

Use of Antibodies to Purified Newcastle Disease Virus Glycoproteins for Strain Comparisons and Characterizations

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The H/N and F₁ glycoproteins responsible for hemagglutinin/neuraminidase (H/N) activity and fusion (F₁) activity were purified from several Newcastle disease virus strains and used to produce antisera. These antisera can be used to clearly distinguish the virus strains by a number of techniques including radioimmunoassay, and so provide a means of classifying new virus isolates. The variations detected between strains are not related to field virulence.

Newcastle disease virus (NDV) is the causative agent of a major disease of poultry which has frequently occurred in Western Europe and the United States (1, 6). In spite of apparently adequate protection measures (i.e., vaccination of birds), the disease still recurs periodically (1).

A large number of isolates of NDV have been reported which show considerable variation in their virulence for chickens (7, 23). However, strains differing in pathogenicity and place of isolation are very similar antigenically. Some strains are indistinguishable, whereas in a few cases minor antigenic differences can be demonstrated by neutralization and immunodiffusion tests (5, 8, 13, 22). This presents a major problem in the study of the disease in the laboratory since there is no sure way of distinguishing isolates except by determining their virulence *in vivo*. Thus, there is clearly a need for greatly improved methods for comparison and classification of new virus isolates.

Newcastle disease virions contain four major proteins (H/N, F₁, NP, and M) of which two are glycosylated (H/N and F₁) (9). NP and M are internal proteins whereas the two glycoproteins are located as spikes on the surface of virions and contain the major antigenic determinants (3, 19). As individual proteins may show greater serological variation than complete virions, we purified the individual glycoproteins from a number of NDV strains, differing in virulence and place of origin, and raised antisera against them. Using these protein and antisera preparations, strain comparisons have been made by a number of techniques including radioimmunoassay. The results of these comparisons are the subject of this report.

MATERIALS AND METHODS

Virus strains. NDV strains La Sota, F (avirulent),

Texas GB, and Herts 33 (virulent) were obtained from N. F. Moore. Avirulent strains were grown in 10-day-old eggs for 72 h; virulent strains were grown in 11-day-old eggs for 40 to 48 h. Stock allantoic fluid was stored at -70°C.

Growth of [³⁵S]methionine-labeled NDV. De-embryonated eggs were prepared as described previously (2) and infected with 0.1 ml of stock virus. A 200- μ Ci amount of [³⁵S]methionine (700 Ci/mmol; Radiochemical Centre, Amersham, England) was added to each egg (in batches of 30), and fluids were harvested after incubation for 30 h at 37°C. All virus preparations were purified essentially as described by Moore and Burke (9).

Plaque, neuraminidase, and hemolysin assays. Plaque, neuraminidase, and hemolysin assays were performed as described previously (4). Neuraminidase inhibition tests were carried out on 50- μ l samples of diluted virus preparations which were incubated with 50 μ l of diluted antiserum for 1 h at 37°C. A 100- μ l volume of fetuin was then added, and a neuraminidase assay was performed.

Hemolysin inhibition tests were done on 500- μ l samples of diluted virus which were incubated with 500 μ l of diluted antiserum for 1 h at room temperature. A 1-ml volume of chicken erythrocytes was then added, and hemolysin activity was determined.

In plaque inhibition tests, various dilutions of virus (1 ml) were incubated with 1 ml of a 1:50 dilution of antiserum for 2 h at room temperature. Volumes (0.5 ml) of each sample were then plaque assayed as usual.

For kinetic neutralization experiments, 2 ml of virus (10^3 plaque-forming units per ml) and 2 ml of antisera (1:1,000 dilution) were brought to 37°C. Antisera and virus were mixed, and 0.2-ml samples were removed at various times for plaque assay.

Protein purification. Purification of the M protein was achieved using Triton high-salt disruption, followed by dialysis, essentially as described by Scheid and Chopin (16). The glycoprotein fraction which was also obtained in the above procedure was further fractionated into H/N and F proteins by the fetuin affinity chromatography method developed for Sendai virus (17) but using an elution temperature of 37°C.

Polyacrylamide gel electrophoresis. Electro-

phoresis and Coomassie blue staining were carried out as described by Moore and Burke (9). Gels containing ^{35}S -labeled proteins were fractionated using a Mickle gel slicer, and 1-mm fractions were solubilized in hydrogen peroxide (200 μl of 100 volumes) at 70°C for 2 h. Triton-toluene scintillation fluid was added, and samples were counted in a Packard scintillation spectrometer.

Preparation of antisera. Purified viral antigens (100 to 200 $\mu\text{g}/\text{ml}$ in saline containing 0.1% Triton X-100) were emulsified by forced mixing with complete Freund adjuvant. The mixture was injected intramuscularly at multiple points into New Zealand White rabbits. This process was repeated after 21 days, and 7 days later the rabbits were bled from the marginal ear vein. After clotting, the serum obtained was clarified by centrifugation at $2,500 \times g$ for 10 min, and the supernatant was stored in portions at -20°C .

Immunoprecipitation of proteins. A 50- μl volume of viral protein (1×10^4 to 2×10^4 cpm) was mixed with 50 μl of antiserum diluted 1:10 with radioimmunoassay (RIA) buffer [20 mM tris(hydroxymethyl)aminomethane-hydrochloride-100 mM NaCl-1 mM EDTA (pH 7.6)]. The mixture was incubated at 37°C for 4 h and then at 4°C for 12 h. A 50- μl volume of sheep anti-rabbit immunoglobulin G was added so that its concentration was equivalent to that of the hyperimmune rabbit serum. After mixing, the sample was incubated for 2 h at 37°C and then for 2 h at 4°C . The precipitate was pelleted by centrifugation at $14,000 \times g$ for 5 min and washed several times with 200 μl of RIA buffer. For electrophoresis, the precipitate was dissolved by boiling for 5 min in 100 μl of RIA buffer containing sodium dodecyl sulfate (2%), urea (1 M), and β -mercaptoethanol (1%).

RIA. Samples (50 μl) of [^{35}S]methionine-labeled antigen in RIA buffer containing 0.1% Triton X-100 were mixed with 50 μl of various dilutions of antiserum and 50 μl of RIA buffer. Samples were incubated for 4 h at 37°C and then for 12 h at 4°C . A 50- μl volume of sheep anti-rabbit immunoglobulin G (10-fold excess) was added, and incubation was continued for 2 h at 37°C and 2 h at 4°C .

Mixtures were centrifuged at $14,000 \times g$ for 5 min, and 180- μl volumes of the supernatant fractions were assayed for radioactivity. The dilution of antiserum precipitating 75% of the labeled antigen was determined and used in subsequent competition assays. These were carried out exactly as described above, except that increasing amounts of cold viral antigen were included in the samples.

RESULTS

Purification of individual NDV proteins.

^{35}S -labeled virus was purified and glycoproteins were prepared as described in Materials and Methods. Separation of the H/N and F_1 glycoproteins was achieved by affinity chromatography on a fetuin column. A typical separation is shown in Fig. 1 for strain Texas. Two peaks of radioactivity (A and B) were resolved, and the one eluted at 37°C (peak B) contains the vast majority of the neuraminidase activity. Both

fractions were analyzed by polyacrylamide gel electrophoresis to identify the proteins present. Fraction A did not represent a single protein, and so it was subjected to a second cycle of chromatography and reanalyzed on gels. Figure 2 shows the gel profiles obtained for fraction B (Fig. 2A) and fraction A after two cycles of chromatography (Fig. 2B). In each case a sample of unlabeled purified virus was co-run as a marker. The gels were run under nonreducing conditions to resolve F and NP proteins (9). It is clear that with NDV, as previously shown for Sendai virus (17) and simian virus 5 (18), H/N and F_1 proteins can be obtained by this procedure in an essentially pure form.

Preparation and characterization of antisera. Antisera were raised in rabbits against the purified individual glycoproteins of NDV strains F, Herts, and Texas. To demonstrate the specificity of each antiserum, a labeled total glycoprotein fraction was prepared from each virus strain and immunoprecipitated as described in Materials and Methods with homologous antiserum. The precipitates obtained were dissolved and analyzed on nonreducing polyacrylamide gels, along with cold virus to act as a marker. Analysis of immune precipitates obtained for strain F is shown in Fig. 3A (anti-H/N) and B (anti- F_1). Precipitates produced with antiserum raised against M protein or preimmune serum contained no detectable virus glycoproteins (data not shown). These results show that anti-H/N selectively precipitated the H/N protein, and anti- F_1 predominantly precipitated

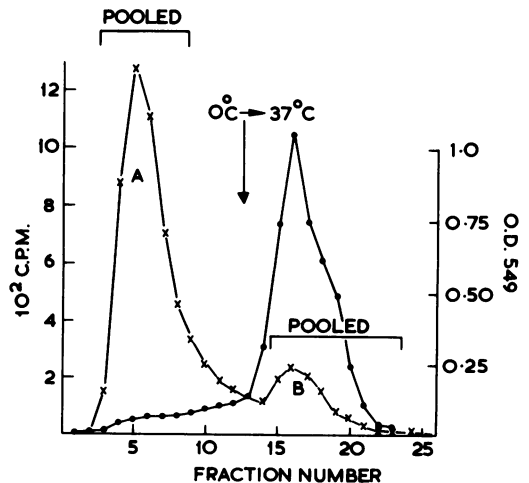


FIG. 1. Elution profile of [^{35}S]methionine-labeled NDV Texas glycoproteins from a fetuin-Sepharose column at 0°C (fractions 1 to 12) and 37°C (fractions 13 to 25). Symbols: \times , radioactivity eluted; \bullet , neuraminidase activity eluted.

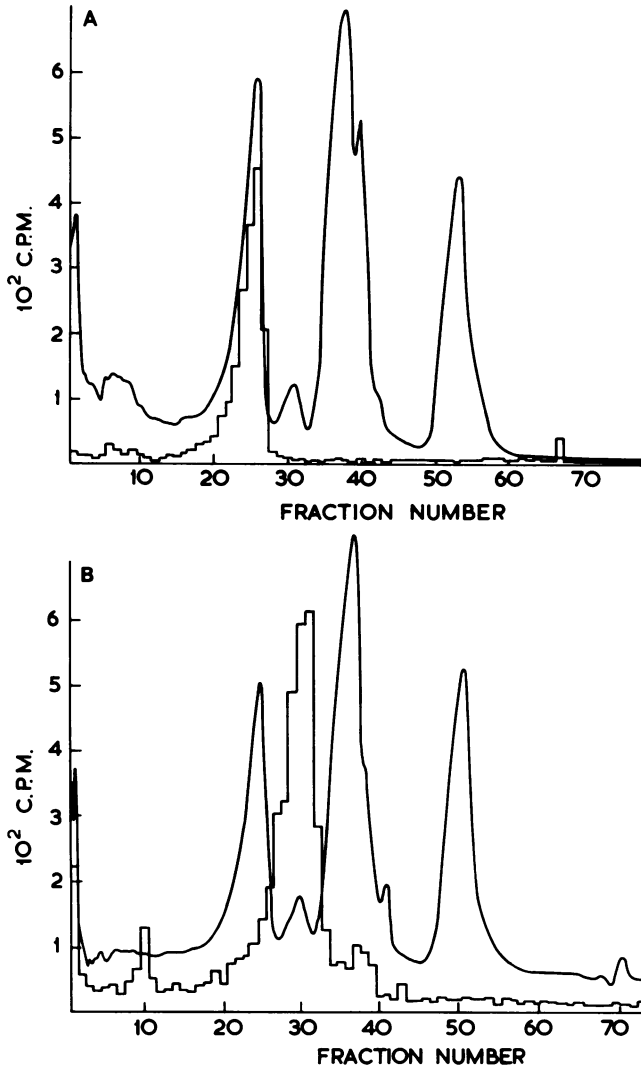


FIG. 2. Polyacrylamide gel electrophoresis of NDV strain Texas proteins under nonreducing conditions. Migration is from left to right. The solid line is optical density at 575 nm (marker virus), and the histogram is ³⁵S radioactivity. (A) Purified H/N protein; (B) purified F₁ protein.

the F₁ protein. An identical conclusion was drawn by analysis of the supernatants remaining after precipitation (data not shown).

The glycoproteins of NDV are known to be responsible for several of the biological activities of the virions. Thus, H/N protein possesses both hemagglutinin and neuraminidase activity, whereas F₁ is responsible for cell fusion and hemolysis (10, 14, 15, 17, 18, 20, 21). We have further characterized our antisera by examining their effect on various biological properties of NDV. Antisera against purified M proteins (data not shown) were used as controls in this series of experiments. Table 1 shows that antisera

against both H/N and F₁ can completely abolish infectivity, whereas antisera against the M protein has very little effect. Neuraminidase activity was used as an indicator of H/N protein biological activity, and Figure 4 shows the effect of increasing amounts of various antisera on this activity. Antisera raised against purified H/N protein was very effective at reducing neuraminidase activity. The effect of antisera raised against M or F₁ proteins was much less and the same as that found with preimmune serum (data not shown).

Hemolysin activity is known to reside on the F₁ protein (10), and Fig. 5 shows that this activ-

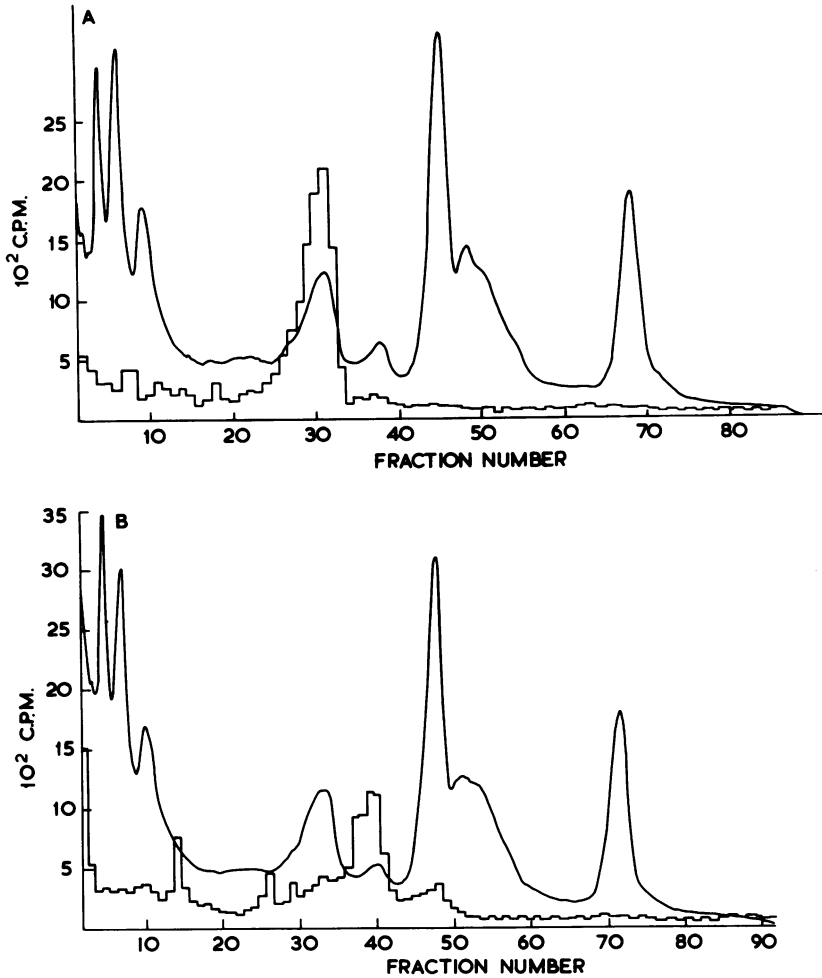


FIG. 3. Polyacrylamide gel electrophoresis of NDV strain Texas glycoproteins after immunoprecipitation (nonreducing conditions). Notations as in Fig. 2. (A) Anti-H/N serum precipitate; (B) anti-F₁ serum precipitate.

TABLE 1. Plaque inhibition of NDV strain Texas by antisera against various virion proteins

Virus dilution	Plaques obtained in the presence of antisera:			
	None	Anti-M ^a	Anti-F ^a	Anti-H/N ^a
10 ^{6.0}	99 ^b	88	0	0
10 ^{6.5}	43	28	0	0
10 ^{7.0}	5	6	0	0
10 ^{7.5}	2	3	0	0

^a Antisera. All antisera were used at a dilution of 1:50.

^b Means of duplicate determinations.

ity is greatly reduced by antisera raised against F₁. Again antiserum against M is not effective. However, antiserum against H/N is able to ef-

fectively reduce hemolysis. This observation accords with those made by Seto et al. (14) and Örvell and Norrby (11). Results similar to those presented above were obtained for all of the virus strains investigated.

Strain comparisons by RIA. ³⁵S-labeled purified H/N and F₁ proteins were immunoprecipitated with their homologous antisera in the presence or absence of unlabeled competing glycoproteins as described in Materials and Methods. The results obtained for strains F, Herts, and Texas glycoproteins are summarized in Table 2. The competition observed is expressed in two ways: (i) the amount of competitor required for 50% competition, and (ii) the degree of competition produced by 10³ ng of competitor. In all cases competition was observed with all competitors, and as expected the homologous cold

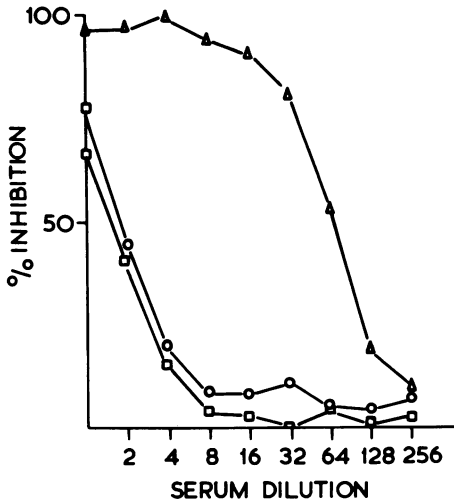


FIG. 4. Inhibition of NDV strain F neuraminidase activity by antisera against H/N protein (Δ), M protein (\square), and F₁ protein (\circ).

glycoproteins were always the best competitors. No competition was observed when Sendai glycoproteins were used as competitors (data not shown).

Strain comparisons by inhibition of biological activities. Antisera raised against the H/N proteins of strains Herts and Texas were used in kinetic neutralization tests as described in Materials and Methods. Figure 6 compares the effect of antiserum against Herts H/N on the plaquing ability of Herts and Texas strains. Both strains are neutralized, but the effect is more rapid in the case of the homologous (Herts) reaction. The reciprocal experiment is also recorded in Fig. 6. Antiserum raised against Texas H/N can neutralize the Herts strain, but less rapidly than the Texas strain. These results indicate that individual strains of NDV can be distinguished in kinetic neutralization tests using antisera against individual virion proteins.

A similar conclusion is reached when other biological activities are studied. Figure 7 compares the effect of antiserum raised against strain Texas H/N protein on the neuraminidase activity of strains F and Texas. In both cases there is inhibition, but the serum is most effective in the homologous case. The reverse situation is also shown in Fig. 7. Again the homologous virus (strain F) is more sensitive to inhibition by the antiserum.

DISCUSSION

We have prepared individual glycoproteins from several NDV strains using affinity chromatography on fetuin-Sepharose as described

by Scheid and Chopping for Sendai virus (17) and simian virus 5 (18). This demonstrates that this method is generally applicable to paramyxoviruses. The proteins we prepared were essentially pure, and antiserum raised against a particular protein would only immunoprecipitate that protein. Additional small peaks seen on gels of F₁ protein (Fig. 2B and 3B) are artifacts of the nonreducing electrophoresis conditions used to resolve H/N and F₁. Reducing gels show a single peak of F₁ protein (data not shown). Antiserum against F₁ protein was shown to inhibit hemolysis (but not neuraminidase) activity, confirming that this protein is responsible for hemolysis. Antiserum against H/N protein inhibited both neuraminidase and hemolysin activities as previously shown by others (11, 14, 21). However, polyacrylamide gel analysis showed that this antiserum only immunoprecipitated H/N protein from a mixture of the two glycoproteins (Fig. 3). Thus, whereas the effect on neuraminidase is a direct effect, that on hemolysis is indirect. The reduction in hemolysis activity brought about by anti-H/N is probably caused by reduced virus/erythrocyte binding resulting from the reaction of the antiserum with H/N protein. Both H/N and F₁ antisera are able to neutralize infectivity. This indicates that the purified glycoproteins are potentially useful as immunogens against NDV infection and are probably the most important antigens involved in eliciting protection in vivo. It also shows that the glycoproteins are required for virus infectivity.

RIA competition is a very sensitive means of

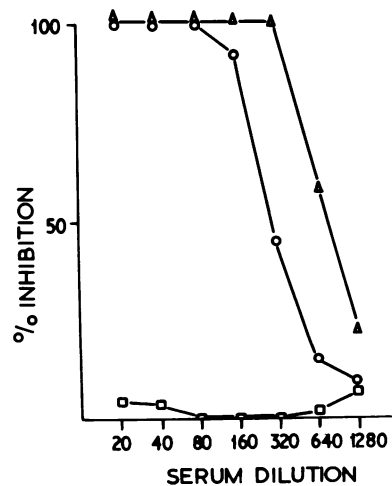


FIG. 5. Inhibition of NDV strain Herts hemolysin activity by antisera against H/N protein (Δ), M protein (\square), and F₁ protein (\circ).

TABLE 2. RIA competition

Strain	H/N proteins		F proteins	
	Antigen giving 50% competition (ng)	Competition by 10^3 ng of antigen (%)	Antigen giving 50% competition (ng)	Competition by 10^3 ng of antigen (%)
Test strain ^a —F; competitor strain ^b :				
F	5.2×10^1	100	6.5×10^2	60.7
Herts	$>10^4$	19	2.1×10^3	44.0
La Sota	6.1×10^2	61.6	3.0×10^3	17.4
Texas	1.2×10^3	48.5	2.8×10^3	36.2
Test strain—Herts; competitor strain:				
F	$>10^4$	12.9	2.5×10^3	31.3
Herts	6×10^2	55.4	9.8×10^2	50.5
La Sota	$>10^4$	6.3	8.9×10^3	6.5
Texas	$>10^4$	7.2	6.4×10^3	9.4
Test strain—Texas; competitor strain:				
F	8.2×10^3	30.6	6.8×10^2	59
Herts	$>10^4$	11.0	$>10^4$	25.4
La Sota	5.9×10^3	29.3	2.6×10^3	20
Texas	2.9×10^2	78.4	3.9×10^2	59.7

^a Strain providing antisera and ³⁵S-labeled glycoproteins.

^b Strains providing unlabeled glycoprotein competitor.

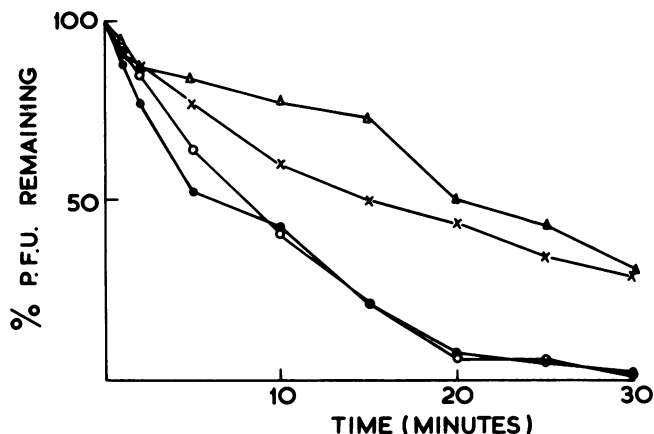


FIG. 6. Kinetic neutralization of NDV strains Texas (Δ) and Herts (\circ) by antiserum raised against H/N protein of strain Herts, and of NDV strains Texas (\bullet) and Herts (\times) by antiserum raised against H/N protein of strain Texas.

distinguishing between related antigens and requires only small amounts of material (12). We have applied this technique to purified glycoproteins from four strains of NDV. Examination of H/N protein showed that all four strains could be distinguished by the use of this technique (Table 2). Strain Herts was the most distinct strain. It showed the least competition for Texas or F antisera, and was itself least competed with by Texas, F, or La Sota glycoproteins. The other three strains appear to be approximately equally related, showing 50 to 60% competition when

the homologous competition was 100% in all combinations studied.

Antigenically dissimilar F proteins are also found with all four strains (Table 2). In this case the divergence is not as great as with H/N, but nevertheless the strains can be clearly and easily distinguished. Again strain Herts appears to be the most distinct of the four strains. We conclude that RIA is feasible for the immunogenically important proteins of NDV and is suitable as a diagnostic procedure for either NDV-specific antigens or antibodies. The method can distinguish

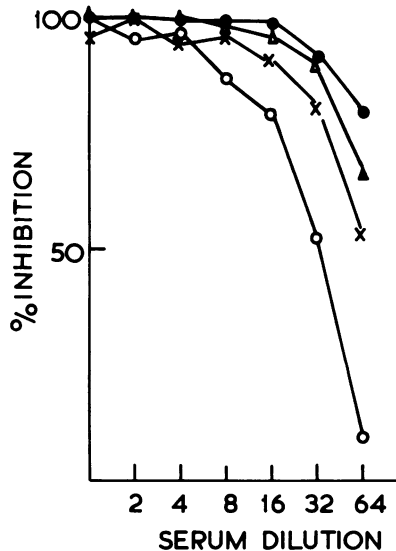


FIG. 7. Inhibition of neuraminidase activity of NDV strains Texas (Δ) and F (\circ) by antiserum raised against H/N protein of strain Texas, and of NDV strains Texas (\times) and F (\bullet) by antiserum raised against H/N protein of strain F.

between the various NDV strains and so it can be used to identify the strain of NDV present in an isolate. Because of the sensitivity of the method, it would also be possible to identify any new variants which might arise. The antigenic variation we have detected between strains has no apparent correlation with the virulence of the strains. This is not surprising since virulence is a function of the virus-host cell relationship rather than characteristic of the virus per se (10).

NDV strains could also be distinguished by the use of antisera against the purified proteins in kinetic neutralization and neuraminidase inhibition tests (Fig. 6 and 7). Antiserum was always more efficient at inhibiting these functions of the homologous rather than the heterologous strain. We conclude that purified proteins of NDV and antisera raised against them are generally more useful than whole virus preparations for strain comparisons and classification by a variety of techniques.

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