 or 100 μg of C179 or PBS, were used. Although the mice that received 100 μg of C179 showed a low survival rate (20%), the mice that received 1,000 μg of C179 showed a high one (80%). Results similar to those described above were obtained by repeated experiments, which confirmed that 1,000 μg of C179 per mouse is an effective treatment for infection by A/FM/1/47.

To investigate the level of virus replication in the lungs of mice receiving 100 μg of C179 or PBS 1 day before the challenge with A/FM/1/47, three mice each per day in the two groups were sacrificed on days 0 to 5, 7, and 10 after the virus inoculation, and their virus titers were determined (Fig. 3A). Even 1 day after the inoculation, the control mice, which had

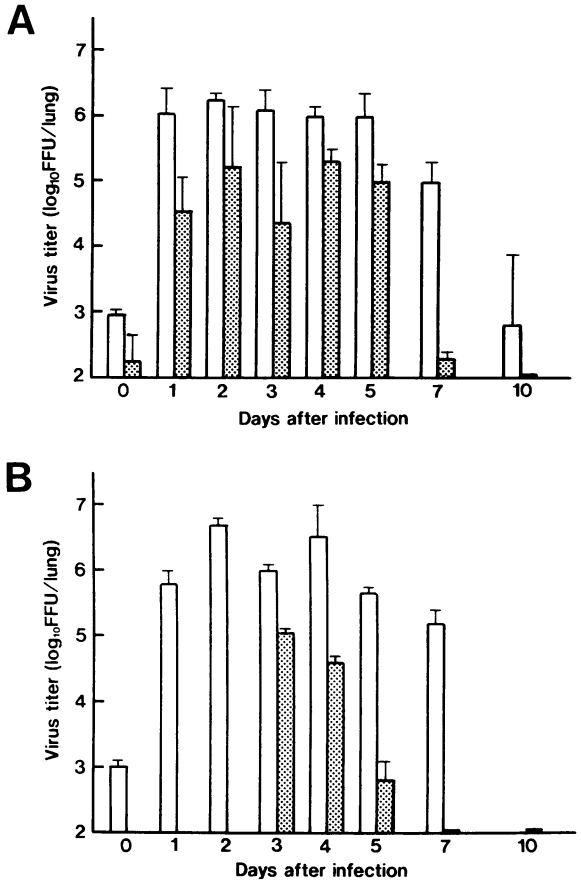

FIG. 3. Titers of virus in the lungs of mice receiving PBS (open bars) or 100 μg of C179 per mouse 1 day before (A; stippled bars) or 1,000 μg of C179 per mouse 2 days after (B; stippled bars) the virus challenge. Three mice each per day in the two groups were sacrificed at days 0 to 5, 7, and 10, and their lungs were harvested. After the homogenization of the lungs, their virus titers were obtained by a previously reported method (16). Means and standard errors are presented.

received PBS alone, had high titers, suggesting that the multiplication of this strain in the mouse lungs was very rapid. The virus titers were almost the same from day 1 to day 5. Although the virus titers in the lungs of mice receiving C179 were significantly lower than those of the control mice, the administration of C179 did not considerably inhibit virus replication. However, on day 7, a remarkable difference in the virus titers between the two groups was observed, with negligible titers in the lungs of mice receiving C179. Similar experiments, in which mice received 1,000 μg of C179 per mouse 2 days after the virus infection, were performed (Fig. 3B). In this case, three mice each per day in the group receiving C179 were sacrificed from day 3 after the infection. All of the control mice died before day 10, which made it impossible to obtain results for the control group on day 10. Even 1 day after C179 administration (day 3), the titers of virus in the lungs of the mice receiving C179 were significantly lower than those of control mice. The virus titers in the group receiving C179 decreased rapidly, with undetectable titers from day 7, indicating that C179 is effective in eradicating infectious virus from the lungs.

The prophylactic effect of C179 against infections with other subtypes of influenza A virus was investigated by use of

TABLE 1. Effect of C179 on the replication of the A/Okuda/57 (H2N2) and A/Aichi/2/68 (H3N2) strains in the lungs of mice

Challenge virus	Infectivity of virus inoculated (FFU/mouse)	Dose of C179 administered (μg/mouse)	No. of mice	Virus titer (mean log_{10} FFU/mouse lung ± SE)
A/Okuda/57 (H2N2)	6.0×10^2	100	5	1.0 ± 0.9
		—[a]	5	3.5 ± 0.1
A/Aichi/2/68 (H3N2)	5.0×10^3	1,000	5	4.5 ± 0.3
		—[a]	5	4.7 ± 0.2

[a] —, One milliliter of PBS instead of C179 was administered.

A/Okuda/57 (H2N2) and A/Aichi/2/68 (H3N2) as the challenge viruses (Table 1). Each mouse received C179 or PBS 1 day before the challenge, and their lungs were harvested 3 days later. As for the challenge with A/Okuda/57, the titers of virus in the lungs of mice receiving 100 μg of C179 were significantly lower than in those of control mice. In contrast, when A/Aichi/2/68 was used as the challenge virus, nearly identical titers in the lungs of mice receiving 1,000 μg of C179 and PBS were observed. The animal experiments described above are consistent with the in vitro studies demonstrating that C179 reacts to H1 and H2 subtypes specifically.

The ability of C179 to neutralize the virus does not seem very high, if the fact that fairly high titers were observed in the lungs of mice receiving C179 is considered (Fig. 3). However, as suggested in the in vitro studies (Fig. 1) in which an inhibition of cell-cell fusion by C179 occurred more effectively than virus neutralization, C179 must have a strong ability to inhibit the spread of infected cells in mouse lungs, which results in high survival rates in the mice receiving C179 (Fig. 2). This idea was also supported by the finding of hemorrhagic lesions in the lungs of mice receiving C179 that were localized only in small areas, in most cases at the sites leading to the bronchi, whereas those of the control mice spread all over the lungs (data not shown).

In the process of obtaining C179, several hundred monoclonal antibodies which showed hemagglutination inhibition activity against the homologous A/Okuda/57 strain were selected. These monoclonal antibodies must recognize the globular head region of HA, because almost all of the HA monoclonal antibodies reported so far recognize the region with hemagglutination inhibition activity (3, 9, 11, 23, 27). Antibodies against the stem region, such as C179, seem to be produced only in small amounts. Since the antigenic determinants in the globular head region are highly variable (17), and, in particular, since antigenic differences in the region among subtypes are very large, it is reasonable that immunity to influenza A virus is subtype specific, which has been reported for natural infections or vaccine trials in humans (4, 21, 24, 25) and for animal experiments (6, 7, 18, 22). However, Mbawuike et al. (10) demonstrated that vaccination of pregnant mice with a high dose of inactivated influenza A virus vaccine protects their offspring not only against the homologous subtype but also against heterologous subtypes and suggested the existence of cross-reactive antibodies which confer cross-protection. In addition, cross-reactive immunity to influenza A virus has been observed in animals (8, 19, 26) as well as humans (2, 14, 20). The observations described above seem to indicate that conserved antigenic sites in the stem region of HA, such as C179, are responsible for a part of the cross-protection.

At present, vaccination with an inactivated influenza virus

vaccine is the only practical measure to control influenza. However, the effect of the current vaccine has incited much controversy, which is mainly due to the antigenic variability of HA. The present study suggests the possibility that the antibody will someday be used for passive immunization against influenza virus. Since influenza virus spreads rapidly in a large population, it is too late to perform a vaccination after the first appearance of the disease in an epidemic season. Therefore, passive immunization with the antibody may be useful for those who have not been vaccinated before. In particular, because the antibody is effective for both prevention and treatment of the disease, it may be most appropriate for use with people with the underlying disease or with the aged who might fall seriously ill if they are infected with the virus. The plan to convert the antibody to a chimeric mouse-human antibody is in progress, and it is hoped that it will be applied for clinical purposes in the near future.

REFERENCES

1. **Arikawa, J., I. Takashima, and N. Hashimoto.** 1985. Cell fusion by hemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. Arch. Virol. **86:**303–313.
2. **Burlington, D. B., P. F. Wright, K. L. Van Wyke, M. A. Phelan, R. E. Mayner, and B. R. Murphy.** 1985. Development of subtype-specific and heterosubtypic antibodies to the influenza A virus hemagglutinin after primary infection in children. J. Clin. Microbiol. **21:**847–849.
3. **Daniels, R. S., S. Jeffries, P. Yates, G. C. Schild, G. N. Rogers, J. C. Paulson, S. A. Wharton, A. R. Douglas, J. J. Skehel, and D. C. Wiley.** 1987. The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-hemagglutinin monoclonal antibodies. EMBO J. **6:**1459–1465.
4. **Frank, A. L., L. H. Taber, and J. M. Wells.** 1983. Individuals infected with two subtypes of influenza A virus in the same season. J. Infec. Dis. **147:**120–124.
5. **Graham, A. K., and M. J. Karnovsky.** 1966. The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of monkey kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. **14:**291–302.
6. **Husseini, R. H., C. Sweet, H. Overton, and H. Smith.** 1984. Role of maternal immunity in the protection of newborn ferrets against infection with a virulent influenza virus. Immunology **52:**389–394.
7. **Jakeman, K. J., H. Sweet, and C. Sweet.** 1989. Mechanisms of immunity to influenza: maternal and passive neonatal protection following immunization of adult ferrets with a live vaccinia-influenza virus hemagglutinin recombinant, but not with recombinants containing other influenza virus proteins. J. Gen. Virol. **70:**1523–1531.
8. **Kurimura, T., A. Hirano, and Y. Okuno.** 1972. The nature of the immunity evoked by infection of mice with avirulent influenza virus. Biken J. **15:**31–37.
9. **Laver, W. G., G. M. Air, R. G. Webster, W. Gerhard, C. W. Ward, and T. A. Dopheide.** 1979. Antigenic drift type A influenza virus: sequence differences in the hemagglutinin of Hong Kong (H3N2) variants selected with monoclonal hybridoma antibodies. Virology **98:**226–237.
10. **Mbawuike, I. N., H. R. Six, T. R. Cate, and R. B. Couch.** 1990. Vaccination with inactivated influenza A virus during pregnancy protects neonatal mice against lethal challenge by influenza A viruses representing three subtypes. J. Virol. **64:**1370–1374.
11. **Nakajima, S., K. Nakajima, and A. P. Kendal.** 1983. Identification of the binding sites to monoclonal antibodies on A/USSR/90/77 (H1N1) hemagglutinin and their involvement in antigenic drift in H1N1 influenza viruses. Virology **131:**116–127.
12. **Nakamura, K.** 1961. Studies on the live vaccine of the influenza A2. Virus **11:**257–266. (In Japanese with English summary.)
13. **Nakamura, K.** 1965. Pathogenicity and immunogenicity of various strains of influenza virus for mice. Biken J. **8:**155–165.
14. **Noble, G. R., H. S. Kaye, A. P. Kendal, and W. R. Dowdle.** 1977. Age-related heterologous antibody responses to influenza virus vaccination. J. Infect. Dis. **136:**S686–S692.
15. **Okuno, Y., Y. Isegawa, F. Sasao, and S. Ueda.** 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J. Virol. **67:**2552–2558.
16. **Okuno, Y., K. Tanaka, K. Baba, A. Maeda, N. Kunita, and S. Ueda.** 1990. Rapid focus reduction neutralization test of influenza A and B viruses in microtiter system. J. Clin. Microbiol. **28:**1308–1313.
17. **Pollock, R. R., J.-L. Teillaud, and M. D. Scharff.** 1984. Monoclonal antibodies: a powerful tool for selecting and analyzing mutations in antigens and antibodies. Annu. Rev. Microbiol. **38:**389–417.
18. **Reuman, P. D., C. M. A. Paganini, E. M. Ayoub, and P. A. Small, Jr.** 1983. Maternal-infant transfer of influenza-specific immunity in the mouse. J. Immunol. **130:**932–937.
19. **Schulman, J. L., and E. D. Kilbourne.** 1965. Induction of partial specific heterotypic immunity in mice by a single infection with influenza A virus. J. Bacteriol. **89:**170–174.
20. **Sonoguchi, T., H. Naito, M. Hara, Y. Takeuchi, and H. Fukumi.** 1985. Cross-subtype protection in humans during sequential, overlapping, and/or concurrent epidemics caused by H3N2 and H1N1 influenza viruses. J. Infect. Dis. **151:**81–88.
21. **Steinhoff, M. C., L. F. Fries, R. A. Karron, M. L. Clements, and B. R. Murphy.** 1993. Effect of heterosubtypic immunity on infection with attenuated influenza A virus vaccines in young children. J. Clin. Microbiol. **31:**836–838.
22. **Sweet, C., R. A. Bird, K. Jakeman, D. M. Coates, and H. Smith.** 1987. Production of passive immunity in neonatal ferrets following maternal vaccination with killed influenza A virus vaccines. Immunology **60:**83–89.
23. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289:**373–378.
24. **Wright, P. F., J. Thompson, and D. T. Karzon.** 1980. Differing virulence of H1N1 and H3N2 influenza strains. Am. J. Epidemiol. **112:**814–819.
25. **Yamane, N., J. Arikawa, T. Odagiri, N. Sukeno, and N. Ishida.** 1978. Isolation of three different influenza A viruses from an individual after probable double infection with H3N2 and H1N1 viruses. Jpn. J. Med. Sci. Biol. **31:**431–434.
26. **Yetter, R. A., W. H. Barber, and P. A. Small, Jr.** 1980. Heterotypic immunity to influenza in ferrets. Infect. Immun. **29:**650–653.
27. **Yewdell, J. W., A. J. Caton, and W. Gerhard.** 1986. Selection of influenza A virus adsorptive mutants by growth in the presence of a mixture of monoclonal antihemagglutinin antibodies. J. Virol. **57:**623–628.