Intracellular Synthesis of Myxovirus Neuraminidase in Chick Embryo Cell Monolayer Culture

I. Neuraminidase Activity, Hemagglutinin Synthesis, and Content of Cell-bound Sialic Acid in Newcastle Disease Virus-infected Cells

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Neuraminidase (Nase) activity of chick embryo monolayer cell homogenates was determined by its rate of splitting of neuraminolactose, free neuraminic acid (NA) being determined by the thiobarbituric acid assay. Noninfected cells were found to have no detectable amount of Nase activity. Newcastle disease virus (NDV)-infected cells (multiplicity of infection, 20 to 75 plaque-forming units per cell) displayed a high level of Nase synthesis, the rate of synthesis being parallel to that of hemagglutinin (HA) synthesis (with a 1.5 hr delay in the latter). An "eclipse" of the Nase and HA activities associated with the virus that was adsorbed onto cells was observed. The data provide evidence that the Nase is not incorporated into the viral envelope from a pre-existing cell supply but that its synthesis is coded by the viral genome. The content of cell-bound sialic acid, determined simultaneously in infected-cell homogenates, showed characteristic features allowing certain conclusions concerning the renewal of NA-terminating cell receptors during the course of infection, and the intracellular action of the Nase of the virus introduced into cells by the inoculum and that of the newly synthesized Nase at different stages of infection.

The origin of myxovirus neuraminidase (Nase) can be accounted for by two alternatives: the Nase present in normal cells is incorporated into the viral envelope during the formation of mature viral particles, or it is a newly formed structural viral component, its synthesis being coded by the viral genome. Both alternatives have been supported by certain experimental results. The support for the first alternative has been provided by three types of evidence. The first one includes well-known facts concerning the insertion of the host cell material into virus particles (8, 13, 14, 20, 24, 25, 42, 43, 46, 50). This group of experiments suggests the possible incorporation of host cell material into virus particles. The second group of findings concerns the wide occurrence of Nase in animal tissues, including those serving as sources of virus multiplication, namely, chick embryo chorioallantoic membrane (CAM) cells (1) and nervous tissue (22, 37). The last group of findings relates to immunological data showing that the soluble enzyme isolated from virus has some immunological relationship to the Nase present in noninfected CAM (2). However, Ishida and Tosava (unpublished data) have demonstrated the immunological specificity of neuraminidases isolated from various myxoviruses.

As to the second alternative, there have been only a few attempts at direct experimental proof of virus-induced Nase synthesis in infected cells (3, 22, 38). However, the cell systems employed by these authors (CAM cells and nervous tissue of mouse brain) both contain Nase as a naturally occurring cell enzyme. Moreover, Nase in both cell systems has been shown to participate in certain functional reactions and, thus, is under the regulating control of certain physiological mechanisms inherent to a whole organism (6, 36, 47). Therefore, the increase in Nase synthesis in infected cells as compared with normal cells may be considered only as quantitative intensification of the synthesis occurring in normal cells, the virus being merely stimulating and not being the synthesis-coding agent.

1 Some of these data were reported at the IXth International Congress of Microbiology, Moscow, 24–30 July 1966 (Abstracts of Papers Presented at Focal Topic Sessions, Moscow, 1966, p. 468).
Thus, the determination of the origin of myxovirus neuraminidase requires further study.

The present work was undertaken to examine the possibility of intracellular synthesis of Nase in myxovirus-infected chick embryo cell monolayer cultures, in which some myxoviruses, e.g., Newcastle disease virus (NDV), reproduce in infectious form and others, e.g., influenza A2 virus, do not.

This work concerns the intracellular synthesis of Nase, determined simultaneously with hemagglutinin (HA) synthesis, and the content of cell-bound sialic acid in NDV-infected cells.

**Materials and Methods**

**Viruses.** The “Tomilinsky” epizootic strain of NDV was kindly supplied by N. V. Syurin (Institute of Veterinary Virology, Moscow). Before use in the present experiments, the virus was cloned three times by single-plaque isolation (11). Some details concerning this strain, such as its cytopathic effect in various cell cultures, plaque-forming ability, description of the early stages of the latent period, and some aspects of reproduction and interference have been recently described (10, 21, 27, 29). Allantoic fluid harvested 48 hr after infection of 10-day-old embryos was used as virus-containing material, the pools usually having a titer of 640 to 2,180 plaque-forming units (PFU) per ml.

**Tissue culture.** The culture of chick embryo cells was prepared from 10-day-old chick embryos by trypsinization (51) and was used for all experiments, including the plaque assay for NDV titration. In the main experiments, large (volume, 1,000 ml; area occupied by the cell monolayer, 175 cm²) Povitskaya flasks (Jena) were used; for NDV plaque assay, small Povitskaya flasks were used. The cell suspension contained 1.5 × 10⁶ cells per milliliter; the volume of cell suspension per flask was 75 ml. The growth medium consisted of 0.5% lactalbumin hydrolysate in Hanks’ balanced salt solution (BSS), 45%; medium 199, 45%; and bovine serum, 10%. A perfect monolayer was formed after 72 hr at 37 C, flasks at this time being taken for experiments. Further details have been described (28).

**Virus infectivity titration.** NDV was titrated by plaque assay in 100-ml Povitskaya flasks. The diluent was medium 199. After washing with Dulbecco and Vogt (11) phosphate-buffered saline (PBS), the monolayers were inoculated with 0.5 ml of successive 10-fold dilutions. After 2 hr of incubation at 37 C, the inocula were removed; the monolayers were washed twice with PBS and covered with nutrient agar. The agar overlay medium consisted of medium 199 (10-fold concentrate) supplemented with bovine serum (5%); neutral red (5% of 0.1% solution in twice-distilled water); sufficient 7.5% bicarbonate solution to adjust the pH to 7.6; tri(hydroxymethyl)-aminomethane buffer (5% of an 0.1 M solution, pH 7.6); chick embryo extract (1%); and antibiotics. The final agar (Difco) concentration in the overlay was 1.35%. After addition of the overlay medium, the flasks were kept in darkness. The plaques were counted 72 hr after inoculation. When calculating PFU, a correction for overlapping plaques was made, the proportion of such plaques depending on the total quantity of virus in the inoculum relative to the flask bottom surface area (5, 11, 30, 31). Quantitative estimation of the reduction in plaque number due to overlapping has been accomplished by Dulbecco and Vogt (11), but only for poliomyelitis virus. We have used our own tables, which were drawn up by means of the formula based on Poissonian distribution. Some other specifics concerning the plaque assay with the “Tomilinsky” strain of NDV have been described (28).

**HA titration.** An 0.4-ml quantity of a 1% suspension of chicken red blood cells in saline (0.9% NaCl) was added to each of a set of holes in a plastic dish containing 0.4 ml of serial twofold dilutions of virus in saline. The virus titer was read after 45 min at room temperature. The titer was determined from the highest dilution of virus which provided 50% agglutination and is expressed as HA units per milliliter.

During the course of infection of a cell monolayer, intracellular viral HA was measured in homogenates prepared from the cells.

**Conditions of cell inoculation and maintenance of infected monolayers.** The large Povitskaya flasks, with monolayers containing 40 to 45 million cells per flask (counted in a hemocytometer after cells were removed from the glass with a 1:1 mixture of ethylenediaminetetraacetic acid-trypsin), were washed once with BSS, inoculated with 2 ml of undiluted virus-containing allantoic fluid. Immediately after inoculation, the flasks were placed at 4 C for 1 hr. Then the inocula were removed from each flask. The monolayers were washed once with cold BSS, covered with 25 ml of the maintenance medium (medium 199 with 2% of bovine serum), held at 37 C, and assayed at intervals.

Input multiplicity of infection fluctuated in different experiments from 45 to 160 PFU per cell. It had been found previously that, under these experimental conditions, about 50 to 55% of the virus initially contained in the inoculum was adsorbed to the cell monolayer.

**Preparation and maintenance of homogenates.** At 6 hr intervals after inoculation, the flasks were placed at 4 C for 0.5 to 1 hr to prevent the further development of infection. The maintenance medium was then removed, and the cell monolayers were washed twice with cold PBS. The cells were removed from the glass, by means of glass beads, and were kept in PBS at 4 C for 6 to 12 hr. The cells were sedimented by centrifugation at 1,000 rev/min for 15 min and suspended in 0.2 M phosphate-citrate buffer (pH 6.1). Homogenates from each sample of cells were prepared in a Potter-Elvehjem homogenizer with a Teflon pestle.

The following determinations were carried out on the homogenates: HA content, Nase activity, content of cell-bound sialic acid, and content of nitrogen. All determinations were carried out with a single sample.

**Determination of Nase activity.** Nase activity of cell homogenates was determined from the value of N-acetylmuraminic acid (NANA) split from a specific substrate, NANA being determined by means of the thiorbarbituric acid assay (49) with certain modifications (48) and expressed as micrograms per milliliter of nitrogen. As a specific substrate for Nase, N-acetyl-

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neuraminyl(2 → 3)lactose (NANL; 15, 26), kindly supplied by R. Kuhn (Max-Planck-Institut für Medizinische Forschung, Institut für Chemie, Germany) was used. The quantity of NANL in the mixture was 150 μg per 0.4 ml of homogenate. The samples were incubated for 20 min at 37°C. The extinction value was measured spectrophotometrically.

We have tried to minimize the contribution from interfering chromophores (deoxyribose compounds) in the homogenates that might react in the thio- radicalic acid assay and result in erroneous values for sialic acid (6, 23, 49). In the procedure used (48), the samples were treated exactly as in Warren’s assay (49), including heating of the samples with thiobarbituric acid; the pH of the samples was then adjusted to 5.0 with 5% NaOH solution prior to the first extraction with cyclohexanone. The maximal absorbance of the extracted product was at 532 μm (the wavelength associated mainly with deoxyribose compounds). The aqueous phase was then adjusted to pH 3.0 with 66% H₂SO₄ solution, and the second extraction with cyclohexanone was carried out. The absorbance peak of the extracted material was observed at 549 μm (the wavelength associated with sialic acid).

Determination of NANA aldolase in the cell homogenate. The purified NANA preparation was added to the cell homogenate, and NANA aldolase activity was determined by the decrease in NANA in the mixture after 20 min of incubation at 37°C. Determination of cell-bound sialic acid. The homogenate was heated in 0.1 N H₂SO₄ at 80°C for 1 hr to release cell-bound sialic acid, which was measured as free sialic acid (48).

Determination of nitrogen. By means of the Kjeldahl method, the final N₂ concentration was determined by microdiffusion analysis (9). All determinations carried out on a homogenate sample (HA and Nase activities and cell-bound sialic acid content) are expressed per 1 ml of the homogenate sample and related to N₂ content in this volume.

RESULTS

The experiments were carried out with two multiplicities of infection: 75 and 20 PFU per cell. (The latter was the minimal multiplicity value providing synchrony of infection.) The large dose required to provide synchronous infection was related to the curvature of the glass surface of the flasks used (28), which caused uneven adsorption of the virus inoculated on the cell monolayer. Thus, the real multiplicity was different along the cell monolayer surface, and only with 20 PFU per cell was each cell of the monolayer infected (i.e., received an effective dose of 1 PFU). The multiplicity of infection used in the experiments is expressed as the amount of virus actually adsorbed onto the cell monolayer (approximately half of the corresponding input multiplicity).

The results presented in Fig. 1 represent a typical experiment with the larger inoculum. The Nase and HA activities were practically absent in the control culture and the amount of cell-bound sialic acid was of the same order as that found in infected cells at the end of the adsorption period, i.e., before the infection really had started.

In contrast, the cells initially (just after the 1-hr period of contact with virus at 4°C) had a Nase activity which could be attributed to the virus in the inoculums which adsorbed onto the cell surface. Within 2 hr at 37°C, a decrease in Nase activity was observed (to 40% of the initial Nase value). Then, starting from this point, Nase activity began to rise, the activity peak being reached at 6 to 8 hr after infection. Then followed a clearly evident decrease of Nase activity, starting at 9 hr.

We examined cells for the possible presence of NANA aldolase activity (7), which might distort the real value of Nase activity by degrading the NANA formed from NANL by infected cell Nase. No NANA aldolase activity was detected in the chick embryo cells under the experimental conditions used.

The curve related to HA synthesis was parallel to that for Nase activity, but lagged by 2 hr the Nase curve. The decrease in the initial HA activity of infected cells (after the adsorption period) was also observed within 1 to 3 hr after infection. Maximal HA synthesis occurred at 9 to 10 hr after infection, followed by a decrease. The difference between the HA value of the inoculum before and after the virus adsorption period (the quantity of HA lost when the cell monolayer was washed after the adsorption period has been taken into account) was 7.6 times the HA value of the cell homogenate prepared just after the adsorption period at 4°C.
FIG. 2. Neuraminidase activity, hemagglutinin synthesis, and cell-bound sialic acid content of chick embryo cell monolayer culture infected with NDV (20 PFU per cell).

Thus, only a small part of the virus adsorbed onto the cell surface was available to provide HA activity.

The values of cell-bound sialic acid in the course of infection also had characteristic features. A clear decrease within the first 2 hr after infection, corresponding to the decrease in Nase activity, was observed. The level of cell-bound sialic acid increased at 3 hr after infection, reaching almost the initial value, and it remained at this level until 6 hr after infection. After 6 hr, a gradual decrease occurred up to 11 hr, at that point reaching 48% of the initial value.

With the lower multiplicity of infection (20 PFU per cell), the results were somewhat different (Fig. 2). The initial value of Nase activity (just after the virus adsorption period at 4 C) was less, and it correlated with the amount of virus adsorbed onto the cell monolayer. The decrease in Nase activity at the beginning of infection was more pronounced, being only 12% of the initial Nase activity value. Nase activity increased 1 hr earlier, and the curve was of the exponential type until 6 hr after infection. The slope of the curve of HA synthesis was less steep than that observed in the previous experiment and showed a lag compared with Nase activity. The content of cell-bound sialic acid in infected cells did not differ significantly from that of the experiment with a higher multiplicity of infection.

DISCUSSION

The principal objective of this work—to demonstrate the intracellular synthesis of Nase in virus-infected cell culture in a manner like other viral components—has been accomplished. It appears that the enzyme is not merely adsorbed onto the viral envelope from a pre-existing intracellular supply.

It should be emphasized that the system employed in our experiments has an advantage over the intracellular synthesis of viral Nase in CAM cells of chick embryos (3, 38) and in mouse brain tissue (22); Nase activity has not been demonstrated in control cells of our system. Evidence of intracellular Nase synthesis after virus infection cannot be evaluated in a tissue system in which NANA is involved (3, 22, 38).

The question is whether there is synthesis of a specific viral component (like HA) that the normal cell cannot provide, or whether Nase synthesis is a result of virus induction of intracellular activity and that the enzyme is coded for by the cells genome.

The present cell culture system does not exclude the possibility that a normal cell can synthesize Nase after some kind of provocation but permits more definitive proof and interpretation.

The important point in this respect is that our noninfected chick embryo monolayer cells did not show significant Nase activity. Thus, there is no pre-existing supply of the enzyme which could be incorporated into newly synthesized virus particles.

Other evidence for the viral nature of Nase is the very close relation of its synthesis to HA synthesis. Both components were found to have a parallel course of synthesis.

In this respect, the decrease in Nase as well as in HA activity within the first few hours after infection is of some interest. The decrease could be considered as an “eclipse” of HA and Nase, if the concept of an eclipse (18), particularly in the case of NDV (39, 41), may be applied in general to viral components. However, does this “eclipse” block a portion of the active sites on the virus particle [a nontemperature-dependent stage which is reversible by high salt concentration, acid, urea, or antibody (16, 19, 32)], or does it relate to the next, temperature-dependent stage, at which virus particles are inaccessible to treatments dissociating the initial virus-cell complex (16, 17, 32, 40) and which ends in disintegration of virus particles (45). Our data allow certain conclusions concerning the problem. The deficit between the viral HA adsorbed onto the cells (the value of viral HA in inoculum before and after adsorption period) and the HA value of the cell homogenate just after the adsorption period at 4 C is caused by a block of most of the HA-active surface sites of the virus particles by cell membrane receptors. The HA activity that remains at that time is due
to HA-active sites which are outside the cell surface and are thus not bound to cell membrane receptors. Therefore, the further decrease in HA and Nase activities which is observed after placing the cells loaded with virus at 37°C has to do with the penetration of virus into cells. There might be two alternative explanations. (i) As virus penetrates into a cell at 37°C, or is engulfed by the cell (45), those HA-active sites which had not reacted with cell receptors at 4°C now also become bound to receptors and thus are not evident in HA reaction after cells are homogenized. (ii) The decrease in HA activity is a result of the disintegration of virus particles, with the liberation of viral subunits (including HA and Nase), and their successive destruction.

The following data lead us to accept the latter alternative. The decrease in activities continues at least for 3 hr with HA and 2 hr with Nase (Fig. 1 and Fig. 2), whereas, after only 5 min, over 95% of NDV becomes refractory to inactivation by specific antibody (44) or to removal from the cell surface with receptor-destroying enzyme (4) or Nase (33); over the same period, the loss of morphological integrity of the virus engulfed is observed (45). Thus, there is no time course parallelism between the decrease in HA and Nase activities found in our experiments, on the one hand, and the engulfment process which the first alternative corresponds to, on the other hand.

The correlation between Nase synthesis kinetics and the content of cell-bound sialic acid gives further support for the above supposition concerning the nature of HA and Nase "eclipse." In this respect, the diphasic shape of the sialic acid curve is of interest. The loss of cell-bound sialic acid within the first 2 hr after infection may result from the splitting of NANA from cell receptors at the cell surface as well as inside the cells (35) by the penetrating virus. This fact supports the assumption that Nase activity (as well as HA) is not blocked after virus engulfment and that its inactivation is caused by the gradual destruction of free-existing Nase subunits.

The ensuing increase in the content of cell-bound sialic acid (almost to the initial level) is assumed to be due to renewal of the NANA-containing receptors (34), which can occur only if the cessation of the Nase activity takes place. Therefore, the duration of the period of decrease (about 2 hr) is to a certain extent a measure of the Nase destruction rate. From 3 to 6 hr (Fig. 1 and Fig. 2), the content of cell-bound sialic acid is rather constant, in contrast to the significant increase in Nase activity. This discrepancy is thought to be caused by the low activity of the newly synthesized intracellular Nase. Whether slight or no Nase activity at this stage of the synthesis cycle reflects an intrinsic defect in the enzyme, or whether there are some structural difficulties inhibiting its action on cell-bound sialic acid, remains a question of interest. Acceleration of the splitting rate later than 6 hr after infection might be connected with the incorporation of the newly synthesized "amorphous" Nase into virus particles. Being "structured" in the virus envelope, the Nase may be in a more suitable steric condition for its action on intracellular sialic acid.

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