Antigenic Characterization of Influenza A Virus Matrix Protein with Monoclonal Antibodies

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Monoclonal antibodies were used to study antigenic variation in three distinct epitopes on the matrix protein of influenza A viruses. We found that two of these epitopes underwent antigenic variation, but in a very limited number of virus strains. A third epitope appeared to be an invariant type-specific determinant for influenza A viruses. Competitive antibody binding assays and Western blot analysis of proteolytically digested matrix protein indicated that at least two of the three epitopes are located in nonoverlapping domains on the matrix protein molecule.

The internal matrix protein of influenza viruses contains a type-specific antigen which allows serological discrimination of type A, B, and C influenza viruses. The matrix proteins of influenza A viruses appear to be very similar. For example, the lengths of the genes coding for matrix proteins are identical in strains isolated 38 years apart (13), and several studies have found >90% homology among matrix genes (13, 22). Peptide mapping has shown that, although regions of the matrix protein are highly conserved, there are a small number of clear-cut differences among strains (3, 6). However, none of these differences has been detected in serological assays with heterogeneous monospecific antisera to the matrix protein (21).

Since antigenic variation has been readily demonstrated in the influenza A virus hemagglutinin (8), neuraminidase (26), and nucleoprotein (24), using monoclonal antibodies, we have produced monoclonal antibodies to the matrix protein. Using these antibodies in enzyme-linked immunosorbent assays (ELISA), we have detected three distinct epitopes on the matrix protein. Two of these epitopes have undergone antigenic variation in naturally occurring field strains.

MATERIALS AND METHODS

Viruses. Viruses were grown in the allantoic cavities of 10-day-old embryonated hen eggs, concentrated by centrifugation, and purified by banding in sucrose gradients, as previously described (14). Human reference strains of the H1N1 subtype used included A/WSN/33, A/PR/8/34, A/FW/1/50, A/HongKong/123/77, A/Columbia/1078, and A/USSR/50/77. H2N2 human strains used were A/Japan/30/57 and A/Taiwan/1/62. H3N2 human strains used included A/Hong Kong/1/68, A/Aichi/2/68, A/Udorn/307/72, A/Alaska/6/67, and A/Peking/2/79. Prototype strains isolated from lower vertebrae were obtained from Virginia S. Hinshaw and included the following: A/Mallard/Alberta/573/78 (H1N1), A/Mallard/NY/675/78 (H2N2), A/Mallard/NY/6874/78 (H3N2), A/Pintail/Alberta/358/79 (H3N6), A/Duck/Ukraine/1/63 (H3N8), A/Pintail/Alberta/119/79 (H4N6), A/Turkey/Mass/65 (H6N2), A/Equine/Cornell/16/74 (H7N7), A/Pintail/Alberta/121/79 (H7N8), A/Turkey/Ontario/6118/68 (H8N4), A/Turkey/Wis/66 (H9N2), A/Turkey/MN/5/79 (H10N7), and A/Gull/Md/704/77 (H13N6).

Production of hybridoma cell lines. BALB/c mice were hyperimmunized to the A/WSN/33 (H1N1) strain of influenza virus by two intraperitoneal injections of 200 μg of purified virus or 50 μg of purified matrix protein (30) in complete Freund adjuvant followed by an intravenous boost 3 days before fusion. Spleen cells from these hyperimmunized mice were fused with MOPC-21 myeloma cells which do not secrete immunoglobulins (NS1/1), using polyethylene glycol (11). The culture fluids were tested for antibody production by hemagglutination inhibition, neuraminidase inhibition, and ELISA. The specificity of hybridomas negative in the hemagglutination inhibition and neuraminidase inhibition tests but positive in ELISA was determined by immunoprecipitation assays. Hybridomas producing antibodies which specifically precipitated the matrix protein were cloned in soft sugar.

Sero logical assays. Hemagglutination inhibition and neuraminidase inhibition tests were performed as previously described (2, 7). Indirect ELISA was performed as previously described (25). Briefly, purified influenza virus in phosphate-buffered saline was adsorbed to polystyrene multwell dishes at 37°C for 12 h (1 μg of viral protein per well). Virus became disrupted during the incubation interval, allowing each of its antigens to become accessible for immunosassay. The ladder of reagents used in these assays was (i) test monoclonal antibodies (50 μl), (ii) horseradish peroxidase-conjugated antimouse globulin (50 μl), and (iii) substrate solution (100 μl) consisting of 0.05 M citrate buffer (pH 4.0) containing 0.008% hydrogen peroxide and 40 mM azino-di-3-ethyl-benzothiazoline-6-sulfuric acid. Wells were washed six times with phosphate-buffered saline between addition of reagents, and the final optical density at 405 nm was read with a multichannel photometer.

Competitive binding ELISAs were performed as previously described (24) with the following modifications. Horseradish peroxidase was conjugated to monoclonal antibodies (20) and titrated directly in ELISA to determine the conjugated antibody concentration required to saturate all available matrix protein binding sites. Competitive binding ELISAs were then performed with mixtures containing a fixed amount of conjugated monoclonal antibodies (determined as above) and increasing amounts of unconjugated, protein A-purified monoclonal antibodies. The concentrations of protein A-purified antibodies were determined by the method of Lowry et al. (17).

Preparation of labeled viral antigens. Confluent Madin-
Darby canine kidney cells were infected with A/WSN/33 (H1N1) influenza virus (multiplicity of infection, 0.1). Virus adsorption was for 30 min, after which the inoculum was replaced with Eagle minimal essential medium containing 70 
\( \mu \)Ci of L-[\(^{35}\)S]methionine (1,270 Ci/mmole; Amersham Corp.) per ml. After 48 h the medium was harvested and virus was banded by sucrose density centrifugation. Labeled virus was lysed in 0.05 M Tris–hydrochloride (pH 7.5)–0.6 M KCl–0.5% Triton X-100 for 20 min at 25°C and used in immunoprecipitation assays.

**Immunoprecipitation.** Tissue culture fluid (100 µl) containing monoclonal antibodies was added to labeled viral antigens in immunoprecipitation buffer (0.005 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. Immune complexes were collected on protein A-Sepharose (100 µl of a 1:1 slurry; Pharmacia Fine Chemicals, Inc.) and washed three times in immunoprecipitation buffer. Pellets were resuspended in polyacrylamide gel electrophoresis sample buffer (12) and heated to 100°C for 2 min to elute bound immune complexes. Eluted samples were electrophoresed in 10% polyacrylamide–sodium dodecyl sulfate (SDS) gels as described by Laemmli (12) for 12 h at 40 V. Gels were processed for fluorography and exposed to Kodak XAR-5 film for 20 h.

**Matrix protein purification and proteolytic digestion.** Puriﬁed A/WSN/33 virus was electrophoresed in 10% polyacrylamide–Laemmli gels under nonreducing conditions for 12 h at 40 V. After Coomassie blue staining, the matrix band was cut from the gel. Matrix protein was recovered and simultaneously concentrated from the gel slice by electroelution at 1 W for 2 h. A total of 45 µg of electroeluted matrix protein was precipitated with 12.5% trichloroacetic acid, washed with absolute ethanol, and suspended in 200 µl of buffer (125 mM Tris, pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromophenol blue). The sample was heated at 100°C for 2 min, and half was digested with 5 µg of Staphylococcus aureus V8 protease at 37°C for 30 min. The samples were boiled again for 2 min and electrophoresed in 15% polyacrylamide–SDS gels for 12 h at 40 V.

**Western blot analysis.** Proteins electrophoresed in 15% polyacrylamide–SDS gels were electrophoretically transferred to nitrocellulose (0.22-µm pore size) sheets (23). Transfer buffer was 25 mM Tris (pH 8.3)–192 mM glycine–20% methanol, and electrophoresis was for 3 h at 55 V (0.2 A). After electrophoretic transfer, the gel was stained with Coomassie blue to locate residual peptides. Nitrocellulose sheets were soaked in 10 mM Tris–0.85% NaCl–5% bovine serum albumin for 2 h at 37°C. Sheets were then cut into 5-mm strips and soaked separately in hybridoma culture supernatants for 2 h at room temperature. Strips were washed in 10 mM Tris–0.85% NaCl and soaked in \(^{35}\)S-labeled protein A (30 mCi/mg; Amersham) for 30 min. After being washed, strips were air dried and exposed to Kodak XAR-5 film for 72 h.

**RESULTS**

**Specificities of the monoclonal antibodies.** Antibodies to any one of the viral proteins might be produced by hybridomas derived by fusion of splenic lymphocytes from mice immunized with WSN virions. Preliminary screening of the culture supernatants by hemagglutination inhibition and neuraminidase inhibition tests eliminated hybridomas directed to the viral surface antigens. Remaining hybridomas were screened by indirect ELISA, using disrupted purified WSN virions to identify culture supernatants reacting with internal virion components. The specificities of these antibodies to internal proteins were determined by immunoprecipitation and polyacrylamide gel electrophoresis analysis of the precipitates. Figure 1 shows the electrophoretic profile of A/WSN/33 (lane 1) and the specific precipitation of matrix protein by three monoclonal antibodies (lanes 3 to 5). A total of five hybridomas producing antimatrix antibodies were identified. These cultures were cloned in soft agar and used in subsequent experiments. The characterization of one of these hybridomas has been described previously (30).

**Monoclonal antibodies directed toward different epitopes on the M protein.** To determine whether the monoclonal antibodies recognized different epitopes on the matrix protein, they were tested in indirect ELISA against a panel of influenza A viruses. Although all antibodies reacted with the immunizing WSN strain, three antibodies reacted with a unique subset of heterologous virus strains (Table 1). These results indicated that each of the three monoclonal antibody preparations is directed toward a different antigenic determinant on the matrix protein of influenza A virus.

The above-described results indicated that some influenza A virus strains do not possess all of the epitopes detectable on the matrix protein of the A/WSN/33 strain. To study the extent of antigenic variation in the matrix protein, we tested a large panel of influenza A viruses by ELISA. Strains tested included representatives of the major human and animal subtypes (see Materials and Methods). Of all strains examined, only three had undergone antigenic alteration rendering their matrix protein unrecognizable by one of the three monoclonal antibodies. These strains were A/Duck/Canada/2/62 (H1N1), A/Aichi/2/68 (H3N2), and A/USSR/90/77 (H1N1). These results indicate that antigenic variation in the matrix protein of influenza A viruses occurs, but very likely to a limited degree.

**Number of nonoverlapping antigenic domains on matrix protein.** Competitive binding ELISA assays were performed to determine the distribution of the three distinct antigenic determinants on the influenza A matrix protein. These
experiments were designed to test the capability of each of three monoclonal antibodies to sterically inhibit the binding of antibodies to other epitopes on the matrix protein molecule. The results of these assays are illustrated by competitive binding curves (Fig. 2). Each of two monoclonal antibody preparations (M2-1C6 and 289/4) was inhibited only by homologous antibodies, indicating that each of these monoclonal antibodies recognizes an epitope which occupies a physically distinct domain on the molecule. In contrast, the binding of antibody preparation 904/6 was also partially inhibited by high concentrations of antibody M2-1C6. This inhibition was not due to non-immunoglobulin contaminants in the preparations, since high concentrations of antibody 289/4 and monoclonal antibodies to the influenza A nucleoprotein or hemagglutinin produced only slight inhibition of 904/6 antibody binding (data not shown).

There are several possible explanations for nonreciprocal binding of monoclonal antibodies. For example, binding of one antibody might induce allosteric effects on the matrix protein, thus partially inhibiting the binding of a second antibody. Alternatively, antibodies of different binding affinities might react nonreciprocally in a competitive binding assay. A third possibility is that the competing antibody, in addition to binding to its corresponding epitope, also binds with reduced affinity to a second cross-reactive determinant which is located in close proximity to the determinant recognized by the conjugated antibody. This situation could produce nonreciprocal competitive inhibition at high competing antibody concentrations and has been suggested to explain similar data obtained with monoclonal antibodies to vesicular stomatitis virus (15). To discriminate among these possibilities, the antibodies were individually evaluated for their abilities to bind to fragments of the matrix protein, since this assay does not depend on competitive binding and would detect a cross-reactive determinant. Intact and proteolytically digested matrix proteins were transferred to nitrocellulose by the Western blot technique, and immunoreactive polypeptides were identified by using the three monoclonal antibody preparations. Figure 3 shows the results of these experiments. Lanes a, b, and c (undigested matrix)

![Image](http://jvi.asm.org/)

**FIG. 2.** Determination of the number of nonoverlapping antigenic matrix protein domains by competitive binding ELISA. Fixed concentrations of conjugated antibodies were mixed with increasing concentrations of purified unconjugated antibody competitors. These mixtures were tested in ELISA with A/WSN/33 (H1N1) virus as described in the text.

**TABLE 1.** Unique reactivity patterns of three antimatrix monoclonal antibodies with influenza A viruses, as determined by ELISA

<table>
<thead>
<tr>
<th>Influenza virus strain</th>
<th>Reactivity of monoclonal antibody (optical density at 404 nm)</th>
<th>M2-1C6</th>
<th>904/6</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33 (H1N1)</td>
<td>0.31, 0.32, 0.32, 0.32, 0.08</td>
<td>0.31</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>A/Duck/Ukr/1/63 (H3N8)</td>
<td>0.06&lt;sup&gt;a&lt;/sup&gt;, 0.46, 0.35, 0.06</td>
<td>0.06</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>A/Aichi/2/68 (H3N2)</td>
<td>0.27, 0.42, 0.08&lt;sup&gt;b&lt;/sup&gt;, 0.05</td>
<td>0.27</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>A/USSR/90/77 (H1N1)</td>
<td>0.34, 0.26, 0.10&lt;sup&gt;b&lt;/sup&gt;, 0.05</td>
<td>0.34</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>B/HongKong/5/72</td>
<td>0.05&lt;sup&gt;a&lt;/sup&gt;, 0.04&lt;sup&gt;b&lt;/sup&gt;, 0.06&lt;sup&gt;b&lt;/sup&gt;, 0.05</td>
<td>0.05</td>
<td>0.05</td>
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<sup>a</sup> The negative control was supernatant from NS/1 cells containing no anti-influenza virus antibodies. Optical densities at 404 nm represent color development due to incomplete removal of enzyme-conjugated rabbit antimouse 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 404 nm produced by negative control supernatant was 0.06. In evaluating the results of the ELISA, therefore, a monoclonal antibody producing an optical density at 404 of >0.18 was considered to be reactive in our assay.

<sup>b</sup> These optical density values of <0.18 indicate that the monoclonal antibody has no reactivity with the viral matrix protein.

These monoclonal antibodies were shown to be nonreciprocal in their competitive inhibition patterns, suggesting that the epitopes recognized by these antibodies are not identical.

![Image](http://jvi.asm.org/)

**FIG. 3.** Autoradiogram showing the antigenic domains of the influenza A matrix protein recognized by monoclonal antibodies M2-1C6 and 289/4. Lane a, intact matrix; lane b, matrix digested with pronase; lane c, matrix digested with protease.

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matrix protein possesses a minimum of three antigenic sites, two of which independently undergo antigenic variation in other strains. The third epitope detected by our monoclonal antibodies was present on each of the 26 strains examined and represents an influenza A type-specific matrix protein determinant.

To examine the extent of antigenic variation the three detectable matrix protein epitopes undergo, the monoclonal antibodies were tested in ELISA against 26 influenza A viruses (see Materials and Methods) isolated from animals and humans over a 49-year period and representing most of the major antigenic subtypes. Antigenic variation was detected in only three of these strains, namely, A/Aichi/2/68 (H3N2), A/Duck/Ukraine/1/63 (H3N8), and A/USSR/90/77 (H1N1) (Table 1). The two 1968 H3N2 strains tested (Hong Kong and Aichi) possessed clearly distinguishable matrix proteins. This corroborates previous studies with monoclonal antibodies which demonstrated that the hemagglutinins and nucleoproteins of these strains are antigenically different (25, 28) and suggests that there were two distinct strains cocirculating during the first year of the H3N2 era. As a result of sequential, cumulative mutations in their hemagglutinins, each of these 1968 strains appears to have given rise to distinct antigenic families of H3N2 viruses (29). In contrast, the pattern of antigenic variation detected in the matrix protein is not likely to be caused by progressive accumulation of point mutations in the matrix gene. Rather, it is more likely that the matrix protein variation described here is an example of genetic dimorphism in influenza virus populations, which has been previously described for the influenza hemagglutinin (4, 9, 10) and neuraminidase (19). Examination of influenza A viruses with a larger number of monoclonal antibodies may answer more clearly whether antigenic variation in the matrix protein arises from progressive changes, genetic dimorphism, or both. An unexpected result of these experiments was that the matrix protein of the A/USSR/90/77 virus differed antigenically from that of the A/Fort Warren/1/50 strain since these two strains have not been distinguished previously with monoclonal antibodies to the hemagglutinin and nucleoprotein (25, 27). Although the data indicate that these two strains possess antigenically distinguishable matrix proteins, they do not address the question of whether or not the A/USSR/90/77 virus arose directly from the A/Fort Warren/1/50 strain; point mutations or genetic dimorphism in strains circulating in 1950 or 1977 could explain the observed antigenic difference. That antigenic variation was detected in only 3 of 26 strains stands in sharp contrast not only to similar studies with monoclonal antibodies to the hemagglutinin and neuraminidase (27), but also to those with the other major structural protein of influenza viruses, namely, the nucleoprotein (25). Although the number of monoclonal antibodies used in the present study is small, the results suggest that matrix protein undergoes a lesser degree of antigenic variation than the nucleoprotein. The reason for this is not clear, since both are internal proteins and neither is subject to antibody selection pressure in the population. It is possible that viruses with unaltered matrix proteins have a selective advantage since the matrix protein appears to play a role in virus assembly and maturation (16).

The antigenic domains of the influenza A virus matrix protein were mapped by testing the monoclonal antibodies in competitive binding ELISA. Results of these assays (Fig. 2) indicated that the matrix protein has at least two physically distinct domains which can bind monoclonal antibodies noncompetitively. Assays with monoclonal antibodies to a
third epitope (904/6) gave ambiguous results; this monoclonal antibody exhibited nonreciprocal competitive binding with monoclonal antibody M2-1C6. To determine whether the nonreciprocal binding was due to allosteric effects or to low-affinity binding of antibody M2-1C6 with a cross-reactive epitope located in near proximity to the epitope recognized by antibody 904/6, Western blot analysis of proteolytically digested matrix protein was performed. Under these conditions antibody 904/6 exclusively bound to one fragment of the matrix protein. According to molecular weight markers this particular fragment was approximately 14 kilodaltons in size, which represents roughly half of the matrix protein. Since antibody M2-1C6 did not bind to this rather large fragment, it seems unlikely that a cross-reactive determinant located near the binding site for antibody 904/6 is responsible for the above-described nonreciprocal inhibition. A more likely explanation of these results is that the binding of antibody M2-1C6 promotes a conformational change in the matrix protein which renders the epitope defined by antibody 904/6 sterically less accessible for antibody binding. The exact mechanism responsible for nonreciprocal binding of monoclonal antibodies to the matrix protein may be elucidated when the topological location of the determinants involved is determined more precisely. These experiments are currently in progress.

ACKNOWLEDGMENTS

We acknowledge the capable technical assistance of E. Wu, and we thank L. Jordan for preparation of the manuscript and R. M. Chanock for helpful discussion.

LITERATURE CITED