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Neuraminidase Activity and Syncytial Formation in Variants of Parainfluenza 3 Virus

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By a sensitive fluorometric assay method, we could definitely demonstrate neuraminidase activity for two variants of parainfluenza 3 virus, M and SC, which were previously shown to have no detectable neuraminidase activity. The enzyme activities of these viruses were very similar to each other, showing a much lower catalytic rate, a much higher K_m value, and a more acidic pH optimum than those of the virus variants of high neuraminidase activity, 910N, LT, and MR. M and SC viruses eluted from guinea pig erythrocytes very poorly, whereas 910N and LT viruses eluted readily. M virus required the aid of a bacterial neuraminidase for effective growth and plaque formation in MDBK cells, but the virus grew well and formed plaques in R66 and Vero cells without the enzyme. SC virus required no exogenous neuraminidase for growth in all of these cell types. Depending on cell type, SC virus induced slight to extensive syncytial formation which was greatly inhibited by exogenous neuraminidase. In contrast, M virus induced extensive syncytial formation in all these cells regardless of the presence or absence of exogenous neuraminidase, although development and disintegration of the syncytia were more or less retarded by the enzyme, especially in MDBK cells. These results indicate that M virus possesses highly potent inducibility of syncytial formation which is further fortified by being low in viral neuraminidase activity.

As reported previously, neuraminidase activity cannot be demonstrated for M and SC strains of bovine parainfluenza 3 virus by the assay method with fetuin or *N*-acetylneuramin lactose as the substrate, whereas the enzyme activity is clearly shown for 910N, LT, and MR strains of the virus (7). M, SC, LT, and MR viruses are plaque-type variants isolated from a wild-type strain, and 910N virus is another wild-type strain (5, 7). M virus requires exogenous neuraminidase for its effective growth and plaque formation in MDBK cells, whereas the enzyme exhibits little effect on growth and plaque formation of 910N, LT, and MR viruses (7). However, SC virus, possessing no detectable neuraminidase activity like M virus, is affected much less markedly by exogenous neuraminidase; SC virus produces small clear plaques in the absence of the enzyme, but large turbid plaques in its presence, whereas the replication of SC virus is enhanced only a little by the enzyme (7). On the other hand, M virus, but not SC virus, induces extensive syncytial formation in MDBK cells (7). We observed that both M and SC viruses can replicate effectively and form plaques in R66 cells in the absence of exogenous neuraminidase as 910N virus. These facts suggest the possibility that M and SC viruses would possess low neuraminidase activity, although undetectable by the conventional assay methods, and that the enzyme activity of M virus would be much lower than that of SC virus, since the lower activity would account for the higher inducibility of syncytial formation as reported for mumps virus (2) and would be insufficient for M virus to release from MDBK cells. Another possibility is that M and SC viruses would possess neuraminidase of similar activity, whereas M virus would possess much higher syncytium inducibility than SC virus.

Recently, Myers et al. (3) developed a highly sensitive and simple assay method for neuraminidase with the 4-methylumbelliferyl-α-ketoside of N-acetylneuraminic acid as a fluorescent substrate. The method was shown to be advantageous for determination of neuraminidases of influenza viruses (8). These reports prompted us to reexamine the neuraminidase activity of our variants of parainfluenza 3 virus by this method. In parallel, we tried to characterize more clearly the syncytium inducibility of these viruses.

In this paper we describe the results of the test for neuraminidase activity of the plaque-type

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variants; the effects of exogenous neuraminidase on the cytopathic effects, replication, and plaque formation of these viruses in MDBK, R66, and Vero cells; and some other observations.

MATERIALS AND METHODS

Cells. Madin-Darby bovine kidney (MDBK) cells, R66 cells from human embryonic lung, and Vero cells from African green monkey kidney were grown in Eagle minimum essential medium (MEM) containing 5% calf serum. The maintenance medium for virus growth was MEM supplemented with 10% tryptose phosphate broth (TPB) (Difco Laboratories, Detroit, Mich.).

Viruses. The 910N strain of parainfluenza 3 virus and clones of plaque-type variants M, SC, LT, and MR, obtained from the YN strain (5, 7), were used. Virus seeds of 910N, SC, LT, and MR viruses were culture fluids harvested from MDBK cells infected at an input of 0.01 PFU per cell and incubated at 37°C for 48 to 72 h. For propagation of M virus, MDBK cells were infected at an input of 1 PFU per cell and incubated at 37°C for 24 h in the medium containing 0.003 U of type V neuraminidase from *Clostridium perfringens* (Sigma Chemical Co., St. Louis, Mo.) per ml (the enzyme units were determined with *N*-acetylneuramin lactose as the substrate). In some experiments, Sendai virus strain Z and Newcastle disease virus strain Miyadera were used in the form of infectious allantoic fluids from embryonated hen's eggs.

Plaque formation. The plaque formation method was previously described (7). Inoculated cultures of MDBK cells were incubated at 37°C for 4 days, and those of R66 and Vero cells for 5 days for plaque development. For plaque formation of M virus in MDBK cells, the agar overlay medium contained type V neuraminidase. The infectious titer of the viruses was expressed in PFU obtained in MDBK cells.

Virus purification. Virus was purified by the method described previously (6) from the culture fluid of MDBK cells infected at an input of 5 PFU per cell and incubated at 37°C for 24 to 48 h. For propagation of M virus the medium contained type V neuraminidase.

Determination of viral protein. Purified virus was appropriately diluted in phosphate-buffered saline (PBS) containing 0.1% sodium dodecyl sulfate. A 20-μl sample of the dilution was mixed with 1 ml of Coomasie brilliant blue G250 solution, and the optical density at 595 nm was measured (1). Bovine serum albumin was used as a standard.

Neuraminidase assay. The fluorescent substrate, 4-methylumbelliferyl-α-ketoside of *N*-acetylneuraminic acid (3), was kindly supplied by Y. Uchida (Kyoto Research Laboratory, Marukin Shoyu, Ltd., Kyoto). A 200-μl sample of the test material diluted in PBS, 20 μg of the substrate in 100 μl of distilled water, and 100 μl of 0.1 M sodium acetate buffer at the appropriate pH were mixed in a 12 by 75-mm Pyrex borosillicate tube (Corning Glass Works, Corning, N.Y.) on ice and gently shaken in a water bath at 37°C. After the incubation time indicated below, 3.6 ml of 0.25 M glycine–NaOH (pH 10.4) was added to the mixture. Fluorescence was measured with a Hitachi 650-40 fluorescence spectrophotometer (excitation, 360 nm; emission, 440 nm). The fluorescence of the mixture containing 200 μl of PBS instead of the test sample was measured for each incubation time as the background to be subtracted from the fluorescence of each determination. The 4-methylumbelliferon (sodium salt; Sigma) solutions in 0.25 M glycine–NaOH (pH 10.4) served as a standard.

Virus adsorption and elution. Virus adsorption to and elution from guinea pig erythrocytes were studied with purified virus dialyzed at 4°C for 16 h against MEM containing 10% TPB (TPB-MEM). After dilution in TPB-MEM, 1.5 ml of the virus suspension was mixed with an equal volume of fresh guinea pig erythrocyte suspension in PBS on ice and shaken gently for 60 min. The ratio of erythrocyte number to virus infectivity was 3×10^9 cells per 3×10^7 PFU (excess erythrocyte condition) and 3×10^8 cells per 3×10^8 PFU (equivalent condition). At intervals, a 200-μl sample of the mixture was removed, diluted with 9 volumes of TPB-MEM, and immediately centrifuged at $1,000 \times g$ for 10 min. Unadsorbed virus in the supernatant fluid was measured by plaque counting. After adsorption for 60 min, the erythrocytes were sedimented by centrifugation at $750 \times g$ for 5 min, suspended in the original volume of TPB-MEM, and shaken at room temperature (23°C). After 60 min and 16 h, 500 μl of the suspension was removed, diluted with 2 volumes of TPB-MEM, and centrifuged at $1,000 \times g$ for 10 min. The supernatant fluid was assayed for infectivity. The input virus minus the unadsorbed virus at the end of adsorption was taken as the total virus adsorbed to the erythrocytes.

Hemagglutination. Hemagglutination was assayed by the pattern method in microtiter plates with a 1% suspension of fresh guinea pig erythrocytes in PBS. In some experiments erythrocytes were treated with 910N virus as follows. A 1-ml sample of 10,000 hemagglutinin units of purified 910N virus in PBS containing 1 mM Ca^{2+} (Ca-PBS) was mixed with 2 ml of 10% fresh erythrocyte suspension in Ca-PBS and shaken gently for 30 min on ice. Then 2 ml of Ca-PBS was added, and the mixture was shaken at room temperature overnight. The erythrocytes were pelleted by centrifugation at $750 \times g$ for 10 min and washed three times with PBS.

Cell fusion. MDBK cells were monodispersed by trypsin and suspended in MEM at a concentration of 10^6 cells per 0.4 ml. Purified viruses diluted in MEM to a concentration of 10 μg/0.4 ml were completely inactivated with irradiation by a 15-W UV lamp at a distance of 10 cm for 2 min. A 0.4-ml sample of cell suspension was mixed with the same volume of irradiated virus. The mixtures were shaken in an ice bath for 20 min and then incubated at 37°C for 60 min with gentle shaking. The mixtures were diluted with 8 ml of 5% calf serum–MEM, seeded onto four-chamber culture slides (Lab-Tek Products, Napervilla, Ill.) (0.5 ml per chamber), and incubated at 37°C overnight. After fixation with methanol-acetic acid (3:1), the cells were stained with Giemsa solution and examined microscopically.

Neuraminidases. Neuraminidase from *Vibrio cholerae* (Calbiochem, La Jolla, Calif.), neuraminidase from *Streptococcus* sp. (Seikagaku Kogyo, Tokyo), highly purified neuraminidase from *Arthrobactor ureafaciens* (Nakarai Chemicals, Tokyo), and type V neuraminidase from *C. perfringens* (Sigma) were used.

RESULTS

Demonstration and properties of viral neuraminidase. In preliminary experiments neuraminidase activity, although low, was definitely demonstrated for M and SC viruses by the fluorometric assay method of Myers et al. (3). Therefore we examined characteristics of neuraminidase activity of M, SC, LT, MR, and 910N viruses.

The 910N virus neuraminidase showed the highest activity at pH 5.1 and exhibited a fairly high activity at pH 5.7. In contrast, the neuraminidase activity of M and SC viruses exhibited a peak at pH 4.5, was slightly reduced at pH 5.1, and was much reduced at pH 5.7 (Fig. 1). The pH profile of LT and MR virus neuraminidases was quite similar to that of 910N neuraminidase (Table 1).

To determine the catalytic rate of viral neuraminidase at optimal pH, the amount of liberated 4-methylumbelliferon from the substrate was plotted against the incubation time at 37°C. The amount of the hydrolyzed substrate was in direct proportion with the incubation time (Fig. 2). The catalytic rate was calculated to be 5.2, 5.9, and 1,000 nmol of 4-methylumbelliferon per min per mg of viral protein for M, SC, and 910N viruses, respectively, indicating the neuraminidase activity of M and SC viruses to be less than 1/100 of that of 910N virus (Table 1). The catalytic rate of LT and MR virus neuraminidases was most similar to that of 910N virus neuraminidase (Table 1).

The Lineweaver-Burke double-reciprocal plot analysis was carried out to estimate the Michaelis-Menten constant (K_m value) of neuraminidases at optimal pH (Table 1). K_m values of M and SC virus neuraminidases were approximate ones because they were markedly higher than those of the other virus neuraminidases, and therefore satisfactory concentrations of the substrate were not available in the test. The results pointed that the affinity of M and SC virus neuraminidases to the substrate was much weaker than that of neuraminidase of the other viruses.

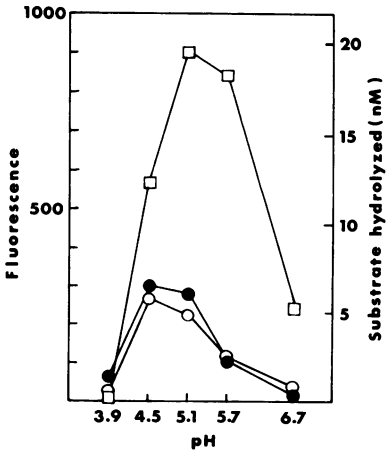

FIG. 1. Neuraminidase pH optimum. The fluorescent substrate was incubated at 37°C for 20 min at various pHs with purified M (●), SC (○), and 910N (□) viruses at respective concentrations of 50, 45, and 1.0 μg per reaction mixture.

TABLE 1. Properties of viral neuraminidase

Neuraminidase	Optimal pH	Catalytic rate[a]	K_m (μM)
910N	5.1	1,000	300
LT	5.1	670	320
MR	5.1	450	520
SC	4.5	5.9	2,600
M	4.5	5.2	2,300
Type V	5.1	620	320

[a] Expressed as nanomoles per minute per milligram of viral protein.

These observations clearly indicate that M and SC virus neuraminidases are very similar in their biochemical properties, but different from 910N, LT, and MR virus neuraminidases.

As described above, the M virus preparations were obtained from virus propagated in MDBK cells in the presence of type V neuraminidase from *C. perfringens*. There were clear differences in the optimal pH, catalytic rate, and K_m value between M virus neuraminidase and type V neuraminidase (Table 1). Moreover, 0.25% Triton X-100 included in the reaction mixture of the neuraminidase assay inhibited viral neuraminidase activity significantly, but enhanced the activity of type V neuraminidase (Table 2). Furthermore, purified M virus preparations obtained from R66 cells as well as primary monkey kidney cells in the absence of exogenous neuraminidase showed the same characteristics of neuraminidase activity as those described above for M virus neuraminidase (data not shown). These findings exclude the possibility that the neuraminidase activity demonstrated for M virus was due to the type V neuraminidase contaminating the virus preparations.

Adsorption and elution pattern of viruses. Previously we showed not only the neuraminidase activity but also the hemagglutinating activity of M and SC viruses to be markedly lower than those of 910N and LT viruses (7). We therefore examined the adsorption as well as elution pattern of these viruses. Purified virus was allowed to adsorb to fresh guinea pig erythrocytes on ice in excess erythrocyte and equivalent conditions and then to elute from the erythrocytes at room temperature (23°C). The ratios of erythrocyte number to the virus amount were 100 and 1 per

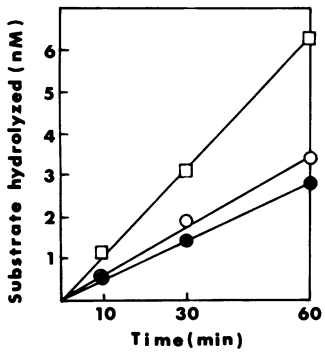

FIG. 2. Time course hydrolysis of fluorescent substrate by viral neuraminidase at optimal pH. The substrate was incubated at 37°C with purified M (●), SC (○), and 910N (□) viruses at respective concentrations of 10, 10, and 0.1 μg per reaction mixture.

PFU for excess erythrocyte and equivalent conditions, respectively (Table 3).

There was no obvious difference in the adsorption rate between the viruses; M and SC viruses adsorbed to erythrocytes as readily as did 910N and LT viruses. In the excess erythrocyte condition the adsorption was rapid and almost complete within 30 min, whereas considerable amounts of virus remained unadsorbed in the equivalent condition. In contrast to the adsorption, marked differences were demonstrated among the viruses in the virus elution. Under the equivalent condition high proportions of 910N and LT viruses eluted from the cells within 60 min, whereas only less than 1% of M and SC viruses eluted. After 16 h the elution of M and SC viruses reached only several percent, whereas that of 910N and LT viruses was complete. Under the excess erythrocyte condition the elution of M and SC viruses was minimal even after 16 h, whereas that of 910N and LT viruses was very small after 60 min, but became more than 50% after 16 h.

Although M and SC viruses were quite different from 910N and LT viruses in the elution pattern, the receptor for M and SC hemagglutinin on the surface of guinea pig erythrocytes did not seem different from that for 910N hemagglutinin (Table 4). The erythrocytes, which were agglutinated with an excess amount of purified 910N virus and then disagglutinated by incubation at room temperature (23°C) for 16 h, were not agglutinated by any of 910N, M, and SC viruses. However, the treated cells were agglutinated by Sendai and Newcastle disease viruses as readily as fresh erythrocytes.

Effect of exogenous neuraminidase on growth and plaque formation of viruses in MDBK, R66, and Vero cells. Previously we demonstrated that type V neuraminidase incorporated into the medium affected the replication and plaque formation of M and SC viruses in MDBK cells (7). These neuraminidase effects were examined for other cell types, R66 and Vero. Cultures of MDBK, R66, and Vero cells prepared in 35-mm wells of a 6-well plastic plate (Costar, Cambridge, Mass.) were infected with M, SC, or 910N virus at an input of 0.03 PFU per cell and incubated at 37°C in the presence or absence of 0.003 U of type V neuraminidase per ml. The culture fluids removed 48 h postinfection were assayed for infectivity (Table 5).

In MDBK cells SC and 910N viruses showed high virus yields that were not influenced by exogenous neuraminidase. On the other hand, M virus required exogenous neuraminidase for its effective growth. These results confirm the previous results (7).

In R66 cells M virus and SC and 910N viruses readily multiplied, and their growth was independent of exogenous neuraminidase. The yield of M virus was not affected by type V neuraminidase.

In Vero cells the growth of M, SC, and 910N viruses was not affected by type V neuraminidase, although yields of M and SC viruses were low as compared with that of 910N virus.

M virus could form clear plaques in R66 and Vero cells without the aid of exogenous neuraminidase. This is a striking contrast to the previous observation that in MDBK cells the exogenous neuraminidase is needed for the plaque formation of this virus (7). The plaque titer of M virus obtained in R66 and Vero cells in the absence of exogenous neuraminidase was almost the same as that in MDBK cells in the presence of the enzyme. However, there was a distinct difference between R66 and Vero cells in the plaque formation of M virus in the presence of exogenous neuraminidase. In Vero cells M virus plaques became smaller and turbid in the presence of type V neuraminidase, and the plaque number decreased by 50% or more, whereas the enzyme exerted no such effect on plaque morphology and plaque number of M virus in R66 cells (Fig. 3). Plaques of M virus in R66 and Vero cells were composed of syncytia, whereas M virus-infected MDBK cells under agar overlay medium did not show syncytial formation, but contracted as a single cell, regardless of the presence or absence of exogenous neuraminidase.

SC virus formed small, clear plaques, and 910N virus formed large, turbid plaques in MDBK cells. The exogenous neuraminidase had little effect on the plaque formation of 910N virus, but it made SC virus form large turbid plaques, confirming the previous results (7). Plaques of SC and 910N viruses in R66 cells were clear and virtually indistinguishable from those of M virus in the absence of type V

TABLE 2. Effect of Triton X-100 on viral and type V neuraminidases

Neuraminidase	Virus protein (μg) per reaction tube	Incubation time (min) at 37°C	Fluorescence[a]	
			No detergent	Detergent
910N	1.0	10	163	40
M	40	60	570	186
SC	40	60	568	168
Type V	0.2	10	36	176

[a] Neuraminidase activity was assayed in the presence and absence of 0.25% Triton X-100 in the reaction mixture.

neuraminidase, although M virus tended to form somewhat larger plaques than SC and 910N virus. However, the plaque formation of SC and 910N viruses in R66 cells, unlike that of M virus, was affected by type V neuraminidase. Plaques of SC and 910N viruses became turbid and smaller by the enzyme, although the plaque number was not significantly affected. In Vero cells plaques of 910N virus tended to be large and turbid, and those of SC virus were small and turbid, being difficult to count. The addition of exogenous neuraminidase made 910N virus plaques somewhat smaller and more turbid and reduced the plaque number by about two-thirds. In the presence of the enzyme SC virus plaques were also small and turbid, being difficult to count.

Effect of exogenous neuraminidase on cytopathic effects of viruses in MDBK, R66, and Vero cells. Cultures of MDBK, R66, and Vero cells were prepared and inoculated with virus in the same way as above experiments.

M virus induced extensive syncytial formation in MDBK cells. One-half or more of the cells in the culture were recruited into a few giant syncytia containing even several hundred to several thousand nuclei by 24 h postinfection. Moreover, a considerable proportion of the syncytia had already detached from the plastic's surface, proceeding rapidly to degradation. Only 10 to 20% of the cells remained mononuclear. By 40 h postinfection the cells remaining on the plastic's surface were all fused into giant syncytia, which completely sloughed off from the plastic's surface by 48 h postinfection.

Type V neuraminidase exerted marked effects on the cytopathic effects of M virus in MDBK cells. After incubation for 24 h the cells in the presence of the enzyme showed many small syncytia containing several to dozens of nuclei and occasionally medium-sized syncytia containing 50 to 100 nuclei. Here again 10 to 20% of the cells remained mononuclear, and detached cells were rare. By 48 h postinfection about one-half of the cells detached from the plastic surface, and the remaining cells formed small syncytia containing 5 to 20 nuclei. These findings indicate that a similar number of the cells were included in syncytial formation in the presence of exogenous neuraminidase as in its absence, but development and degradation of syncytia were distinctly retarded by the enzyme.

SC virus induced less marked syncytial formation in MDBK cells in the absence of the enzyme. At 24 h postinfection there were scattered small syncytia containing several to 30 nuclei, which comprised 25 to 30% of the cells in the culture. The remaining cells were mononuclear, and a few detached cells were observed. Even at 48 h, about 80% of the cells remained attached,

TABLE 3. Adsorption and elution patterns of viruses

Condition	Virus	% Adsorption[a]			% Elution[b]	
		10 min	30 min	60 min	60 min	16 h
Excess erythrocyte[c]	910N	1.2	0.5	0.5	1.7	57
	LT	3.6	1.1	1.5	5.7	65
	SC	2.2	0.2	0.3	0.05	0.02
	M	1.4	0.1	0.1	0.07	0.00
Equivalent[d]	910N	55	36	24	60	100
	LT	36	55	43	88	100
	SC	86	15	25	0.3	6.3
	M	49	22	27	0.5	8.5

[a] Percentage of unadsorbed virus PFU over input virus PFU.
[b] Percentage of eluted virus PFU over adsorbed virus PFU.
[c] Erythrocyte/virus ratio, 100.
[d] Erythrocyte/virus ratio, 1.

TABLE 4. Effect of treatment with purified 910N virus on hemagglutinability of guinea pig erythrocytes

Virus	Hemagglutinin titer with erythrocytes	
	Fresh	910N-treated
910N	4,096	<4
M	128	<4
SC	128	<4
Sendai	4,096	4,096
Newcastle disease	1,024	1,024

forming mainly small syncytia and leaving about 10% of the cells mononuclear; syncytia never became large. Type V neuraminidase almost completely inhibited the syncytial formation of SC virus. Cell rounding was the major change of infected cells, and almost all of the cells still remained attached at 48 h.

Cytopathic effects of 910N virus in MDBK cells were mild and consisted of cell rounding. No syncytia were produced. The exogenous neuraminidase exerted no obvious effect on the cytopathic effects of 910N virus.

Cytopathic effects of the viruses in R66 cells were greatly different from those in MDBK cells (Fig. 4). Not only M virus, but also SC and 910N viruses, induced extensive syncytial formation. Syncytia developed as rapidly as in MDBK cells infected with M virus. By 48 h postinfection all of the cells in the culture had become involved in a single giant cell. In contrast to MDBK cells infected with M virus, giant syncytia of R66 cells infected with any of M, SC, and 910N viruses remained attached to the plastic's surface even at 48 h postinfection. Type V neuraminidase markedly inhibited the syncytial formation of SC and 910N viruses, but it did not show discernible effect on the syncytial formation of M virus. Thus, in the presence of the enzyme, M virus induced extensive syncytial formation in R66 cells, whereas almost all R66 cells infected with SC or 910N virus remained mononuclear and became flattened and of polygonal appearance. The only effect of the enzyme on M virus-infected R66 cells was that the giant syncytia continued to be attached in the presence of the enzyme.

Cytopathic effects of the viruses in Vero cells differed from those in MDBK and R66 cells (Fig. 4). SC and 910N viruses induced syncytial formation of low grades. At 48 h postinfection a few small syncytia containing no more than 20 nuclei were observed. The syncytial formation of SC and 910N viruses was completely abolished by type V neuraminidase. M virus induced syncytia in Vero cells regardless of the presence or absence of the enzyme. However, even in the absence of the enzyme, syncytia developed much more slowly than those in MDBK and R66 cells. At 48 h postinfection a small number of syncytia containing 30 to 50 nuclei were observed, and it took several days for these syncytia to fuse into a giant syncytium. Type V neuraminidase retarded the development of the syncytia.

Effects of neuraminidases from different sources on M virus in MDBK cells. Growth-enhancing and plaque-forming effects of type V neuraminidase from *C. perfringens* on M virus were demonstrated only in MDBK cells among the cell types examined. We examined these effects for neuraminidases from different sources. The enzymes tested were preparations from *V. cholerae*, *Streptococcus* sp., and *A. ureafaciens*. All of the enzymes were tested at a concentration of 0.003 U/ml and were shown to enhance yields and to enable plaque formation and to reduce cytopathic effects of M virus in MDBK cells.

Cell fusing activity of viruses. As the difference in syncytium inducibility was marked among the viruses, they were estimated for activity of cell fusion from without. Samples (10 μg) of purified and UV-irradiated virus were mixed with suspensions of 10^6 MDBK cells, and the mixtures were subjected to the cell fusion procedures. Numbers of cell nuclei (A) and numbers of cells (B) were counted, and the degree of cell fusion was expressed as a fusion index, i.e., $[(A - B) \times 100]/B$. About 2,000 nuclei were counted for each estimation. The fusion indexes were 97, 109, and 193 for M, SC, and 910N viruses, respectively, indicating that all the viruses have distinct cell fusing activity for MDBK cells.

DISCUSSION

In the previous study (7) we could not demonstrate neuraminidase activity for M and SC viruses even though a large sample of purified virus was used for the assay. In the present study a more sensitive fluorometric assay method could clearly demonstrate the enzyme activi-

TABLE 5. Effect of exogenous neuraminidase on virus growth in MDBK, R66, and Vero cells[a]

Virus	MDBK		R66		Vero	
	NA(−)	NA(+)	NA(−)	NA(+)	NA(−)	NA(+)
M	4.6	7.8	7.2	7.3	5.2	4.9
SC	8.0	8.3	7.2	6.9	5.6	5.6
910N	8.6	8.3	7.5	7.5	7.1	7.1

[a] Cells were infected with virus at an input of 0.03 PFU per cell and incubated at 37°C for 48 h in the absence [NA(−)] or presence [NA(+)] of 0.003 U of type V neuraminidase per ml. The results are expressed as log PFU per milliliter of culture fluid.

ty for these viruses. The neuraminidase activities of M and SC viruses were very similar to each other, showing much lower catalytic rates, much higher K_m values, and more acidic pH optima than those of 910N, LT, and MR viruses.

As previously reported (7), M and SC viruses show lower hemagglutinating activity for guinea pig erythrocytes than did 910N and LT viruses. However, the erythrocytes were shown to adsorb M and SC viruses as readily as 910N and LT viruses. Coinciding with the level of neuraminidase activity, on the other hand, M and SC viruses eluted very poorly from the erythrocytes, whereas 910N and LT viruses readily eluted, although the receptor for M and SC hemagglutinin on the surface of the erythrocytes seems not to differ from that for 910N hemagglutinin.

These findings indicate that there is virtually no difference between M and SC viruses in the biological and biochemical properties of their low neuraminidase activity. The essential role of viral neuraminidase has been shown to be mediation of virus release from infected cells, as evidenced for influenza virus (4). The low neuraminidase activity of M and SC viruses seems fundamentally sufficient for the viruses to release from infected cells, since viruses in culture fluids of R66 and Vero cells attained fairly high titers and were not affected by exogenous neuraminidase. However, the enhancing effect of exogenous neuraminidase on the growth of M virus in MDBK cells was impressive, but that on SC virus was much less marked. The idea that exogenous neuraminidase may activate M virus grown in MDBK cells is not adequate, since the virus propagated in MDBK cells under one-step growth conditions in the absence of the enzyme shows the cleaved form of glycoproteins, and the treatment of the virus preparation with the enzyme does not result in enhancement of infectivity of the virus (7). Therefore, the question is why M virus neuraminidase cannot contribute to effective virus release from MDBK cells.

Another characteristic feature of M virus is its inducibility of extensive syncytial formation in MDBK, R66, and Vero cells. The addition of exogenous neuraminidase did not inhibit syncytial formation itself, but retarded more or less the development and degradation of the syncytia induced by M virus. These effects of exogenous neuraminidase were dramatic in MDBK cells, but were not obvious in R66 cells. The level of syncytial formation of SC and 910N viruses greatly depended on cell type. SC virus induced extensive syncytial formation in R66 cells, moderate syncytial formation in MDBK cells, and slight syncytial formation in Vero cells. The 910N virus also induced extensive syncytial formation in R66 cells, slight syncytial formation in Vero cells, and practically no syncytia in MDBK cells. Exogenous neuraminidase inhibited markedly or almost completely the syncytial formation of SC and 910N viruses.

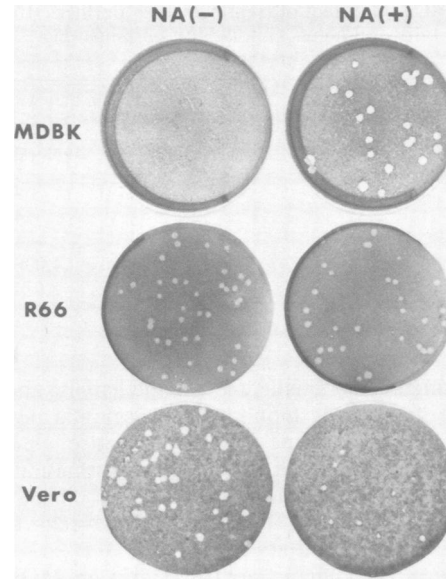

FIG. 3. Plaques of M virus formed in the absence [NA(−)] or presence [NA(+)] of type V neuraminidase in MDBK cells at 37°C in 4 days and in R66 and Vero cells at 37°C in 5 days.

These observations can be summarized as follows. There are two phases in syncytial formation of parainfluenza 3 virus, the neuraminidase-sensitive phase and the neuraminidase-insensitive phase. Both phases concern syncytial formation of M virus, whereas only the neuraminidase-sensitive phase contributes to that of SC and 910N viruses.

In MDBK cells, M virus neuraminidase is insufficient for both virus release and inhibition of the neuraminidase-sensitive phase, and the expression of both phases results in extensive syncytial formation. Therefore, the inhibitory effects of exogenous neuraminidase on the syncytial formation is marked, but far from complete. The limited neuraminidase activity of SC virus is sufficient for virus release, but insufficient for the inhibition of the neuraminidase-sensitive phase, resulting in moderate syncytial formation. The high neuraminidase activity of 910N virus is effective for both virus release and inhibition of neuraminidase-sensitive phase, resulting in no syncytial formation.

In R66 cells, which readily form syncytia, the neuraminidase activity of all these viruses is sufficient for virus release, but not effective for inhibition of the neuraminidase-sensitive phase of syncytial formation. Therefore, exogenous neuraminidase is required to abolish syncytial

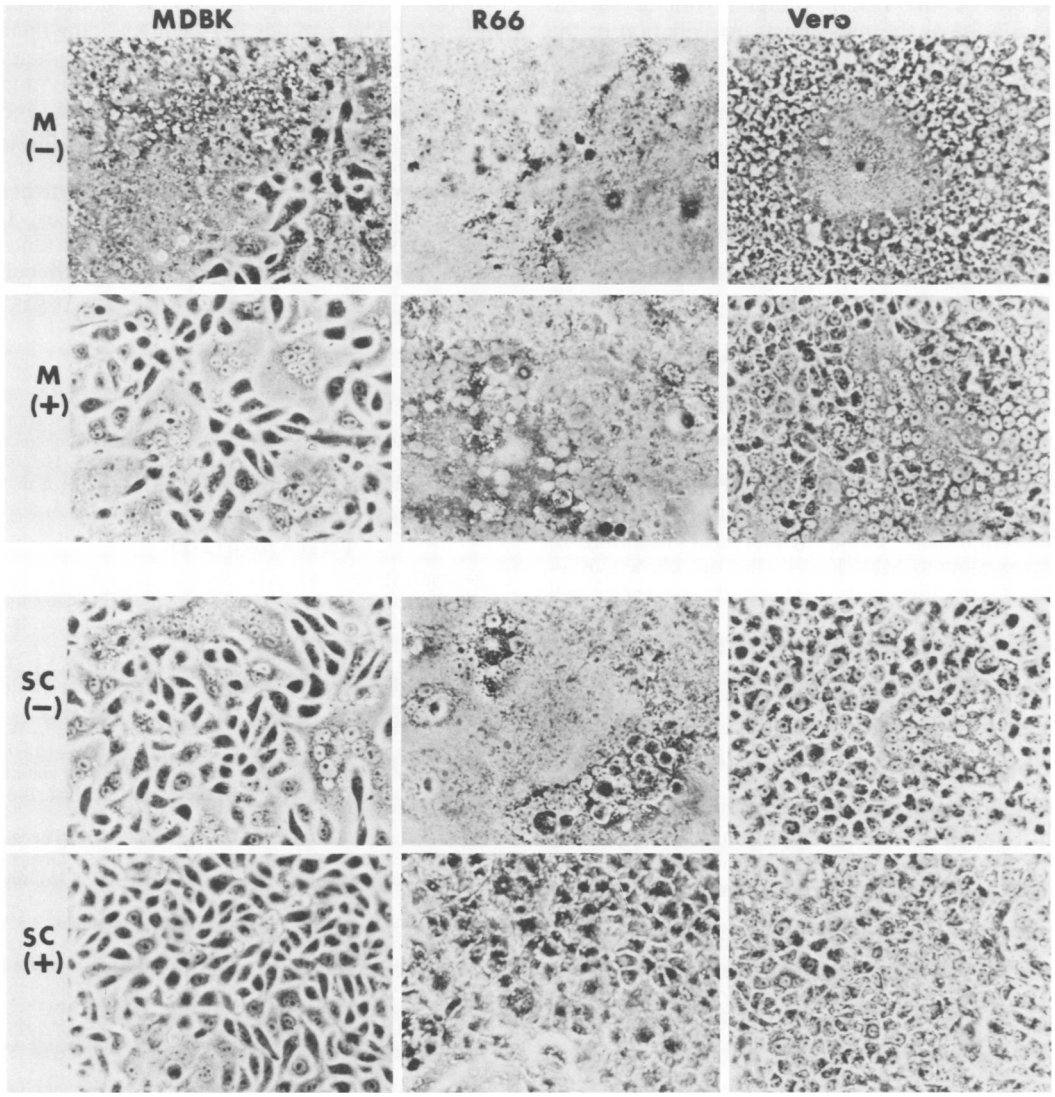

FIG. 4. Syncytial formation of M and SC viruses in MDBK, R66, and Vero cells in the absence [(−)] or presence [(+)] of type V neuraminidase. Cells infected at an input of 0.03 PFU per cell were incubated at 37°C. Phase-contrast photographs (×100) show MDBK cells at 24 h postinfection and R66 and Vero cells at 48 h postinfection.

formation of SC and 910N viruses. In syncytial formation of M virus, however, the neuraminidase-insensitive phase is expressed extensively, hence no obvious effect of exogenous neuraminidase. Similar considerations also account for Vero cells.

Thus, it is clear that M virus is much more potent than SC and 910N viruses in its ability to form syncytia, and the cytopathology of M virus is fortified by its being limited in viral neuraminidase activity. The neuraminidase-sensitive phase of syncytial formation seems to depend on the quantity of virions or viral proteins accumulated on the surface of infected cells, and viral as well as exogenous neuraminidase would tend to inhibit this phase by reducing the quantity, that is, accelerating virus release, although this notion should be confirmed by kinetic studies of virus release. On the other hand, the neuraminidase-insensitive phase is considered to depend on the quality of virions or viral proteins on the surface of infected cells. As previously reported (6), parainfluenza 3 virus has two glycoproteins. By analogy with the other paramyxoviruses, one would be hemagglutinin-neuraminidase protein, and the other

would be the fusion protein. Among several possibilities, the most likely explanation of the neuraminidase-insensitive phase is that the biological activity of the M virus fusion protein is greater than that of other parainfluenza 3 virus fusion proteins. The fusion index of M virus, estimated by using MDBK cells, was not greater than that of SC and 910N viruses. However, this estimation cannot be regarded as a proper evaluation of the activity of fusion protein in syncytial formation, but only indicates that all of these viruses have distinct cell-fusing activity in spite of the difference in syncytial formation.

The above question of why M virus neuraminidase is not able to contribute to effective virus release from MDBK cells is left unexplained. However, it is conceivable that the severe cytopathology of M virus makes MDBK cells deteriorate too early to yield expected amounts of progeny virus without the aid of exogenous neuraminidase. Under agar overlay medium, M virus-infected MDBK cells did not show syncytial formation, but contracted as single cell regardless of the presence or absence of exogenous neuraminidase, whereas plaques of M virus in R66 and Vero cells were composed of syncytia. This indicates that the direct cell-to-cell spread of M virus through syncytial formation does not occur in MDBK cells under agar overlay medium; therefore, enhanced virus release effected by exogenous neuraminidase is required for plaque formation of M virus in MDBK cells. On the contrary, the cytopathology and virus yields of M virus in R66 cells were little affected by exogenous neuraminidase, resulting in no obvious change of plaque formation. In Vero cells the slow development and degradation of syncytia of M virus was further retarded by the enzyme, resulting in reduction of size and number of plaques.

Recently Merz and Wolinsky (2) reported that strains of mumps virus with low neuraminidase activity show extensive syncytial formation as well as high virulence for hamsters. Coinciding with these observations for mumps virus strains, M virus was shown to have low neuraminidase activity and to induce extensive syncytial formation. Furthermore, only M virus among our variants of parainfluenza 3 virus induces an acute fatal illness in newborn and suckling mice (5). The causal relationship of the pathogenicity of M virus with its neuraminidase activity and syncytium inducibility should be clarified, and molecular studies of fusion proteins and hemagglutinin-neuraminidase proteins of M virus and the other viruses would be essential for further understandings of biology of these viruses. It remains also to be approached for understandings of viral pathogenesis and biology of cell surface why syncytial formation of the viruses greatly varied among different host cells.

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