Newcastle Disease Virus Neuraminidase Inhibitions: Differences Among Strains and a ro osed echanism for the lution Inhibition Antibod eaction

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ABSTRACT

Newcastle disease virus (NDV) neuraminidase (NA) inhibition with elution-inhibition (EI) antibodies was estimated with the EI reaction using strain 575 isolated from a mute swan (*Cygnus olor*). The NA inhibition was assessed by persistent red blood cell (RBC) agglutination patterns, rather than by the standard test involving the reduction in the cleavage of added artificial substrates. The more numerous antibody determinants for the hemagglutinin (HA) of strain 575 enriched the antiserum for EI antibodies. Thus, the proposed mechanism for NA inhibition is governed by the relative amounts of HA and NA antibody determinants. The NA of strains Cg and Roakin, but not the B1 vaccine, were inhibited only if the antiserum was enriched for EI antibody with strain 575. The antiserum contained complement-dependent antibody. The EI antibody determinants were indirectly titrated by progressively increasing strain 575 in the antiserum until the NA of the Cg and then Roakin strains were inhibited. The decreasing order of NA activities estimated by the cleavage of fetuin was: 1) B1, 2) Cg, 3) 575, and 4) Roakin. To characterize each strain, numerical values were constructed. The 2 attributes of the NA, the amounts of 575 required to titrate NA determinants and the NA activity estimated with cleavage of fetuin, were multiplied. B1 differed from strain 575 by a factor of ≥370 (237/0.64 = ≥370). Values for strains Cg and Roakin were 45 and 48, respectively. Thus, B1 had more NA determinants than strain 575. Fewer NA determinants were reflected in no NA inhibition and perhaps increased NA activity when tested with fetuin. The HA-sialic acid configuration specificity for binding strain 575 to the RBC was not involved in the mechanism because the NA of strains Cg and Roakin could also be inhibited. Correlations of NA values with virulence were not apparent. The antibody determinant estimates combined with NA activities provide a more complete evaluation of the strains.

INTRODUCTION

A new assay for Newcastle disease virus

(NDV) anti-neuraminidase (NA) elution-inhibition (EI) antibodies was used to estimate prevalences.[1] The populations tested were European mute swan (*Cygnus olor*), tundra swan (*Cygnus columbianus*), and Canada geese (*Branta canadensis*). Differences in EI antibody prevalence were found between adult mute and tundra swan as well as between tundra swan and Canada geese. Seroconversions as well as persisting or declining titers were found in mute swan.

Thus, using the field strain 575 isolated from a mute swan, the differences in individuals and populations were delineated.[2] The EI assay that employs persistent red blood cell (RBC) agglutination patterns is less complicated than the standard test,[3] is inexpensive, and is suitable for testing large numbers of polyclonal sera.

The purpose of this report is to examine how strain 575 functions. Two explanations for the NA inhibition will be explored. One possibility is that the swan strain has more hemagglutinin (HA) determinants than strains that failed to respond to EI antibody. Enrichment for EI antibodies would result in NA inhibition. Another possibility is that the infrequent HA-sialic acid configuration required for the EI reaction occurs only with the strain 575.[4–7] Enriching antiserum for EI antibody with the swan strain and then testing if the NA of other strains could be inhibited was the approach for resolving the possibilities.

The reactions leading to antibody enrichment and NA inhibition are described. Additionally, combining NA activities[3] with the results from the indirect antibody determinant titrations provide numerical values that help characterize the NA variation among the 4 strains examined.[8–11]

MATERIALS AND METHODS

NDV Strains ol clonal Antiserum and linical Signs

Partial purification of PMC-1/Mute swan/Maryland/575/1977 (strain 575) with sephadex filtration and centrifugation has been described.[1] Biologic plaque purification on chicken embryo cells and terminal end-point dilution tests in embryonating eggs[12] showed that strain 575 is genetically stable.[1,13] The Roakin strain and homologous, post-infection chicken antiserum were obtained from the Centers for Disease Control (CDC), Atlanta, GA. Ten NDV strains were recovered from 4 avian species.[2] The 17th passage in embryonating eggs of the B1 vaccine and the 13th passage of the virulent Cg 179 (Cg) strains were obtained from the late F. B. Bang, The Johns Hopkins University, Baltimore, MD; he also donated sera from rabbits immunized with the Beaudette B strain. The Beaudette B strain, like Cg, is a strain lethal for chicken embryos.[14–16] Rabbit sera were drawn 17 days post-immunization and contained IgG and IgM antibodies; either will mediate the HA-inhibition (HI) and EI reactions.[1] Red blood cells from 2 leghorn chickens were selected for the tests by trial and error[6,14] and suspended (0.3% v/v) in phosphate (0.05 M) buffered (pH 7.2-7.4) NaCl containing 0.1% NaN_3 (PBS). Before testing, all antisera were adsorbed with RBC.

All birds were without signs of infection. The flightless, juvenile 575 swan was resighted with no apparent clinical disease during 24 months following isolation of the strain from feces. No deaths occurred in 72 hours among embryonated eggs inoculated with strain 575.

Serologic ests ocali ation of NDV on ith Normal and luorescent Antibodies and ests for NDV in Antiserum

Inhibition of NA was estimated in a direct assay by persistent agglutination patterns of RBC[1,2,5,12,13,17,18] rather than indirectly by a reduction in the cleavage of added artificial substrates.[3] Strain 575 elutes from RBC above the level of effective EI antibody. Following the incomplete elution, the RBC became sensitized.[12,19,20] The NA on sensitized RBC is inactive whereas the HA is functional and agglutinates up to 7 volumes of normal RBC in the agglutination-separation (AS) reaction. During 37°C incubation

of normal RBC-sensitized RBC, the sialic acid on the normal RBC reactivates the NA and separation occurs. Products of the AS reaction, consisting of altered NDV and dimers of hemagglutinin-neuraminidase glycoprotein spikes (HN spike), are released. The proportions of each product is governed by the normal RBC sialic acid concentrations in the AS reaction. Altered NDV has fewer HN spikes than allantoic NDV.[5]

The HA, NA, and antibody determinants are on the globular head of the HN spike.[10,21,22] The EI reaction is dependent on the heat-labile NA antibody determinant.[1] The activities of HA and NA are separated with mild heat as well as with EI and HI antibodies.[1,2,5,12,13] Crystals of the HN spike show 2 sialic acid binding sites. The HN may be pliable allowing switches in HA and NA activities by conformational change.[8,23,24] Thus, the HI, EI, and AS reactions are compatible with the 2 binding sites and the proposed conformational changes.

All tests were conducted in microtiter wells with a conical bottom. Reagent volumes were 0.025 mL. Assays for HI and EI use suspensions of NDV and are analogous to agglutination-inhibition (AI) and separation-inhibition (SI) tests, which use sensitized RBC.[5,12,14] Usually, normal RBC are added to sensitized RBC in diluted antiserum. Here, normal RBC were added to RBC in the HI and EI tests after the enriched serum was removed. The RBC addition allowed an estimate of the AI and SI titers and located NDV bound to RBC. Following washing with PBS, the NDV bound to RBC was also located with fluorescent anti-IgG antibodies.[1]

Eluted NDV, dimers of HN spikes, and incompletely sensitized RBC do not respond to SI or EI antibody; allantoic NDV was required to test for EI antibody.[5] The more heat-resistant HA[12] remains functional; thus, the control test for NDV in all sera was agglutination of RBC. Unless otherwise stated, the reaction times were not specified; endpoints were usually reached within 16 hours of 37˚C-humidified incubation.

The complement-dependent, single-radial–hemolysis test was used to examine if antibodies were present in the antiserum; other substances could be entering the reactions. Antibodies were assayed with sensitized RBC as well as NDV bound to RBC (NDV-RBC). Washed sensitized RBC were mixed at a final concentration of 1% with 2.7 mL of melted 1.5% agarose in PBS at 40˚C. Complement from guinea pigs (0.15 mL) was added before dispensing the mixture into a 6-cm diameter plastic dish. Wells 2 mm in diameter were cut in the agarose and filled with 0.05 mL of rabbit antiserum. Serum drawn pre-immunization and 5 and 17 days post-immunization was adsorbed with chicken RBC, then heat inactivated at 56˚C for 30 minutes. Control preparations contained normal RBC and complement in agarose. An additional test was adsorbing the antibody with sensitized RBC. Rabbit serum (0.05 mL) was adsorbed with packed normal RBC (0.05 mL), then again 3 times with sensitized RBC. Hemolysis was complete in 15 hours of 37˚C incubation.

NA Acti it Assa

Fetuin (Sigma Chemical Corporation, Saint Louis, MO) was cleaved yielding N-acetylneuraminic acid (Neu5Ac) in the standard indirect NA assay. One to 16 HA units (HAU) of NDV were incubated at 37˚C with 1.25 mg fetuin for 3 hours. The NA activities are expressed in optical density (OD) units recorded at 549 nm.[1,3]

itration of NA Determinants of Strains b rogressi el Increasing the oncentration of Strain in Antiserum

The titration was an estimate of the number of HAU of strain 575 required to enrich the antiserum to the level where another strain could respond to EI antibodies. Thus, the assay is an indirect titration of the antibody determinant on the strain added to the enriched antiserum. Rabbit antiserum received 128, 256, and 512 HAU. All preparations were brought to a constant volume containing a 1/32 dilution of the antiserum, then incubated for 2 hours at 37˚C. The mix-

tures were diluted, and 8 HAU of 575, Cg, Roakin, and B1 strains were added. Although antibody binding was rapid (unpublished results), for convenience the tests were held for 16 hours at 5˚C, and then normal RBC were added. The HI titers were recorded, and the tests were incubated again until the EI antibody endpoints were attained.

Assignment of Numerical Values for ach NA

One attribute of the NA is the activity estimated by the release of Neu5Ac from fetuin. Another is the HAU required for enrichment of the antisera for EI antibody that results in NA inhibition. When multiplied, the product of the 2 attributes is a numerical value that compares the NA of the 4 strains. For purposes of comparison, a numerical value was assigned to a strain if the NA was not inhibited with the enriched antiserum.

RESULTS

es onse of NDV Strains to I Antibod

Roakin, Cg, B1, and 9 field strains isolated from 4 species did not respond to EI antibody in CDC chicken or rabbit antiserum. In tests with Roakin and Cg strains, the rates of elution from RBC were reduced indicating some, although not total, NA inhibition. The B1 strain showed rapid elution from RBC. Only strain 575 responded to EI antibody after the initial isolation as well as following 3 subsequent passages in embryonating eggs.

eactions esulting in nrichment for I Antibodies in abbit and hic en Antiserum Shown b the NA Inhibitions of the oa in and Strains

The reactions involved in the NA inhibition of the Roakin strain are detailed in Table 1. Location of the 575 NDV on RBC was with fluorescent antibody at 1/40–1/320 dilutions, also following addition of normal RBC at 1/80–1/640 dilutions of antisera. Altered NDV and HN spikes were released from the sensitized RBC in the AS reaction at dilutions of 1/320–1/1280. With antiserum enriched for EI antibodies, the Roakin strain now showed NA inhibitions at 1/80–1/160 dilutions whereas without enrichment the EI titer was negative.

Tests with strain 575 and CDC chicken antiserum showed HI and EI titers of 1/40 and 1/320; thus, serum other than rabbit could be enriched. Following the second addition of strain 575 to the antiserum, the titers decreased to 1/30 and 1/40, respectively. The reduction in HI and EI titers was unequal; thus, the HA and NA determinants responded to their respective antibodies independently.

rogressi e nrichment of Antisera for I Antibod

The EI antibodies were detected with strain 575 without enrichment; with the Cg strain when the HI titer was reduced from 1/1024 to 1/128 by the addition of 256 HAU; and with the Roakin strain, which required the addition of 512 HAU. The HI titer was reduced from 1/512 to 1/64 before EI antibodies were detected. The B1 strain failed to respond to EI antibodies, despite the reduction of HI antibody titers from 1/256 to <1/64. Thus, the order of NA inhibition was: 1) 575, 2) Cg, 3) Roakin, and 4) B1, which showed no NA inhibition (Table 2).

Differences Among NDV Strains in the elease of Neu Ac rom etuin

The release of Neu5Ac by each strain resulted in the following decreasing order: 1) B1, 2) Cg, 3) 575, and 4) Roakin (Table 3). Linearity in the release was most apparent in tests with 8 HAU; thus, 8 HAU was used in comparisons of each NA as detailed below.

Numerical Differences Among Strains Shown b ombining the Amounts of Strain e uired for itrations and the elease of Neu Ac rom etuin

The HAU of strain 575 required for enrichment resulting in NA inhibition (Table 2) and the NA activity (Table 3) were multiplied. The NA of the B1 strain was not inhibited; thus, the number >1032 was assigned. The multiplication product was greatest with the B1 strain. Comparison of

Ta Reactions of Strain 575 Newcastle Disease Virus that Enrich Antiserum for Elution-Inhibition Antibodies Resulting in Neuraminidase Inhibition of the Roakin Strain

	Reactions*									
	Strain 575 + Antisera			RBC From EI Tests + Normal RBC			Enriched Sera From EI Tests†			
Antisera Dilutions				Tests for HA and NA Inhibitions§		Release of AS	Strains			
							575		Roakin	
	HI	EI	FA‡ on RBC	AI	SI	Products	HI	EI	HI	EI
20	+	–	–	+	–	–	+	–	+	–
40	+	–	+	+	–	–	+/–	+	+	–
80	–	+	+	+/–	+/–	–	–	–	–	+
160	–	+	+	–	+	–	–	–	–	+
320	–	+	+	–	+	+‖	–	–	–	–
640	–	–	–	–	+	+	–	–	–	–
1280	–	–	–	–	–	+	–	–	–	–

HI = hemagglutination-inhibition; EI = elution-inhibition; AI = agglutination-inhibition; SI = separation-inhibition; FA = fluorescent antibody (anti-IgG); AS = agglutination-separation (a test for neuraminidase [NA] function, no antibody involved; AS products released as described below‖).

*The HI test contained 32 hemagglutinin (HA) units of strain 575 and rabbit antisera. Incubation of the HI tests resulted in an EI titer of 1/320, which decreased to 1/40 in the tests with enriched sera when 575 and Roakin strains were compared. Elution of NDV from red blood cells (RBC) resulted in sensitization of RBC at ineffective levels of EI antibody. Sensitized RBC has NDV, HN spikes, and an inactive NA.

†The supernatant fluids of the EI tests were divided, and 32 HA units of strains 575 or Roakin were added and incubated before normal RBC was added. The Roakin strain in other tests failed to respond to EI antibody whereas the titer was 1/160 with antisera enriched for EI antibody. Titers in a replicate experiment with the Roakin strain were also HI = 1/40 and EI = 1/160.

‡FA tests showed staining and "foci" indicating aggregation of NDV at antiserum dilutions of 1/40-1/320. At the 1/40 HI endpoint, the FA test was also positive. The anti-IgG stains all IgG antibodies; thus, the test was not specific for EI antibodies.

§After the enriched antisera were removed, normal RBC were mixed with the RBC from the EI tests. Endpoints designate the reciprocal of the antisera dilutions (+). SI antibody was detected at dilutions of +/-1/80-1/640. The +/-1/80 is the titer of a partial AI or SI endpoint or both.

‖The AS products, altered NDV and HN spikes, were released in the AS reactions after the sialic acid on normal RBC reactivated the NA. Despite SI antibody titer of 1/640, the AS products were detected in the supernatant fluids at 1/320-1/1280 dilutions. This indicates incomplete neutralization of the NA at 1/320-1/640 dilutions.

Ta Titration of Neuraminidase Antibody Determinants on Newcastle Disease Virus (NDV) Strains Using Antiserum Adsorbed With Progressively Increasing Hemagglutinin Units (HAU) of Strain 575

	NDV Strains							
HAU	**575**		**Cg**		**Roakin**		**B1**	
in Antisera*	**HI†**	**EI**	**HI**	**EI**	**HI**	**EI**	**HI**	**EI**
None	128‡	1024	1024	-§	512	-	256	-
128	64	512	256	-	256	-	128	-
256	64	256	128	512	128	-	64	-
512	<64	512	64	512	64	512	<64	-

*Strain 575 added to rabbit antisera.
†The hemagglutination-inhibition (HI) tests used 8 HAU of each strain.
‡Reciprocal of the dilution of the sera where the HI and elution-inhibition (EI) endpoints were observed.
§Negative (-) EI tests that failed to maintain an agglutination pattern following incubation of the HI tests.

Ta Activities of the Neuraminidase of Four Strains of Newcastle Disease Virus (NDV) Estimated by the Release of Neu5Ac From Fetuin

	Strains of NDV			
HAU	**B1**	**Cg**	**575**	**Roakin**
16	0.36*	0.23	0.16	0.11
8	0.23	0.11	0.08	0.06
4	0.08	0.07	0.05	0.04
2	ND	0.03	0.02	0.02
1	0.03	0.02	0.01	0.01
Average	**0.148**	**0.092**	**0.064**	**0.048**

HAU = hemagglutinin units; ND = not done.
*Optical density following 3 hours of incubation at 37˚C of fetuin and NDV.

strains B1 with 575 showed a numerical value of >370, whereas lesser values of 45 and 48 were found for the Cg and Roakin strains, respectively (Table 4).

ests for om lement De endent Antibod

Single-radial-hemolysis tests for antibodies in antiserum drawn 17 days post-immunization showed hemolysis with both the sensitized RBC and NDV-RBC preparations. No hemolysis was seen with pre-immunization or serum drawn 5 days post-immunization. Adsorption of antibodies with sensitized RBC was complete following 2 exposures to sensitized RBC. Control tests were uniformly negative for hemolysis.

DISCUSSION

Two possibilities regarding the mechanism of inhibition of the NA in the EI reaction were examined: 1) determinant concentrations or 2) a unique HA-sialic acid configuration. The greater concentration of HA determinants than that of NA antibody determinants on strain 575 is the attractive explanation. Enrichment for EI antibody resulted.

Only with enrichment did the inhibition of the NA of Cg and Roakin strains, but not B1, become apparent (Tables 1 and 2). Thus, the Roakin and Cg strains also formed the infrequent HA-sialic acid configuration necessary for EI antibody response. Regarding the configuration infrequency, an average of approximately 400,000 out of 20 million sialic acid-containing projections on a RBC are required for the attachment of each NDV.[5] Clearly, the configuration is not unique since it is not restricted to strain 575. Thus, the second explanation involving the configuration may be rejected. However, the inhibition of the NA with non-enriched sera is thus far apparent only with strain 575 (Table 1).[1,2]

Strain 575 is avirulent for mute swan and, like B1, for the embryonating chicken egg. A proposed increasing virulence order is: 1) B1, 2) 575, 3) Roakin, and 4) Cg, which correlates with neither the values for the NA activities nor the combined activities and titration results (Tables 2–4). However, other studies have correlated virulence with NA activity and with certain antigens.[25–28]

Lower NA determinant concentrations on Cg, Roakin, and 575 strains correlate with

Ta Differences Among Strains of Newcastle Disease Virus (NDV) Calculated From the Number of the Hemagglutinin Units (HAU) of Strain 575 Required to Titrate the Neuraminidase Antibody Determinant Times the Neuraminidase Activity of Each Strain

NDV Strain	HAU Required in Titration*	HAU × OD†	Comparison With Strain 575 (HAU × OD/0.64)
B1	≤1032‡	1032 × 0.23 = ≥237	≥237/0.64 = ≥370
Roakin	520	520 × 0.06 = 31	31/0.64 = 48
Cg	264	264 × 0.11 = 29	29/0.64 = 45
575	8	8 × 0.08 = 0.64	0.64/0.64 = 1

*The number is the sum of the HAU of strain 575 required to enrich the serum for elution-inhibition (EI) antibodies detailed in Table 2 plus the 8 HAU used in the EI tests.
†The optical density (OD) was from the 8 HAU series where the tests gave the most linear responses as detailed in Table 3.
‡The B1 vaccine strain did not respond to EI antibodies when 520 HAU were used to enrich the sera (Table 2); therefore, the number ≥1032 was assigned.

Ta Single-Radial–Hemolysis Tests for Newcastle Disease Virus (NDV) Complement-Dependent Antibodies Estimated With Sensitized Red Blood Cells (RBC) and NDV-RBC Preparations

	Area of Hemolysis (cm^2)		
NDV Antisera Adsorbed With:	**Control RBC***	**Sensitized RBC†**	**RBC-NDV‡**
Chicken RBC§	0	1.4	1.4
Sensitized RBC 1×	0	0.95	1.1
Sensitized RBC 2×	0	0.07	0.2
Sensitized RBC 3×	0	0	0
Total area	**0**	**2.42**	**2.7**

*Control tests without NDV contained normal RBC and complement.
†RBC were sensitized with strain 575.
‡The RBC-NDV preparations were RBC agglutinated with NDV and incorporated into the agarose prior to elution. The hemolysis area was somewhat greater than in the sensitized RBC tests. However, the difference was not considered significant. Both preparations responded to antibodies that were completely removed with adsorption indicating the specificity for NDV.
§The rabbit immune sera was adsorbed initially with chicken RBC. The sensitized RBC were removed by centrifugation following a 30-minute period of adsorbtion of the antiserum.

low NA activities (Table 3). In contrast, the negative determinant titration with B1 correlates with higher NA activity. If the number of active NA sites were proportional to the quantities of NA determinants, the B1 strain would have a preponderance of NA sites and few HA sites. This disproportion of NA sites would be reflected by 1) failure to respond to EI antibodies in enriched antiserum (Table 2), 2) a greater cleaving activity for fetuin (Table 3), 3) incomplete sensitization, 4) a greater separation rate in the AS reaction,[5] and 5) a greater elution rate from RBC.[15] In addition, strain B1 lacks covalent bonds in the dimer,[8] and the HN spike has a difference of 3 amino acid residues.[29] Further studies may show that certain of these properties contribute to the vaccine characteristics.[11]

The B1 is a candidate strain wherein the catalytic NA may also serve mainly as the attachment HA.[24] Strain 575, in contrast to B1, has a preponderance of HA determinants and presumably HA sites. These contrasting characteristics suggest that the number of determinants is closely related to the number of active HA and NA sites.[10,11,21,22,30–32]

As exemplified by the N9 of avian influenza virus,[33] more than one NA site on the HN spike could account for the variations in the quantities of antibody determinants. Duplication would result in a disproportionate number of HA or NA determinants on strain 575 and B1 and, less remarkably, on the Roakin and Cg strains (Table 4). Thus, influenza N9 may illustrate

site and perhaps determinant differences. However, regarding NDV, the 3-dimensional characterization[24,33] is needed.

Genetic stability of strain 575 was further shown by the response to EI antibody. At all passage levels and following each biological purification, the resulting NDV responded to EI antibody. In all likelihood antibody is mediating the reactions, as indicated by complement-dependent antibody tests (Table 5). Newcastle disease virus has a spontaneous overall mutation rate of 1%–2%. Despite this rate, the EI and HI domains are conserved.[12,34,35] Thus, the antibody determinants vary among strains but are a stable quantity for a given strain.

The proposed HN monomer structure shows the active NA site overlapping with antigenic site 23[21,29,30] and mapping closely to the interface region.[8,9,31] Site 23 is formed last and is associated with the HN dimer.[22,32] The likely precursor for the NA determinant is site 23. However, the dimer as well as altered NDV do not respond to EI antibody.[5] Perhaps the newly described sialic acid binding site at the dimer interface[24] was altered[31] during separation of the tetramer in the AS reaction.[5] Further steps in the synthesis of tetrameric allantoic NDV are required before the NA can be inhibited with EI antibodies.

Strain differences in antibody determinants are useful in understanding the mechanism for measuring EI antibodies. The assay based on this mechanism estimated the seroconversions and population differences in the initial studies of NA inhibition with EI antibodies.[1,2]

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