Cells Persistently Infected with Newcastle Disease Virus

III. Thermal Stability of Hemagglutinin and Neuraminidase of a Mutant Isolated from Persistently Infected L Cells

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Data were obtained which indicated the possible cause of the defective elution from erythrocytes of the mutant virus (NDV_{pi}) isolated from L cells persistently infected with the Herts strain of Newcastle disease virus (NDV_o). The chicken erythrocyte receptors for the mutant and wild-type viruses were equally sensitive to the action of *Vibrio cholera* filtrate neuraminidase; this suggests that the failure of NDV_{pi} to elute from chicken erythrocytes is not due to a specific neuraminidase-resistant receptor for this virus on the erythrocyte membrane. There was no difference in the enzyme content of the intact virions of NDV_o and NDV_{pi} when tested with a soluble substrate, indicating that the inefficient elution of NDV_{pi} was not due to a reduced enzyme content. The neuraminidase activity of intact NDV_{pi} virions was significantly more stable at 55 C than the enzyme of NDV_o virions, whereas the dissociated enzymes of the two viruses were inactivated at the same rate. On the basis of these findings, it seems likely there is a structural difference between the two viruses. The neuraminidase protein of the mutant NDV_{pi} may be incorporated into the viral envelope in such a manner that it is prevented from reacting with the substrate in the erythrocyte membrane, although it can react with a soluble substrate. The hemagglutinin activity of both intact and disrupted NDV_{pi} was significantly more resistant to thermal inactivation than that of the wild-type NDV_o. This finding suggests a genetic difference in the hemagglutinin protein of the two viruses.

We reported earlier that the mutant NDV_{pi}, isolated from L cells persistently infected with the Herts strain of Newcastle disease virus (NDV_o), differed markedly from the wild type in a number of properties, including efficiency of elution from chicken erythrocytes (6). Although 80% of adsorbed NDV_o eluted from chicken erythrocytes at 37 C, only 20% of the mutant NDV_{pi} was recoverable under similar conditions. No significant difference was found in the neuraminidase activity of the two viruses when tested with a soluble substrate (fetuin). The present work was undertaken to study some properties of the hemagglutinin (HA) and the neuraminidase of the wild-type NDV_o and of the mutant NDV_{pi} in an attempt to elucidate the cause of the defective elution of NDV_{pi} from chicken erythrocytes.

MATERIALS AND METHODS

Viruses. The Herts strain of NDV (NDV_o, clone 19) and the small plaque mutant NDV_{pi} (clone 2)

were used in this study. The viruses were grown in the allantoic cavity of 9-day-old chick embryos and infected fluids were concentrated by centrifugation at $105,000 \times g$ for 60 min at 4 C as described earlier (7). The virus concentrate (20-fold) in phosphate-buffered saline (PBS, pH 7.2) was stored at 4 C. The infectivity of the 20-fold virus concentrate was usually about 9×10^{10} and 8×10^9 plaque-forming units (PFU)/ml for NDV_o and NDV_{pi}, respectively.

Infectivity assays. Infectivity assays for PFU were carried out in primary cultures of chick embryo cells as described elsewhere (8).

HA assays. HA assays were performed in an ice bath as follows. Two series of twofold dilutions of virus were prepared by using PBS as diluent; one series started with a 1:10 dilution, and the other with a 1:15 dilution. To 0.5 ml of each dilution in a tube (13 by 100 mm) was added an equal volume of 0.5% (v/v) washed chicken erythrocytes. The suspensions were kept in an ice bath, and the hemagglutination patterns were recorded after 60 min. The HA titer was expressed as the reciprocal of the highest dilution which caused agglutination of erythrocytes.

Neuraminidase assays. Neuraminidase assays were carried out by incubating virus samples (0.1 ml) with 0.05 ml of a 2.4% water solution of fetuin (Colorado Serum Co., Denver, Colo.) plus 0.1 ml of 0.4 M sodium phosphate buffer (pH 5.9). The liberated *N*-acetyl neuraminic acid was estimated by the procedure of Osborn (5). One unit of enzyme was the amount which liberated 10^{-9} moles of *N*-acetyl neuraminic acid per min from fetuin under the above conditions (4).

Disruption of virus. Disruption of virus with Tween 80 and ether was carried out by the method of Haslam et al. (3). To 4 ml of a 20-fold virus concentrate was added 0.1 ml of Tween 80 (Atlas Chemical Industries, Inc., Wilmington, Del.); the solution was stirred for 30 min at 0 C. Two milliliters of chilled diethyl ether was added, and stirring was continued for another 60 min. The ether was then evaporated by passing a stream of nitrogen through the fluid for 20 min, and the fluid was dialyzed overnight against PBS at 4 C. Although the infectivity of both viruses was completely destroyed under these conditions, the HA activity was completely stable, and 60 to 90% of the neuraminidase activity was recovered from the disrupted virus preparations.

Thermal inactivation of virus activities. Intact- or disrupted-virus preparations were diluted in PBS, and portions (0.6 to 2.0 ml) were distributed in 5-ml sleeve-stoppered vials. The vials were completely immersed in a thermostatically controlled water bath which was adjusted to the desired temperature (± 0.05 C). Vials were removed from the water bath at appropriate intervals and chilled rapidly in ice; unheated control samples were kept in an ice bath for the duration of the experiment. All samples were stored at 4 C until assayed for infectivity, HA and neuraminidase.

RESULTS

Neuraminidase sensitivity of erythrocyte receptors for NDV_o and NDV_{pi}. Choppin and Tamm (1), working with strains of influenza A₂ virus, showed that the chicken erythrocyte receptors for strains RI/4⁺ and RI/5⁺ (non-eluting strains) were more resistant to *Vibrio cholera* filtrate neuraminidase than were the receptors for the rapidly eluting strains RI/4⁻ and RI/5⁻. To test the possibility that the difference in elution of NDV_o and NDV_{pi} from chicken erythrocytes could be due to a difference in the sensitivity to neuraminidase of the receptors for the two viruses, a modification of the experiment described by Choppin and Tamm was carried out. Two sets of two-fold dilutions of *V. cholera* filtrate (Sigma Chemical Company, St. Louis, Mo.) were made in PBS. To 0.5 ml of each dilution in a tube (13 by 100 mm) was added an equal volume of washed 0.5% chicken erythrocytes in PBS. Control tubes contained erythrocytes in PBS only. After incubation at 37 C for 60 min, the erythrocytes were removed by centrifugation at $1,000 \times g$ for 10

min at 4 C and were suspended in 0.5 ml of PBS. To one set of dilutions was added 0.5 ml of NDV_o (15 HA units), to the other set, NDV_{pi} (29 HA units). The tubes were incubated at 4 C, and HA patterns were recorded after 60 min. Erythrocytes which had been incubated with dilutions of cholera vibrio filtrate up to and including 1:32 were not agglutinated by either NDV_o or NDV_{pi}; higher dilutions of the enzyme preparation had no effect on the agglutinability of the erythrocytes. These results indicate that the receptors for NDV_o and for NDV_{pi} are equally sensitive to the action of *V. cholera* filtrate, and suggest that the failure of NDV_{pi} to elute from chicken erythrocytes is not due to a specific neuraminidase-resistant receptor for this virus on the erythrocyte membrane. For this reason, we turned our attention to the properties of the neuraminidase and HA proteins of the wild-type and mutant viruses.

Rate of inactivation at 50 C of infectivity, HA, and neuraminidase of NDV_o and NDV_{pi}. The virus concentrates were diluted in PBS to contain about 10^9 PFU/ml; replicate samples were heated at 50 C for various times as previously described. There was a marked difference in the rate of inactivation of the infectivity of the two viruses at 50 C (Fig. 1, top panel). After 40 min of heating, the infectivity of NDV_{pi} was reduced by 3.1 logs, whereas NDV_o showed only a 0.8 log drop. By 120 min, the infectivities of NDV_{pi} and NDV_o were reduced by 5.0 logs and 1.7 logs, respectively. These data confirm our earlier report that the infectivity of NDV_o was more stable at 48 C than that of NDV_{pi} (6).

The inactivation rates at 50 C of HA and neuraminidase were strikingly different than the inactivation rates of infectivity (Fig. 1, lower panel). In the case of NDV_{pi}, which showed rapid inactivation of infectivity at 50 C, the HA and neuraminidase of the intact virus were unaffected by 120 min of heating. With NDV_o, 83 and 43% of HA and neuraminidase activities, respectively, were destroyed after 2 hr at 50 C without significantly affecting the infectivity of the virus.

These data reveal the paradox of the more rapid thermal inactivation of the infectivity of the mutant, although its neuraminidase and HA are more stable than those of the wild-type virus. It is likely that there is a difference in the heat stability of a component of the ribonucleoprotein core of the two viruses, unrelated to the stabilities of the protein components of their envelopes.

These results indicate that (i) the rapid loss of infectivity of NDV_{pi} at 50 C is not associated with the inactivation of HA and neuraminidase activities (2), and (ii) the HA and the neuraminidase proteins of NDV_{pi} are more heat stable than those

FIG. 1. Rate of inactivation at 50 C of infectivity, hemagglutinin, and neuraminidase activities of NDV_o and NDV_{pi}. Concentrated virus preparations were diluted in PBS to contain approximately 10^9 PFU/ml. Portions (2 ml) were distributed in sleeve-stoppered vials and heated for different times at 50 C as described in the text. Unheated control samples were kept at 0 C. The activities in the control samples were 560 and 400 hemagglutinin units per ml for NDV_o and NDV_{pi}, respectively, and 68 and 62 neuraminidase units per ml for NDV_o and NDV_{pi}, respectively. Symbols: ●, NDV_o; ▲, NDV_{pi}.

of NDV_o. The heat stability of the neuraminidase of intact NDV_{pi} could be due to a protected structural state of the enzyme in the viral envelope. This physical location also could account for lack of accessibility of the enzyme to the receptors in the erythrocyte membrane, thereby explaining inefficient elution. On the other hand, the heat stability could be the result of a genetic alteration of the properties of the neuraminidase protein itself. To permit a choice between these alternatives, the virions were disrupted by chemical treatment, and the thermal stabilities of the separated HA and neuraminidase were tested.

Stability of HA and neuraminidase of disrupted NDV_o and NDV_{pi}. To determine the completeness of the physical separation of the HA and neuraminidase separated as previously described, the following experiment was conducted. Disrupted NDV_o and NDV_{pi} preparations (1.0 ml) containing 160 and 320 HA units, respectively, were mixed with washed chicken erythrocytes (0.1 ml, packed volume after centrifugation at $1,000 \times g$ for 10 min). After the mixture was incubated at 0 C for 5 min, the erythrocytes were removed by centrifugation at $1,000 \times g$ for 10 min, and the supernatant fluid was tested for unadsorbed HA and neuraminidase. The results showed that the HA activity of both viruses was completely adsorbed to the erythrocytes and removed from the fluid, whereas all of the neuraminidase activity remained unadsorbed. This finding indicated a complete separation of the two activities by Tween 80-ether treatment.

The disrupted-virus preparations were diluted in PBS and heated at different temperatures (37 to 55 C) for 60 min as described in the legend of Fig. 2. Control samples were kept at 0 C. The data in Fig. 2 show that, in contrast to the findings with intact virus particles, the HA and neuraminidase from the two viruses could not be distinguished by thermal inactivation. However, the dissociated neuraminidases were more stable than the hemagglutinins. For example, when kept at 37 C for 1 hr, 65 and 83% of HA activity of NDV_o and NDV_{pi}, respectively, was destroyed, whereas the neuraminidase activity of both the viruses was stable under these conditions (Fig. 2).

Since heating at 55 C for 1 hr completely destroyed both HA and neuraminidase activities of NDV_o and NDV_{pi}, experiments were carried out at this temperature to study the rates of inactivation of the two activities in preparations of disrupted virus particles.

Rate of inactivation at 55 C of HA of intact and disrupted NDV_o and NDV_{pi}. Virus concentrates and the disrupted preparations were heated at 55 C as previously described. At various times, samples were removed and rapidly chilled in ice; the HA was measured.

The infectivity of both viruses was rapidly inactivated at 55 C; after 10 min at this temperature, no residual infectivity could be detected. However, with intact virus the HA activity of NDV_{pi} was much more stable than that of NDV_o. After 20 min of heating, the HA activity of NDV_{pi} was unchanged, whereas the HA titer of intact NDV_o had fallen from 800 HA units per ml to 70 HA units per ml, a 92% loss of activity (Fig. 3A). After 30 min at 55 C, NDV_{pi} had only lost about 50% of its HA activity, whereas the HA of NDV_o was completely destroyed.

FIG. 2. Stability of hemagglutinin and neuraminidase activities of disrupted NDV_o and NDV_{pi} at different temperatures. Disrupted-virus preparations were diluted in PBS, and portions (0.6 ml) were distributed in vials. The samples were kept at the appropriate temperatures for 1 hr and then rapidly chilled in ice. Unheated control samples, kept at 0 C, contained 200 and 800 hemagglutinin units per ml for NDV_o and NDV_{pi}, respectively, and 43 and 30 neuraminidase per units ml for NDV_o and NDV_{pi}, respectively. Symbols: ●, NDV_o; ▲, NDV_{pi}.

Although the inactivation of HA in disrupted-virus suspensions was much more rapid than in intact virus, the same relation between the stability of HA of NDV_{pi} and NDV_o was observed (Fig. 3A). With NDV_o, the disrupted HA was inactivated at a very rapid rate; all activity was destroyed by 5 min of heating at 55 C. On the other hand, about 25% of the HA activity of disrupted NDV_{pi} was retained after 5 min at 55 C, and the inactivation proceeded at a much slower rate than for NDV_o.

The rates of inactivation of HA from disrupted NDV_o and NDV_{pi} during the first 4 min at 55 C are shown in Fig. 3B. All of the HA activity (1,120 HA units/ml) of disrupted NDV_o was destroyed by 4 min of heating at 55 C, whereas the HA activity of NDV_{pi} fell from 800 to 280 HA units per ml, a 65% loss.

Rate of inactivation at 55 C of neuraminidase of intact and disrupted NDV_o and NDV_{pi}. Virus concentrates and disrupted preparations were heated at 55 C as previously described. The re-

sults (Fig. 4) show that, with intact NDV_o, the neuraminidase activity was completely destroyed by 30 min; the enzyme of intact NDV_{pi}, however, was completely stable for 50 min and was only reduced to 80% of the original activity by 60 min. The rates of inactivation of the neuraminidases of disrupted NDV_o and NDV_{pi} were similar to the inactivation rate of the enzyme activity of intact NDV_o. These results indicate that, although the neuraminidase of intact NDV_{pi} was stable at 55 C for 50 min, the rate of inactivation of the dissociated NDV_{pi} enzyme was the same as the rate observed for the enzyme of either intact or disrupted NDV_o.

DISCUSSION

A possible explanation of the defective elution of NDV_{pi} from erythrocytes is suggested by the results of this investigation. In contrast to the neuraminidase of the wild-type NDV_o, the enzyme of the mutant virus may be incorporated into the viral envelope in such a manner that it is unable to interact with the substrate present in the erythrocyte membrane. Evidence for such structural hindrance is provided by the following observations. First, there is no difference in the enzyme content of the intact virions of NDV_o or NDV_{pi} when tested with a soluble substrate, despite the fact that NDV_{pi} virions do not elute efficiently from erythrocytes (6). Second, the thermal stability of the neuraminidase activity of NDV_o and NDV_{pi} virions is markedly different (NDV_{pi} is more stable), whereas the enzymes dissociated from the two viruses are inactivated by heat at similar rates. The similarity of the enzymes from the wild-type virus and from the mutant is further borne out by our unpublished observations that the pH and temperature optima of the enzymes from the two virus populations are the same.

This evidence leads us to the conclusion that, in the maturation process, the neuraminidase protein of the mutant NDV_{pi} is incorporated into the viral envelope in such a way that it is inhibited from reacting with the substrate in the erythrocyte membrane, although it can react with soluble substrate. Direct evidence of such a difference in the structure and composition of the virus envelopes of NDV_o and NDV_{pi} is being sought by electron microscopy and lipid analysis.

The data obtained also suggest that there may be a difference in the hemagglutinins of the two viruses. The HA activity of NDV_{pi} was significantly more resistant to thermal inactivation than that of NDV_o; greater stability of the HA of NDV_{pi} was observed with intact virions and with disrupted virus particles. Studies of highly purified

FIG. 3. Rate of inactivation at 55 C of hemagglutinin activity of intact and disrupted NDV_o and NDV_{pi}. Virus concentrates and disrupted-virus preparations

FIG. 4. Rate of inactivation at 55 C of neuraminidase activity of intact and disrupted NDV_o and NDV_{pi}. Virus concentrates and disrupted-virus preparations were heated at 55 C for various times as described in the text. Unheated control samples were kept at 0 C. The original enzyme activity was 70 and 65 units/ml for intact NDV_o and NDV_{pi}, respectively, and 130 and 126 units/ml for disrupted NDV_o and NDV_{pi}, respectively. The rates of inactivation of neuraminidase activity are shown for intact NDV_o (●); disrupted NDV_o (○); intact NDV_{pi} (▲); and disrupted NDV_{pi} (△).

HA proteins from the two viruses are required to understand the basis of this difference.

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were heated at 55 C for various times as described in the text. The rates of inactivation of hemagglutinin activity are shown for intact NDV_o (●), disrupted NDV_o (○), intact NDV_{pi} (▲), and disrupted NDV_{pi} (△). Panel B shows the inactivation during the first 4 min at 55 C.

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