Antigenic Relationships Among Influenza Virus A Neuraminidase (N2) Antigens by Immunodiffusion and Postinfection Neutralization Tests

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The antigenic relationships among the neuraminidases of influenza A strains from 1957 to 1973 were examined by postinfection application of neuraminidase antisera. This procedure causes inhibition of virus spread and apparent neutralization. Neuraminidase (apparent) neutralization and neuraminidase inhibition tests with chicken antisera gave similar results. Neuraminidase inhibition tests were more discriminating than neuraminidase neutralization tests when rabbit and goat antisera were used. Antibody absorption studies revealed that the neuraminidase, like the hemagglutinin, may possess two kinds of antigenic determinants, which can give rise to "common," or "cross-reacting," and "specific" antibodies. "Specific" antibody appears to be more effective in the inhibition of enzyme activity than in the inhibition of virus spread.

Hemagglutinin (HA) antibodies added to influenza virus suspensions before inoculation of susceptible cells will neutralize virus infectivity; neuraminidase (NA) antibodies will not. However, NA antibodies added to cell culture media after virus inoculation will cause apparent virus neutralization by preventing virus release and spread from infected cells (2, 3, 6, 15, 16, 19). NA antibody has been postulated to act by interfering with an essential enzyme function (3, 6, 15, 16, 19). More recent evidence suggests that postinfection addition of antibodies to both NA and HA may act to prevent virus release and spread by binding newly formed virions to each other and to the antigenically altered cell membrane (2, 4).

Antibodies to the NA antigens are usually assayed by the neuraminidase inhibition (NI) test. Antigenic relationships between NA enzymes of different virus strains are also determined by the NI test. If postinfection neutralization, that is, inhibition of virus spread, by NA antibody is dependent on antigen binding rather than on inhibition of enzyme activity, then antigenic relationships by the NI test may not be relevant for expressing antigenic differences, which may have a bearing on host protection. In this paper we report on the use of postinfection application of NA antisera for examining the NA antigenic relationships of influenza virus A strains representing isolates from 1957 to 1973. We show that with some sera NI and apparent neutralization results were

similar; with others, NI tests were more discriminating than apparent neutralization tests. We also show that the NA antigen, like the HA antigen (8, 13, 17), may possess more than one kind of antigenic determinant, which can give rise to "common," or "cross-reacting," and "specific" antibodies.

MATERIALS AND METHODS

Virus strains. A/Japan/305/57(H2N2), A/Aichi/2/68(H3N2), A/England/42/72(H3N2), A/Port Chalmers/1/73(H3N2), and A/Puerto Rico/8/34(H0N1) were from the collection of the World Health Organization Collaborating Center for Influenza, Atlanta. All strains were grown in 10- or 11-day-old embryonated eggs.

Antisera. Chicken antisera were produced with A/equine/Prague/1/56(Heq1) recombinant viruses containing the N2 antigens indicated in Tables 1 and 2. Recombinant viruses grown in 10- or 11-day-old embryonated eggs were concentrated and purified by sucrose density gradient centrifugation. Two milliliters of a purified virus suspension containing approximately 10^5 HA units/ml was mixed with equal quantities of Freund complete adjuvant. A 0.5-ml amount was injected into each thigh and into the back of the chicken. An intravenous injection of 1 ml of a 10^4 HA units/ml of suspension was given 30 days later. Chickens were bled 7 days after the aqueous injection.

Rabbit anti-NA serum was prepared with NA subunits isolated by electrophoresis of sodium dodecyl sulfate-disrupted virus particles (7). Immunization schedules have been previously described (4). Goat anti-NA serum prepared in the same manner

was provided by R. G. Webster, St. Jude Children's Research Hospital, Memphis.

Absorption of serum and dissociation of antibody. Rabbit antiserum prepared against isolated A/Singapore/1/57(H2N2) NA subunits was absorbed with intact particles of A/Port Chalmers/1/73(H3N2) as described previously (8). Virus-antibody complexes were isolated, and antibody was dissociated by treatment at pH 3 (8).

NI test. Assays for NA-inhibiting antibodies were performed according to the procedure recommended by the World Health Organization (1).

NN test. The basis of the NA (apparent) neutralization (NN) test is the allantois-on-eggshell virus assay system described by Fazekas de St. Groth and White (5) and the adaptation of the system for selection of recombinants as described by Webster (18). Allantoic fluid suspensions of viruses to be used in the test were assayed for infectivity by inoculating 0.05 ml of serial 10-fold dilutions of virus into four wells (Limbro tray 96-U-CV), each containing 0.45 ml of Eagle minimum essential medium. An "egg bit" (EB) was added to each well. After incubation for 1 h at room temperature, the trays were placed on a slow rotation shaker for 72 h at 35°C. EBs were removed by forceps, and 0.05 ml of 5% chicken erythrocytes was added to each well. Virus replication was detected by hemagglutination. Virus titers (50% EB infectious dose) were calculated by the Karber method (9).

For assay of NA antibody, virus suspensions were adjusted to contain 100 to 300 EB infectious doses/0.05 ml and added to wells containing 0.4 ml of Eagle minimum essential medium. EBs were added, and the trays were incubated for 1 h at room temperature. To triplicate wells was added 0.05 ml of serial twofold dilutions of serum in Eagle minimum essential medium. Virus titration was done to confirm proper dosage. Controls consisted of wells containing virus plus EB without serum and wells containing serum plus EB without virus. The trays were incubated as described previously for virus titration. Virus replication was detected by testing for HA formation. Serum neutralization titers were calculated by the Karber method. Nonspecific neutralization of some viruses, which occurred with low dilutions of goat and rabbit sera, was eliminated by prior treatment of the sera with potassium periodate (10). No treatment was required for chicken sera.

Single radial immunodiffusion test. Quantitative determinations of NA antibody were performed with purified virus incorporated into 1.5% agar according to the procedure described by Schild et al. (12).

Double immunodiffusion test. Immunodiffusion tests were performed in 1% agar gels in 1% NaCl after disruption of purified virus ($>10^6$ HA units/ml) with sodium dodecyl sulfate as described by Schild (11).

RESULTS

The effect of virus dosage on serum NN titers was investigated with recombinant and wild-type viruses and their homologous N2 antisera. Two extremes of NN patterns were observed: a

flat response, where a large change in virus dosage resulted in little change in serum titer. This pattern seemed to be characteristic of the virus rather than the antiserum since different antisera gave the same pattern.

A/Japan/57 and A/Aichi/68 viruses, which represented the extremes of NN patterns, were selected for further study. NN titers of chicken anti-NA sera were determined with serial 10-fold dilutions of virus. Each test was performed on three separate occasions, and virus infectivity titers were calculated for each test. A 1,000-fold change in dosage of A/Japan/57 virus resulted in an approximately twofold change in 1957(N2) antibody titer (Fig. 1). In contrast, a 10-fold change Aichi/68 virus dosage resulted in an approximately twofold change in 1968(N2) antibody titer.

The relationships of NA (N2) antigens by NI tests with chicken antisera are shown in Table 1. Antigenic relationships by NN tests using the same antisera are shown in Table 2. Homologous titers by the NN test were approximately 10- to 20-fold lower than titers by the NI test, but titer ratios of homologous/heterologous antigens were not substantially different for the two tests.

Rabbit and goat antisera were more discriminating than chicken antisera by the NI test (Table 3). Antisera prepared against the 1957(N2) antigen(s) had homologous/heterologous titer ratios of ≥ 78 with A/Port Chalmers/73. Ratios for the two viruses were 15.6 with chicken sera. Rabbit and goat antisera were also more discriminating by the NI than the

FIG. 1. NN tests: the effect of virus dosage on serum titers. Symbols: $\circ$, 1968 NA (N2) chicken antiserum titrated against increasing dosages of A/Aichi/68 virus; $\bullet$, 1957 NA (N2) chicken antiserum titrated against increasing dosages of A/Japan/57 virus. EBID₅₀, 50% EB infectious dose.

TABLE 1. Relationship of NA (N2) antigens by NI tests^a with chicken antisera

Antisera ^b	Homologous titer	Titer ratio of homologous/heterologous antigen				
		A/Japan/ 305/57	A/Aichi/2/ 68	A/Eng./42/ 72	A/Pt.Ch./ 1/73	A/PR/8/34
A/RI/5/57(N2) ^c	5,693	—	3.4	7.8	15.6	∞ ^d
A/HK/16/68(N2) ^c	24,035	6.4	—	2.7	6.2	∞
A/Eng./42/72(N2) ^c	7,127	3.4	2.9	—	7.3	∞
A/Pt.Ch./1/73(N2) ^c	13,520	12.3	8.9	2.5	—	∞
A/PR/8/34(N1) ^c	263	∞	∞	∞	∞	—

^a Average of two tests.^b Antisera produced against A/equine/Prague/1/56(Heq1) recombinant viruses containing indicated N2 antigen.^c Viruses received from E. D. Kilbourne, Mt. Sinai School of Medicine, New York, N. Y.^d No titer with heterologous antigen.^e Viruses received from R. G. Webster, St. Jude Children's Hospital, Memphis, Tenn.TABLE 2. Relationship of NA (N2) antigens by NN tests^a with chicken antisera

Antisera ^b	Homologous titer	Titer ratio of homologous/heterologous antigen				
		A/Japan/ 305/57	A/Aichi/2/ 68	A/Eng./42/ 72	A/Pt.Ch./ 1/73	A/PR/8/34
A/RI/5/57(N2) ^c	468	—	2.4	8.5	7.4	∞ ^d
A/HK/16/68(N2) ^c	3,990	12.9	—	2.3	14.5	∞
A/Eng./42/72(N2) ^c	900	9	2	—	9	∞
A/Pt.Ch./1/73(N2) ^c	617	7.7	8	1.1	—	∞
A/PR/8/34(N1) ^c	<10	<10	<10	<10	<10	—

^a Average of two tests with virus dosage of 100 to 1,000 50% EB infectious doses.^b Antisera produced against A/equine/Prague/1/56(Heq1) recombinant viruses containing indicated N2 antigen.^c Viruses received from E. D. Kilbourne, Mt. Sinai School of Medicine, New York, N. Y.^d No titer with heterologous antigen.^e Viruses received from R. G. Webster, St. Jude Children's Hospital, Memphis, Tenn.TABLE 3. Relationship of NA (N2) Antigen by NI and NN tests with rabbit and goat N2 (57) antisera^a

Test	Antisera	Ho- mo- logous titer	Titer ratio of homologous/heterologous antigen		
			A/Ai- chi/2/ 68	A/ Eng. 42/72	A/ Pt.Ch./ 1/73
NI	Rabbit				
	N2	72,440	3.2	19.2	78
	Normal	<10			
	Goat				
NN	N2	37,055	5	17	88
	Normal	<10			
	Rabbit				
	N2	3,548	2.6	4	10
NN	Normal	<40			
	Goat				
	N2	708	4	3	10
	Normal	<40			

^a Antisera produced against A/Singapore/1/57 NA subunits. The goat antiserum was kindly provided by R. G. Webster, St. Jude Children's Hospital, Memphis, Tenn.

NN test. By the latter test homologous/heterologous titer ratios of 10 for the 1957(N2) antisera and A/Port Chalmers/73 virus were obtained for both rabbit and goat antisera.

The nature of the cross-reaction between the 1957 and 1973 N2 antigens was examined by antibody absorption studies. From these studies we have obtained evidence which suggests that NA subunits, like the HA subunits, possess more than one kind of antigenic determinant. Thus, when antiserum to the 1957 NA was tested in double immunodiffusion tests against the 1957 and 1973 NA antigens, marked spur formation was obtained (Fig. 2). Absorption of the 1957 NA antiserum with concentrated A/Port Chalmers/73 virus removed antibody reacting with the "common" determinant(s) on the two different NA antigens and left antibody reacting only with the "specific" determinant(s) on the 1957 NA subunits (Fig. 2).

The common antibody dissociated from

A/Port Chalmers/73 virus particles reacted equally well with the 1957 and 1973 NA subunits in the single radial immunodiffusion test (Fig. 3). However, in NI and NN tests the dissociated common antibody gave much higher titers with the 1957 NA than with the 1973 NA antigens (Table 4).

DISCUSSION

If, as postulated, NA antibody does not directly neutralize virus infectivity and has no

effect on the production of virus by an infected cell but acts by restricting virus spread, then NA antibody titers by NN tests would be expected to be affected by the number of infected cells as well as by the yield of virus per cell. Differences in total yield of antigenic units may explain the difference in the neutralization patterns seen with various strains. This deserves further study. Such differences in patterns of apparent neutralization theoretically could distort antigenic relationships, but we have no

FIG. 2. Double immunodiffusion tests showing the relationship in precipitin reactions between 1957 and 1973 NA molecules and the preparation of antibody to the "specific" antigenic determinant(s) on the 1957 NA molecule. Antiserum prepared against isolated 1957 NA (left side, center well) and the same antiserum absorbed with intact particles of A/Port Chalmers/73 virus (right side, center well) were tested against sodium dodecyl sulfate-disrupted particles of X-7F1 (containing A/1957 NA) and A/Port Chalmers/73 viruses in the peripheral wells. Absorption removed antibody reacting with the "common" determinant(s) on the two different NA molecules, leaving antibody reacting only with the specific determinant(s) on the 1957 NA molecule.

FIG. 3. Single radial diffusion tests with purified antibodies reacting with the "common" and "specific" determinant(s) on the 1957 and 1973 NA molecules. The antibodies were prepared from hyperimmune rabbit antiserum to purified 1957 NA subunits. Specific antibodies were prepared by absorption of antiserum with 1973 virus particles. Common antibodies were prepared by absorption to and dissociation from 1973 virus particles followed by centrifugation through a sucrose gradient as described. The amount of virus incorporated into each of the agar gels was adjusted so that the concentration of NA was the same in each gel.

TABLE 4. NI and NN test results on antiserum fractions

Test	Antiserum ^a	Antigen ^b	
		A/Japan/ 305/ 57(N2)	A/ Pt.Ch./ 1/ 73(N2)
NI	A/Singapore/57	107,200	1,047
	Absorbed	50,120	<10
	Dissociated	12,590	209
NN	A/Singapore/57	3,906	320
	Absorbed	1,955	<20
	Dissociated	1,085	320

^a Hyperimmune rabbit antiserum to the isolated NA of A/Singapore/57(H2N2) virus was absorbed with purified A/Port Chalmers/1/73(H3N2) virus. The absorbed serum and antibody dissociated from Port Chalmers virus were tested.

^b A/equine/Prague/1/56(Heq1) recombinant viruses containing indicated N2 antigen.

evidence that this occurred. The asymmetric relationship between A/Japan/57 and A/Aichi/68 N2 antigens seen in the NN test with chicken antisera was also seen in the NI test.

Schulman and Kilbourne (14) used a plaque size reduction test to demonstrate differences between the 1957 and 1968 N2 antigens. They reported an asymmetric relationship, which was also in the same direction as reported here. In their tests with rabbit antisera, however, they showed at least fivefold greater differences between antigens than observed by us with chicken sera and using the NN test.

Our findings of minimal antigenic difference by NN tests with chicken antisera produced with recombinant viruses are consistent with findings by NI tests with the same sera. Under these conditions the results of the two tests were similar. This was not the case with rabbit or goat antisera produced with isolated NA subunits. The striking difference between the two tests with rabbit or goat antisera suggested that the two tests may be assaying antibody of different quality or antibody to different antigenic determinants.

The HA antigen has previously been shown to have both common and specific antigenic determinants (8, 13, 17). We now have evidence to suggest that the NA antigen also possesses common and specific antigenic determinants. Absorption of antiserum to the 1957(N2) NA with A/Port Chalmers/73 virus removed antibodies reacting with both the 1957 and 1973 NA antigens (common antibody), leaving behind antibody reacting only with the 1957 NA (specific antibody). However, antibody dissociated from A/Port Chalmers/73 virus was not common in the classic sense. This antibody reacted

to the same extent with the 1957 and 1973 neuraminidase antigens in single radial immunodiffusion tests, but titers by NI and NN tests were much higher for the 1957 than for the 1973 NA antigen. The common antibody is therefore best described as cross-reacting.

In the NI test with the rabbit sera the common or cross-reacting antibody represented a small subset of the total antibody population. In the NN test the common antibody was essentially equal in titer to the specific antibody. This suggests to us that specific antibody is more effective in the inhibition of enzyme activity than in the inhibition of virus spread in EBs. In this respect NN test results more closely resemble double immunodiffusion test results.

The broad activity of chicken antisera is not unique for the NA. Chicken antisera have also been recognized as being less discriminating than rabbit or ferret antisera in HA antigenic studies with the HI test.

Based on NI test results with goat and rabbit antisera and those reported by others (13), we must conclude that antigenic drift over the past 18 years has been extensive. Based on NN tests with all sera, we must conclude that antigenic drift among the N2 antigens has been minimal. Whether antibody capable of *in vitro* inhibition of virus spread has more significance *in vivo* than antibody capable of *in vitro* inhibition of enzyme activity is open to speculation. However, our results would suggest that NI tests with rabbit and goat antisera produced with isolated NA subunits detect antigenic differences among N2 antigens that are not apparent by the NN test.

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