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Effect of Antibody to Neuraminidase on the Maturation and Hemagglutinating Activity of an Influenza A_2 Virus

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Antiserum to a recombinant between an A_o and an A_2 influenza virus had no detectable antibody against an A_2 virus in standard hemagglutination-inhibition tests, and inhibited 95% of viral neuraminidase activity at a 1 to 400 dilution. However, on mixing virus with antiserum, a drop of up to 90% in hemagglutinin titer was observed. The effects of ultrasonication and direct electron microscopic examination indicated that the antiserum caused aggregation of virus particles. When antiserum was added to A_2 virus-infected chick embryo fibroblasts, release of virus appeared markedly inhibited. After ultrasonication to disrupt aggregates, an increase in released hemagglutinin was observed, but the resulting level was considerably lower than that in control cultures containing normal rabbit serum. In thin sections of infected cells, similar numbers of virus profiles were observed in control and antiserum-treated cultures. A marked increase in release of hemagglutinin was noted if receptor-destroying enzyme was added to antiserum-treated cultures. The results indicate that antibody to neuraminidase does not exert a direct effect on viral maturation, but inhibits the detachment of viral progeny from cell surface receptors.

The neuraminidase of influenza virus is a morphologically distinct type of projection on the surface of the virion (6). The function of this enzyme in the replicative cycle of the virus has not been established. Recently, the application of immunological methods to the study of neuraminidase function has been facilitated by the use of recombinant viruses containing hemagglutinin and enzyme from different parents, and it has been suggested that the enzyme may function in the final stages of the infectious process (3, 7, 9). Antibody to neuraminidase did not prevent virus from infecting cells, but only low levels of released virus were detected in cultures containing antibody. Antibody to neuraminidase inhibited hemagglutination by some strains of virus, but not others with serologically related enzymes (3, 10).

The present paper describes experiments which provide further information as to the mechanism by which antibody to neuraminidase inhibits virus release. No information concerning this mechanism has been obtained in previous studies, but the possibility has been considered that antibody may exert a direct effect on the process of viral maturation (7).

MATERIALS AND METHODS

Virus. A stock of A_2/Jap/305/57 virus with a titer of 1.5×10^{10} egg infectious doses (EID_{50}) per ml, and 10^4 hemagglutinin units (HAU) per ml, was used for most experiments. In some experiments, as indicated in the text, the A_2/NT/65/68 strain, an Australian isolate of the 1968 Hong Kong strain, and the A_o/NWS strain were also used. For preparation of antiserum, the X7F1 virus (4), a recombinant of the A_o/NWS and A_2/RI5$^+$ strains, was used. Virus stocks were prepared by inoculating about 10^6 EID_{50} into the allantoic cavity of 10- or 11-day-old embryonated eggs, which were incubated for 24 to 48 hr at 35 C before harvesting.

Cells. Chick embryo fibroblast (CEF) cells were prepared from 10-day-old embryos and grown in Eagle's medium containing 0.05% sodium bicarbonate and 5% calf serum. Monolayers were rinsed once with Hank's saline before inoculation. Virus was adsorbed at 35 C for 30 min and removed by washing three times with warm saline. The monolayers were incubated for the required time with Eagle's medium containing 0.002% sodium bicarbonate and 0.02 M tris (hydroxymethyl) aminomethane (Tris) buffer.

Infectivity titrations were made by inoculating groups of five embryonated eggs with serial 10-fold

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dilutions of virus. Allantoic fluids were tested for hemagglutinin after 72 hr of incubation at 35 C. Titers were calculated by the Spearman-Kärber method.

Hemagglutinin (HA) was assayed by making doubling dilutions using 0.25-ml volumes in plastic trays and detected with 0.5% chicken erythrocytes. The end point was estimated by interpolation between agglutination and no agglutination.

Antiserum was prepared by R. G. Webster by inoculating two lots of 5,000 HAU of X7F1 virus, purified by adsorption to and elution from erythrocytes followed by sedimentation in a 10 to 40% sucrose density gradient, into the marginal ear vein of a rabbit at an interval of 3 weeks. Antiserum was obtained 8 days after the second inoculation, and it and a normal rabbit serum control were treated with 0.0007 M potassium periodate, dialyzed and heated at 56 C for 30 min before use.

Hemagglutinin inhibition tests. Hemagglutinin inhibition tests in plastic trays were performed as described by Webster and Laver (9), by diluting the antiserum and incubating with 4 HAU of virus.

Receptor-destroying enzyme (RDE). RDE from *Vibrio cholerae* was provided by M. F. Warburton. The enzyme preparation was dialyzed against Eagle's bicarbonate-Tris medium before use. It was titrated by the method of French and Ada (2) and used at a concentration of 500 units/ml.

Neuraminidase assay. Virus samples were incubated with fetuin for 1 hr at 35 C, and liberated *N*-acetyl neuraminic acid was estimated by the method of Warren (8).

Sonication of virus suspensions. A probe powered by an ultrasonic generator (MEL Equipment Co. Ltd., England) at an output of 1.5 amp was used to disrupt virus aggregates. Maximum disaggregation was achieved by sonication for 30 sec. This procedure was carried out in the cold and infectivity titrations indicated that virions were not disrupted by sonication.

Negative staining. A drop of virus suspension was placed on a Formvar-coated grid, excess liquid was removed, and a drop of 4% sodium phosphotungstate, *p*H 7.0, was applied.

Electron microscopy of infected cells. Cells were scraped from petri dishes and pelleted at 800 × g for 5 min. The pellets were fixed for 5 min in 1% glutaraldehyde in phosphate-buffered saline [PBS, (1)], postfixed in 2% osmium tetroxide in PBS, dehydrated in alcohol and propylene oxide, and embedded in an epoxy resin mixture (Durcupan, obtained from Fluka A. G., Buchs, Switzerland). Thin sections were stained with lead citrate and uranyl acetate, and examined in a Philips EM 200 microscope.

RESULTS

Effect of antineuraminidase antibody on hemagglutination by A_2 viruses. The X7F1 recombinant virus (4) contains the HA of its A_0 parent (NWS) and the neuraminidase of its A_2 parent ($RI5^+$). We observed that the neuraminidase activity of the A_2/Jap strain was 95% inhibited by antiserum to X7F1 virus at a 1 to 400 dilution (Fig. 1), and at a 1 to 40 dilution no hemagglutination-inhibiting antibody was detectable against the A_2/Jap strain in standard tests. However, in the course of control experiments in a study on the effect of this serum on release of virus from infected cells, we observed a marked and reproducible drop in HA titer when virus suspensions were mixed with a 1 to 200 dilution of serum (Fig. 2). A loss of 50% in HA titer was observed on titrating the virus-antiserum mixture immediately, and with time the titer dropped further to 12.5% of controls in the experiment shown. This loss did not appear to be caused by cross-reaction of antibody to A_0 HA with the A_2/Jap virus, because the same drop

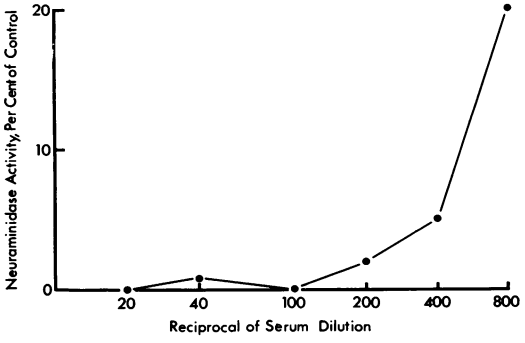


FIG. 1. *Inhibition of A_2/Jap neuraminidase by antiserum to X7F1 virus. Samples of 0.1 ml of virus with a titer of 5 × 10^3 HAU/ml were incubated with the serum dilutions indicated for 1 hr at 20 C and neuraminidase activity was determined. The optical densities of control reactions containing dilutions of normal rabbit serum were taken as 100%, and the readings of antiserum-virus mixtures are expressed as a percentage of the control.*

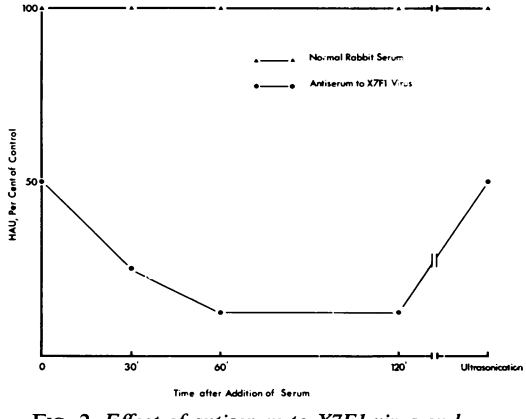


FIG. 2. *Effect of antiserum to X7F1 virus on hemagglutination by A_2/Jap virus. Samples of 0.2 ml of virus with a titer of 10^4 HAU/ml were incubated at room temperature with an equal volume of serum at a 1 to 200 final dilution. At the times indicated, HA titers were determined.*

was observed after 99% of antibody to NWS hemagglutinin was removed by adsorbing with NWS virus.

A similar drop in HA titer was observed on mixing X7F1 antiserum with the A_2/NT strain of influenza virus. This strain has a neuraminidase serologically similar to other A_2 viruses, but an HA which is serologically dissimilar (N. J. Dimmock, *unpublished data*). The fact that a drop in HA titer is observed with this virus provides additional evidence that antibody reacting with hemagglutinin is not responsible for the drop in HA titer, and that it is due to the interaction of antibody with the A_2 neuraminidase.

The effect of sonication on virus-antiserum mixtures was determined because it seemed likely that aggregation of virions by antibody was responsible for the drop in HA titer. A fourfold rise in the HA titer of virus-antiserum mixtures was observed after sonication, whereas no increase was found in control mixtures containing normal rabbit serum. However, the HA titer of the virus-antiserum mixture was only 50% of the control value after sonication, and sonication for longer periods did not result in a further increase in titer.

Electron microscopy of virus-antiserum mixtures. Direct demonstration of virus aggregation by antiserum was easily possible with the electron microscope. A plot of the per cent of virions observed in aggregates of various sizes, for mixtures of virus and antiserum or virus and normal serum, is illustrated in Fig. 3. In control preparations, 82% of the particles observed were found singly, and most of the remainder were in groups of two or three. When a sample of the same virus stock was mixed with antiserum to X7F1 virus, all but 7% of the virions were aggregated after 1 hr. The observed aggregates were heterogeneous in size, with up to 24 virions found in a group. A mean value of 1.1 virus particles per group was calculated for the control mixture, and 4.3 particles per group for the antiserum-treated sample. These values indicate a reduction of about fourfold in the number of structures capable of hemagglutination.

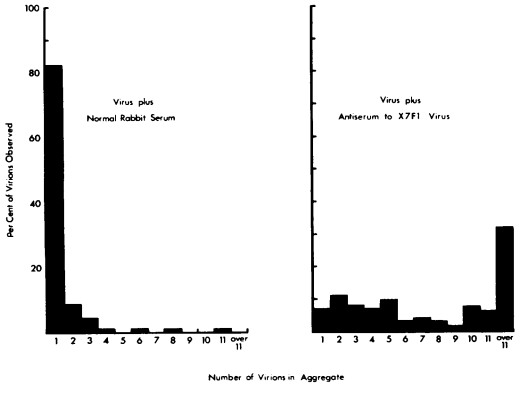


FIG. 3. *Aggregation of virions by antibody to neuraminidase. Virus was centrifuged at 8,000 rev/min for 20 min in a Spinco model SW-39 rotor, and the supernatant fraction, which contained no large aggregates, was used. Mixtures of virus and sera at a 1 to 200 final dilution were prepared as in Fig. 2, and after 1 hr, samples were taken for electron microscopy. A total of 586 virions were counted in the sample with normal serum, and 555 in that with antiserum.*

In addition to the striking differences in the distribution of virions in control and antiserum-treated preparations, a morphological alteration at the viral surface was also clearly evident (Fig. 4–6). In control preparations, the distinct spikes characteristic of influenza virions could be seen clearly. Virions were only occasionally found in contact, and in such instances the spike layers of the adjoining particles appeared to be in direct contact (Fig. 4). In preparations of virus mixed with antiserum, the individual surface spikes could not be distinguished on some parts of viral envelopes, particularly in regions of contact between virions (Fig. 5 and 6). It seems likely that the spikes in such regions are coated with antibody, and that antibody causes aggregation by forming bridges between virions. Similar aggregation has been noted previously when influenza virus was mixed with antibody to the whole virus particle (5). In the study with antiviral antibody, the entire surfaces of the virus particles appeared coated with antibody. However, with the antiserum specific for neuraminidase, we find some parts of the viral spike layer to be of normal appearance, possibly because they are devoid of neuraminidase subunits (Fig. 6). This finding raises the possibility that neuraminidase may be localized in patches on the viral envelope, rather than being distributed in a regular manner on the viral surface.

A plot of the distribution of virions after sonication is shown in Fig. 7. The mean aggregate size in antiserum-treated preparations was 1.7 virions after sonication, compared with 1.1 in the control sample. This observation may explain the failure to recover HA completely after sonication of antiserum-treated preparations.

Effect of antibody to neuraminidase on release of hemagglutinin. The levels of released and cell-associated HA at 7, 12.5, and 24 hr after inoculation with a multiplicity of 80 EID_{50}/cell in control and antiserum-treated CEF cultures are summarized in Table 1. In cultures containing normal rabbit serum, released HA was first detected at 3 to 4 hr; by 24 hr, a titer of over 1,000 HAU per culture was observed. In cultures con-

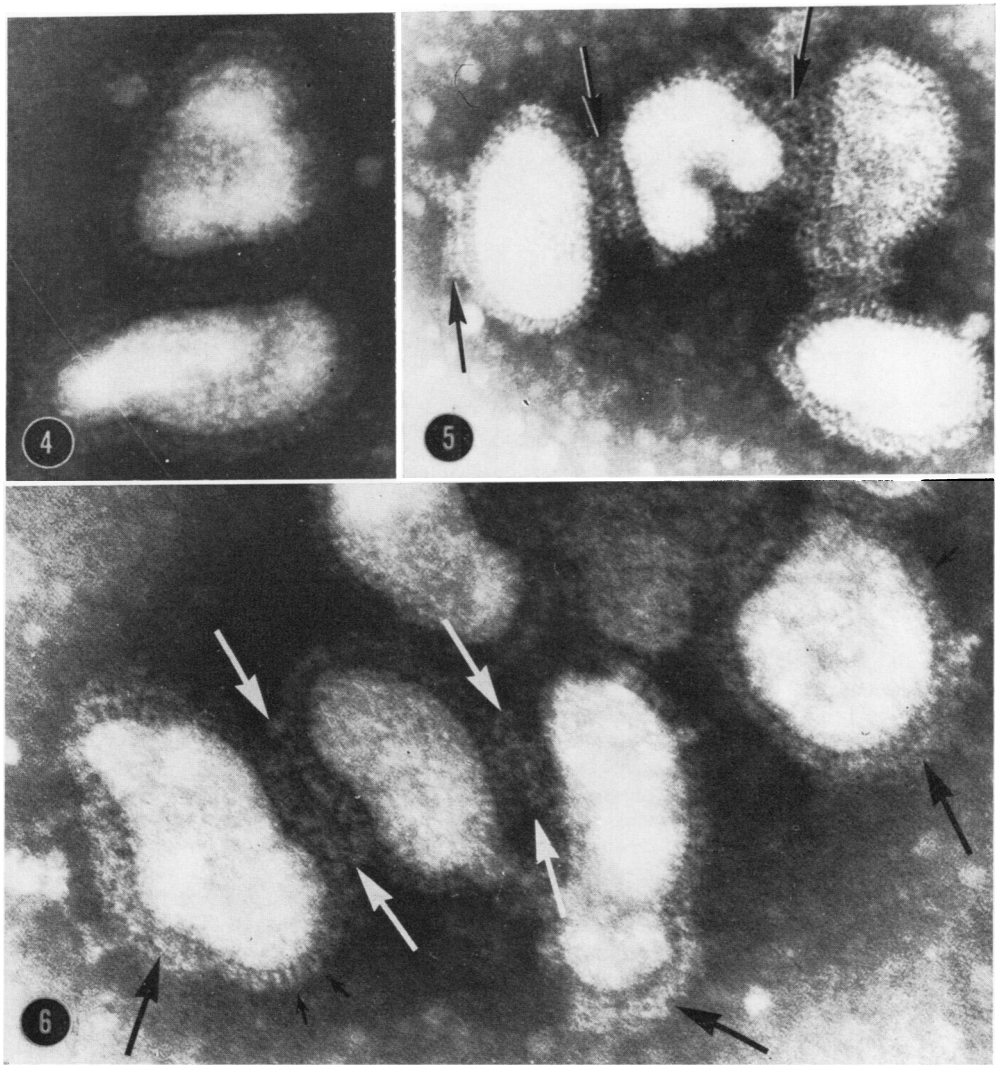


FIG. 4. *A_2/Jap virions selected to show contact between viral surfaces, from a sample incubated with normal rabbit serum at a 1 to 200 final dilution for 1 hr. The surface spikes characteristic of the viral envelope can be seen clearly, and there appear to be direct contacts between the spike layers of the two particles. × 300,000.*

FIG. 5 and 6. *Virions from a sample incubated with X7F1 antiserum at a 1 to 200 final dilution for 1 hr. In* Fig. 5, *the aggregated virions appear to be spaced further from one another than those in Fig. 4, and it is suggested that material (antibody) may be attached to the spikes in some regions (arrows); × 200,000. In Fig. 6, the viral spikes cannot be distinguished on some parts of the viral envelopes (large black arrows) and in regions of contact between virions (white arrows); it is suggested that antibody is attached to the spikes in such regions. On other parts of the viral envelopes (small black arrows), the spike layer is of normal appearance. Such regions may contain no neuraminidase subunits. × 300,000.*

taining X7F1 antiserum, the level of released HA was markedly lower, as expected from previous studies of the effects of antibody to neuraminidase (7, 9). When virus released from antiserum-treated cultures was sonicated, a fourfold or greater increase in HA titer was observed; in some samples HA was only detected after sonication. However, even after sonication, the resulting HA titer was never more than 10% of that in cultures containing normal rabbit serum. Titers of cell-associated HA and the development of cytopathic changes were similar in control and antiserum-treated cultures.

Formation of virions. We could not detect any

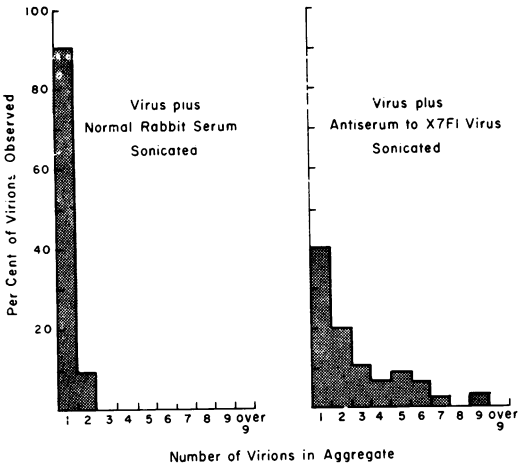


FIG. 7. *Effect of sonication on the distribution of virions. Samples were prepared as in Fig. 3, sonicated, and immediately applied to grids for negative staining. A total of 127 virions were counted in the sample with normal serum, and 276 in that with antiserum.*

qualitative differences between control and antiserum-treated cultures in the process of virion formation. The progeny virus particles were of similar appearance, and both particles in the process of budding as well as others which appeared to have completed budding were found (Fig. 8 and 9). Apparent aggregates of virus particles were sometimes found in antiserum-treated cultures, but aggregates of similar size were found occasionally in control cultures.

In order to obtain a more quantitative estimate of the levels of virus particle formation in control and antiserum-treated cultures, we used the electron microscope to count the number of virus profiles associated with infected cells (Table 2). At 2 hr, virus particles were rarely observed, indicating that those seen at later times do not represent residual inoculum virus. At later times, the number of virus profiles per cell profile was strikingly similar in control and antiserum-treated cultures. Thus, the antiserum does not appear to exert a direct inhibitory effect on the process of viral maturation.

Release of hemagglutinin by receptor-destroying enzyme. It seemed likely that antibody to neuraminidase caused virus particles to remain attached to receptors at the cell surface. We, therefore, treated cells with RDE to determine whether enhanced release of virus could be demonstrated. There was no inhibition of RDE by antiserum, and preincubation of antiserum with RDE had no effect on the ability of the serum to cause virus aggregation. Exposure of CEF cells to RDE markedly increased the level of released HA in

TABLE 1. *Effect of antineuraminidase on release of hemagglutinin from CEF cells*

Serum in culture medium[a]	Hr after inoculation	Released hemagglutinin[b]		Cell-associated hemagglutinin[b] (sonicated)
		Not sonicated	Sonicated	
Normal rabbit serum.....	7	1.7	2.0	4.2
	12.5	2.5	2.6	4.3
	24	3.2	3.5	4.1
X7F1 anti-serum...	7	<0.9	<0.9	4.2
	12.5	<0.9	1.3	4.5
	24	1.3	2.0	4.4

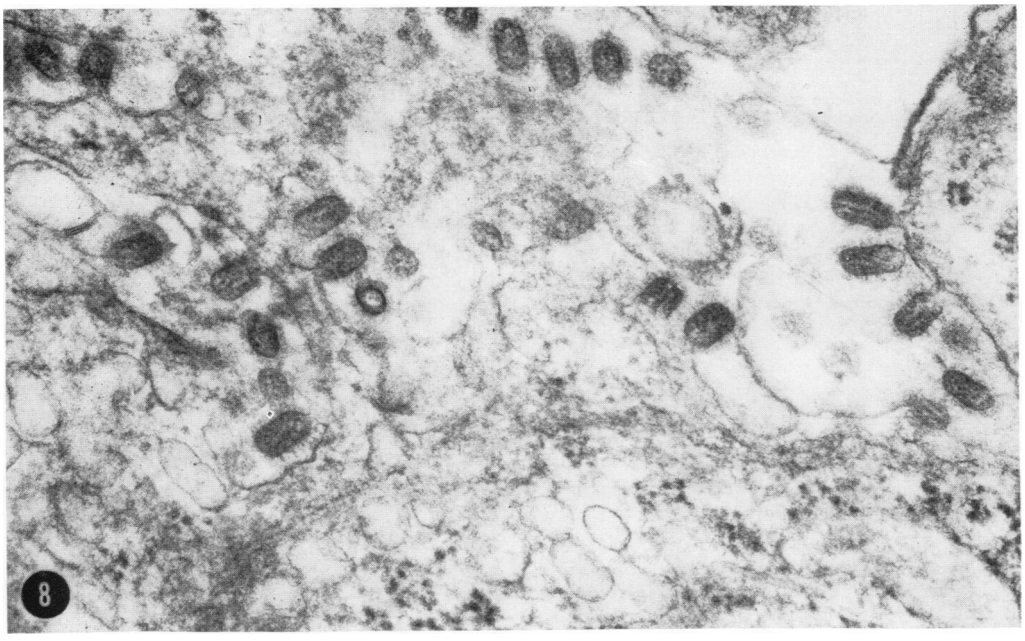
[a] Dilution, 1:200.
[b] Log_{10} hemagglutinin titer per 25×10^6 cells.

antiserum-treated cultures, whereas only a slight increase was found in control cultures (Table 3). Sonication of the virus released by RDE caused only a slight increase in titer in antiserum-treated cultures, which was similar to that in control cultures. Thus, it appears that RDE treatment permits virus release in antiserum-treated cultures, and that this virus is not in the form of large aggregates susceptible to disruption by sonication.

DISCUSSION

The present studies were undertaken to examine the mechanism by which antibody to neuraminidase inhibited the release of influenza virus from infected cells. In the course of control experiments, it was discovered that an antiserum mixed with virus caused a marked drop in HA titer, although the serum had no detectable anti-HA antibody in standard tests. Our investigation of this phenomenon indicates that the drop in titer is due to aggregation of virions by antibody to neuraminidase. Such aggregation probably does not occur in the standard hemagglutination-inhibition test, because dilute preparations of virus and antibody are used. The formation of aggregates by antibody may contribute to the low levels of released virus detected in cultures containing antiserum to X7F1 virus, but the relatively low level of virus recovered after sonication indicated that aggregation is not the sole cause of the low yields.

The present demonstration that virions are formed readily in the presence of antibody to neuraminidase appears to rule out any possibility that such antibody exerts any direct effect on the process of maturation. The finding of increased amounts of released virus in cultures treated with RDE suggests that virions remain attached to cell surfaces in antiserum-treated cultures. The fact



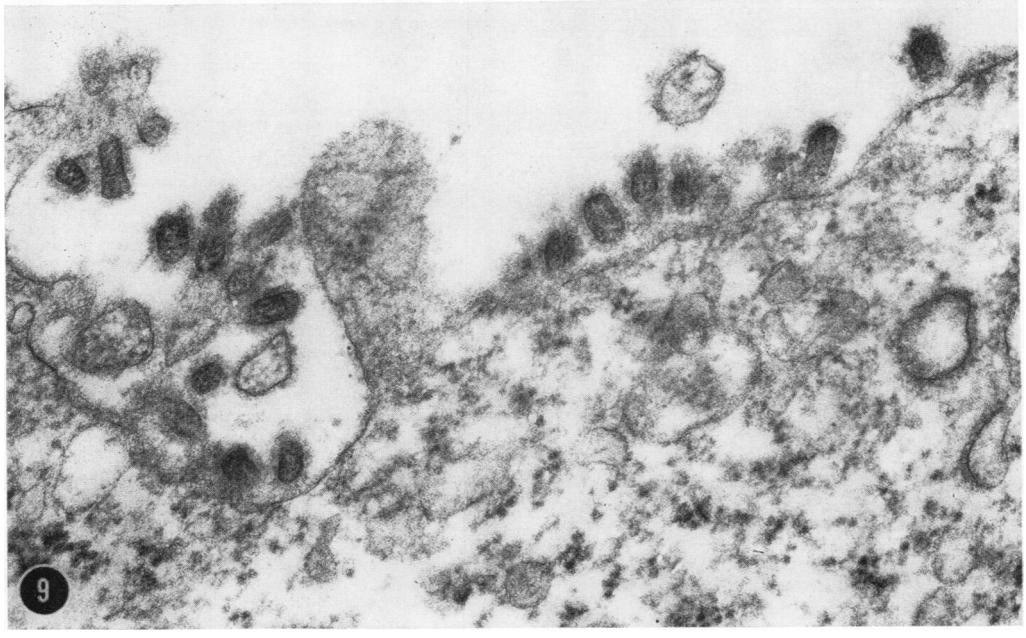


FIG. 8. *A_2/Jap virus particles at the surface of a cell 7.5 hr after inoculation, incubated in the presence of normal rabbit serum at a 1 to 100 dilution. Some virus particles are in the process of budding, while others show no continuity with the cell surface.* × *82,000.*

FIG. 9. *A_2/Jap virus particles at the surface of a cell 7.5 hr after inoculation, incubated in the presence of X7F1 antiserum at a 1 to 100 dilution. The virus particles appear indistinguishable from those seen with normal serum.* × *82,000.*

TABLE 2. *Effect of antineuraminidase on virion formation*[a]

Serum in culture medium[b]	Hr after inoculation	Number observed		Virus profiles per cell profile
		Cell profiles	Virus profiles	
Normal rabbit serum...	2	110	2	0.02
	7	98	645	6.5
	11.5	80	1437	18.0
X7F1 antiserum.....	2	134	6	0.05
	7	105	667	6.3
	11.5	116	1845	15.9

[a] Electron micrographs were taken, and counts were made of sections through cells (cell profiles) and virus particles (virus profiles) in samples which were fixed for microscopy at the times indicated.

[b] Dilution, 1:200.

TABLE 3. *Release of hemagglutinin by receptor-destroying enzyme*

Serum in culture medium[a]	Hemagglutinin titer[b] of untreated cultures		Hemagglutination titer[b] of RDE-treated cultures[c]	
	Not sonicated	Sonicated	Not sonicated	Sonicated
Normal rabbit serum.....	3.0	3.1	3.1	3.4
X7F1 antiserum...	<0.9	1.5	2.6	2.9

[a] Dilution, 1:200.

[b] Log_{10} per 25×10^6 cells in medium harvested at 11.75 hr after inoculation.

[c] One thousand units per 25×10^6 cells, added with serum following removal of inoculum at 30 min after inoculation.

that virus released with RDE in antiserum-treated culture was only one-third of that in controls could be due in part to virus aggregation. We have observed that at least 50% of the drop in HA titer due to aggregation may not be recoverable by sonication. It is also possible that some of the virions may not agglutinate erythrocytes because of steric interference of neuraminidase antibody with the viral HA.

Our results showing similar levels of cell-associated HA in control and antiserum-treated A_2/Jap-infected cells differ from those of Seto and Rott (7) with fowl plague-infected cells, where antibody to neuraminidase caused a marked reduction in cell-associated HA. Since we observed similar numbers of virions in the electron microscope in control and antiserum-treated cells, there was no indication that antiserum caused an accumulation of virus particles at the cell surface. It is possible that virus which remains in contact with the cell surface for a prolonged period is degraded as a result of such contact, and a buildup of intact virus is thus prevented. Alternatively, cell surfaces may become saturated with virions at a given level, and a type of feedback may inhibit further viral maturation.

The present results with antineuraminidase antibody indicate that the viral neuraminidase functions in permitting the elution of virions from receptors at the cell surface after they are formed. It is possible that the enzyme may play an additional intracellular role in viral replication, which would not be affected by the presence of antibody.

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