Archives of Virology 84, 251—259 (1985)

Archives of Virology

Antigenic Analysis of H4 Influenza Virus Isolates Using Monoclonal Antibodies to Defined Antigenic Sites on the Hemagglutinin of A/Budgerigar/Hokkaido/1/77 Strain

By

T. Ito, H. Kida, and R. Yanagawa

Department of Hygiene and Microbiology,
Faculty of Veterinary Medicine, Hokkaido University,
Sapporo, Japan

Accepted July 24, 1984

Summary

Three non-overlapping antigenic sites were defined on the hemagglutinin of avian influenza virus A/budgerigar/Hokkaido/1/77 (H4N6) by competitive binding assay of monoclonal antibodies to the virus and comparative antigenic analysis of variants selected with monoclonal antibodies. Antigenic relationship among 25 H4 influenza viruses of different bird origin was examined by ELISA with the monoclonal antibodies to each of defined antigenic sites. Two of the three antigenic sites contained epitopes specific to the H4 influenza viruses of budgerigar and mynah origin, and the remaining site contained an epitope which was cross-reactive with almost all of the H4 influenza viruses.

Introduction

Attention has been focused on avian influenza viruses since evidences were found suggesting that new pandemic human influenza viruses arise from influenza viruses infecting lower mammals or birds either by direct transmission or by genetic recombination between human and animal viruses (11, 18, 19). Therefore, antigenic analyses of the hemagglutinin, with the aid of monoclonal antibodies, like those which have been done with human influenza viruses (H1 and H3) (3, 13, 20) and seal influenza virus (H7) (9), are required for avian influenza viruses.

Influenza A viruses possessing H4 hemagglutinin are one of the most frequently isolated subtype of viruses from bird species of a wide host range and geographic distribution (6). Fukushi *et al.* (2) prepared monoclonal

antibodies to the hemagglutinin of A/budgerigar/Hokkaido/1/77 (Bg/77) (H4N6) and A/duck/Czechoslovakia/56(H4N6) influenza viruses and used those antibodies for the study of antigenic comparison of H4 influenza virus isolates. Twenty-five H4 avian influenza viruses of different bird origin were divided into 4 groups based on the reactivity patterns with these monoclonal antibodies in HI tests.

In the present study, an operational antigenic map of the hemagglutinin of Bg/77 virus was established using the monoclonal antibodies to the hemagglutinin of Bg/77 virus. The antigenic relationship among H4 avian influenza viruses of different bird origin was examined with the monoclonal antibodies to each of the defined antigenic sites. ELISA was used for the examination because the presence of epitopes on the hemagglutinin molecules, which are not detectable by HI test, has been shown (9, 12, 23), and antibody binding to antigen is directly detectable by ELISA.

From the results obtained, on the hemagglutinin of Bg/77 virus three non-overlapping antigenic sites were defined. Two of the three antigenic sites contained epitopes specific to the group I H4 influenza viruses (2), and the remaining site contained an epitope which was cross-reactive with almost all of the H4 influenza viruses.

Materials and Methods

Viruses

The H4 influenza viruses used in this study are given in Table 4; they had been maintained in the respository at the Department of Hygiene and Microbiology, Faculty of Veterinary Medicine, Hokkaido University. The viruses were grown in the allantoic cavity of 11-day-old chicken embryonated eggs for 48 hours at 35° C. The allantoic fluids of the eggs were used as the intact virus antigens. Purification of the virus was done by differential centrifugation and sedimentation through a sucrose gradient (10—50 per cent in phosphate buffered saline, pH 7.2) (7). Hemagglutinin rosettes were prepared from the purified virus by treatment with an equal volume of ether for 30 minutes at 20° C in the presence of 0.1 per cent Tween 20 (23).

Monoclonal Antibodies

Ascitic fluids obtained from BALB/c mice which had been inoculated with the hybridoma cell lines producing monoclonal antibodies to the hemagglutinin of Bg/77 virus (2) were used. The immunoglobulin subclass was determined by double immunodiffusion using specific antisera to each immunoglobulin isotype (Miles Lab., Inc., Elkhart, Ind., U.S.A.).

Serological Tests

Hemagglutination-inhibition (HI) tests were done by the micromethod (15). Enzyme-linked immunosorbent assays (ELISA) were performed according to Kida *et al.* (9). Neutralization assays were done in Mardine Darby canine kidney (MDCK) cells (17). Titers were expressed as the reciprocals of antibody dilutions in agar overlay which caused 50 per cent plaque reduction of 100—200 PFU of virus (9).

Competitive Binding Assay

The competitive binding assays were performed as described by KIMURA-KURODA and YASUI (10). The purified Bg/77 virus at a limiting concentration was used as antigen. Serial dilutions of each competing antibody from the ascitic fluids were added to Bg/77 virus-coated wells in a microplate. Binding of purified antibody conjugated with horseradish peroxidase to the virus was examined. Normal ascitic fluids were prepared from mice inoculated with Sp2/0-Ag14 cells (16) and used as a control. The percentage of competition was determined by the formula [100(A—n)/(A—B)], where A is optical density at a wave length of 405 nm (OD_{405}) in the absence of competing antibody, B is OD_{405} in the presence of the homologous antibody and n is OD_{405} in the presence of competitor. The competition titers were expressed as the reciprocal of the dilution of the competitors that caused 50 per cent competition.

Selection of Antigenic Variants

Selection of antigenic variants in the presence of monoclonal antibodies and determination of frequency of the variants were preformed as described previously (9, 22).

Results

Characterization of Monoclonal Antibodies to the Hemagglutinin of Bg/77 Virus

In the present study, 9 monoclonal antibodies were used (Table 1). These antibody preparations all inhibited hemagglutination of homologous virus and reacted with the virus at high titers in ELISA binding assay, but did not bind to A/chicken/Germany'N'/49(H10N7) virus of a different subtype (data not shown). Eight of the 9 monoclonal antibodies effectively neutralized infectivity of Bg/77 virus, while the remaining one, B10, showed only a low titer of neutralization activity.

Table 1. *Characterization of the monoclonal antibodies to A/budgerigar/Hokkaido/1/77 hemagglutinin*

Monoclonal antibodies	Isotype	HI[a] titers	ELISA titers ($\times 10^{-2}$)	Neutralization titers ($\times 10^{-2}$)
B3	IgG1	640	12,416	1,280
B6	IgG1	320	16,384	640
B7	IgG1	5,120	14,265	2,560
B9	IgG1	320	18,820	640
B14	IgG1	640	8,192	1,940
B18	IgG2a	2,560	21,618	3,380
B19	IgM	320	16,384	2,940
B21	IgG1	2,560	2,712	750
B10	IgM	160	14,203	60

Titers are expressed as the reciprocals of antibody dilution

[a] Intact virus particles of Bg/77 were used as antigen in HI tests

Antigenic Mapping of the Hemagglutinin Molecule of Bg/77 Virus

In order to define antigenic sites on the Bg/77 hemagglutinin molecule, 9 monoclonal antibodies were examined by cross competitive binding assay with Bg/77 virus. On the basis of the results obtained, 9 monoclonal antibodies were divided into 2 groups (8 antibodies except B10 and B10) (Table 2). This result shows that these antibodies bind to the epitopes in

Table 2. *Competitive binding of the monoclonal antibodies to the hemagglutinin of Bg/77 virus*

Competitors	Competition titers[a] with the following labeled monoclonal antibodies ($4^n \times 10^{-2}$)								
	B3	B6	B14	B9	B19	B7	B18	B21	B10
B3	7	—	—	—	—	—	—	—	—
B6	±	1	1	1	1	1	1	1	—
B14	±	4	5	3	2	±	±	±	—
B9	1	2	5	4	4	5	3	6	—
B19	4	4	5	5	5	6	4	7	—
B7	±	1	1	4	5	3	4	4	—
B18	4	4	5	5	5	3	5	6	—
B21	±	1	±	4	3	3	2	1	—
B10	—	—	—	—	—	—	—	—	5

[a] Titers are expressed as the reciprocal of competitor dilution that cause 50 per cent inhibition of labeled antibody binding; — indicates no competition, ± indicates that the competitor partially interfered with the binding of the labeled antibody

Table 3. *Reactivity patterns of antigenic variants of Bg/77 virus selected with monoclonal antibodies*

Sites	Monoclonal antibodies no.	Reactivity of antigenic variants selected with the following monoclonal antibodies								Frequency ($\log_{10}$) of variants in Bg/77 virus selected with monoclonal antibody
		B3	B6	B14	B9	B19	B7	B18	B21	
A	B3	—[a]	—	—						—6.84
	B6	—	—	—						—7.00
	B14	—	—	—	—	—				—5.33
B	B9				—	—	—	—	—	—5.17
	B19				—	—	—	—	—	—6.00
	B7				—	—	—	—	—	—6.67
	B18				—	—	—	—	—	—5.33
	B21						—	—	—	—6.17
C	B10[b]									

[a] Each of the monoclonal antibodies was titrated by ELISA against each of the variants; no entry indicates significant binding to variants, — indicates no binding of the monoclonal antibody to the variant

[b] B10 antibody showed only a low titer of neutralization activity

two different areas on the Bg/77 virus hemagglutinin. A panel of antigenic variants was then selected in the presence of the neutralizing monoclonal antibodies, and the reactivity of these viruses with each of the antibodies was examined to define antigenic sites. Using the 8 antigenic variants

Table 4. *Cross reaction among H4 influenza viruses with monoclonal antibodies to the three different antigenic sites on the hemagglutinin molecule of Bg/77 virus revealed by ELISA and HI test*

		Monoclonal antibodies								
		Site A			Site B					Site C
Groups[a]	Viruses	B3	B6	B14	B9	B19	B7	B18	B21	B10
I	A/budgerigar/Hokkaido/1/77	EH[b]	EH	EH	EH	EH	EH	EH	EH	EH
	A/budgerigar/Hokkaido/3/78	EH	EH	EH	EH	EH	EH	EH	EH	E
	A/budgerigar/Hokkaido/6/78	E(H)	EH	EH	EH	EH	EH	EH	EH	E
	A/budgerigar/Tottori/1/77	EH	EH	EH	E(H)	EH	EH	EH	EH	E(H)
	A/budgerigar/Hokkaido/1/78	EH	EH	EH	EH	EH	EH	EH	EH	E
	A/mynah/Tokyo/21/77	EH	EH	EH	EH	EH	EH	EH	EH	E(H)
	A/mynah/Tokyo/22/77	EH	EH	EH	EH	EH	EH	EH	EH	E(H)
II	A/duck/Hong Kong/200/77									E
	A/duck/Alberta/211/79									
	A/duck/Czechoslovakia/56									E
	A/duck/Hokkaido/6/80									E
III	A/duck/Miyagi/46/77									E
	A/duck/Alberta/119/79									E
	A/turkey/Minnesota/1277/81									E
	A/duck/Alberta/24/76									
	A/duck/Minnesota/978/80									E
	A/duck/Hokkaido/2/81									E
	A/duck/Alberta/26/76									E
	A/duck/Alberta/157/77									E
	A/duck/Alberta/209/77									E
	A/duck/Alberta/280/77									E
	A/turkey/Minnesota/833/79									E
	A/duck/Minnesota/965/80									E
	A/duck/Alberta/286/78									E
IV	A/chicken/Alabama/1/75									E

[a] FUKUSHI *et al.* (2)

[b] 'H' represents positive ($\geq$80) in HI test with the virus
'E' represents positive ($\geq$400) in ELISA with the virus. Blank represents negative in both serological tests
'(H)' represents positive ($\geq$80) in HI test using isolated hemagglutinin rosettes of the virus as antigen

obtained, reactivity of a series of monoclonal antibodies was determined by ELISA (Table 3). The monoclonal antibodies were divided into 3 groups based on the reactivity patterns. The 8 monoclonal antibodies, which were included in one group on the basis of the results obtained by competitive binding assay, were further divided into 2 groups. The remaining monoclonal antibody, B10, bound to all variants, indicating that the epitope recognized by B10 is located on an independent site on the hemagglutinin. On the hemagglutinin molecule of Bg/77 virus, three different antigenic sites, designated as sites, A B, and C, were thus defined (Table 3).

Antigenic Analysis of the Hemagglutinin of H4 Influenza Viruses of Different Bird Origin Using Monoclonal Antibodies to Each of the Defined Antigenic Sites by ELISA and HI Tests

Antigenic relationship among 25 H4 influenza viruses which were isolated from a variety of bird species in different districts of the world was examined using monoclonal antibodies to each of three different antigenic sites by ELISA and HI tests (Table 4). By ELISA all 8 antibodies to sites A and B bound to group I H4 influenza viruses (2) of budgerigar and mynah origin. The results showed that two of three antigenic sites contained epitopes specific to the group I viruses. The remaining antibody B10 bound to almost all of the H4 influenza viruses, indicating that site C contained a cross-reactive epitope. By HI tests using intact virus as antigen, some of the monoclonal antibodies which were positive in ELISA showed negative (only 'E' designated in Table 4). Especially, the antibody B10 reacted only with homologous Bg/77 virus in HI test, whereas it reacted with almost all of the H4 influenza viruses in ELISA. Following application of isolated hemagglutinin rosettes as antigens to HI tests, some epitopes became detectable ['(H)' designated in Table 4]. These epitopes belonged to group I viruses.

Discussion

An operational antigenic map of the hemagglutinin molecule of an avian influenza virus A/budgerigar/Hokkaido/1/77(H4N6) using monoclonal antibodies defined three non-overlapping antigenic sites on the molecule. Comparative analysis of the reactivity of a panel of antigenic variants selected in the presence of monoclonal antibodies to the hemagglutinin with each of the antibodies revealed more detailed antigenic configuration of the hemagglutinin compared to the assay of cross competitive ELISA binding to the virus between the antibodies. This fact may be explained by the following possibilities. In competitive binding, epitopes which are closely located to each other on the hemagglutinin molecule, even in distinct sites, may not be distinguished since the physical bulk of the antibody molecules

sterically interferes with mutual binding. In addition, the binding of some antibodies may induce conformational changes in the hemagglutinin molecule which alter the epitopes recognized by another antibody (1, 4, 13, 14).

The isolation of antigenic variants from avian influenza viruses with monoclonal antibodies has not been accomplished before. The figure of the frequency at which variants were isolated in the present study is in agreement with the range with human and seal strains (9, 20, 22). This finding indicates that under the pressure of antibodies, antigenic drift may occur in an avian influenza virus, similar to that in human strains. FUKUSHI *et al.* (2), however, showed that epitopes of A/duck/Czechoslovakia/56 virus have been conserved on the hemagglutinin of A/duck/Hokkaido/6/80 on the basis of the reactivity pattern with the monoclonal antibodies in HI test. The conservation of epitopes on the hemagglutinin of avian strains may be due to rapid spread of viruses from one bird to others in gregarious birds which do not possess the antibodies to the virus, or poor antibody response to avian influenza viruses in some species of birds (5, 8).

Using the monoclonal antibodies to the different antigenic sites defined in the present study, the antigenic relationship among H4 influenza viruses of different bird origin was examined by ELISA. All antibodies to the three antigenic sites bound to group I viruses of budgerigar and mynah origin, indicating an intimate relationship among the hemagglutinins of these viruses. The antibodies to two of the three antigenic sites (sites A and B) bound only to group I viruses. The remaining antibody to site C reacted with almost all of the H4 influenza viruses tested (group I—IV). This figure may be correlated to the fact that the antibody to site C did not effectively neutralize the infectivity of Bg/77 virus, in contrast to those to sites A and B, which showed the neutralization activity.

In the present study we used ELISA for antigenic analysis, since it has been shown that on the hemagglutinin molecule of influenza viruses there are epitopes which are not detectable by HI test (9, 12, 23). The comparison between the results in ELISA binding assay and in HI test revealed that also on the H4 influenza virus hemagglutinin are epitopes which are not detected by HI test. Especially, antibody B10 to site C inhibited hemagglutination only of homologous Bg/77 virus, yet bound to almost all of the H4 influenza viruses tested, as shown by ELISA. Therefore, it was presumed that site C is located in an area which is sterically farther away from the receptor binding site on the hemagglutinin molecule of these viruses than the sites which were defined on the hemagglutinin of human H3 virus (21). KIDA *et al.* (9) showed that in HI tests the use of isolated hemagglutinin rosettes as antigen made more epitopes detectable. On the hemagglutinin of H4 influenza viruses, epitopes undetectable even by using hemagglutinin rosettes in HI tests were found. For detailed antigenic analysis, therefore, ELISA binding assay is more advisable. When ELISA

is used for screening assay of hybridoma cells producing monoclonal antibodies, antigenic site(s) other than those defined in the present study might be recognized on the hemagglutinin molecule of Bg/77 virus.

References

1. Clegg, J. C. S., Chanas, A. C., Gould, E. A.: Conformational changes in sindbis virus E 1 glycoprotein induced by monoclonal antibody binding. J. gen. Virol. **64**, 1121—1126 (1983).
2. Fukushi, H., Yanagawa, R., Kida, H.: Host species-related antigenic groups of avian influenza viruses possessing H 4 hemagglutinin revealed by monoclonal antibodies. Arch. Virol. **72**, 217—221 (1982).
3. Gerhard, W., Yewdell, J., Frankel, M. E., Webster, R.: Antigenic structure of influenza virus haemagglutinin defined by hybridoma antibodies. Nature **290**, 713—717 (1981).
4. Heinz, F. X., Mandl, C., Berger, R., Tuma, W., Kunz, C.: Antibody-induced conformational changes result in enhanced avidity of antibodies to different antigenic sites on the tick-borne encephalitis virus glycoprotein. Virology **133**, 25—34 (1984).
5. Hinshaw, V. S., Bean, W. J., Webster, R. G., Sriram, G.: Genetic reassortment of influenza A viruses in the intestinal tract of ducks. Virology **102**, 412—419 (1980).
6. Hinshaw, V. S., Webster, R. G., Rodriguez, R. J.: Influenza A viruses: Combinations of hemagglutinin and neuraminidase subtypes isolated from animals and other sources. Arch. Virol. **67**, 191—201 (1981).
7. Kida, H., Yanagawa, R.: Isolation and characterization of influenza A viruses from wild free-flying ducks in Hokkaido, Japan. Zbl. Bakt. Hyg., I. Abt. Orig. **A 244**, 135—143 (1979).
8. Kida, H., Yanagawa, R., Matsuoka, Y.: Duck influenza lacking evidence of disease signs and immune response. Infect. Immun. **30**, 547—553 (1980).
9. Kida, H., Brown, L. E., Webster, R. G.: Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/seal/Massachusetts/1/80 (H 7 N 7) influenza virus. Virology **122**, 38—47 (1982).
10. Kimura-Kuroda, J., Yasui, K.: Topographical analysis of antigenic determinants on envelope glycoprotein V 3 (E) of Japanese encephalitis virus, using monoclonal antibodies. J. Virol. **45**, 124—132 (1983).
11. Laver, W. G., Webster, R. G.: Studies on the origin of pandemic influenza. III. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology **51**, 383—391 (1973).
12. Lu, B. L., Webster, R. G., Hinshaw, V. S.: Failure to detect hemagglutination-inhibiting antibodies with intact avian influenza virions. Infect. Immun. **38**, 530—535 (1982).
13. Lubeck, M. D., Gerhard, W.: Topological mapping of antigenic sites on the influenza A/PR/8/34 virus hemagglutinin using monoclonal antibodies. Virology **113**, 64—72 (1981).
14. Lubeck, M., Gerhard, W.: Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. Virology **118**, 1—7 (1982).
15. Sever, J. L.: Application of a microtechnique to viral serological investigations. J. Immunol. **88**, 320—329 (1962).
16. Shulman, M., Wilde, C. D., Köhler, G.: A better cell line for making hybridomas secreting specific antibodies. Nature **276**, 269—270 (1978).

17. Tobita, K., Sugiura, A., Enomoto, C., Furuyama, M.: Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med. Microbiol. Immunol. **162**, 9—14 (1975).
18. Webster, R. G., Campbell, C. H.: Studies on the origin of pandemic influenza. IV. Selection and transmission of "new" influenza viruses *in vivo*. Virology **62**, 404—413 (1974).
19. Webster, R. G., Laver, W. G., Tumova, B.: Studies on the origin of pandemic influenza viruses. V. Persistence of asian influenza virus hemagglutinin (H2) antigen in nature? Virology **67**, 534—543 (1975).
20. Webster, R. G., Laver, W. G.: Determination of the number of nonoverlapping antigenic areas in Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. Virology **104**, 139—148 (1980).
21. Wiley, D. C., Wilson, I. A., Skehel, J. J.: Structural identification of the antibody-binding sites on Hong Kong influenza haemagglutinin and their involvement in antigenic variations. Nature **289**, 373—378 (1981).
22. Yewdell, J. W., Webster, R. G., Gerhard, W. U.: Antigenic variation in three distinct determinants of an influenza type A hemagglutinin molecule. Nature **279**, 246—248 (1979).
23. Yoden, S., Kida, H., Yanagawa, R.: An avian influenza virus of which infectivity is neutralized by antisera lacking hemagglutination-inhibition activity. Arch. Virol. **74**, 205—210 (1982).

Authors' address: Dr. R. Yanagawa, Department of Hygiene and Microbiology, Faculty of Veterinary Medicine, Hokkaido University, Kita 18-Nishi 9, Kita-Ku, Sapporo 060, Japan.

Received June 22, 1984