

Antibody-Mediated Growth of Influenza A NWS Virus in Macrophagelike Cell Line P388D1

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We investigated the internalization and growth of influenza A NWS virus in macrophagelike P388D1 cells. Flow cytometric analysis using fluorescein isothiocyanate-labeled virus showed that the attachment of normal rabbit serum-exposed virus (NS-V) to neuraminidase (NA)-treated cells was noticeably limited compared with that to untreated cells. However, rabbit antiserum-exposed virus (AS-V) could attach equally well to both cells. Virus coated with Fab prepared from antiviral immunoglobulin G could not attach. These data suggest that the NWS virus can infect P388D1 cells in one of two ways, via viral or via Fc receptors, depending on the presence of antibodies. The NS-V could grow in the untreated cells, but not in the NA-treated cells. The highest growth of AS-V in the NA-treated cells was observed at an antibody concentration showing 50% plaque reduction titer. Growth was exponentially decreased toward the lower and higher dilutions of antibodies. By using three different immunoglobulin G subclasses of monoclonal antibodies against hemagglutinin, it was demonstrated that both Fc receptors I and II could take part in this phenomenon. The presence of 20 mM NH₄Cl inhibited the growth of both AS-V and NS-V, suggesting that the intracellular pathways after internalization via Fc or viral receptors are similar. These data indicate that the concentration of antibodies has a critical role on the antibody-mediated growth of influenza virus in macrophages.

In the early stage of virus infection, nonspecific host defense factors represent the main line of defense. Macrophages especially have a key role in preventing the establishment of infection (25). Indeed, the growth of many viruses has been shown to be restricted in macrophages (1, 24, 34).

It has been recognized that macrophages are able to effectively internalize antibody-coated particles through Fc receptors and digest them (21, 37). In contrast, it has also been demonstrated that the enhanced internalization of antibody-coated viruses via Fc receptors results in enhanced infections under several conditions (7). Early studies *in vitro* have shown that plaque production in chicken embryo fibroblast monolayers is increased after inoculation with partially neutralized vesicular stomatitis virus (13), influenza A Melbourne virus (16), rabbitpox, and some arboviruses (10). Thereafter, Kliks and Halstead (14) provided evidence that antibody-dependent plaque enhancement of Murray Valley encephalitis virus is due to the existence of Fc receptor-bearing phagocytes in chick fibroblast monolayers. Recently, enhanced infection of cultured peripheral macrophages and continuous macrophagelike cell lines in the presence of subneutralizing antibody has been demonstrated for dengue virus (8, 9), West Nile virus (27), Venezuelan equine encephalitis virus (18), yellow fever virus (29, 30), and reovirus (2). These unbeneficial and paradoxical infectious pathways might contribute to the pathogenicity of viruses (8, 9).

In the case of influenza virus, in addition to Lafferty's report (16) described above, Webster and Askonas (40) have recently demonstrated *in vivo* experiments that the very low antibody levels in mice immunized with inactivated whole virus or subunit vaccines of A/USSR/90/79 (H1N1) increase virus infectivity. Although the paper by Webster and Askonas made no reference to the participation of macrophages, their findings permit speculation that anti-

body-enhanced influenza virus infection may actually occur in macrophages under specific conditions *in vitro* and *in vivo*. Therefore, we focused our investigations on the interactions among influenza virus, antibodies, and macrophages.

Two different immunities to influenza virus have been reported; one is subtype-specific immunity mediated by humoral antibodies raised against hemagglutinin and neuraminidase (NA) (31), and the other is heterotypic immunity in the different subtypes of influenza A virus in the absence of antibodies of appropriate specificity (32). During recent decades, the central role of macrophages in specific humoral and cell-mediated immune responses has been recognized (26). If antibody-mediated growth of influenza virus occurs in macrophages, it might be important in regulation or modification of such immune responses to the infection, as well as in possible viral persistence or as a source of antigenic variant strains of influenza virus.

In the present study, we have investigated the internalization and growth of influenza A NWS virus in cells of the macrophagelike cell line P388D1, with or without viral receptors, and in the presence or absence of antiviral antibody.

MATERIALS AND METHODS

Virus. Influenza A virus NWS (H1N1) strain was propagated in the allantoic cavity of 10-day-old embryonated hen eggs for 48 to 72 h at 35°C. The allantoic fluids were collected and then stored in small portions at -80°C after clarification at 1,000 × g for 20 min. The virus titer of allantoic fluids was 1.5 × 10⁸ PFU/ml.

Cells. P388D1 cells, a murine macrophagelike cell line derived from methylcholanthrene-induced neoplasm of a DBA/2 mouse (4, 15), were kindly supplied by Hidehiko Suzuki (Toyama Medical and Pharmaceutical University, Toyama, Japan). The cells were grown in Dulbecco modified Eagle minimal essential medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 µg/ml). Madin-

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Darby canine kidney (MDCK) cells were grown in Eagle minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 µg/ml). The P388D1 and MDCK cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 and 34°C, respectively.

Preparation of FITC-labeled virus. Purification of virus from fluids was carried out by differential centrifugation and sedimentation through a sucrose gradient (15 to 60% sucrose in phosphate-buffered saline [PBS]) (17). The purified viruses were washed twice with PBS by centrifugation and then suspended in 1 to 2 ml of PBS. The fluorescence labeling of the viruses was carried out by the method of Yoshimura and Ohnishi (42) with minor modifications. Briefly, the virus (2.65 mg of virus protein per ml) was reacted with an equal volume of 0.1-mg/ml fluorescein isothiocyanate (FITC) dissolved in 0.5 M bicarbonate buffer (pH 9.5) for 1 h at room temperature. The reaction mixture was dispersed in PBS and centrifuged at $77,400 \times g$ for 1 h. This process was repeated, and the final pelleted FITC-labeled viruses were suspended in PBS (530 µg of virus protein per ml). The fluorescence labeling did not significantly alter the hemagglutination (HA) titer (65,536). Amounts of virus protein were measured by the method of Lowry et al. (20).

Antiserum and monoclonal antibody. The purified viruses were disrupted twice by vigorous shaking with an equal volume of 0.05% Tween-ether for 60 min at room temperature. For preparation of rabbit anti-NWS antiserum, New Zealand White rabbits were subcutaneously injected with 0.5 ml of the disrupted viruses emulsified in Freund complete adjuvant and given several intravenous booster injections of antigen in PBS (0.2 ml) every 4 weeks. A week after the last booster, the rabbits were bled for collection of sera. After the treatment of the antiserum with receptor-destroying enzyme (Denka Institute of Biological Science, Tokyo, Japan) followed by incubation at 56°C for 30 min, the antiserum which showed an HA inhibition (HI) titer of 12,000 was stored at -20°C. For preparation of monoclonal antibodies, BALB/c mice were intraperitoneally injected with 0.2 ml of disrupted virus (80 µg of virus protein) emulsified in Freund complete adjuvant and given an intravenous booster injection of antigen (0.1 ml) 4 weeks later. Four days after the booster injection, immune spleen cells were fused with myeloma cells (P3X68Ag8U.1) by using polyethylene glycol 4000. The fused cells were cultured in HAT medium (39). Supernatants of growing hybrid cells were screened for production of virus protein-specific antibodies by enzyme-linked immunosorbent assay using the MonoAB screening kit G (Zymed Laboratories, Burlingame, Calif.) in microtiter plates coated with disrupted viruses. The cell supernatants were further screened for production of virus HA-specific antibodies by HI test and plaque reduction assay on MDCK cells. For determining the specificity of antibodies, immunoprecipitation was carried out as described previously (38) using radiolabeled cell lysates prepared from infected MDCK cells in the presence of [³⁵S]methionine (5 µCi/ml) from 0 to 6 h postinfection. Subcloning of positive clones was performed by the limiting-dilution method in microtiter plates. Ascitic fluids were obtained by intraperitoneal injection of 10⁶ to 10⁷ cells into BALB/c mice that had been pristane (2, 6, 10, 14-tetramethylpentadecane) primed 2 weeks earlier.

Preparation of IgG and Fab. The antiserum was applied to a column of protein A-Sepharose CL-4b (10 by 110 mm, Pharmacia) equilibrated with PBS. Rabbit anti-NWS immu-

noglobulin G (IgG) was eluted with 0.58% acetic acid-0.15 M NaCl after thorough washing with the starting buffer. The eluted IgG fraction was dialyzed against H₂O and then lyophilized. The purified IgG (48 mg) was digested with 0.5 mg of papain (Cooper Biochemical) in 0.1 M sodium phosphate (pH 7.0)-0.01 M cysteine-2 mM EDTA for 18 h at 37°C. The resulting digests were applied to a column of carboxymethyl cellulose (1.6 × 230 mm, Whatman) equilibrated with 0.01 M sodium acetate (pH 5.5) by the method of Porter (28). The digested IgG fractions were eluted with 100 ml of the equilibrating buffer and then with a linear gradient (250 ml) of 0.01 to 1 M sodium acetate. The Fab fragments eluted with the starting buffer and in the first peak at A₂₆₀ in the gradient were collected and repurified by using protein A affinity column chromatography, as described above, to remove Fc and undigested IgG from Fab fragments. The Fab fraction in PBS (2.83 mg/ml) had an HI titer of 32,768.

Cell treatment. P388D1 cells, dispensed into 24-well microplates at 4×10^5 cells per well, were incubated at 37°C overnight. The cells were washed with PBS and treated with 2.5 mg of NA (*Clostridium perfringens*, type V, Sigma) per ml in 10 mM sodium phosphate (pH 6.1)-150 mM NaCl-5 mM CaCl₂ for 30 min at 37°C (36). Buffer containing 2% heat-inactivated fetal bovine serum was used as a control.

Flow cytometry. The FITC-labeled virus (HA titer, 1,024) was mixed with an equal volume of rabbit anti-NWS antiserum (HA titer, 200), rabbit normal serum, purified IgG (HI titer, 512), or Fab (HI titer, 512), dissolved in PBS containing 1% bovine serum albumin, and then incubated for 30 min at 37°C. After each treatment, the HA titer of the FITC-labeled virus was less than 16, except for normal serum. The NA-treated cells were washed twice with cold PBS and infected with the FITC-labeled virus for 1 h at 0°C. After the virus suspension was removed, the cells were collected by pipetting, washed with cold PBS, and applied to an EPICS C flow cytometer (Coulter) equipped with a 5-W argon laser (488-nm excitation, 500-mW output) and with a computer for data collection and flow cytometric assay. Emission was measured using 515-nm long-pass and 560-nm short-pass filters.

Growth assay. P388D1 cells (3×10^5 per well) were inoculated into a 24-well plate and incubated at 37°C overnight. The cells were washed twice with PBS and then infected with 0.2 ml of antiserum-exposed NWS virus (AS-V) at various concentrations for 30 min at 37°C at a multiplicity of infection (MOI) of 2 or 0.2 PFU/cell. After infection for 45 min at 37°C, the cells were washed four times with PBS and then further cultured in Dulbecco modified Eagle minimal essential medium supplemented with 2% heat-inactivated fetal bovine serum at 37°C. This time was designated 0 h postinfection. At 24 h postinfection, the culture fluids were collected and centrifuged at $500 \times g$ for 10 min. The virus yield in the supernatants, the residual infectivity of antibody-exposed viruses, and amounts of unadsorbed viruses were assayed by plaque titration on MDCK cells. Finally, virus yields were calculated by subtracting the unadsorbed virus titers from released virus titer at 24 h postinfection.

Serological tests. HI tests were done by the standard method (3). Hemolysis inhibition tests were carried out by the method described by Kida et al. (12) with slight modifications. Briefly, 50-µl samples of virus concentrates containing 3,000 HA units per 0.8 ml were incubated with 50 µl of serial twofold dilutions of antibodies for 30 min at room temperature, and then 500 µl of 1% chicken erythrocytes in saline buffered with 0.1 M citrate acid-sodium citrate (pH

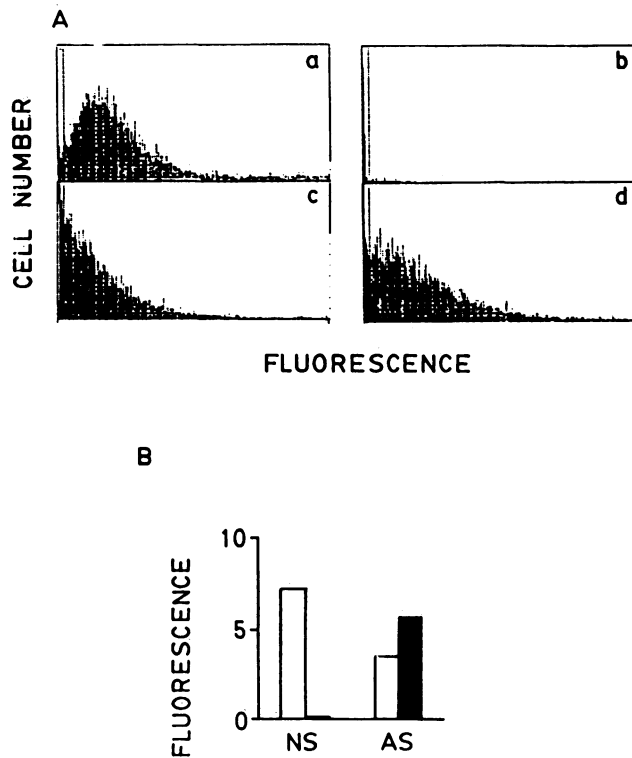


FIG. 1. Flow cytometric analyses of attachment of FITC-labeled virus to P388D1 cells. (A) Flow cytometry profiles of P388D1 cells infected with FITC-labeled viruses exposed to normal rabbit (a) or rabbit anti-NWS serum (c). NA-treated cells were infected with viruses exposed to normal serum (b) or antiserum (d) for 1 h at 0°C, as described in Materials and Methods. (B) Comparison of total amounts of viruses attached to 10^4 P388D1 cells. Untreated (open bars) or NA-treated (solid bars) cells were infected with FITC-labeled viruses exposed to normal serum (NS) or antiserum (AS). Total amounts of attached viruses were calculated by multiplying mean fluorescence intensity per cell by the peak area in the flow cytometry data shown in panel A. Fluorescence in the ordinate is expressed as arbitrary units.

5.5) was added. After incubation of the mixtures at 0°C for 60 min and then at 37°C for 60 min with mixing every 10 min, the cells were sedimented by centrifugation (1,500 rpm for 10 min), and the supernatants were measured for hemoglobin at 540 nm. Titers were expressed as antibody dilutions which inhibit 50% of hemolysis.

RESULTS

Attachment of NWS virus to P388D1 cells with or without viral receptors. P388D1 cells were permissive for growth of influenza NWS virus (see Fig. 3 and 4), indicating that P388D1 cells have influenza viral receptors which contain sialic acids (6). When the P388D1 cells were treated with NA (2.5 mg/ml for 30 min at 37°C), the virus failed to replicate in the cells (see Fig. 3 and 4). The NA treatment released 30.2% of total cellular sialic acid (data not shown). The released amount was similar to the maximum amount released from polymorphonuclear leukocytes by treatment with NA derived from *Vibrio cholerae* (11). Moreover, the phagocytic activity of P388D1 cells was not affected by the NA treatment; P388D1 cells equally phagocytosed IgG-coated or uncoated latex particles (mean diameter, 2 μ m) regardless of

the NA treatment (data not shown). These results suggest that the phagocytic activity of P388D1 cells was maintained under the NA treatment.

Figure 1A shows fluorescence distributions of P388D1 cells with FITC-labeled viruses. The normal rabbit serum-exposed viruses (NS-V), with an HA titer of 512, could attach to the untreated cells (96.8% of total cells counted by a flow cytometer were fluorescence positive), but the attachment to NA-treated cells (only 1.9% of counted cells were fluorescence positive) was extremely limited. On the other hand, rabbit antiserum-exposed influenza viruses (AS-V), which showed an HA titer of <16, could attach not only to the untreated cells but also to the NA-treated cells. Fluorescence-positive cells made up 72.6 and 85.4% of the untreated and NA-treated cells, respectively. In addition, to compare the efficiency of viral attachment to NA-treated or untreated cells, we calculated the total fluorescence intensity per 10^4 cells (Fig. 1B). The attachment of NS-V to NA-treated cells was much less than that of AS-V; the total fluorescence intensity of NS-V was only 1.2% of the AS-V. The AS-V could significantly attach to both types of cells (Fig. 1B). These results indicate that the NA treatment can remove the viral receptors from the membrane surfaces of cultured P388D1 cells and that AS-V can attach to P388D1 cells without viral receptors.

Fc receptor-mediated viral attachment. To clarify that the attachment of AS-V to P388D1 cells is mediated by Fc receptors, the attachment of purified IgG- or Fab fragment-coated influenza NWS viruses to the cells was examined. The antiviral IgG was purified from rabbit antiserum, and then the Fab fragment was prepared from the IgG as described in Materials and Methods. The purified IgG and Fab fragment were pure by sodium dodecyl sulfate-gel electrophoretic analysis. Figure 2 shows the total fluorescence intensity of P388D1 cells with IgG- or Fab-coated viruses per 10^4 cells. When FITC-labeled viruses were incubated with IgG or Fab fragments, the HA titer was less than 16 in either case. The attachment of the Fab-coated viruses to both NA-treated and untreated cells was extremely limited as compared with the attachment of IgG-coated viruses. This result indicates that the antibody-coated influenza virus can mainly attach to P388D1 cells via Fc receptors. These observations suggest that influenza NWS virus can infect P388D1 cells in one of two ways: via NA-sensitive viral receptors in the absence of the antibody

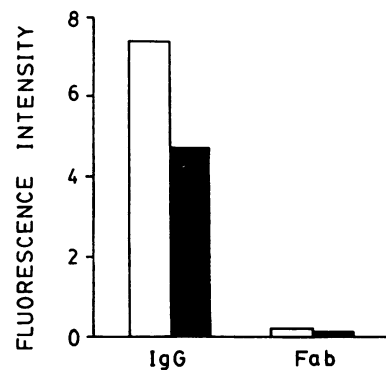


FIG. 2. Attachment of IgG- or Fab-coated viruses to P388D1 cells. Untreated (open bars) and NA-treated (solid bars) cells were infected with FITC-labeled viruses exposed to IgG or Fab from prepared from rabbit anti-NWS serum for 1 h at 0°C. Fluorescence intensity in the ordinate is expressed as arbitrary units.

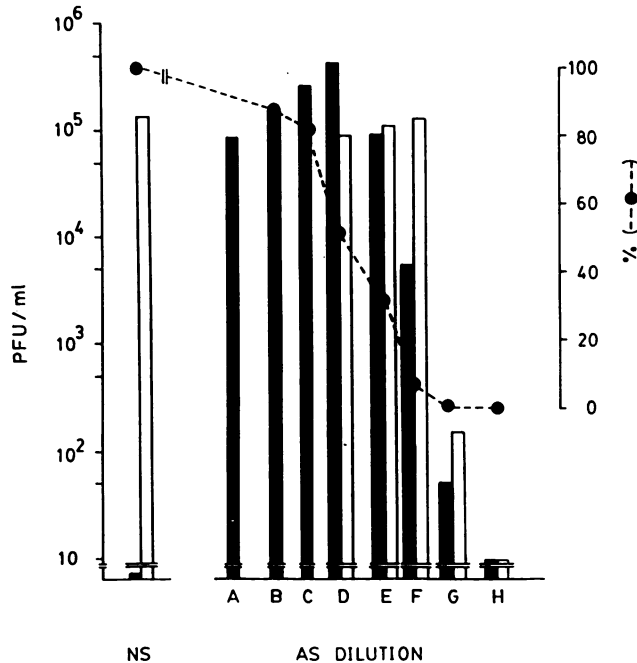


FIG. 3. Viral growth in P388D1 cells. Viruses were exposed to various concentrations of anti-NWS serum (AS) (dilutions: A, 1:24,000; B, 1:12,000; C, 1:6,000; D, 1:3,000; E, 1:1,200; F, 1:600; G, 1:300; H, 1:120) or normal serum (NS; 1:120 dilution) for 30 min at 37°C. Untreated (open bars) or NA-treated (solid bars) P388D1 cells were infected with these viruses at an MOI of 2 PFU/cell for 45 min at 37°C. The virus yield in culture fluid at 24 h postinfection and the residual infectivities of AS-V (dashed lines) were measured by plaque assay on MDCK cells. The value of 100% corresponds to the infective virus amounts (2.1×10^6 PFU/ml) for NS-V.

or via NA-resistant Fc receptors in the presence of the antibody.

Antibody-mediated viral growth in P388D1 cells. Viral growth after internalization via one of the two ways described above was examined (Fig. 3). To assay viral growth via Fc receptors, the NA-treated and untreated cells were infected with AS-V at various antiserum concentrations or with NS-V at an MOI of 2 PFU/cell for 45 min at 37°C. The viral growth via viral receptors was examined by infection with NS-V of the NA-untreated cells. The residual infectivities of the NS-V and AS-V used were also assayed by plaque titration on MDCK cells (Fig. 3). Growth of NS-V in untreated cells was observed, with a virus yield of 1.1×10^5 PFU/ml. However, no viral growth of NS-V in NA-treated cells was observed. Concerning the growth of AS-V in NA-treated cells, the highest virus yield (4.2×10^5 PFU/ml) was observed at a 1:3,000 dilution of antiserum, which showed an HI titer of 4 and corresponded to 50% plaque reduction titer as assayed on MDCK cells. This virus yield was slightly higher than that from untreated cells infected with NS-V. At the lower antibody dilution ranges, between 1:1,200 and 1:120, the virus yields from NA-treated and untreated cells were logarithmically decreased, although the virus yield from untreated cells was slightly higher than that from NA-treated cells (Fig. 3). Finally, with antiserum diluted 1:120, a level which corresponded to 100% plaque reduction titer, no viral growth was observed in either NA-treated or untreated cells. On the other hand, at the higher antibody dilution ranges, between 1:6,000 and

1:24,000, a relatively high virus yield could be maintained (Fig. 3).

We also examined viral growth in much higher antiserum dilutions to clarify the antibody-mediated growth in NA-treated cells (Fig. 4). We observed high virus yields only at antiserum dilution ranges between 1.3×10^4 and 1.3×10^5 . At less than 1.3×10^5 dilution, the virus yields from NA-treated cells were much lower than those from untreated cells (Fig. 4A). Viral growth in NA-treated cells was exponentially reduced at low antiserum concentrations (1.3×10^6 to 1.3×10^4), suggesting that the binding effect of antiserum could be diluted out for NA-treated cells. When P388D1 cells were infected with the viruses at an MOI of 0.2 PFU/cell to detect the potential enhancement of viral growth in P388D1 cells in the presence of antibody, antibody-mediated growth was observed not only for NA-treated cells but also for untreated cells (Fig. 4B). In this case, the virus yields from untreated cells were higher than those from NA-treated cells at each antiserum dilution. This phenomenon could be due to a lower binding of AS-V to NA-treated cells (Fig. 4B) as compared with infection at an MOI of 2 PFU/cell (Fig. 4A). These results suggest that influenza NWS virus, internalized via Fc receptors in the presence of appropriate concentrations of antibody, can grow in P388D1 cells.

Monoclonal antibody-mediated viral growth. Using monoclonal antibodies, we investigated the antibody-mediated

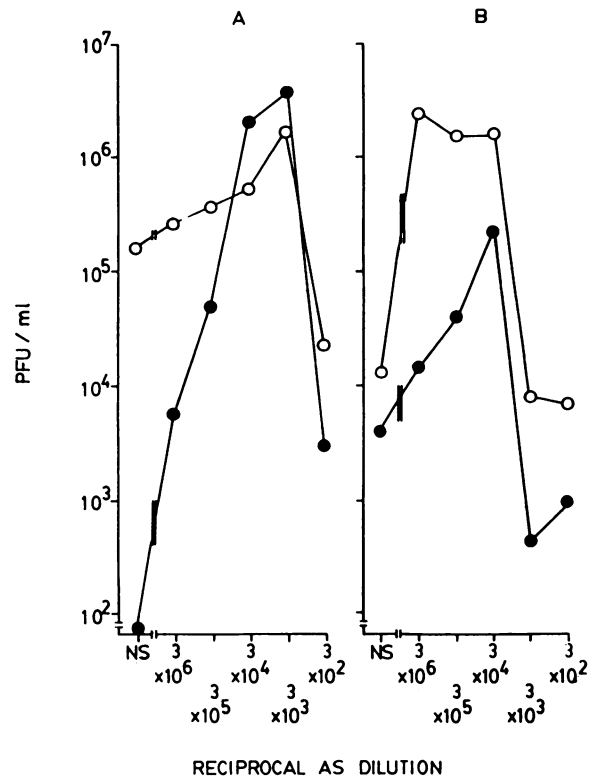


FIG. 4. Antibody-mediated growth of influenza NWS virus exposed to a wider range of antiserum concentrations. Viruses were exposed to various concentrations of anti-NWS serum (AS; 1.3×10^2 to 1.3×10^6) or normal serum (NS; 1.3×10^2 dilution) for 30 min at 37°C. Untreated (O) or NA-treated (●) P388D1 cells were infected with these viruses at an MOI of 2 (A) or 0.2 (B) PFU/cell for 45 min at 37°C. The virus yield in culture fluid at 24 h postinfection was measured by plaque assay on MDCK cells.

TABLE 1. Characterization of antibody

Antibody	HI titer	50% plaque reduction titer	50% hemolysis inhibition titer	IgG subclass
Rabbit antiserum	1.2×10^4	3.0×10^3	NT ^a	
Anti-HA monoclonal antibody				
B-4	3.3×10^5	2.1×10^4	143	IgG1
G-6	3.3×10^5	2.5×10^4	164	IgG2b
I-2	3.3×10^5	8.1×10^3	87	IgG2a

^a NT, Not tested.

growth in P388D1 cells. The IgG subclasses of three monoclonal antibodies were as follows: B-4, IgG1; G-6, IgG2b; I-2, IgG2a (Table 1). It was found that all three monoclonal antibodies specifically bound to hemagglutinin molecules by immunoprecipitation. The 50% plaque reduction and 50% hemolysis inhibition titers of I-2 monoclonal antibody were lower than those of B-4 and G-6, in spite of showing the same HI titers. Figure 5 shows the virus yields from NA-treated P388D1 cells infected with viruses that had been exposed to various concentrations of mouse ascitic fluids. In each case, using B-4, G-6, or I-2, the highest virus yield was observed at the dilution range of 1:20,000 to 1:10,000, which was a slightly higher dilution than the 50% plaque reduction titer of the corresponding monoclonal antibody (arrows in Fig. 5). At lower dilutions, viral proliferation was remarkably inhibited. Thus, the antibody-mediated growth, depending on the concentrations of the monoclonal antibodies, was similar to that of rabbit polyclonal antiserum as shown in Fig. 3 and 4. There was no significant difference in viral growth mediated

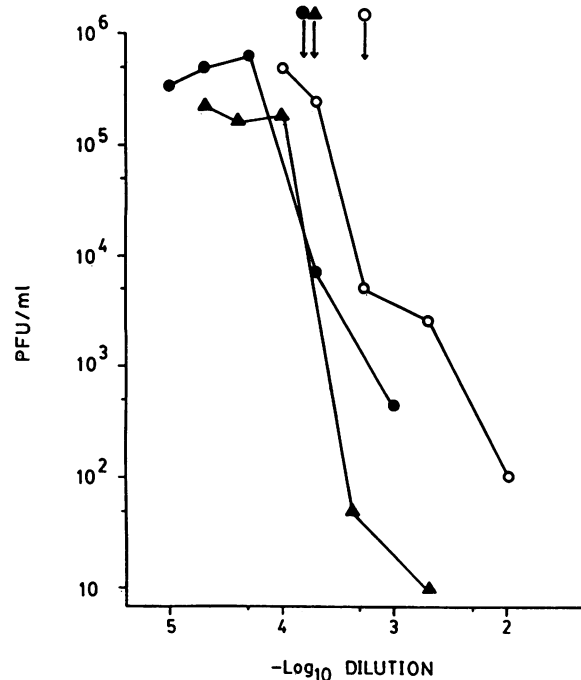


FIG. 5. Effect of monoclonal antibody on viral growth in NA-treated P388D1 cells. NA-treated P388D1 cells were infected with viruses exposed to various concentrations of ascitic fluids containing monoclonal antibodies (B-4, ▲; G-6, ●; I-2, ○) at an MOI of 2 PFU/cell for 45 min at 37°C. The virus yield in culture fluid at 24 h postinfection was measured by plaque assay on MDCK cells. Arrows indicate the dilution of each monoclonal antibody showing 50% plaque reduction titer as described in the legend for Fig. 3.

by the IgG subclasses used in this study (IgG1, IgG2a, and IgG2b), suggesting that both murine Fc receptors, FcRI (for IgG2a) and FcRII (for IgG1 and IgG2b), might be responsible for the antibody-mediated growth.

Effect of NH₄Cl on antibody-mediated viral growth. To compare the early intracellular pathways of the influenza NWS virus through viral and Fc receptors in P388D1 cells, the cells were maintained in the presence of 20 mM NH₄Cl after virus infection at 0°C. This concentration of NH₄Cl for 24 h did not influence cell viability, as measured by the trypan blue exclusion test. The NS-V and AS-V dramatically lost their growth abilities in the untreated and NA-treated cells, respectively (Table 2). When the cells were infected with the viruses and then maintained at 37°C in the presence of 20 mM NH₄Cl, this phenomenon was also observed. In either case, acid-catalyzed fusion reactions could be inhibited by NH₄Cl.

DISCUSSION

Antibody-mediated growth has been shown with several enveloped viruses (8, 9, 18, 27, 29, 30) and, with one exception, nonenveloped reovirus (2) (for a review, see reference 7). In this study, we have demonstrated antibody-mediated growth of influenza NWS virus in the cultured macrophagelike P388D1 cell line in the presence of subneutralizing antiviral IgG. This phenomenon resulted from the attachment of virus-antibody complexes to Fc receptors, followed by internalization and then the undergoing of proliferative steps.

Sialic acid-containing cell surface components have receptor activity towards influenza virus (6); also, these components are distributed on the surfaces of all vertebrate cells (33). In this study, flow cytometric analysis (Fig. 1) showed that P388D1 cells have influenza virus receptors as well as Fc receptors. The amounts of NS-V attached to untreated cells were almost equal to those of AS-V to NA-treated cells. Moreover, we have observed similar behavior with another macrophagelike cell line, J774 cells, and with thioglycolate-elicited peritoneal macrophages (unpublished data). These data suggest that the number of viral receptor sites is almost equal to that of Fc receptors on murine macrophages. Lonberg-Holm (19) has stated that the density of NA-sensitive receptors for certain viruses may be more than 10⁵ on a cell. In addition, it has been estimated that there are 4.4 × 10⁵ and 2.9 × 10⁵ sites per cell for murine FcRI and FcRII, respectively (35). We consider our observations to be consistent with these data.

The influenza NWS virus can grow in P388D1 cells after establishment of infection via viral receptors. This finding indicates that P388D1 cells are originally permissive for the growth of NWS virus. At lower antiserum concentrations, the virus yield from the NA-treated cells infected with AS-V decreased in proportion to the decrease of the antibody concentrations (Fig. 3) and became much lower than that

TABLE 2. Effect of NH₄Cl on proliferation of influenza NWS virus in P388D1 cells

Cells (treatment)	Virus ^a	Temp (°C) of virus attachment	Virus yield (PFU/ml) in NH ₄ Cl concn ^b :	
			0 mM	20 mM
P388D1 (untreated)	NS-V	0	3.6 × 10 ⁶	<10
		37	3.1 × 10 ⁵	<10
P388D1 (NA treated)	AS-V	0	1.3 × 10 ⁶	<10
		37	2.9 × 10 ⁵	<10

^a NS-V, Virus exposed to normal serum; AS-V, virus exposed to antiserum diluted 1:3,000.

^b Virus yield in culture fluids at 24 h postinfection. NH₄Cl presented at 0 and 24 h postinfection.

from untreated cells at similar low concentrations of antiserum such as 1:3 × 10⁵ dilution (Fig. 4A). This phenomenon is probably due to limited attachment of virus-antibody complexes to Fc receptors. In the presence of sub-neutralizing concentrations of antibodies (1:300 to 1:1,200 dilutions), viral growth was observed on both NA-treated and untreated P388D1 cells, although the virus yield from untreated cells was slightly higher than that from the treated cells (Fig. 3). Judging from the curve of percent reduction of infectivity on MDCK cells (Fig. 3), viral growth on the untreated cells after infection might predominantly occur via Fc receptors but also, in some part, via viral receptors, resulting in a higher virus yield than that on the NA-treated cells. Using a lower concentration of the viruses (Fig. 4B), we observed higher virus yields from untreated cells than from NA-treated cells at each antiserum dilution. This could be due to attachment of the viruses to both receptors on untreated cells. In addition, viral growth on NA-untreated cells probably occurs via multiple steps, because the progeny viruses can infect untreated cells, which have intact viral receptors, in the absence of antibody. These results suggest that antibody-mediated enhancement of infection of influenza virus on Fc receptor-bearing cells actually occurs. On the other hand, at higher concentrations of antiserum, the virus yield decreased exponentially in proportion to the reduction of infectivity to MDCK cells, but in reverse proportion to the efficiency of attachment via Fc receptors on P388D1 cells (Fig. 3). These results indicate that antibody concentrations have a critical role in infection either via Fc receptors on P388D1 cells or via viral receptors on MDCK cells. It has been shown that HI antibody for influenza virus acts as a neutralizing antibody through the inhibition of attachment to viral receptors on permissive cells (31). However, because of the occurrence of viral attachment to P388D1 cells via Fc receptors in NA-treated cells in the presence of appropriate concentrations of antibodies, a question arises as to what kinds of antibodies could exhibit such inhibitory effects on viral growth even after attachment is finished.

Murine Fc receptors have been identified for IgG2a (FcRI), IgG1 and IgG2b (FcRII), and IgG3 (FcRIII) (35). In this study, three monoclonal antibodies belonging to different IgG subclasses were used to examine the participation of these Fc receptors in antibody-mediated growth. However, there was no significant difference among the monoclonal antibodies we used. Thus, at least, we can say that both FcRI and FcRII could be involved in this phenomenon (Fig. 5).

It has been demonstrated that lysosomotropic weak bases such as ammonium chloride and chloroquine inhibit an acid-catalyzed fusion reaction between the membranes of

the virus particles and those of endosomes or lysosomes by elevating pH, thus causing the infectious viral core to become unable to enter the cytosol (22, 41). In our studies (Table 2), the growth of influenza NWS virus was inhibited by 20 mM ammonium chloride whether infections were established via viral or Fc receptors. Thus, the intracellular pathways after internalization via Fc receptors are considered to be similar to those after internalization via viral receptors in P388D1 cells, and also similar to the behavior of influenza virus in MDCK cells (23, 42). Recently, Gollins and Porterfield (5) have shown a new neutralizing mechanism for the West Nile virus, one of the enveloped viruses, in which antiviral antibodies inhibit the intraendosomal acid-catalyzed fusion step in P388D1 cells. Therefore, in the case of influenza virus, this inhibitory mechanism might occur at the higher antiserum concentrations, as demonstrated in Fig. 3, 4, and 5. It should be further studied by extended analysis using monoclonal antibodies against viral surface antigens such as HA, NA, and possibly M protein to identify such antibodies for influenza viruses.

The antibody-mediated infection of influenza NWS virus demonstrated in this study does not have a recognized counterpart in influenza epidemics in human beings (7, 40). However, as pointed out by Halstead (7), the unusual susceptibility of infants and elderly persons permits speculation that insufficient immunity may not always provide a benign or protective influence and also may actually enhance influenza infection. In addition, it is considered that the antibody-mediated growth of influenza virus in Fc receptor-bearing phagocytes might be important in possible viral persistence or as a source of antigenic variant strains of the influenza virus. All these problems remain to be solved.

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