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Antigenic Variation of Influenza A Virus Nucleoprotein Detected with Monoclonal Antibodies

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Monoclonal antibodies were used to study antigenic variation in the nucleoprotein of influenza A viruses. We found that the nucleoprotein molecule of the WSN/33 strain possesses at least five different determinants. Viruses of other influenza A virus subtypes showed antigenic variation in these nucleoprotein determinants, although changes in only one determinant were detected in H0N1 and animal strains. The nucleoprotein of human strains isolated from 1933 through 1979 could be divided into six groups, based on their reactivities with monoclonal antibodies; these groups did not correlate with any particular hemagglutinin or neuraminidase subtype. Our results indicate that antigenic variation in the nucleoproteins of influenza A viruses proceeds independently of changes in the viral surface antigens and suggest that point mutations and genetic reassortment may account for nucleoprotein variability.

The three types of influenza viruses (types A, B, and C) are distinguished by the lack of serological cross-reactivity among the type-specific antigens of their internally located matrix proteins and nucleoproteins (NP). Type A influenza viruses are classified further into antigenic subtypes on the basis of the antigenic relatedness of their highly variable surface proteins, hemagglutinin and neuraminidase (4). Initial complement fixation and immunodiffusion tests indicated that the NP and matrix proteins of influenza A viruses are antigenically stable (18, 20). However, reports of differing electrophoretic migration rates of NP (1, 13, 14) and of RNA segments coding for NP (15) in polyacrylamide gels and reports of differences in the oligonucleotide maps of these NP genes (25) from different influenza A strains indicate that mutations occur in the genetic information for the NP. Until recently however, the only evidence that these mutations may be reflected in antigenic alterations in NP molecules was a report that slight differences among influenza A virus NP could be detected by modified complement fixation tests (5).

The first direct evidence for antigenic variation in the NP was reported by Schild et al. (19). Using immune sera to purified NP in double-immunodiffusion tests, they found that the NP of the PR/8/34 (H0N1) strain differs antigenically from the NP of the H3N2 strains. Although their results indicate that antigenic variation in the NP of human strains occurred during the period from 1934 to 1968, interpretation of their data is limited by the source of antibody used and the low sensitivity of the serological assay employed. Because immune serum is a heterogeneous mixture of antibodies, small changes in the NP would not be detected in serological tests of such antisera. Furthermore, immunodiffusion tests are insensitive and detect only antibodies which form visible precipitates; minor subpopulations of antibodies to different determinants are not detectable.

To examine antigenic variation in NP more precisely, we tested homogeneous monoclonal antibodies to NP in enzyme-linked immunosorbent assays (EIA) with human and "animal" influenza strains isolated between 1933 and 1979. Monoclonal antibodies are highly specific reagents capable of detecting a single amino acid change in a determinant (10), and the sensitivity of the EIA is on the order of that of a radioimmunoassay (7). Using these powerful methods in combination, we showed that the WSN/33 (H0N1) influenza virus NP possesses at least five distinct antigenic determinants and that variation in some of these determinants has occurred in other influenza A strains.

MATERIALS AND METHODS

Viruses. Reference strains of the H0N1, H1N1, H2N2, and H3N2 human subtypes and some representative animal strains were used (see Table 2). The production of recombinant strains (see Table 3) followed published procedures (23). Viruses were grown in the allantoic cavities of 10-day-old embryonated hen eggs, concentrated by adsorption to and elution from chicken erythrocytes, and purified by banding in sucrose gradients, as previously described (11).

Production of hybridoma cell lines. BALB/c mice were hyperimmunized to the A/WSN/33 (H0N1) strain of influenza virus by three injections of either 500 μg of purified virus or with 0.5 ml of a lung suspension from WSN-infected BALB/c mice. Spleen cells from these hyperimmunized mice were fused with

the 8-azaquanine-resistant clone of MOPC-21 myeloma cells (P3/X63-Ag8), using polyethylene glycol (8). The culture fluids were tested for antibody by hemagglutination inhibition and neuraminidase inhibition tests, using as antigen a clone of WSN virus selected for inhibitor resistance (24), and by EIA using purified WSN virus. The specificity of hybridomas negative in the hemagglutination inhibition and neuraminidase inhibition tests but positive in the EIA was determined by radio-immunoprecipitation assays. Hybridomas producing antibodies which specifically precipitated the NP were cloned in soft agar.

Serological assays. Hemagglutinin inhibition and neuraminidase inhibition tests were performed as described previously (2, 6). Indirect EIA were performed by the method of Ruitenberg et al. (17), with the following modifications. Purified influenza virus in lysis buffer (0.5 M Tris-hydrochloride buffer, pH 7.5, containing 0.5% Triton X-100 and 0.6 M KCl) was adsorbed to polystyrene multiwell dishes (15 μg of viral protein per well). Any remaining protein-adsorptive capacity of the plastic was eliminated before the assays by treatment with 100 μl of 10% fetal bovine serum in phosphate-buffered saline. Test antibodies and negative controls (culture supernatants from P3/X63-Ag8 cells) (50 μl) were added to the wells and incubated for 30 min, after which unbound antibodies were removed by six phosphate-buffered saline washes. Each well was incubated for 30 min with 50 μl of anti-mouse globulin which had been conjugated to horseradish peroxidase (EC 1.11.1.71; Sigma Chemical Co.) by the method of Nakane and Kawaoi (12). After washing to remove unbound anti-mouse globulin, 100 μl of substrate solution (0.05 M citrate buffer, pH 4.0, containing 0.008% hydrogen peroxide and 40 mM azino-di-3-ethyl-benzothiazoline-6-sulfuric acid) was added. The color development was stopped after 10 min by the addition of 100 μl of 0.1 M hydrogen fluoride (pH 3.3), and the optical density at 405 nm was read with a multichannel photometer.

Preparation of labeled viral antigens. Primary chicken embryo fibroblasts were infected with A/WSN/33 (H0N1) influenza virus. Virus absorption was for 30 min, after which the inoculum was replaced with minimal essential medium containing 70 μCi of L-[^{35}S]methionine (1,270 Ci/mmol; Amersham) per ml. The medium was harvested after 48 h, and virus was banded by equilibrium centrifugation. Labeled virus was lysed in 0.05 M Tris-hydrochloride (pH 7.5)–0.6 M KCl–0.5% Triton X-100 for 30 min at 25°C. Protein aggregates were removed by centrifugation at 150,000 $\times g$ for 45 min. The soluble labeled viral proteins were used in immunoprecipitation assays.

Radio-immunoprecipitation. Tissue culture fluid (200 μl) containing monoclonal antibodies was added to labeled viral antigens (50,000 cpm/100 μl) in radio-immunoprecipitation buffer (0.05 M Tris-hydrochloride, pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.25% bovine serum albumin). The reaction mixture was incubated for 2 h at 25°C and then centrifuged at 800 $\times g$ for 15 min. This was necessary to prevent trace amounts of matrix protein from adhering non-specifically to precipitates. Rabbit anti-mouse immunoglobulin (50 μl) at equivalence was added, and the mixture was incubated at 25°C for 2 h and then at 4°C for 15 h. Precipitates were washed three times with radio-immunoprecipitation buffer and dissolved in polyacrylamide gel electrophoresis sample buffer containing 1% 2-mercaptoethanol. Immunoprecipitates were electrophoresed in 8% polyacrylamide sodium dodecyl sulfate-gels as described by Laemmli (9) for 15 h at 70 V. The gels were processed for fluorography and exposed to Kodak XR-5 film for 20 h.

RESULTS

Specificities of the monoclonal antibodies. Hybridomas produced by fusion of myeloma cells with splenic lymphocytes from mice immunized with WSN virions might produce antibodies to any one of the viral proteins. Therefore, it was necessary to identify the cultures producing antibodies specific for the NP. A preliminary screening of the culture supernatants by hemagglutination inhibition and neuraminidase inhibition tests eliminated those supernatants reacting with the viral surface proteins. Supernatants which were negative by the hemagglutination inhibition and neuraminidase inhibition tests were screened for reactivity with internal viral components by EIA, using detergent-lysed WSN virions. The specificities of these antibodies to internal proteins were determined by radio-immunoprecipitation and polyacrylamide gel electrophoresis analysis of the precipitates. In several precipitates there was a protein which comigrated with the NP band of marker virus. For undetermined reasons the influenza virus NP migrated as a doublet, as observed by others (16). Figure 1 shows the migration profile of WSN viral proteins (Fig. 1, lane 1) and the specific precipitation of the NP by five monoclonal antibodies (lanes 2 through 6). To rule out the possibility that the antibodies were directed toward one of the viral polymerase proteins, resulting in co-precipitation of the NP, these antibodies were tested in in vitro polymerase inhibition assays (21) (data not shown). It has been demonstrated that influenza virus polymerase activity is not inhibited by antibodies to the NP (21). None of our monoclonal antibodies inhibited the WSN/33 virus polymerase, confirming their specificities for the NP as determined by the immunoprecipitation assay. A total of 11 hybridomas producing anti-NP antibodies were identified in this manner. These cultures were cloned and used in subsequent experiments.

Monoclonal antibodies directed toward different antigenic determinants on the NP. To determine whether the monoclonal antibodies were directed toward the same or different antigenic determinants on the NP molecule, they were tested in EIA (Table 1). Tests of the monoclonal antibodies with a panel of influenza A viruses showed that although all reacted with the homologous WSN strain, five antibody prep-

arations reacted with a different subset of heterologous virus strains (Table 1). For example, antibody 3/1 reacted only with the PR/8/34, FLW/1/52, and equine strains, whereas antibody 7/3 reacted only with the FM/1/47, FLW/1/52, and equine strains. Thus, each of the five monoclonal antibodies recognized a different antigenic determinant on the influenza virus NP molecule.

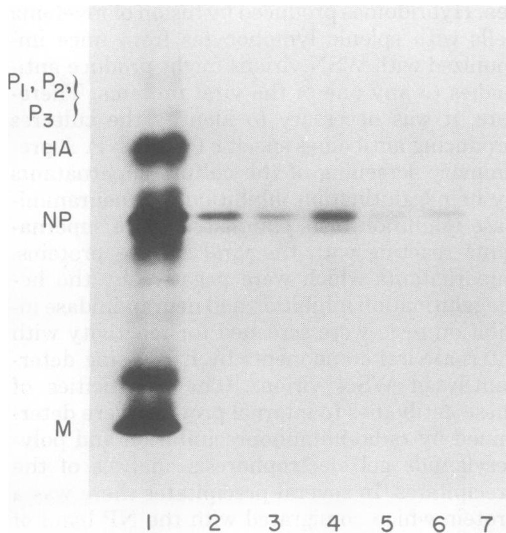

FIG. 1. *Fluorogram of sodium dodecyl sulfate-polyacrylamide gel showing electrophoresed ^{35}S-labeled WSN/33 viral proteins (lane 1) and NP immunoprecipitated by five hybridoma antibodies (lanes 2 through 6). Immunoprecipitation assays included a control for nonspecific precipitation of viral proteins (culture supernatants from P3/X63-Ag8 cells) (lane 7). M, Matrix protein; HA, hemagglutinin.*

Antigenic variation in NP determinants. The above-described results indicated that some influenza A viruses do not possess all five of the determinants detectable on the NP of the WSN/33 strain. To study antigenic variation in the influenza A virus NP, we tested a larger panel of viruses by EIA against the monoclonal antibodies. The viruses examined included representative field strains of the major human subtypes, animal strains, and laboratory strains (Table 2). The WSN, WSA, and NWS viruses, which are laboratory strains of the first H0N1 human influenza virus isolate, were included in the study because they were isolated independently in different laboratories and have experienced multiple and diverse passage histories. Despite these differences, however, no antigenic variation was detected among these strains. In contrast, antigenic variation was detected in two of three subsequently isolated H0N1 field strains. Monoclonal antibodies 7/3 and 469/6 did not react with the PR/8/34 and Bellamy/42 strains, respectively, indicating a change in one of the five antigenic determinants in each of these viruses. These data indicate that some, but not all, of the H0N1 field strains have antigenic alterations in the NP epitopes defined by these monoclonal antibodies.

Variation also occurred in the NP determinants of H1N1, H2N2, and H3N2 influenza viruses. Every strain examined had undergone antigenic alterations rendering their NP unrecognizable by two of the five monoclonal antibodies (150/4 and 469/6). Some of these strains (FM/1/47 and Aichi/2/68) also showed antigenic variation in a third determinant, that defined by antibody 3/1. In addition to these alter-

TABLE 1. *Unique reactivity patterns of five anti-NP monoclonal antibodies with influenza A viruses, as determined by EIA*

Influenza virus strain	Reactivity of monoclonal antibody:					
	3/1	5/1	7/3	150/4	469/6	Reactivity of negative control[a]
WSN/33 (H0N1)	0.26[b]	0.27	0.22	0.32	0.27	0.04
PR/8/34 (H0N1)	0.15	0.16	0.03[c]	0.13	0.14	0.04
FM/1/47 (H1N1)	0.04[c]	0.27	0.19	0.05[c]	0.05[c]	0.04
FLW/1/52 (H1N1)	0.18	0.20	0.26	0.05[c]	0.05[c]	0.04
USSR/90/77 (H1N1)	0.01[c]	0.19	0.03[c]	0.03[c]	0.02[c]	0.04
Equine/Miami/63 (Heq2 Neq2)	0.21	0.19	0.22	0.25	0.05[c]	0.03
B/Hong Kong/5/72	0.01[c]	0.04[c]	0.02[c]	0.02[c]	0.04[c]	0.02

[a] The negative control was supernatant from P3/X63-Ag8 cells containing no anti-influenza virus antibodies. Optical densities at 405 nm represent color development due to incomplete removal of enzyme-conjugated rabbit anti-mouse globulin. Such controls were essential for distinguishing nonspecific color development due to residual conjugate from that due to specific antibody attachment. The average optical density at 405 nm produced by a control culture (P3/X63-Ag8) supernatant was 0.035. In evaluating the results of the EIA, therefore, a monoclonal antibody producing an optical density at 405 nm of ≧0.07 was considered to be reactive in our assay.

[b] Optical density at 405 nm, as obtained by EIA.

[c] These optical density values of <0.07 (less than twice that of negative controls) indicate that the monoclonal antibody has no reactivity with the viral NP.

TABLE 2. *Antigenic variation in NP determinants of influenza A viruses detected by monoclonal antibodies*

Influenza A subtype	Strain	Reactivity in EIA with monoclonal antibody preparation:				
		3/1	5/1	7/3	150/4	469/6
H0N1						
	WSN/33	+[a]	+	+	+	+
	WSA/33	+	+	+	+	+
	NWS/33	+	+	+	+	+
	PR/8/34	+	+	−	+	+
	Mel/35	+	+	+	+	+
	Bellamy/42	+	+	+	+	−
H1N1						
	FM/1/47	−	+	+	−	−
	FW/1/50	−	+	−	−	−
	FLW/1/52	+	+	+	−	−
	USSR/90/77	−	+	−	−	−
	Brazil/11/78	−	+	−	−	−
	Fukushima/103/78	+	+	+	−	−
	California/10/78	+	+	+	−	−
H2N2						
	Japan/305/57	+	+	+	−	−
	Ned/84/68	+	+	+	−	−
H3N2						
	Hong Kong/1/68	+	+	+	+	−
	Aichi/2/68	−	+	+	−	−
	England/878/69	+	+	+	−	−
	England/187/70	+	+	+	−	−
	England/42/72	+	+	+	−	−
	Port Chalmers/1/73	+	+	+	−	−
	Scotland/840/74	+	+	+	−	−
	Victoria/3/75	+	+	+	−	−
	New Jersey/745/76	+	+	+	−	−
	Texas/1/77	+	+	+	−	−
Hsw1N1	Swine/Iowa/30	+	+	+	+	+
Hsw1N1	Duck/Alb/35/76	+	+	+	+	+
Hav1Nav2	Turkey/Oregon/71	+	+	+	+	+
H2Nav1	Duck/Germany/1215/74	+	+	+	+	+
Heq1Neq1	Equine/Prague/1/57	+	+	+	+	−
Heq2Neq2	Equine/Miami/1/63	+	+	+	+	−

[a] +, Optical density at 405 nm threefold higher than the optical density at 405 nm of a P3/X63-Ag8 culture supernatant in EIA; −, optical density at 405 nm less than twice the optical density at 405 nm of a P3/X63-Ag8 culture supernatant in EIA.

ations, the FW/1/50, USSR/90/77, and Brazil/11/78 strains were not recognized by monoclonal antibody 7/3, indicating that these strains had undergone antigenic variation in four of the five NP determinants examined.

In contrast to the variability detected in the NP of the human influenza viruses, we found only two examples of antigenic variation among animal strains. Although only a limited number of animal strains were tested, all but the equine strains were identical to the WSN virus in five determinants. The equine strains were not recognized by monoclonal antibody 469/6, indicating that this NP determinant had undergone antigenic variation.

Characterization of the NP of recombinant influenza A viruses. A panel of recombinant influenza A viruses was tested in EIA to evaluate the usefulness of monoclonal antibodies for antigenic analysis of the NP of new influenza virus strains (Table 3). Included in the panel was the X-31 strain, a recombinant which derived its hemagglutinin and neuraminidase genes from the Hong Kong/1/68 (H3N2) strain and its genes coding for internal proteins from the PR/8/34 (H0N1) virus (22). Other recombinants were produced from parental influenza A viruses whose NP were readily distinguishable by our monoclonal antibodies (Table 2). These recombinant strains were shown to be surface antigen recombinants by hemagglutination inhibition and neuraminidase inhibition tests. The parental donors of their NP genes were identified by EIA of the recombinants with monoclonal antibodies.

The X-31 strain had a reactivity pattern in EIA which was identical to that of the PR/8/34 (H0N1) virus, but clearly different from the pattern of the Hong Kong/1/68 (H3N2) virus (Ta-

TABLE 3. *Characterization of NP of recombinant influenza A viruses by monoclonal antibodies in EIA*

Parental viruses	Surface antigen subtype of recombinant strain[a]	Reactivity in EIA with monoclonal antibody: 3/1	5/1	7/3	150/4	469/6	Parental donor of NP gene
Hong Kong/1/68 (H3N2) ×		+[b]	+	+	+	−	
PR/8/34 (H0N1)		+	+	−	+	+	
	H3N2[c]	+	+	−	+	+	PR/8/34
USSR/90/77 (H1N1) ×		−	+	−	−	−	
Equine/Prague/1/57 (Heq1Neq1)		+	+	+	+	−	
	H1Neq1	−	+	−	−	−	USSR/90/77
Aichi/2/68 (H3N2) ×		−	+	+	−	−	
Bellamy/42 (H0N1)		+	+	+	+	−	
	H3N1	+	+	+	+	−	Bellamy/42
Aichi/2/68 (H3N2) ×		−	+	+	−	−	
Swine/Iowa/30 (Hsw1N1)		+	+	+	+	+	
	H3N1	+	+	+	+	+	Swine/Iowa/30

[a] Surface antigen subtypes were determined by hemagglutination inhibition and neuraminidase inhibition tests with reference antisera.

[b] +, Optical density at 405 nm threefold higher than the optical density at 405 nm of a P3/X63-Ag8 culture supernatant in EIA; −, optical density at 405 nm less than twice the optical density at 405 nm of a P3/X63-Ag8 culture supernatant in EIA.

[c] The recombinant produced from Hong Kong/1/68 and PR/8/34 has been designated X-31.

ble 3). These results indicate that the PR/8/34 (H0N1) parent contributed the NP gene to the X-31 recombinant, confirming the RNA analysis of X-31 (22). The other strains examined were known to be surface antigen recombinants, but the parental donor of the NP gene was unknown. By comparing the reactivity patterns of the parental viruses with those of the recombinants (Table 3), we were able in every case to unambiguously identify the parental donor of the NP gene. These results illustrate the usefulness of monoclonal antibodies for antigenic characterization of new strains of influenza viruses.

DISCUSSION

In contrast to the highly variable surface proteins, the influenza NP was characterized originally as antigenically invariant (20). Since the NP is located internally in the virion and antibodies to the NP do not neutralize virus infectivity, the apparent antigenic stability of this molecule seemed reasonable; it would not be exposed to immune selection pressure. Our results, however, clearly demonstrate that antigenic variation does occur in the influenza virus NP. We produced five monoclonal antibodies, each of which reacts with a different NP determinant. The topographical location on the NP molecule of the determinants defined by these five monoclonal antibodies and the fraction of the total complement of NP antigenic sites which they represent are not known. However, it appears that one of the epitopes must be present on a region of the NP molecule which has been conserved in every influenza A virus strain examined and may be necessary for either the functional or structural integrity of the molecule. This particular epitope, which is identical in human and animal strains circulating during the past 45 years, may be a type-specific determinant for influenza A viruses.

In addition to the invariant type-specific determinant, we found four other NP epitopes which differ antigenically. Monoclonal antibodies to these epitopes detected a minor amount of variation in the animal strains and human H0N1 influenza A viruses. In these strains variation was limited to only one of the five determinants; the other determinants were indistinguishable from the WSN strain. In contrast, the H1N1, H2N2, and H3N2 viruses showed more extensive alterations in their NP determinants. For example, four of the five NP epitopes of the WSN/33 strain have not been conserved in the USSR/90/77 strain.

Based on their reactivity with the monoclonal antibodies, all of the strains tested fell into six NP epitope groups (Table 4). Since the only NP alterations detected were those affecting five antibody-binding sites, the antigenic similarities among the viruses may be restricted to a minor, but variable, portion of the NP and do not necessarily reflect the total homologies among the NP genes of strains within an NP group. Antigenic variation in the NP appears to proceed independently of major antigenic changes in the hemagglutinin and neuraminidase. The NP epitope groups do not correlate with any particular hemagglutinin or neuraminidase subtype; most NP epitope groups are composed of strains rep-

TABLE 4. *Combinations of NP epitopes found in influenza A viruses circulating between 1933 and 1978*

Group	NP epitopes recognized in EIA by monoclonal antibody:					Group members	Subtype
	3/1	5/1	7/3	150/4	469/6		
I	+	+	+	+	+	WSN/33	H0N1
						Swine/Iowa/30	Hsw1N1
						Turkey/Oregon/71	Hav1Nav2
						Duck/Germany/1225/74	H2Nav1
II	+	+	−	+	+	PR/8/34	H0N1
III	+	+	+	+	−	Bellamy/42	H0N1
						Equine/Prague/1/57	Heq1Neq1
						Equine/Miami/1/63	Heq2Neq2
						Hong Kong/1/68	H3N2
IV	+	+	+	−	−	FLW/1/52	
						Fukushima/103/78	H1N1
						California/10/78	
						Japan/305/57	H2N2
						England/878/69	H3N2
						England/178/70	
						Texas/1/77	
V	−	+	+	−	−	FM/1/47	H1N1
						Aichi/2/68	H3N2
VI	−	+	−	−	−	FW/1/50	
						USSR/90/77	H1N1
						Brazil/11/78	

resenting several subtypes. For example, the WSN/33 (H0N1), Swine/Iowa/30 (Hsw1N1), Turkey/Oregon/71 (Hav1Nav1), and Duck/Germany/1225/74 (H2Nav1) viruses are all in group I, which indicates that their NP are closely related. The NP of the group I strains are readily distinguished by monoclonal antibodies from the PR/8/34 (H0N1) strain, which is in group II. These results conflict with those of Schild et al. (19), who found the PR/8/34 (H0N1), Turkey/Eng/63 (Hav1Nav2), and Swine/Iowa/30 (Hsw1N1) viruses antigenically indistinguishable. The sensitivity of the EIA and the specificities of the monoclonal antibodies are the most likely explanations for these discrepancies.

In some instances viruses of the same surface antigen subtype have NP epitopes which are closely related to viruses of other subtypes. For example, the NP of some recent H1N1 isolates (California/10/78 and Fukushima/103/78) are more similar to the Texas/1/77 (H3N2) strain than to other currently circulating H1N1 strains (USSR/90/77 and Brazil/11/78). That the California/10/78 (H1N1) field strain has arisen by genetic reassortment between cocirculating H1N1 and H3N2 viruses and has derived its NP gene from an H3N2 source has been demonstrated (26). The mechanism of genetic reassortment provides a reasonable explanation for the close antigenic similarity between the California/10/78 (H1N1) NP and the Texas/1/77 (H3N2) NP. An alternative mechanism for antigenic variation in the influenza virus NP is point mutations in the NP gene. The minor amount of antigenic variation detected among the H0N1 strains (groups I, II, and III) could be due to different point mutations in the NP gene. However, this is probably not the sole mechanism operative in NP variability, since it is unlikely that strains isolated during a period of 25 years (group IV) would have independently undergone identical point mutations resulting in NP indistinguishable at the five epitopes examined.

The data reported here suggest that both point mutations and genetic reassortment contribute to antigenic variation in influenza A virus NP, as in the hemagglutinin and neuraminidase. The selective advantage of influenza viruses with altered NP antigens is not known. However, since the NP is intimately involved in the viral replication complex and has been associated with virulence characteristics of the virus (3), alterations in this molecule may affect important biological properties, such as viral replication, transmission, and virulence. Some changes could provide a survival advantage in nature and could explain why strains circulating early in the H3N2 era, e.g. Aichi/2/68 (group V) and Hong Kong/1/68 (group III), were replaced

by H3N2 strains of group IV. This also may account for the recent emergence of the recombinant H1N1 strain California/10/78, which has apparently derived its NP and polymerase genes from a group IV H3N2 virus, Texas/1/77 (26).

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