

IMMUNOLOGIC STUDIES ON THE INFLUENZA A VIRUS NONSTRUCTURAL PROTEIN NS₁*

BY MICHAEL W. SHAW,‡ EDDIE W. LAMON, AND RICHARD W. COMPANS

From the Departments of Microbiology and Surgery and The Veterans Administration Hospital, University of Alabama in Birmingham, Birmingham, Alabama 35294

The eight RNA genome segments of influenza A viruses have been shown to encode at least 10 polypeptides(1-6). Previous immunologic studies on these polypeptides have been limited primarily to the major virion structural proteins, hemagglutinin, nucleocapsid protein (NP),¹ neuraminidase, and the membrane protein. Such studies have included analyses of antigenic cross-reactivities (7-10) and localization of antigens within and on the surfaces of virus-infected cells (11-17). These studies have shown that in addition to the major glycoprotein antigenic shifts that are thought to result from molecular reassortment of the viral genome segments during double infection, influenza viruses undergo a gradual antigenic drift due to mutations in the RNA coding for the viral proteins (8). Such antigenic drift has been detected in the hemagglutinin and neuraminidase glycoproteins (7, 10) and, to a lesser extent, in the NP polypeptide (9).

The smallest influenza viral genome segment (segment 8) contains overlapping genes (18) coding for two nonstructural proteins designated NS₁ (~23,000 mol wt) and NS₂ (~14,000 mol wt). The antigenic properties of these nonstructural proteins have not been investigated previously. Further, there is little information available on the function of these polypeptides, although the largest nonstructural protein (NS₁) is often the most abundant viral polypeptide in influenza A virus-infected cells (1-3). Cell fractionation experiments have shown that NS₁ is associated with cell fractions containing polysomes (19, 20) and with nucleoplasm and nucleolar fractions (1, 21-23). NS₁ has also been shown to form electron-dense cytoplasmic inclusions in association with RNA (21, 24). We have previously shown (20) that milligram quantities of purified influenza A virus NS₁ protein can be obtained in the form of paracrystalline inclusions isolated from the cytoplasm of infected cells. This has made it feasible to produce antiserum specific for this polypeptide. This report describes the first immunologic characterization of purified NS₁ protein, including the demonstration of antigenic cross-reactivity between the NS₁ proteins from different influenza virus strains by immunoprecipitation and competition radioimmunoassay.

* Supported by grants AI 12680 and CA 17273 from the U. S. Public Health Service.

‡ Supported by training grant T32 CA09202 from the National Cancer Institute. Present address is The Rockefeller University, New York 10021.

¹ *Abbreviations used in this paper:* BBS, barbitone-buffered saline; BSA, bovine serum albumin; MEM, Eagle's minimal essential medium; NP, nucleocapsid protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; REM, reinforced Eagle's medium; RIA, radioimmunoassay; RSB, reticulocyte standard buffer; SDS, sodium dodecyl sulfate; T cells, thymus-dependent lymphocytes; WSN, A/WSN/33 virus.

Materials and Methods

Virus and Cells. Stock A/WSN/33 (H₁N₁) virus was grown in MDBK cells (25). Stock A/PR/8/34 (H₁N₁), A/FW/1/50 (H₁N₁), A/USSR/90/77 (H₁N₁), A/R1/5+/57 (H₂N₂), A/Jap/305/57 (H₂N₂), A/Victoria/3/75 (H₃N₂), A/Swine/1976/31 (H_{sw}N₁), and B/Lee/40 viruses were grown in the allantoic cavity of embryonated hens' eggs. Seed virus diluted into Eagle's minimal essential medium (MEM) with 1% bovine serum albumin (BSA) was injected into the allantoic cavity of 10–11-d-old embryonated eggs using a volume of 0.2 ml per egg. After a 2-d incubation at 37°C, the infected eggs were transferred to 4°C overnight. The allantoic fluid was then harvested, and cells and cell debris were removed by pelleting for 20 min at 1,000 *g*. The supernatant was frozen in aliquots and stored at –80°C. MDBK (bovine kidney), MDCK (canine kidney), and BHK21-F (baby hamster kidney) cells were grown according to previously described procedures (25–27).

Isolation of Cytoplasmic Inclusions. Monolayer cultures of BHK21-F cells grown in 100-mm plastic petri dishes were harvested by scraping at 20–24 h after infection with A/WSN/33 (WSN) virus, as described previously (20). The cells were pelleted and resuspended in RSB (0.01 M NaCl, 0.005 M MgCl₂, 0.01 M Tris HCl, pH 7.4) and kept on ice for 20 min before homogenizing for 40 strokes with a Dounce homogenizer. The extract was centrifuged at 800 *g* for 10 min, and the pellet was resuspended to 10 ml in Tris-EDTA (0.01 M Tris-HCl, 0.02 M EDTA) and 1% Triton X100 and homogenized for an additional 20 strokes. The nuclei were pelleted at 800 *g* for 10 min, and the supernatant fractions from the two homogenizations were pooled and extracted with trichlorotrifluoroethane (28), first with twice the sample volume of fluorocarbon and then three times with equal volumes of sample and fluorocarbon.

The aqueous extract was pelleted at 20,000 rpm for 30 min in an SW27 rotor and resuspended in 2.5 ml of Tris-EDTA and 1% Triton X100. After an 18-h incubation at 4°C, the sample was layered onto discontinuous sucrose gradients prepared as follows (wt/wt) in Tris-EDTA (top to bottom: 2.5-ml sample; 2.5 ml each of 45%, 50%, 55%, and 60% sucrose) and centrifuged at 35,000 rpm for 90 min in an SW41 rotor. The band of inclusions forming at the 55/60% sucrose interface was collected, diluted in Tris-EDTA, and washed by pelleting (three times for 15 min at 25,000 rpm in an SW41 rotor) in Tris-EDTA before resuspension in that buffer.

Immune Sera. The purity of NS₁ from batches of inclusions was ascertained by polyacrylamide gel electrophoresis (PAGE) (see below). For immunization, purified inclusions were solubilized by addition of urea to 6 M and KCl to 1 M and incubating at 60°C for 24 h. An aliquot of 500 µg of protein in 6 M urea was emulsified in Freund's complete adjuvant and injected subcutaneously into adult rabbits. At 2-wk intervals, second and third injections of 250 µg each in Freund's incomplete adjuvant were given subcutaneously. After another 2 wk, 250 µg of protein from inclusions solubilized as above and readjusted to physiologic conditions by dialysis against phosphate-buffered saline (PBS) was injected intravenously into the marginal ear vein. Bleeding was done 1 wk after the final injection.

Antiserum to the structural proteins of WSN virus was obtained by immunization of rabbits with ether-disrupted purified virions. The first intravenous injection consisted of 200 µg of protein. After 2 wk, three intraperitoneal injections of 500 µg each were given at 2-mo intervals. Serum was collected 2 wk after the final injection.

All serum samples were divided into 0.5–1.0 ml aliquots and stored at –60°C.

Complement Fixation Assay. Serial twofold dilutions of rabbit anti-NS₁ serum, previously heat inactivated at 56°C for 30 min, were prepared in 0.15 M barbitone-buffered saline, pH 7.6, containing 10 mM MgCl₂ (BBS) (29) in a volume of 40 µl each. To the antiserum dilution were added 20 µl of antigen (10 µg protein) in BBS or BBS alone and two minimum hemolytic doses of guinea pig complement in 40 µl of BBS. After a 30-min incubation at 37°C, 50 µl of IgM-sensitized sheep erythrocytes labeled with ⁵¹Cr, as previously described (30), and adjusted to 2 × 10⁸ cells/ml in BBS was added. After a second 30-min incubation at 37°C, 75 µl of supernate from each sample was assayed for radioactivity. For the determination of spontaneous release, erythrocytes were incubated as above in BBS alone. Maximum release was determined by the incubation of sensitized erythrocytes with complement and BBS in the place of antiserum and antigen. Percent lysis was calculated by the following formula: percent lysis = [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] × 100.

Immunoprecipitation of Cell Extracts. Monolayer cultures of MDCK or MDBK cells grown in

60-mm plastic petri dishes were inoculated with influenza A or influenza B virus at a multiplicity of 10–20 plaque-forming units/cell in 1 ml of MEM with 1% BSA per culture. After a 2½-h adsorption period at 37°C, 2.5 ml of MEM supplemented with 2% calf serum was added to each culture.

At 4½ h after infection, the medium was removed and the monolayers rinsed with PBS and incubated for 30 min in 1 ml per dish of MEM without methionine or without leucine. The cells were radiolabeled with 1 ml per dish of methionine or leucine-free MEM supplemented with 50 µCi [³⁵S]methionine or 100 µCi [³H]leucine, respectively. At 8 h after infection, the cell monolayers were rinsed with cold PBS, overlaid with 0.5 ml per dish of cell dissociation buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.02 M EDTA, 1% Triton X100, 1% sodium deoxycholate, pH 7.5), and incubated for 30 min at 4°C. The nuclei were pelleted out at 12,000 g for 4 min and the supernatants stored at –80°C until use.

For immunoprecipitation, extracts were mixed with 5 µl of rabbit antiserum and incubated at 37°C for 30 min. Then 100 µl of a 10% (wt/vol) suspension of *Staphylococcus aureus* (31) in cell dissociation buffer was added, and the mixture was incubated for 30 min at room temperature. The samples were then pelleted at 12,000 g for 3 min and the pellets washed three times, first with cell dissociation buffer with 0.1% sodium dodecyl sulfate (SDS) and then twice with cell dissociation buffer without SDS.

The washed pellet was resuspended in 25 µl of deionized water and 50 µl of 2X PAGE dissociation buffer (4% SDS, 4% 2-mercaptoethanol in 0.125 M Tris-HCl, pH 6.8) and incubated at 60°C for 60 min. The slurry was pelleted at 12,000 g for 4 min and the supernate run on PAGE.

Protein PAGE. Samples were heated for 1 min at 100°C in 2% SDS and 2% 2-mercaptoethanol in 0.0625 M Tris-HCl, pH 6.8. Then, 14% (or 17.5%) acrylamide, 0.093% (or 0.116%) N,N'-methylenebisacrylamide gels containing SDS were prepared and run at a constant 15 mA per 3-mm slab gel, as described by Laemmli (32). Processing and exposing of gels for fluorography was as described by Bonner and Laskey (33).

Radioiodination of NS₁ Protein. Purified NS₁ inclusions from WSN-infected BHK21-F cells were dissociated by incubation at 60°C for 60 min in 0.01 M Tris-HCl, pH 8.5, 0.001 M EDTA, 6 M urea, and 1 M KCl (TEU buffer). The dissociated protein and RNA were then separated by centrifugation (SW50.1 rotor, 48,000 rpm for 23 h) through a 10–20% (wt/vol) sucrose gradient prepared in TEU buffer as described elsewhere (24). The gradients were collected by dripping 0.4-ml fractions from the bottom of the tubes. Those fractions containing RNA-free NS₁ protein were pooled and dialyzed against deionized water for 18 h at 4°C. The protein was then lyophilized and stored at –20°C until use.

The RNA-free NS₁ protein was radiolabeled with ¹²⁵I by the chloramine T method (34) modified as follows: 5 µg of protein in 10 µl of 1 M sodium phosphate buffer (pH 7.2) with 8 M urea was mixed with 1 mCi of [¹²⁵I]Na and 10 µl of chloramine T (2.5 mg/ml). After 15 min at room temperature, an additional 10 µl of chloramine T (2.5 mg/ml) was added. After a further 15 min at room temperature, the reaction was mixed with 40 µl of sodium metabisulfite (2.5 mg/ml), 100 µl of KI (100 mg/ml), and 50 µl of calf serum, and the labeled NS₁ protein was desalted through a column of Sephadex G-25 with TEU buffer containing 1 mg/ml of BSA.

Radioimmunoassay (RIA) for NS₁. Antibody was titrated for 50% precipitation endpoint by mixing 50 µl of antiserum dilutions in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% BSA, 0.1% Sarkosyl, pH 7.4 (RIA buffer), with 50 µl of cell dissociation buffer and incubation for 1 h at 37°C before addition of 50 µl of [¹²⁵I]NS₁ (~10,000 trichloroacetic acid-precipitable cpm in RIA buffer). After incubation for 1 h at 37°C, a 10% wt/vol suspension of *S. aureus* in RIA buffer (75 µl) was added, and the mixture was incubated at room temperature for 60 min. The suspension was then diluted with 1 ml of RIA buffer and pelleted by centrifugation. The pellets were washed twice with RIA buffer before assaying for radioactivity. Specific precipitation was determined by subtracting cpm precipitated with normal rabbit serum from that precipitated with an equivalent dilution of immune serum.

Cell extracts for use in competition radioimmunoassays were prepared by disrupting cells (uninfected or 24 h postinfection) in cell dissociation buffer and removing the nuclei by pelleting at 1,000 g for 20 min. The cytoplasmic extracts were stored at –20°C until use.

Competition radioimmunoassays for NS₁ were conducted by initially mixing 50 μ l of antiserum diluted to its 50% precipitation titer in RIA buffer with 50 μ l of unlabeled NS₁ or cell extract dilutions in cell dissociation buffer and incubating 60 min at 37°C. Then 50 μ l of [¹²⁵I]NS₁ (~10,000 TCA-precipitable cpm in RIA buffer) was added and the mixture incubated further for 60 min at 37°C. A 10% (wt/vol) suspension of *S. aureus* in RIA buffer (50 μ l) was then added and the mixture incubated at room temperature for 60 min. After dilution with 1 ml of RIA buffer, the samples were pelleted and washed twice with RIA buffer before assaying for radioactivity. Specific precipitation was determined as above for antibody titration.

Results

Titration of Rabbit Antiserum. The specificity and titers of antisera were determined by complement fixation tests with inclusions purified from influenza WSN virus-infected BHK21-F cells or structural proteins from purified WSN virions as antigen. Antiserum raised against urea-dissociated NS₁ inclusions was found to be specific for NS₁ by the complement fixation test (50% inhibition of hemolysis titer = 600), with no detectable activity against virus structural proteins (Fig. 1). Preimmune serum from the same rabbit showed no discernable reactivity with virus structural or nonstructural proteins. Antisera raised against intact inclusions (data not shown) gave a higher anti-NS₁ titer (1,200) but also had a high anti-NP titer (300).

To characterize the specificity of antisera by another approach, we analyzed the radiolabeled polypeptides in immune precipitates from cytoplasmic extracts of WSN virus-infected MDBK cells. The antiserum raised using dissociated inclusions as immunogen was observed to be specific for NS₁ protein, as shown in Fig. 2. In some experiments, variable amounts of NP were also seen in immune precipitates obtained with this antiserum; however, the demonstration of a single polypeptide band corresponding to NS₁, as in Fig. 2, lane b, suggests that the occasional presence of virion structural proteins in immune precipitates is due to nonspecific coprecipitation rather than to reactivity of the antibody preparations with virion structural proteins.

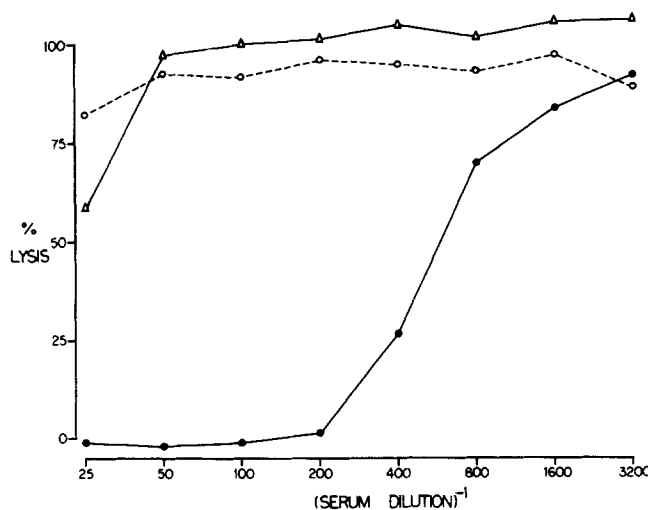


FIG. 1. Titration of rabbit antiserum to urea-dissociated NS₁ inclusions by complement fixation. Samples used for titration were intact NS₁ inclusions (●), purified WSN structural proteins (Δ), or buffer alone (○).

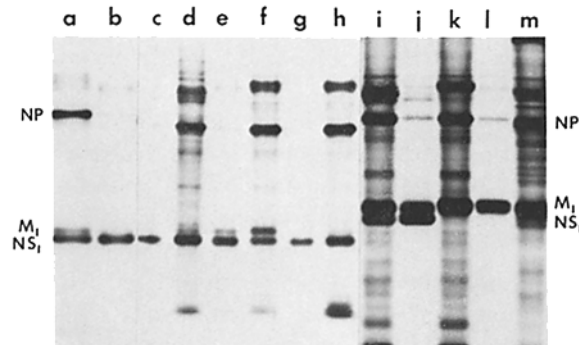


FIG. 2. Analysis of polypeptides precipitated by antiserum to the WSN NS₁ protein from [³H]leucine-labeled extracts from MDCK cells infected with different influenza A virus serotypes. Radiolabeled polypeptides in cell extracts or immune precipitates were analyzed by PAGE and fluorography, as described in Materials and Methods. (a) WSN-infected cell extract; (b) immune precipitate from WSN-infected cell extract; (c) immune precipitate from PR8-infected cell extract; (d) PR8-infected cell extract; (e) immune precipitate from FW-infected cell extract; (f) FW-infected cell extract; (g) immune precipitate from USSR-infected cell extract; (h) USSR-infected cell extract; (i) RI-infected cell extract; (j) immune precipitate of (i); (k) Victoria-infected cell extract; (l) immune precipitate of (k); (m) WSN-infected cell extract.

Furthermore, this antiserum precipitated no virion structural polypeptides when reacted with detergent-disrupted purified virus (not shown).

Immunoprecipitation of Infected Cell Extracts. To detect possible cross-reactivity in the antigenic determinants among the NS₁ proteins of different influenza virus strains, we prepared labeled cell extracts for immune precipitation as described in Materials and Methods. Immunoprecipitates were analyzed by electrophoresis in 14% polyacrylamide gels in parallel with the corresponding unprecipitated cell extracts, as shown in Fig. 2, lanes c–m. In all cases, the antiserum used for immunoprecipitation was that raised against dissociated NS₁ inclusions from WSN-infected cells shown to be specific for NS₁ by complement fixation (above). The virus strains used for comparison were representative of all human influenza A antigenic subtypes and included PR8 (A/PR/8/34, H₁N₁), FW (A/FW/1/50, H₁N₁), USSR (A/USSR/90/77, H₁N₁), RI (A/RI/5+/57, H₂N₂), and Victoria (A/Victoria/3/75, H₂N₂).

No specific immunoprecipitation was detectable when extracts of uninfected MDCK cells were treated with the anti-NS₁ serum (data not shown). However, the antiserum to the WSN NS₁ protein appears to recognize and precipitate virus-coded NS₁ polypeptides from cells infected with all influenza A virus strains tested, including PR8 virus-infected MDCK cells (Fig. 2c), cells infected with either FW (Fig. 2e) or USSR (Fig. 2g) viruses, and cells infected with RI (Fig. 2j) and Victoria (Fig. 2l) virus. With some samples, the NP polypeptides and minor amounts of some higher molecular weight polypeptides were found in the immune precipitates. Also, because the M₁ and NS₁ proteins of USSR (Fig. 2h) and Victoria (Fig. 2k) viruses are not resolved in this gel system, the possibility that M₁ is also present in the immunoprecipitates cannot be excluded. These results are consistent with the conclusion that antibody to the WSN-NS₁ protein is recognizing and precipitating the NS₁ protein synthesized by all the other influenza A virus serotypes tested.

Radioimmunoassay for NS Protein. We used a competition radioimmunoassay to quantitatively compare the antigenic relationships of NS₁ polypeptides from different

influenza virus serotypes. The NS₁ polypeptide from purified inclusions isolated from WSN virus-infected MDCK cells was separated from RNA and radiolabeled *in vitro* with ¹²⁵I, as described in Materials and Methods. The modifications used (increased oxidation time with chloramine T in the presence of 8 M urea) were found to be necessary to maximize the radiolabeling of the single tyrosine residue per molecule shown to be present in NS₁ protein by amino acid analysis (20) and nucleotide sequencing (35). Under these conditions, the specific activity of the labeled sample was 2×10^4 cpm/ng.

Titration of the antiserum prepared against dissociated NS₁ inclusions with the radioiodinated NS₁ sample is shown in Fig. 3. Complete (100%) specific precipitation was determined by subtraction of the radioactivity precipitated by equivalent dilutions of normal rabbit serum. Similar experiments using anti-virion serum showed no specific precipitation, indicating the absence of appreciable quantities of radiolabeled structural antigens in the [¹²⁵I]NS₁ preparation. The 50% precipitation endpoint of

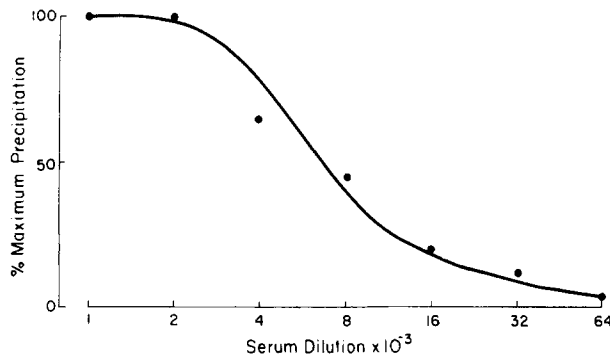


FIG. 3. Titration of antiserum to the NS₁ protein of influenza A/WSN virus with radioiodinated WSN NS₁ protein. A 50- μ l aliquot of each antiserum dilution in RIA buffer was incubated with 50 μ l of cell dissociation buffer and 50 μ l of [¹²⁵I]NS₁ (10,000 TCA-precipitable counts in RIA buffer) and the antigen-antibody complexes precipitated with *S. aureus*.

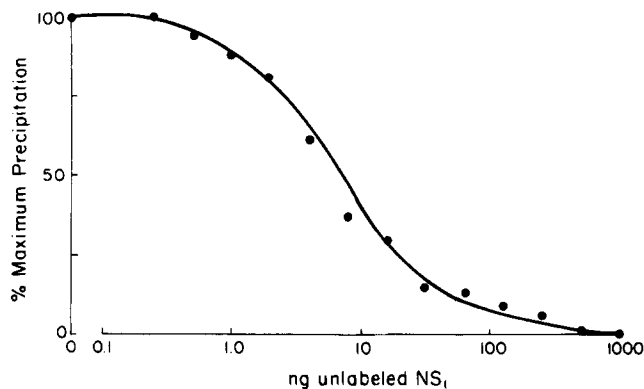


FIG. 4. Standardization of competition radioimmunoassay for NS₁ protein. The antiserum titered in Fig. 3 was used at a 1:6,000 dilution to precipitate [¹²⁵I]NS₁ after preincubation with various concentrations of unlabeled NS₁. 50- μ l aliquots of a 1:6,000 dilution of antiserum in RIA buffer was reacted with 50- μ l samples of purified NS₁ at varying concentrations in cell dissociation buffer before the addition of 10,000 TCA-precipitable counts of [¹²⁵I]NS₁ in RIA buffer.

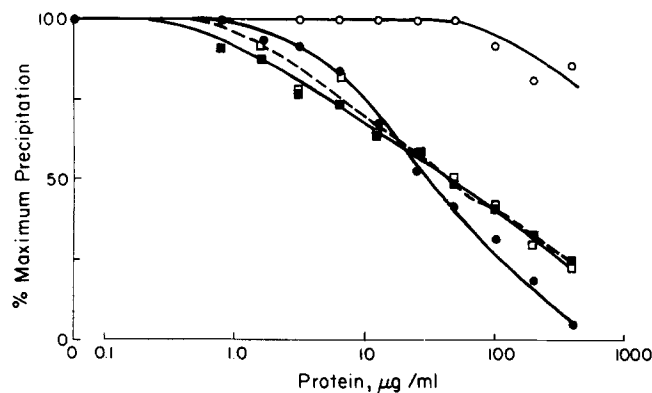


FIG. 5. Competition radioimmunoassay for NS₁ using extracts of MDCK cells infected with different influenza virus serotypes as competitors. The assay was performed as in Fig. 4 using infected cell extracts in cell dissociation buffer as competitors. ●, A/WSN/33; ■, A/PR/8/34; □, A/Swine/1976/31; ○, B/Lee/40.

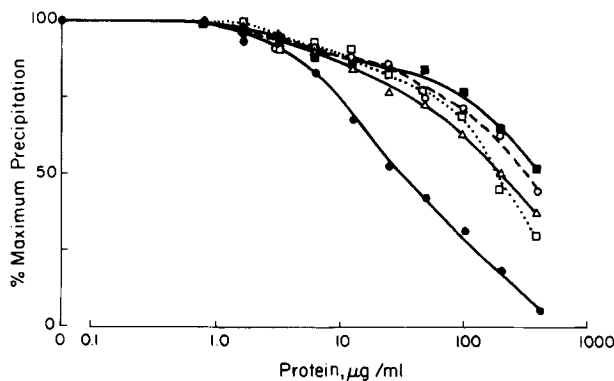


FIG. 6. Competition radioimmunoassay for NS₁ using extracts of MDCK cells infected with different influenza A virus serotypes as competitors. The assay was performed as in Fig. 4. ●, A/WSN/33; ■, A/FW/1/50; □, A/USSR/90/77; ○, A/Jap/305/57; △, A/Victoria/3/75.

the serum (1:6,000) determined as in Fig. 3 was used in subsequent competition assays.

Competitive binding curves were obtained using known quantities of purified NS₁ protein from inclusions obtained from BHK21-F cells infected with WSN. As shown in Fig. 4, the serum used at a 1:6,000 dilution titrates over a range from ~1–100 ng of purified NS₁. 50% inhibition was obtained with ~6.5 ng of NS₁ when 50 µl of diluted serum was used.

The competition curves obtained using cytoplasmic extracts of MDCK cells infected with homologous and heterologous influenza viruses are shown in Figs. 5 and 6. Titrations containing >20 µg of total protein in the cell extract competitors (400 µg/ml) were not possible because of nonspecific inhibition at the higher concentrations. The competition curve obtained with WSN virus-infected cell extract (Fig. 5) was almost identical in shape to that obtained with purified NS₁. Because the 50% endpoint of the cell extract competition occurred at ~1.75 µg of total protein, we

calculated that 0.4–0.5% of the total cytoplasmic extract protein is NS₁ at 8 h after the infection of MDCK cells with WSN virus.

Also shown in Fig. 5 are competition curves using extracts of MDCK cells infected with PR8, Swine, and B/Lee, which are all viruses isolated within 10 yr of A/WSN/33. The two other influenza A viruses exhibited substantial competition, indicating extensive cross-reactivity. The polyphasic nature of the curves indicates the presence of at least two, and possibly more, independent antigenic determinants interacting with this rabbit antiserum. The apparent low level of competition seen with B/Lee/40 may be artifactual because, unlike the titration curves seen with the type A influenza viruses, repeated experiments gave inconsistent results. Further study would be required before any definite conclusions could be drawn about possible antigenic cross-reactivity between nonstructural antigens of type A and B influenza viruses.

Fig. 6 shows the competition curves obtained with human influenza A virus subtypes isolated from 1950 to 1977. All show varying degrees of competition, with appreciable cross-reactivity evident in all cases. The positions of the curves with respect to each other suggests that the degree of cross-reactivity with the NS₁ protein encoded by WSN is more closely related to the date of isolation rather than to the serotype of the surface glycoproteins; i.e., the competition curve obtained with the 1950 isolate FW (H1N1) is closer to that obtained with the 1957 Jap (H2N2) virus; however, USSR, another H1N1 virus, appears more closely related to Victoria, an H3N2 subtype similar to viruses with which it circulated concurrently in 1977.

Discussion

We prepared monospecific antiserum to the influenza A virus nonstructural polypeptide designated NS₁ and carried out the first analysis of antigenic properties of this polypeptide in influenza A viruses. While cross-reactive nonstructural antigens were detected previously in cells infected with influenza A viruses by the use of mouse convalescent serum, the serum used might have reacted with NS₂ in addition to NS₁ (12, 13). Our antigenic analyses of influenza virus NS₁ protein indicate that extensive cross-reactivity exists between all subtypes that were examined of human influenza A viruses isolated from 1934 to 1977. The immunoprecipitation studies show that antibody to WSN NS₁ protein recognized NS₁ in cells infected with five different serotypes of influenza A virus. As revealed in these immunoprecipitation studies, NS₁ protein encoded by all subtypes of influenza A virus tested is recognized by antibody raised against purified NS₁ polypeptides obtained from inclusions isolated from cells infected with the A/WSN strain.

The results of competition radioimmunoassays using infected or uninfected cell extracts as competitor indicated that such assays are useful for the comparison of the antigenic relationships of the NS₁ proteins synthesized by different influenza virus strains. The use of purified NS₁ protein from all the subtypes selected for comparison was not possible because the isolation technique was not successful with all strains. As a consequence of this lack of purified competitors, the use of infected cell extracts in competition radioimmunoassay analyses precluded exact comparison of antigenic determinants because of nonspecific inhibition at higher total protein concentrations. In the assays described here, maximum inhibition levels could not be determined for competitors except in the homologous system. The radioimmunoassay competition curves obtained suggested the presence of at least two independent antigenic deter-

minants on the NS₁ molecule. The differences in the slopes of these curves further suggest that the various competitors bind with different affinities. It is likely that monoclonal antibodies to NS₁, when available, will allow more precise definition of antigenic relationships as has been the case for the virion glycoproteins (36).

Recent studies on influenza A virus genome segments and mRNA species support the conclusions reached in the present study. RNA:RNA hybridization studies conducted on genome segment 8 of influenza A virus (37) indicate that all the non-avian type A viruses studied fall into a single group possessing a sequence homology of >90%. Studies using nucleotide sequencing techniques to deduce the amino acid sequences of the NS₁ proteins encoded by A/PR/8/34 (H1N1), A/FPV/Rostock/34 (Hav1N1), and A/Udorn/307/72(H3N2) viruses show that the predicted amino acid sequences of the NS₁ proteins encoded by these strains vary by only 10–15% (35, 38, 39).

The rabbit antiserum used in the present study appeared to precipitate variable amounts of the viral NP protein from infected cell extracts. However, because this precipitation of NP was not a constant occurrence and the results of the complement fixation assays indicated no activity against virion structural proteins, it seems likely that the presence of NP in these immunoprecipitates is the result of nonspecific coprecipitation. In addition, the antiserum did not precipitate any virion proteins from purified virus preparations, and immunodiffusion studies using infected cell extracts as antigen (not shown) showed a single precipitin line using this antiserum. The reason for such coprecipitation is uncertain, but it is conceivable that it could be due to the association of both NS₁ and NP with the same cytoplasmic structures. It was reported previously that both polypeptides are associated with cell fractions containing polysomes (19, 20). If the antibody recognized and bound to NS₁ while it was associated with polysomes, then the resulting immunoprecipitate would probably contain any other proteins associated with those structures.

Recent studies in this laboratory indicate that NS₁ antigen is expressed on the surfaces of infected cells (17), suggesting that an immune response to this protein could conceivably be of importance. Because the immunoprecipitation and radioimmunoassay results clearly show extensive antigenic cross-reactivity in the NS₁ proteins produced by different influenza A virus serotypes, NS₁-related antigens should be considered as possible targets for cross-reactive cytotoxic T cells generated during infection. These cytotoxic T cells are capable of extensive cross-reactivity that is not limited by the surface glycoproteins serotype (41–43). Other studies have, however, also indicated the surface expression of other cross-reactive viral proteins including M (15, 44) and NP (14), although monoclonal anti-M₁ IgG was not capable of blocking the observed cross-reactive cytotoxicity (16).

Also, our preliminary experiments comparing acute and convalescent human sera indicate that anti-NS₁ activity might increase during infection with the H3N2 viruses currently in circulation. Further information is needed to evaluate the immune response to NS₁ and its possible significance.

Summary

We purified the major influenza virus nonstructural protein, designated NS₁, from cytoplasmic inclusions that were solubilized and used to raise antisera in rabbits. One of the antisera was found to be specific for NS₁ by complement fixation tests and

analyses of immune precipitates. Antiserum to NS₁ isolated from cells infected with A/WSN/33 virus specifically precipitated NS₁ from extracts of cells infected with seven distinct isolates of influenza A virus representing five different antigenic subtypes. These included A/WSN/33, A/PR/8/34, A/FW/5/50, A/USSR/90/77, A/RI/5+/57, A/Victoria/3/75, and A/Swine/1977/31; however, NS₁ from cells infected with B/Lee/40 virus was not precipitated. Radioimmunoassays using radioiodinated NS₁ protein from A/WSN virus-infected cells and unlabeled cytoplasmic extracts of cells infected with various strains of influenza virus as competitors indicated significant antigenic cross-reactivities for the NS₁ proteins of all influenza A viruses tested. The results suggest a gradual antigenic drift over the 45 yr separating the earliest and most recent virus isolates examined. Thus, compared with the virion neuraminidase and hemagglutinin antigens, NS₁ appears to be highly conserved in different influenza A virus isolates.

Received for publication 6 April 1982.

References

1. Lazarowitz, S. G., R. W. Compans, and P. W. Choppin. 1971. Influenza virus structural and nonstructural proteins in infected cells and their plasma membranes. *Virology*. **46**:830.
2. Skehel, J. J. 1972. Polypeptide synthesis in influenza virus infected cells. *Virology*. **49**:23.
3. Lamb, R. A., P. R. Etkind, and P. W. Choppin. 1978. Evidence for a ninth influenza viral polypeptide. *Virology*. **91**:60.
4. Lamb, R. A., and P. W. Choppin. 1981. Identification of a second protein (M₂) encoded by RNA segment 7 of influenza viruses. *Virology*. **112**:729.
5. McGeoch, D., P. Fellner, and C. Newton. 1976. Influenza virus genome consists of eight distinct RNA species. *Proc. Natl. Acad. Sci. U. S. A.* **73**:3045.
6. Desselberger, U., and P. Palese. 1978. Molecular weights of RNA segments of influenza A and B viruses. *Virology*. **88**:394.
7. Paniker, C. K. J. 1968. Serological relationships between the neuraminidases of influenza viruses. *J. Gen. Virol.* **2**:385.
8. Webster, R. G., and W. G. Laver. 1971. Antigenic variation in influenza virus. Biology and chemistry. *Progr. Med. Virol.* **13**:271.
9. Schild, G. C., J. S. Oxford, and R. W. Newman. 1979. Evidence for antigenic variation in influenza A ribonucleoprotein. *Virology*. **93**:569.
10. Six, H. R., and J. A. Kasel. 1979. Assessment of the antigenic relatedness among H3 hemagglutinins of type A influenza viruses by competition radioimmunoassay. *Virology*. **96**:588.
11. Maeno, K., and E. D. Kilbourne. 1970. Developmental sequence and intracellular sites of synthesis of three structural protein antigens of influenza A₂ virus. *J. Virol.* **5**:153.
12. Dimmock, N. J. 1969. New virus-specific antigens in cells infected with influenza virus. *Virology*. **39**:224.
13. Dimmock, N. J., and D. H. Watson. 1969. Proteins specified by influenza virus in infected cells: analysis by polyacrylamide gel electrophoresis of antigens not present in the virus particle. *J. Gen. Virol.* **5**:499.
14. Virelizier, J. L., A. C. Allison, J. S. Oxford, and G. C. Schild. 1977. Early presence of ribonucleoprotein antigen on surface of influenza virus-infected cells. *Nature (Lond.)*. **266**:52.
15. Biddison, W. E., P. C. Doherty, and R. G. Webster. 1977. Antibody to influenza virus matrix protein detects a common antigen on the surface of cells infected with type A influenza virus. *J. Exp. Med.* **146**:690.

16. Hackett, C. J., B. A. Askonas, R. B. Webster, and K. van Wyke. 1980. Quantitation of influenza virus antigens on infected target cells and their recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* **151**:1014.
17. Shaw, M. W., E. W. Lamon, and R. W. Compans. 1981. Surface expression of nonstructural antigen on influenza A virus-infected cells. *Infect. Immun.* **34**:1065.
18. Lamb, R. A., and P. W. Choppin. 1979. Segment 8 of the influenza virus genome is unique in coding for two polypeptides. *Proc. Natl. Acad. Sci. U. S. A.* **76**:4980.
19. Compans, R. W. 1973. Influenza virus proteins. II. Association with components of the cytoplasm. *Virology.* **51**:56.
20. Shaw, M. W., and R. W. Compans. 1978. Isolation and characterization of cytoplasmic inclusions from influenza A virus-infected cells. *J. Virol.* **25**:608.
21. Taylor, J. M., A. W. Hampson, J. E. Layton, and D. O. White. 1970. The polypeptides of influenza virus. IV. An analysis of nuclear accumulation. *Virology.* **42**:744.
22. Krug, R. M., and P. R. Etkind. 1973. Cytoplasmic and nuclear virus-specific proteins in influenza virus-infected MDCK cells. *Virology.* **56**:334.
23. Krug, R. M., and R. Soeiro. 1975. Studies on the intranuclear localization of influenza virus-specific proteins. *Virology.* **64**:378.
24. Yoshida, T., M. W. Shaw, J. F. Young, and R. W. Compans. 1981. Characterization of the RNA associated with influenza A cytoplasmic inclusions and the interaction of NS₁ protein with RNA. *Virology.* **110**:87.
25. Choppin, P. W. 1969. Replication of influenza virus in a continuous cell line: high yield of infective virus from cells inoculated at high multiplicity. *Virology.* **39**:130.
26. Holmes, K. V., and P. W. Choppin. 1966. On the role of the cell membrane in determining virus virulence. Contrasting effects of the parainfluenza virus SV5 in two cell types. *J. Exp. Med.* **124**:501.
27. Nakamura, K., and R. W. Compans. 1978. Glycopeptide components of influenza viral glycoproteins. *Virology.* **86**:432.
28. Nagayama, A., and S. Dales. 1970. Rapid purification and the immunological specificity of mammalian microtubular paracrystals possessing an ATPase activity. *Proc. Natl. Acad. Sci. U. S. A.* **66**:464.
29. Hudson, L., and F. C. Hay. 1976. "Appendix I. Buffers and Media." In *Practical Immunology*. L. Hudson and F. C. Hay, editors. Blackwell Scientific Publications, Oxford, England. 329.
30. Fuson, E. W., M. W. Shaw, R. A. Hubbard, and E. W. Lamon. 1979. Antibody-antigen complex stimulated lysis of nonsensitized sheep red cells by human lymphocytes. I. Requirement for IgG complexes. *Clin. Exp. Immunol.* **38**:158.
31. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617.
32. Laemmli, V. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. **227**:680.
33. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46**:83.
34. Hunter, W. M., and F. C. Greenwood. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature (Lond.)*. **194**:495.
35. Lamb, R. A., and C.-J. Lai. 1980. Sequence of interrupted and uninterrupted mRNAs and cloned DNA coding for the two overlapping nonstructural proteins of influenza virus. *Cell.* **21**:475.
36. Gerhard, W., J. Yewdell, and M. Frankel. 1980. "An experimental approach to define the antigenic structures of the hemagglutinin molecule of A/PR/8/34." In *Structure and*

- Variation in Influenza Virus. W. G. Laver and G. Air, editors. Elsevier/North-Holland, Inc. 273.
37. Scholtissek, C., and V. Von Hoyningen-Huene. 1980. Genetic relatedness of the gene which codes for the nonstructural (NS) protein of different influenza A virus strains. *Virology*. **102**:13.
 38. Porter, A. G., J. C. Smith, and J. S. Emtage. 1980. The sequence of influenza virus RNA segment 8 indicates that the coding regions for the NS₁ and NS₂ proteins overlap. *Proc. Natl. Acad. Sci. U. S. A.* **77**:5074.
 39. Baez, M., R. Tavssig, J. J. Zazra, J. F. Young, and P. Palese. 1980. Complete nucleotide sequence of the influenza A/PR/8/34 virus NS gene and comparison with the NS genes of the A/Udorn/72 and A/FPV/Rostock/34 strains. *Nuc. Acids Res.* **8**:5845.
 40. Doherty, P. C., R. B. Effros, and J. Bennink. 1977. Heterogeneity of the cytotoxic response of thymus-derived lymphocytes after immunization with influenza viruses. *Proc. Natl. Acad. Sci. U. S. A.* **74**:1209.
 41. Effros, R. B., P. C. Doherty, W. Gerhard, and J. Bennink. 1977. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. *J. Exp. Med.* **145**:557.
 42. Zweerink, H. J., S. A. Courtneidge, J. J. Skehel, M. J. Crumpton, and B. A. Askonas. 1977. Cytotoxic T cells kill influenza virus infected cells but do not distinguish between serologically distinct type A viruses. *Nature (Lond.)*. **267**:354.
 43. Braciale, T. J. 1977. Immunological recognition of influenza virus infected cells. II. Expression of influenza A matrix protein on the infected cell surface and its role in recognition by cross-reactive cytotoxic T cells. *J. Exp. Med.* **146**:673.
 44. Reiss, C. S., and J. L. Schulman. 1980. Influenza type A virus M protein expression on infected cells is responsible for cross-reactive recognition by cytotoxic thymus-derived lymphocytes. *Infect. Immun.* **29**:719.