Predicting Antigenic Variants of Influenza A/H3N2 Viruses

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Current inactivated influenza vaccines provide protection when vaccine antigens and circulating viruses share a high degree of similarity in hemagglutinin protein. Five antigenic sites in the hemagglutinin protein have been proposed, and 131 amino acid positions have been identified in the five antigenic sites. In addition, 20, 18, and 32 amino acid positions in the hemagglutinin protein have been identified as mouse monoclonal antibody–binding sites, positively selected codons, and substantially diverse codons, respectively. We investigated these amino acid positions for predicting antigenic variants of influenza A/H3N2 viruses in ferrets. Results indicate that the model based on the number of amino acid changes in the five antigenic sites is best for predicting antigenic variants (agreement = 83%). The methods described in this study could be applied to predict vaccine-induced cross-reactive antibody responses in humans, which may further improve the selection of vaccine strains.

Influenza viruses cause substantial medical and social problems throughout the world, and vaccination is the primary method for preventing influenza and its complications. Of the three types of influenza viruses (A, B, and C), only influenza A and B viruses cause epidemic human disease. Hemagglutinin (HA) and neuraminidase proteins are the two surface antigens that induce protective antibody responses and are the basis for subtyping influenza A viruses. Influenza B viruses are not categorized into subtypes (1). Since 1977, influenza A/H1N1, A/H3N2, and B viruses have been in global circulation, and these three viruses are currently included as vaccine components. Current inactivated vaccines provide essential protection when the vaccine antigens and the circulating viruses share high degree of similarity in the HA protein. Since new influenza virus antigenic variants emerge frequently from accumulation of point mutations in the HA protein (i.e., antigenic drift), influenza vaccine antigens need to be updated frequently, based on the results of global influenza surveillance (1), which includes clinical, virologic, and immunologic surveillance. In virologic surveillance, influenza viruses are characterized antigenically on the basis of ferret serum antibody cross-reactivity. Antigenic variants selected serologically are then tested for antibody cross-reactivity in human sera to evaluate the potential cross-protection against the antigenic variants provided by the current vaccines and to select vaccine strains for the next season (2,3).

The HA protein of influenza viruses is synthesized as a single polypeptide (HA0) that is subsequently cleaved into two polypeptides (HA1 and HA2) and forms into homotrimers. The HA1 polypeptide mutates more frequently than the HA2 polypeptide and plays a major role in natural selection (4,5). Three-dimensional (3-D) structure of the HA protein of A/Aichi/2/68 (H3N2) has been determined, and five antigenic sites on the HA1 polypeptide have been proposed conceptually (4–6). Of the 329 amino acid positions on HA1, 131 lie on or near the five antigenic sites (7,8). Twenty amino acid positions on HA1 have been mapped, based on laboratory variants selected in the presence of mouse monoclonal antibodies (9,10). In addition, 18 amino acid positions have been identified as being under positive selection by comparing 357 viruses isolated from 1984 to 1996 (7). In a recent study, 32 amino acid positions have been identified as diverse codons by comparing 525 viruses isolated from 1968 to 2000 (11). However, the importance of these amino acid positions in terms of predicting antibody cross-reactivity is unclear. Therefore, we conducted this study to explore the usefulness of these amino acid positions for predicting antigenic variants of influenza A/H3N2 viruses. The methods described in this study could be used to predict vaccine-induced cross-reactive antibody responses in humans, which may further improve the selection of vaccine strains.

Methods

Cross-Reactive Antibody Data

In the current global influenza surveillance system, influenza viruses are characterized antigenically based on ferret serum hemagglutinin-inhibition (HAI) antibody cross-reactivity. We first screened publications for
influenza H3N2 virus cross-reactive antibody data. Then, we searched the H3N2 viruses with cross-reactive antibody data for their amino acid sequences of the HA1 polypeptide (www.flu.lanl.gov) (8). Table 1 shows the full name, abbreviation, identification (ID) by type, and accession code of the H3N2 viruses (12–16). Six sets of ferret serum HAI cross-reactivity data were available for analysis. The first set included 11 viruses (55 pairwise comparisons, virus ID: A to K) isolated from 1971 to 1979 (12). The second set included 8 viruses (28 pairwise comparisons, virus ID: J, L to R) isolated from 1979 to 1987 (17). The third set included 10 viruses (45 pairwise comparisons, virus ID: S to AB) isolated from 1989 to 1994 (13). The fourth set included 8 viruses (28 pairwise comparisons, virus ID: AC to AJ) isolated from 1994 to 1996 (18). The fifth set included 5 viruses (10 pairwise comparisons, virus ID: AE, AK to AN) isolated from 1995 to 1999 (15). The sixth set included 6 viruses (15 pairwise comparisons, virus ID: AN to AT) isolated from 1999 to 2002 (16). A mathematical method had been proposed to calculate “antigenic relatedness” between two viruses (presented as a percentage) as a geometric mean of two ratios between antigenic distances of the 55 pairwise comparisons among the four viruses are 640 and 640 and two heterologous titers against each other are 320 and 320, the antigenic relatedness between these two viruses is \((320 \times 320)/(640 \times 640) = 50\%\), and the antigenic distance between these two viruses is \((640 \times 640)/(320 \times 320) = 2\). Table 2 shows the antigenic distances of the 55 pairwise comparisons among the 11 viruses in the first set. In total, 181 pairwise comparisons among 45 viruses were available for analysis. Among the 181 pairwise comparisons, 56 (31%) have an antigenic distance <4 (i.e., similar antigenicity), and 125 (69%) have an antigenic distance ≥4 (i.e., antigenic variant) (21).

Sequence Alignment

Amino acid sequences of the HA1 polypeptide were downloaded from the Los Alamos Influenza Sequence Database (8) or entered from the original publications if they were not available from the Los Alamos Influenza Sequence Database. Amino acid sequences of the 45 viruses were harmonized to same length (329 residues) and were numbered according to A/Aichi/2/68 HA1 sequence because the 3-D structure of the A/Aichi/2/68 hemagglutinin protein has been determined (4–6). Pairwise alignments among the 45 sequences were conducted by using S-Plus 2000 (Insightful Corporation, Seattle, WA). Pairwise-aligned amino acid sequence data were transformed into 0 (without change) and 1 (with change) and were further linked with the pairwise antigenic distance data for predicting analyses.

Predicting Antigenic Variants

The first model was based on amino acid differences in the whole HA1 polypeptide (329 residues). The second
The unusual patterns between antigenic distances and amino acid differences may be due to interaction between amino acid changes in the hemagglutinin or laboratory variability, which needs further experiments to clarify. In addition, A/Victoria/3/75 and A/Victoria/112/76 had only two amino acid differences from A/Sichuan/2/87 (K156E, V186S) and A/Sydney/1/87 (A138S, N193K), but these three viruses were antigenically different (antigenic distance <4). A/Victoria/7/87 had only two amino acid differences from A/Sichuan/2/87 (K156E, V186S) and A/Sydney/1/87 (S138A, K193N), but A/Victoria/7/87 was antigenically different from these two viruses (Table 3). The positions 156, 186, and 193 are located at the antigenic site B and the position 138 is located at the antigenic site A. Moreover, the positions 156 and 193 are also located at the mouse monoclonal antibody-binding sites (online Appendix).

The unusual patterns between antigenic distances and amino acid differences may be due to interaction between amino acid changes in the hemagglutinin or laboratory variability, which needs further experiments to clarify. In addition, A/Victoria/3/75 and A/Victoria/112/76 had only two amino acid differences (L3F, R229G), but they were antigenically different (antigenic distance = 5.7) (Table 3), which also requires further experiments to clarify. The position 3 is not located at any antigenic site, and the position 229 is located at the antigenic site D. We found that 3 of 80 pairwise comparisons with ≥12 amino acid changes had antigenic distance <4 (Figure A).

A/Sydney/5/97 and A/Panama/2007/99 had 12 amino acid differences, but these two viruses were antigenically similar (antigenic distance = 1.4) based on ferret serum HAI titers (Table 3). However, inactivated vaccines containing A/Sydney/5/97 induced low serum antibody titers against A/Panama/2007/99 in humans; therefore, A/Sydney/5/97 was replaced by A/Panama/2007/99 as the vaccine strain for the 2000–01 season (3). A/HK/1550/2002 had 12 amino acid differences from A/Chile/6416/01 and 14 amino acid differences from A/Fujian/140/2000, but A/HK/1550/2002 was antigenically similar to these two viruses (Table 3). These three com-

Table 2. Antigenic distance (upper right) and number of amino acid changes in the HA1 (lower left) in 55 pairwise comparisons among 11 influenza H3N2 viruses

| Virus ID | A   | B   | C   | D   | E   | F   | G   | H   | I   | J   | K   |
|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A. HK/71 | 27.7| 19.6| 39.2| 55.4| 39.2| 48.0| 39.2| 110.9| 67.9| 110.9|
| B. ENG/72 | 15  |     | 4.0 | 26.1| 16.0| 64.0| 156.8| 4.0 | 64.0| 78.4| 181.0|
| C. PC/73  | 16  | 7   | 8.0 | 16.0| 32.0| 27.7| 22.6| 37.0| 55.4| 90.5|
| D. MC/75  | 21  | 12  | 12  | 9.2 | 32.0| 45.3| 32.0| 90.5| 55.4| 90.5|
| E. VIC/75 | 30  | 19  | 19  | 15  | 11.3| 27.7| 1.9  | 5.7 | 78.4| 128.0|
| F. TOLK/75| 20  | 17  | 18  | 16  | 20  | 78.4| 45.3| 26.1| 39.2| 90.5|
| G. ENG/75 | 27  | 18  | 17  | 8   | 17  | 22  | 32.0| 4.6  | 6.9 | 19.6|
| H. AC/76  | 31  | 20  | 18  | 16  | 6   | 21  | 19  | 9.2  | 78.4| 73.9|
| I. VIC/76 | 32  | 21  | 19  | 17  | 2   | 22  | 19  | 4   | 27.7| 32.0|
| J. BAN/1/79| 36  | 24  | 24  | 22  | 26  | 33  | 20  | 28  | 28  | 3   |
| K. BAN/2/79| 36  | 24  | 24  | 22  | 26  | 33  | 20  | 28  | 28  | 3   |

1ID, identification.
comparisons may indicate that interaction of multiple amino acid changes could potentially preserve the 3-D structure of HA1. Alternatively, the ferret serum HAI assay system is not sensitive enough to detect the antigenic difference.

Model Two

Figure B shows the scatterplot between antigenic distance and number of amino acid changes in the five antigenic sites (131 amino acid positions). Among the 181 pairwise comparisons, amino acid changes in the five antigenic sites ranged from 1 to 32. Overall, the antigenic distance correlated to number of amino acid changes in the five antigenic sites (R = 0.77, p < 0.001). Different cutoffs of amino acid changes in the previously defined 20 amino acid positions were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥7 amino acid changes, which shows that the NPV was 71% (42/59), PPV was 89% (108/122), and agreement was 83% (150/181) (Figure B).

Model Three

Figure C shows the scatter plot between antigenic distance and number of amino acid changes in the 20 amino acid positions related to mouse monoclonal antibody binding. Overall, the antigenic distance correlated to number of amino acid changes in the 20 amino acid positions (R = 0.74, p < 0.001). Different cutoffs of amino acid changes in the 20 amino acid positions were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥2 amino acid changes, which shows that the NPV was 64% (32/50), PPV was 82% (107/131), and agreement was 77% (139/181) (Figure C).

Model Four

Figure D shows the scatterplot between antigenic distance and number of amino acid changes in the 18 amino acid positions under positive selection. Overall, the antigenic distance correlated moderately to number of amino acid changes in the 18 amino acid positions (R = 0.43, p < 0.001). Different cutoffs of amino acid changes in the 18 amino acid positions were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥1 amino acid changes, which shows that the NPV was 55% (6/11), PPV was 71% (120/170), and agreement was 70% (126/181) (Figure D).

Model Five

Figure E shows the scatter plot between antigenic distance and number of amino acid changes in the 32 codons with substantial diversity. Overall, the antigenic distance correlated moderately to number of amino acid changes in the 32 codons (R = 0.68, p < 0.001). Different cutoffs of amino acid changes in the 32 codons were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥2 amino acid changes, which shows that the NPV was 72% (13/18), PPV was 74% (120/163), and agreement was 74% (133/181) (Figure E). Overall, the model based on the number of amino acid changes in the five antigenic sites has the highest correlation to the antigenic distance (R = 0.77) and the best performance for predicting antigenic variants (agreement = 83%).

Discussion

Wilson and Cox proposed that a drift variant of epidemiologic importance usually contains ≥4 amino acid changes in the five antigenic sites were evaluated for predicting antigenic variants. The highest agreement was found by using a cutoff of ≥7 amino acid changes, which shows that the NPV was 71% (42/59), PPV was 89% (108/122), and agreement was 83% (150/181) (Figure B).
changes located on ≥2 of the five antigenic sites, but they did not specify the amino acid positions in the five antigenic sites (5). Our study further showed that the model based on the number of amino acid changes in the 131 amino acid positions in the five antigenic sites had the highest correlation to the antigenic distance and the best performance for predicting antigenic variants. Theoretically, not all 131 amino acid positions in the five antigenic sites play a critical role in determining antigenicity, and some immunodominant positions (i.e., major antibody-binding sites) could be identified by using bioinformatics models and reverse genetic techniques (23–25). A model based on the immunodominant positions can potentially have a better performance than the model based on the five antigenic sites.

The model based on the 20 amino acid positions related to mouse monoclonal antibody binding only have moderate performance for predicting antigenic variants (R = 0.74, agreement = 77%), which indicates that mouse and ferret antibodies may recognize different B-cell epitopes. In addition, that models four and five have a low performance for predicting antigenic variants is not surprising, since these two models identified the amino acid positions only on the basis of virus sequence data without incorporating antigenic properties.

Antigenic variants of influenza viruses are currently determined with the ferret serum HAI assay. The ferret serum HAI assay works well to distinguish major drift variants, but moderate differences are difficult to define reliably (26). As shown in Table 3, some unusual patterns between antigenic distance and amino acid changes in the HA1 may be caused by laboratory variability of the ferret serum HAI assay. The prediction models proposed in the present study may perform better if a more reliable assay system is used. Several studies have shown that neutralization assays are more sensitive for detecting influenza virus antibody responses than HAI assays (27,28). However, traditional neutralization assays based on cytopathic effect are labor-intensive and not suitable for a large-scale surveillance system. A simplified EIA-based neutralization assay may be the potential solution (29).

Several studies have documented that one to three amino acid changes in the HA1 of influenza H1N1 and H3N2 viruses could possibly reduce the antigenicity and efficacy of inactivated vaccines in animal models (30–33), which are consistent with our results (Table 3). In animal studies, single mutation at amino acid position 156 of the HA1 of two H3N2 viruses was linked to the reduced antigenicity (32,33). The position 156 is located at the antigenic site B and the mouse monoclonal antibody-binding site (see online Appendix). Overall, this evidence may indicate the existence of immunodominant positions in the HA1 and emphasize the importance of identifying the immunodominant positions to monitor the selection of vaccine strains and the process of vaccine manufacturing.

The current global surveillance system largely relies on ferret serum HAI data for selection of influenza vaccine strains and the process of vaccine manufacturing. Several studies have documented that one to three amino acid changes in the HA1 of influenza H1N1 and H3N2 viruses could possibly reduce the antigenicity and efficacy of inactivated vaccines in animal models (30–33), which are consistent with our results (Table 3).

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References

1. Bridges CB, Fukuda K, Uyeki TM, Cox NJ, Singleton JA. Prevention and control of influenza. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recomm Rep. 2002;51:1–31.

2. Klimov A, Simonsen L, Fukuda K, Cox N. Surveillance and impact of influenza in the United States. Vaccine. 1999;17(Suppl 1):S42–6.

3. World Health Organization. Recommended composition of influenza virus vaccines for use in the 2000–2001 season. Wkly Epidemiol Rec. 2001;75:61–8.

4. Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature. 1981;289:373–8.

5. Wilson IA, Cox N. Structural basis of immune recognition of influenza virus hemagglutinin. Annu Rev Immunol. 1990;8:737–71.

6. Kilbourne ED. Future influenza vaccines and the use of genetic recombinants. Bull World Health Organ. 1969;41:643–5.

7. Bush RM, Bender CA, Subbarao K, Cox NJ, Fitch WM. Predicting the evolution of human influenza A. Science. 1999;286:1921–5.

8. Macken C, Lu H, Goodman J, Boykin L. The value of a database in surveillance and vaccine selection. In: Osterhaus ADME, Cox N, Hampson AW, editors. Options for the control of influenza IV. Amsterdam: Elsevier Science; 2001. p. 103–6.

9. Air GM, Luver WG. Antigenic structure of influenza viruses. In: van Regenmortel MHV, Neurath AR, editors. Immunobiology of viruses. Oxford: Elsevier; 1985. p. 213–38.

10. Thomas DB, Patera AC, Subbarao K, Cox NJ, Fitch WM. Predicting the evolution of human influenza A. Science. 1999;286:1921–5.

11. Plotkin JB, Dushoff J. Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. Proc Natl Acad Sci U S A. 2003;100:7152–7.

12. Both GW, Sleigh MJ, Cox NJ, Kendal AP. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J Virol. 1983;48:52–60.

13. Ellis JS, Chakraverty P, Clewley JP. Genetic and antigenic variation in the haemagglutinin of recently circulating human influenza A (H3N2) viruses in the United Kingdom. Arch Virol. 1995;140:1889–1904.

14. Besselaar TG, Schoub BD, Blackburn NK. Impact of the introduction of A/Sydney/5/97 H3N2 influenza virus into South Africa. J Med Virol. 1999;59:561–8.

15. Coiras MT, Aguilar JC, Galiano M, Carlos S, Gregory V, Lin YP, et al. Rapid molecular analysis of the haemagglutinin gene of human influenza A H3N2 viruses isolated in spain from 1996 to 2000. Arch Virol. 2001;146:2133–47.

16. Centers for Disease Control and Prevention. Information for the Vaccines and Related Biological Products Advisory Committee, CBER, FDA, Atlanta: The Centers; 2003. p. 28. [cited 29 Jan 2004] Available from http://www.fda.gov/ohrms/dockets/ac/03/briefing/3922B1_2.pdf

17. World Health Organization. Recommended composition of influenza virus vaccines for use in the 1988–1989 season. Wkly Epidemiol Rec. 1988;63:57–9.

18. Centers for Disease Control and Prevention. Information for FDA vaccine advisory panel meeting. Atlanta: The Centers; 1997. p. 30.

19. Archetti I, Horsfall FL. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J Exp Med. 1950;92:441–62.

20. Kilbourne ED, Johansson BE, Grajower B. Independent and disparate evolution in nature of influenza A virus hemagglutinin and neuraminidase glycoproteins. Proc Natl Acad Sci U S A. 1990;87:786–90.

21. Schild GC, Henry-Aymard M, Pereira MS, Chakraverty P, Dowdle W, Coleman M, et al. Antigenic variation in current human type A influenza viruses: antigenic characteristics of the variants and their geographic distribution. Bull World Health Organ. 1973;48:269–78.

22. Greiner M, Sohr D, Gobé P. A modified ROC analysis for the selection of cutoff values and the definition of intermediate results of sero-diagnostic tests. J Immunol Methods. 1995;185:123–32.

23. Lee MS, Chen J. Identifying potential immunodominant amino acid positions in hemagglutinin protein of influenza A H3N2 viruses. In: Options for the control of influenza V, Okinawa, Japan, October 7–11, 2003. Okinawa, Japan: International Organising Committee of Options; 2003.

24. Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A. Rescue of influenza A virus from recombiant DNA. J Virol. 1999;73:9679–82.

25. Hoffmann E, Neu mann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A. 2000;97:6108–13.

26. Smith DJ. Applications of bioinformatics and computational biology to influenza surveillance and vaccine strain selection. Vaccine. 2003;21:1758–61.

27. Belshe RB, Gruber WC, Mendelman PM, Mehta HB, Mahmood K, Reisinger K, et al. Correlates of immune protection induced by live, attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine. J Infect Dis. 2000;181:1133–7.

28. Lee MS, Mahmood K, Adhikary L, August MJ, Cordova J, Cho I, et al. Measuring antibody responses to a live-attenuated influenza vaccine in children. Pediatr Infect Dis J. Sept. 2004.

29. Lee MS, Cohen B, Hand J, Nokes DJ. A simplified and standardized neutralization enzyme immunoassay for the quantification of measles neutralizing antibody. J Virol Methods. 1999;78:209–17.

30. Wood JM, Oxf ord JS, Una D, Newman RW, Major D, Robertson JS. Influenza A (H1N1) vaccine efficacy in animal models is influenced by two amino acid substitutions in the hemagglutinin molecule. Virology. 1989;171:214–21.

31. Newman RW, Jennings R, Major DL, Robertson JS, Jenkins R, Potter CW, et al. Immune response of human volunteers and animals to vaccination with egg-grown influenza A (H1N1) virus is influenced by three amino acid substitutions in the haemagglutinin molecule. Vaccine. 1993;11:400–6.

32. Katz JM, Webster RG. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. J Infect Dis. 1989;160:191–8.

33. Kodihalli S, Justewicz DM, Gubareva LV, Webster RG. Selection of a single amino acid substitution in the hemagglutinin molecule by chicken eggs can render influenza A virus (H3) candidate vaccine ineffective. J Virol. 1995;69:4888–97.

34. Nolan T, Lee MS, Cordova JM, Cho I, Walker RE, August MJ, et al. Safety and immunogenicity of a live-attenuated influenza vaccine blended and filled at two manufacturing facilities. Vaccine. 2003;21:1224–31.

35. Lee MS, Yang CF. Cross-reactive H1N1 antibody responses to a live-attenuated influenza vaccine in children: implication for selection of vaccine strains. J Infect Dis 2003;188:1362–6.

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### Appendix. Amino acid positions compared in this study

| aa | Antigenic sites | MAb-20 | PS-18 | D-32 | aa | Antigenic sites | MAb-20 | PS-18 | D-32 |
|----|----------------|--------|-------|------|----|----------------|--------|-------|------|
| 2  |                | X      | 172   | D    |    |                |        |       |      |
| 3  |                | X      | 173   | D    |    |                |        |       |      |
| 5  |                | X      | 174   | D    |    |                |        |       |      |
| 31 |                | X      | 175   | D    |    |                |        |       |      |
| 44 |                | C      | 176   | D    |    |                |        |       |      |
| 45 |                | C      | 177   | D    |    |                |        |       |      |
| 46 |                | C      | 179   | D    |    |                |        |       |      |
| 47 |                | C      | 182   | D    |    |                |        |       |      |
| 48 |                | C      | 186   | B    | X  |                |        |       |      |
| 50 |                | C      | 187   | B    |    |                |        |       |      |
| 51 |                | C      | 188   | B    | X  |                |        |       |      |
| 53 |                | C      | 189   | B    | X  |                |        |       |      |
| 54 |                | C      | 190   | B    | X  |                |        |       |      |
| 57 |                | E      | 192   | B    |    |                |        |       |      |
| 59 |                | E      | 193   | B    | X  |                |        |       |      |
| 62 |                | E      | 194   | B    | X  |                |        |       |      |
| 63 |                | E      | 196   | B    | X  |                |        |       |      |
| 67 |                | E      | 197   | B    | X  |                |        |       |      |
| 75 |                | E      | 198   | B    | X  |                |        |       |      |
| 78 |                | E      | 201   | D    |    |                |        |       |      |
| 80 |                | E      | 203   | D    |    |                |        |       |      |
| 81 |                | E      | 205   |      | X  |                |        |       |      |
| 82 |                | E      | 207   | D    |    |                |        |       |      |
| 83 |                | E      | 208   | D    |    |                |        |       |      |
| 86 |                | E      | 209   | D    |    |                |        |       |      |
| 87 |                | E      | 212   | D    |    |                |        |       |      |
| 88 |                | E      | 213   | D    |    |                |        |       |      |
| 91 |                | E      | 214   | D    |    |                |        |       |      |
| 92 |                | E      | 215   | D    |    |                |        |       |      |
| 94 |                | E      | 216   | D    |    |                |        |       |      |
| 96 |                | D      | 217   | D    |    |                |        |       |      |
| 102|                | D      | 218   | D    | X  |                |        |       |      |
| 103|                | D      | 219   | D    |    |                |        |       |      |
| 109|                | E      | 220   |      | X  |                |        |       |      |
| 112|                | X      | 226   | D    |    |                |        |       |      |
| 117|                | D      | 227   | D    |    |                |        |       |      |
| 121|                | D      | X      | X    | 228 | D    |        |       |      |
| 122|                | A      |        |      | 229 | D    |        |       |      |
| Position | Amino Acid |.a | .b |
|----------|------------|---|---|
| 124      | A          | X | X |
| 126      | A          |   | 238 | D |
| 128      | B          |   | 240 | D |
| 129      | B          |   | 242 | D |
| 130      | A          |   | 244 | D |
| 131      | A          | X | 246 | D |
| 132      | A          |   | 247 | D |
| 133      | A          | X | X | X | 248 | D |
| 135      | A          | X | X | X | 260 | E |
| 137      | A          |   |   | 261 | E |
| 138      | A          | X |   | 262 | E | X | X |
| 140      | A          |   |   | 265 | E |   |   |
| 142      | A          | X | X | X | 271 |   | X |
| 143      | A          | X |   | 273 | C |
| 144      | A          | X | X |   | 275 | C | X | X |
| 145      | A          | X | X | X | 276 | C | X |   |
| 146      | A          | X |   | 278 | C | X |
| 150      | A          |   |   | 279 | C |
| 152      | A          |   |   | 280 | C |
| 155      | B          |   |   | 294 | C |
| 156      | B          | X | X | X | 297 | C |   |
| 157      | B          | X | X |   | 299 | C | X |
| 158      | B          | X | X | X | 300 | C |   |
| 159      | B          |   |   | 304 | C |
| 160      | B          |   |   | 305 | C |
| 163      | B          |   |   | 307 | C |
| 164      | B          |   |   | 308 | C |
| 165      | B          | X |   | 309 | C |
| 167      | D          |   |   | 310 | C |
| 168      | A          |   |   | 311 | C |
| 170      | D          |   |   | 312 | C |
| 171      | D          |   |   |   |   |

*a, amino acid; MAb, monoclonal antibody.

b Among the 329 amino acid residues in the HA1 polypeptide, 131, 20, 18, and 32 amino acid positions have been identified as in the five antigenic sites (A, B, C, D, E), the mouse monoclonal antibody-binding sites (MAb-20), the positively selected codons (PS-18), and the codons with substantial diversity (D-32), respectively.