Neutralization of Influenza Virus by Low Concentrations of Hemagglutinin-Specific Polymeric Immunoglobulin A Inhibits Viral Fusion Activity, but Activation of the Ribonucleoprotein Is also Inhibited

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High concentrations of hemagglutinin-specific neutralizing polymeric monoclonal immunoglobulin A (IgA) inhibit attachment of the majority of type A influenza virus virions to cell monolayers and tracheal epithelium (H. P. Taylor and N. J. Dimmock, *J. Exp. Med.* 161:198–209, 1985; M. C. Outlaw and N. J. Dimmock, *J. Gen. Virol.* 71:69–76, 1990). A minority of virions attaches but is not infectious. Here, we report that a different mechanism operates when influenza virus A/Puerto Rico/8/34 (H1N1) is neutralized by low concentrations of monoclonal polymeric IgA or when A/fowl plague virus/Rostock/34 (H7N1) is neutralized by low concentrations of polyclonal rat secretory IgA. Under these conditions, neutralized virus attaches to cells and is taken up by them. However, upon entering the cell, the nucleoprotein (NP) of neutralized virus is found in the perinuclear cytoplasm, whereas NP from nonneutralized virus is concentrated in the nucleus itself. Further data show that the low-pH-mediated cell fusion activity of virions is inhibited by IgA in proportion to loss of infectivity. The possibilities that neutralization by low amounts of polymeric IgA is caused by inhibition of the virion fusion activity and that the aberrant distribution of NP from neutralized virus results from its failure to escape from the endosomal system were investigated by using A/PR/8/34 and the fusogenic agent polyethylene glycol (PEG) at pH 5.4. A/PR/8/34 attached to cells at 4°C, with minimal internalization of the virus; treatment with PEG at pH 5.4 and 4°C for 1 min led to infectious fusion of nonneutralized virus with the plasma membrane and, under these conditions, was more efficient than PEG at pH 7 or medium at pH 5.4. Neutralized virus which was attached to cells and treated with acidified PEG appeared to undergo primary and secondary uncoating, with its NP protein becoming concentrated in the nucleus and M1 becoming concentrated in the perinuclear cytoplasm. Although the distribution of NP and M1 was indistinguishable from infectious virus, infectivity was not restored. Thus, even when IgA-induced inhibition of fusion is reversed, virus is still neutralized. We suggest that infectious influenza virus undergoes an activation stage which may be the relaxation of the ribonucleoprotein structure needed to permit transcription or may be the removal of M1 bound to the ribonucleoprotein. This paper emphasizes that the apparent mechanism of neutralization may vary according to the number of immunoglobulin molecules per virion and that more than one mechanism may be operative at the same time, so that inhibition of an early stage of infection may obscure an independent effect on a later function.

Polymeric immunoglobulin A (IgA) directed against the hemagglutinin (HA) of influenza virus has neutralizing activity. It is one of the principal immunoglobulins in the respiratory secretions of humans and is probably an important factor in preventing reinfection (23). Structurally, a polymeric IgA molecule consists of two or more identical immunoglobulin units arranged with their Fc regions end to end and disulfide linked to the J chain polypeptide (8, 9, 12). Secretory IgA (sIgA) has an additional polypeptide, the secretory component.

Studies with high concentrations of polyclonal polymeric IgA directed against the HA show that three mechanisms contribute to loss of infectivity: inhibition of attachment, inhibition of internalization, and inhibition of a postinternalization event (39). Thus, even though there was 99.9% neutralization, about half of the virus particles attached to BHK cells and of this half about 40% were resistant to removal by protease digestion; the latter 40% were presumably internalized but failed to initiate infection. More recently, these data were confirmed with a monoclonal polymeric IgA and organ cultures of primary mouse tracheal cells

(28). This work also showed that both infectious and neutralized virus use a sialic acid cell receptor.

In the present study, we have investigated the mechanism of neutralization of type A influenza virus by a concentration of polymeric IgA which does not inhibit attachment of virus to cells. We find that neutralization is paralleled and probably caused by loss of virion fusion activity, a postinternalization event. However, polyethylene glycol (PEG), which promotes infectious fusion of nonneutralized virus to cells, does not restore infectivity to neutralized virus even though the intracellular distribution of NP and M1 antigens is indistinguishable between the two groups. We conclude that IgA, in addition to inhibiting fusion, inhibits a novel activation event of virion ribonucleoprotein (RNP) and that this event is a prerequisite for transcription. The requirement for this activation event is in accord with earlier results obtained with both rat polymeric polyclonal sIgA and mouse polymeric monoclonal IgA (28, 39).

MATERIALS AND METHODS

Preparation of viruses. Influenza viruses A/Puerto Rico/8/34 (H1N1) (A/PR/8) and A/fowl plague virus/Rostock/34

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(H7N1) (A/FPV/R) were grown in 11-day-old embryonated hen's eggs and purified on sucrose gradients (19).

Preparation of antibodies. Monoclonal IgA specific for the HA of A/PR/8 was purified from ascitic fluid produced in BALB/c mice in response to H37-66-1 hybridoma cells (the gift of W. Gerhard). It has both neutralizing and hemagglutination inhibition activities and is polymeric (28). It binds to site Sb (36) on the globular head of the HA; this equates to site B of the H3 crystal structure (6). The preparation contained 6.69 mg of protein per ml, and the hemagglutination inhibition unit titer was 28,000. For all experiments, the dilution quoted is that of the antibody before mixing with an equal volume of virus containing 10^5 hemagglutinating units (HAU) per ml. After incubation for 1 h at room temperature (RT), the mixture was diluted as appropriate for each experiment. In some experiments, polyclonal sIgA produced by inoculation of rats with A/FPV/Dutch/27 (H7N7) and purified from bile (39) was used to neutralize A/FPV/R.

Infectivity assays. A/PR/8 was plaque assayed on Madin-Darby canine kidney (MDCK) cell monolayers under agar containing minimal essential medium, no serum, 2 μ g of trypsin per ml, 100 μ g of DEAE-dextran per ml, 1.5 mg of bovine serum albumin per ml, and A/FPV/R on primary chicken embryo fibroblast (CEF) cell monolayers prepared as described previously (27).

For neutralization studies, a final concentration of 50,000 HAU of virus was incubated with dilutions of IgA at RT for 1 h. Aliquots were taken for electron microscopy (EM), and samples were then vortexed, diluted, and assayed for residual infectivity on the appropriate cells.

For some experiments, an infectious-center assay was used. Virus was adsorbed to BHK cell monolayers at 4°C (5 PFU per cell of nonneutralized virus or an equivalent amount of neutralized virus) and then treated with PEG 6000 (Sigma) at pH 7 or 5.4 or with medium at pH 5.4 at 4°C for various lengths of time for up to 4 min and then washed and incubated in medium at 37°C for 30 min to permit fusion of virus to cell membranes. Cells were trypsinized and seeded onto MDCK cell monolayers and overlaid with agar as for the A/PR/8 plaque assay.

Aggregation of virus by IgA. Virus or virus-IgA mixtures were prepared for EM by mixing with an equal volume of 3% aqueous sodium silicotungstate (pH 6.5) and were put on Formvar-coated grids. Counts were made of the numbers of single virions and of those in aggregates of various sizes with a JEOL JEM 100S transmission electron microscope. In control preparations, roughly half of the virions were single and the rest were in aggregates of two to five. The means and standard deviations of the number of virions per aggregate were calculated, and the antibody preparations were compared with the controls. Significance was determined by the Student *t* test. The data are expressed as histograms for each point, displaying the percentages of single virions and of virions in aggregates of 2 to 5, 6 to 20, and more than 20.

Assay for the presence of IgA. Virus-IgA mixtures on Formvar-coated grids were treated for 30 min at RT with goat anti-mouse IgA tagged with 10-nm particles of gold colloid (E.Y. Laboratories Inc.). The grids were washed and stained with 3% sodium silicotungstate (pH 6.5) and examined under the electron microscope. The numbers of grains over individual virions were counted and compared with the control. Individual virions were visible even in aggregates; these had become flattened on the Formvar, presumably because of surface tension effects during drying. Significance was determined by the Student *t* test. Replicate experiments gave similar end points.

Attachment of virus to cells. A/PR/8 was incubated together with a 1/4 or a 1/40 dilution of either anti-HA IgA or an irrelevant monoclonal antibody (MAb) for 1 h at RT and then cooled at 4°C. The mixtures were vortexed and inoculated onto CEF, MDCK, or BHK monolayers at a multiplicity of infection of 100 PFU per cell for 1 h at 4°C. The cells were prefixed with 0.5% glutaraldehyde for 15 min at 4°C and then by 0.5 M ammonium chloride for 15 min. To ensure labelling of both nonneutralized and neutralized virus, virus-inoculated cells were incubated with an HA-specific monoclonal IgG for 1 h at room temperature and then washed and incubated with a mixture of gold-labelled anti-mouse IgA and IgG (E.Y. Laboratories Inc.) for 30 min at RT. The cells were scraped off, pelleted, and fixed overnight in 2% glutaraldehyde. The pellets were then stained with osmium tetroxide, embedded, and sectioned. The sections were stained with uranyl acetate and lead citrate. The numbers of virions on each of 50 cell sections were counted.

Fusion of virus-coated cells by lowering the pH. The method used for fusion of virus-coated cells is based on that of Yoshimura et al. (40). A/PR/8 was assayed in MDCK cells at its optimum pH of 5.4 for fusion, and A/FPV/R was assayed in CEF cells at its optimum pH of 5.7. Dilutions of antibody and virus were incubated for 1 h at RT and then divided for infectivity and fusion assays. For the latter, a suspension of 2×10^7 cells per ml was mixed with 6,000 HAU of vortexed virus or virus previously incubated with antibody per ml, for 1 h on ice. The cells were pelleted and resuspended in Hank's medium buffered with 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid] at the chosen pH and incubated at 37°C with shaking. The cell concentrations before and after fusion were determined with a hemocytometer, and a fusion index was calculated as follows:

$$\text{Fusion index} = \left(\frac{\text{total cell number before incubation}}{\text{total cell number after incubation}} \right) - 1$$

The fusion index calculated for the nonneutralized virus control was 0.69.

Fusion of virus to cells with PEG and/or acid. The method for fusion of virus to cells with PEG and/or acid was adapted from Sarmiento et al. (33). Cells were inoculated with nonneutralized or neutralized virus for 1 h at 4°C and then washed, drained, and treated with PEG or Eagle's medium at pH 5.4 at 4°C for the desired length of time. This length of time was measured from the time of addition of PEG to the addition of the first phosphate-buffered saline, pH 7 (PBS), wash. A stock solution of PEG 6000 was prepared by melting 40 g of PEG, which was then sterilized and mixed with 36 ml of Eagle's medium and adjusted to pH 7.0 or 5.4 at 4°C. Virus was fused to MDCK cells by treatment with this solution and then by successive rinses in 33% stock PEG in Eagle's medium, 12.5% stock PEG, and several rinses with PBS. Optimum fusion of virus to BHK cells required treatment with 33% stock PEG, followed by rinses with 12.5% stock PEG and several rinses with PBS. The procedure was designed to facilitate virus-cell fusion but not cell-cell fusion.

Immunofluorescence. MDCK and BHK cells on glass coverslips were inoculated with 100 and 200 preneutralization PFU per cell, respectively, for 1 h at 4°C, incubated at 37°C for various lengths of time, and then fixed. The presence of virus in cells was detected by using mouse monoclonal IgG to the nucleoprotein (NP) or to the matrix (M1) (kindly supplied by R. G. Webster and Kathy Coelingh) and goat anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (Nordic). Nucleoprotein (NP) from inoculated vir-

FIG. 1. Determination of the titer of polymeric monoclonal IgA to the HA of A/PR/8 by neutralization (A), with gold-labelled anti-mouse IgA (B), and by aggregation (C). Equal volumes of dilutions of IgA were mixed with 10^5 HAU of A/PR/8 per ml and incubated for 1 h at RT. The three assays were performed with the same sample, except that virus was mixed vigorously before assaying for infectivity so as to break up aggregates. Panel C charts the percentages of single virions and those of virions in aggregates of 2 to 5, 6 to 20, or more than 20. VC, virus control preparation without IgA.

ions was detected in cells fixed in methanol at -20°C , and matrix protein (M1) was detected in cells fixed with formaldehyde and permeabilized with Triton X-100 (25). Coverslips were mounted in Citifluor and examined under a Nikon microscope with UV light.

RESULTS

Parameters of IgA neutralization. Figure 1 correlates aggregation of virus (Fig. 1C; detected by EM) with presence of antibody (Fig. 1B; detected by gold labelling and EM) and compares it with neutralization brought about by mechanisms other than aggregation (Fig. 1A; plaque assays of samples vortexed to break up aggregates). Dilutions of IgA were incubated with virus, and the same samples were subjected to the three assays.

There was gold labelling of virions detectable at an IgA dilution of 1/640. Examination of 104 virions (n) showed that the mean number of grains ($\bar{x}$) was 2.67 and the variance (σ) was 2.81. These values were significantly different from those for the control, for which $\bar{x}$ was 1.94 and σ was 2.57 ($n = 100$). The probability of the samples being the same was between 1 and 0.2%. At a dilution of 1/1280, $\bar{x}$ was 1.49 and σ was 1.89 ($n = 115$), lower than the control values.

Similarly, when aggregation was examined (without vortexing), the 1/640 dilution values ($\bar{x} = 2.90$; $\sigma = 3.25$; $n =$

TABLE 1. Neutralization, gold-label detection of bound IgA, and aggregation

Highest dilution of anti-HA IgA for each condition	End-point dilution ^a	End-point dilution/neutralization end-point ratio ^b
Neutralization detected	160	1.0
IgA detected by gold labelling	640	4.0
Virus aggregated	640	4.0
Maximum aggregation	10	0.06

^a Data are reciprocals.

^b Data from Fig. 1.

406) were significantly different from those of the control ($\bar{x} = 1.52$; $\sigma = 0.75$; $n = 267$) ($P < 0.1$), but the 1/1280 dilution values ($\bar{x} = 1.64$; $\sigma = 1.36$; $n = 332$) were not ($20 > P < 10$). Similarly, all of the values at higher concentrations were significantly different from those for the control. In the preparation showing maximum aggregation (1/10), 1.8% of the virions were single, and the largest single aggregate contained 47 virions.

Table 1 summarizes data showing that gold labelling of IgA and aggregation occurred with an end-point dilution (1/640) only fourfold higher than the last dilution of detectable neutralization (1/160). The data also show that maximum aggregation of virus, at a dilution of 1/10, involves virus which is already neutralized and suggest that aggregation is not an important mechanism of neutralization in this system.

Negative staining of virus-IgA complexes. After incubation with a 1/4 dilution of IgA, A/PR/8 was covered completely with a tangled layer of IgA (Fig. 2B). However, A/PR/8 incubated with a 1/20 dilution of IgA, which was sufficient to neutralize virus by $\geq 99\%$ (Fig. 1A), had no visible fringe of antibody, although occasional IgA molecules were seen silhouetted against the background stain (Fig. 2C). These antibodies were usually attached to the virion by both immunoglobulin units, and their Fc regions spanned two or three viral spikes (Fig. 3). No IgA could be seen on virus incubated with higher dilutions of antibody, even though there was significant neutralization.

The orientation of IgA at higher concentrations was not obvious because of the large number of molecules bound to the virion (Fig. 2B). If the molecules were rigid, fully extended dimers and bound to the virion by one end only,

FIG. 2. A/PR/8, negatively stained with 3% aqueous sodium silicotungstate (pH 6.5), nonneutralized (A) and neutralized with a 1/4 dilution of IgA (B) and with a 1/20 dilution of IgA to $\geq 99\%$ (C).

FIG. 3. A negatively stained electron micrograph of A/FPV/R incubated with rat sIgA showing a single immunoglobulin molecule attached by both ends (arrow). Bar, 25 nm.

the fringe of antibody would measure the same as the length of a free mouse dimeric IgA molecule (26.2 nm; 8). The depth of the IgA fringe was calculated by measuring the distance from the viral envelope to the distal edge of the IgA layer and subtracting the distance from the viral envelope to the distal part of the HA spike on nonneutralized virus. This gave a mean of 12.3 nm and σ of 3.9 nm ($n = 104$). Since this measurement is much less than that of an extended IgA dimer, the fringe must consist of molecules attached at both ends, of flexible antibodies attached at one end, or probably of a mixture of both.

Attachment of neutralized virus to cells. Since negative staining of mixtures of A/PR/8 and IgA (at a dilution of 1/20) showed little physical evidence of the presence of antibody on the surface of virus despite neutralization of $\geq 99\%$ of infectivity (Fig. 2C), we felt that steric hindrance was unlikely to be involved at this level, and we investigated the ability of such virus-antibody complexes to attach to cells.

All inocula were vortexed to disperse virions cross-linked by antibody and were then allowed to attach to several cell types at 4°C. After processing for EM, the numbers of virions on the surfaces of each of 50 cell sections were counted. Virions were present as single particles with very few aggregates visible. Table 2 shows that the 1/40 dilution of IgA had little effect on the attachment of virus to any of the three cell types, whereas the 1/4 dilution strongly interfered. This confirms the previous findings (28, 39) that high concentrations of IgA prevent the majority of radiolabelled influenza virus from attaching to cells but suggests that this is unlikely to be the primary mechanism of neutralization.

Entry of neutralized virus into cells as determined by immunofluorescence. After nonneutralized A/PR/8 was allowed to attach at 4°C, the cells were warmed to 37°C. A few minutes later, grains of NP antigen could be detected in the cytoplasm by immunofluorescence. Later, the fluorescence appeared as larger cytoplasmic accumulations, and by 4 h the majority of NP was concentrated in the nucleus (Fig. 4). This sequence presumably represents movement of NP from endocytic vesicles in which virus enters cells to endosomes and then to nuclei. To confirm that the nuclear fluorescence represented the NP of the inoculum, cells were pretreated with 1 μ g of actinomycin D per ml, which inhibits viral transcription and subsequent expression of newly synthesized viral proteins (35). The extent of fluorescence was unaltered by actinomycin D treatment. Furthermore, we determined that newly synthesized NP is not detected until 12 h postinfection (data not shown). Cells inoculated with A/PR/8 neutralized with a 1/4 dilution of IgA fluoresced only

TABLE 2. Effect of different concentrations of IgA on the attachment of A/PR/8 to cells^a

Cell type	1/IgA dilution	No. of virions ^b		IgA/control (%) ^c
		Neutralized virus	Control	
MDCK	4	38	119	33
	40	132	134	99
BHK	4	83	274	30
	40	231	243	95
CEF	4	26	229	11
	40	226	238	95

^a IgA and A/PR/8 were incubated together at 25°C and then added to the indicated cell monolayers at 4°C for 1 h. After prefixation, virions were tagged with gold-labelled antibody to aid identification; the cells were then fixed and processed for EM.

^b Numbers of virions counted per 50 cell sections.

^c Percentages are numbers of virions counted for IgA-neutralized virus divided by numbers of virions counted for control.

weakly, which is consistent with the EM data described above showing that most such virus-IgA complexes do not bind to the cell. On the other hand, cells inoculated with virus neutralized by a 1/40 dilution of IgA showed, after 4 h of incubation, perinuclear staining of a sort not seen in infected cells (Fig. 4). At 6 h, there was staining generally over the cytoplasm; there was no nuclear fluorescence at either 4 or 6 h. Thus, neutralization with relatively low neutralizing concentrations of IgA allows a substantial proportion of virus to attach to and enter MDCK cells but interferes with the normal progression of virus through the cytoplasm and prevents entry into the nucleus. A similar sequence of events was seen for BHK cells infected with nonneutralized and neutralized A/PR/8 virus (data not shown).

Acid-induced fusion of cells by influenza virus. Since influenza virus neutralized by a 1/40 dilution of IgA attached to and entered cells, it was of interest to determine whether neutralized virus was still capable of carrying out the low-pH-induced fusion process. Figure 5 shows that fusion was inhibited progressively by increasing concentrations of IgA with a close correspondence with loss of infectivity. Similar data were obtained for rat sIgA and A/FPV/R by using a pH of 5.7 and CEF cells (not shown).

PEG-induced fusion of virus to cells. PEG is commonly used to fuse cells together and can also be used to artificially fuse virus with cells. It restores infectivity to neutralized herpes simplex virus by overcoming antibody-inhibited virion-cell fusion (10, 11, 33). If, at the concentration used here, IgA neutralizes solely by inhibiting fusion, then artificial fusion of the virion envelope and the plasma membrane might similarly allow entry of the influenza virion core into the cytoplasm and restore infectivity. To allow this hypothesis to be tested, we optimized fusion of nonneutralized virus to BHK cells at 4°C (Fig. 6). Exposing inoculated cells to medium at pH 5.4 for 3 to 4 min (values taken from replicate experiments) gave maximal infectivity. PEG at pH 7 gave only about 50% infectivity after 2.5 min of exposure, but a short exposure to PEG at pH 5.4 for only 1 min restored full infectivity. Longer exposures were inhibitory. The reason why the lower pH gave better recovery of infectivity is discussed later.

Applying this methodology to IgA-neutralized virus showed very clearly that there was no reactivation of infec-

FIG. 4. Immunofluorescence of MDCK cells inoculated with nonneutralized A/PR/8 or A/PR/8 neutralized with IgA. Virus and cells were incubated together for 4 h, fixed, and stained with monoclonal IgG to the NP. (A) Virus plus a 1/4 dilution of IgA; (B) virus plus a 1/40 dilution of IgA; (C) nonneutralized virus.

tivity (Table 3). Experiments with MDCK cells gave essentially similar results. We conclude that inhibition of fusion is not the primary reason why IgA-neutralized virus loses infectivity. The nature of this IgA-induced defect is investigated in the next section.

Intracellular distribution of M1 and NP proteins after nonneutralized and IgA-neutralized viruses have been artificially fused with BHK cells. The viral core released into the cytoplasm by fusion consists of a shell of M1 containing the RNP. M1 is removed in the cytoplasm, releasing the RNPs, which move through the nuclear pores into the nucleus (25). In normal infections, input M1 can be detected in the cytoplasm by immunofluorescence and NP can be detected in the nucleus.

Figure 7E shows that the majority of M1 of nonneutralized virus infecting by the natural route is in the cytoplasm, although there is a concentration of fluorescence in the perinuclear region. The M1 from neutralized virus is also cytoplasmic. This is consistent with the conclusion reached earlier in this paper that neutralized virus is endocytosed but does not fuse with the vesicle envelope (Fig. 7I). Thus, we suggest that M1 of neutralized virus is still inside the virion, whereas M1 from infectious virus will have been released into the cytoplasm proper by fusion of the virion envelope with the vesicle membrane. Artificial fusion at the plasma membrane with nonneutralized virus shows that M1 also becomes distributed throughout the cytoplasm and that this

FIG. 5. Inhibition of A/PR/8-induced low-pH-mediated fusion of MDCK cells by IgA. Dilutions of IgA and virus were incubated together at RT for 1 h, allowed to attach to cells at 4°C, and then warmed to 37°C at pH 5.4. ▲, percentage of infectivity surviving neutralization; ●, fusion index (where 0.69 is the value obtained with nonneutralized virus).

was most efficient with PEG at pH 5.4 (Fig. 7F to H), in agreement with infectivity data (Table 3). Immunofluorescence with artificially fused neutralized virus is very similar, showing that neutralization does not prevent artificially induced fusion and uncoating or the subsequent distribution of M1 in the cell (Fig. 7J to L), even though the virus is noninfectious (Table 3). The perinuclear distribution of M1 is particularly clear after fusion with PEG at pH 5.4 (Fig. 7H and L).

NP from nonneutralized virus was present largely in the nuclei of cells which were infected naturally (Fig. 8E), while NP of neutralized virus was predominantly perinuclear (Fig. 8I). This is consistent with the interpretation that neutralized virus is endocytosed but remains in vesicles. After artificial fusion with nonneutralized virus at the plasma membrane at pH 5.4, there is NP in the cytoplasm, in the perinuclear region, and in the nucleus (Fig. 8F). The same pattern emerges after fusion with PEG at pH 7 (Fig. 8G). However, fusion with PEG at pH 5.4 (Fig. 8H) gives a pattern indistinguishable from that of natural infection. With neutralized

FIG. 6. Infectivity of A/PR/8 on BHK cells after treatment to induce virus-cell fusion with PEG at pH 7 or 5.4 or medium at pH 5.4. Virus was attached to cell monolayers for 1 h at 4°C and then treated as described in Materials and Methods for 1 to 4.5 min at 4°C. The cells were incubated for 30 min at 37°C to allow internalization and then trypsinized and plated on MDCK cells in an infectious-center assay. Δ, PEG at pH 7.0; ▲, PEG at pH 5.4; ●, medium at pH 5.4.

TABLE 3. Infectivity obtained in BHK cells by inducing fusion between the viral envelope and plasma membrane of non-neutralized A/PR/8 and virus neutralized with IgA^a

Treatment	Growth of virus as % of control ^a	
	Nonneutralized	Neutralized
None	100.0	<1
Medium (pH 5.4)	13.9	<1
PEG (pH 7)	11.1	<1
PEG (pH 5.4)	91.8	<1

^a Control virus and virus neutralized with a 1/40 dilution of IgA were attached to BHK cells at 4°C and then treated for 1 min with neutral or acidified medium or neutral or acidified PEG. The cells were trypsinized and plated as infectious centers on MDCK cells, as described in Materials and Methods. Results are expressed as percentages of the number of infectious centers obtained with the untreated virus control.

virus fused at the plasma membrane at pH 5.4 or with PEG at pH 7, NP is cytoplasmic (Fig. 8J and K), but after fusion with PEG at pH 5.4, NP is seen concentrated in the nucleus (Fig. 8).

These data are summarized in Table 4. The distribution of M1 and NP implies that normal uncoating of both nonneutralized and IgA-neutralized virus can be achieved by fusion with PEG at pH 5.4 but not with neutral PEG or medium at pH 5.4. However, since this does not restore the infectivity of neutralized virus, there must be another antibody-induced defect further down the line.

DISCUSSION

Influenza virions bind to *N*-acetylneuraminic acid residues on the cell surface, enter the cell by endocytosis (7, 30), and are uncoated by fusion between the viral and endosomal membranes (29, 40, 41). The viral cores move toward the nucleus and are uncoated of M1 in the nucleus-associated cytoplasm (4, 5, 25). The RNP migrates into the nucleus (17, 25, 37), in which RNA replication takes place (1, 2, 15, 18, 21, 24, 34).

How does IgA neutralize influenza virus? We have shown by direct EM observation that high concentrations of polymeric IgA prevented attachment of virus to monolayers of three different cell types. The antibody could be seen as a fringe about the particle and presumably interferes sterically

FIG. 7. Intracellular distribution of M1 shown by immunofluorescence after nonneutralized and neutralized virus has been artificially fused with BHK cells. Cells were inoculated for 1 h at 4°C with A/PR/8 or virus neutralized with IgA and then treated for 1 min at 4°C with medium at pH 5.4 or PEG at pH 7 or 5.4. The monolayers were then washed and incubated for 3 h at 37°C before being fixed with formaldehyde and permeabilized. M1 was detected by incubation with a mixture of MAbs followed by fluorescein isothiocyanate-labelled goat anti-mouse IgG. Shown are noninfected cells (A to D), cells infected with A/PR/8 (E to H), cells inoculated with neutralized virus (I to L), and cells treated with Eagle's medium (A, E, and I), with medium at pH 5.4 (B, F, and J), and with PEG at pH 7.0 (C, G, and K) and at pH 5.4 (D, H, and L).

FIG. 8. Intracellular distribution of NP shown by fluorescence (from the experiment described in the legend to Fig. 7). Cells were fixed with methanol at -20°C . NP was detected by incubation with MAb followed by fluorescein isothiocyanate-labelled goat anti-mouse IgG. The photograph panels are labelled as described in the legend to Fig. 7.

with its attachment to *N*-acetylneuraminic acid. This is comparable to IgM-neutralized influenza virus, in which a thick layer of antibody can be seen (3). However, EM study showed that a lower (but still neutralizing) dilution of IgA allowed attachment of most virus to cells. These data agree in principle with those obtained by using neutralized radio-

labelled virus and BHK or primary tracheal epithelial cells (28, 39). Evidently, binding of critical amounts of polymeric monoclonal IgA to antigenic site Sb (36), which lies close to the virus attachment site (6), does not inhibit attachment. IgA aggregated virus at a concentration higher than that which neutralized virus. Thus, aggregation is not responsible for neutralization in this system.

How can IgA-neutralized virus attach to cells and not be infectious? The data presented above show that neutralization correlated closely with inhibition of acid-induced fusion. Immunofluorescence indicated that NP of neutralized virus remained in the cytoplasm instead of becoming nuclear; it first concentrated in the perinuclear region and then dispersed. This pattern is similar to that seen in influenza virus-infected cells treated with ammonium chloride to prevent acid-induced fusion (41). Yoshimura and Ohnishi (41) observed virions in perinuclear lysosomes; later, virions were degraded with some of the resulting material being exported from the cell. This is consistent with the partial loss of fluorescence from our cells at 6 h postinfection (2a).

Taylor and Dimmock (39) have shown that monomeric IgA derived by differential reduction neutralizes virus but allows apparently normal attachment, uncoating, and migration of the viral genome into the nucleus. Inhibition of fusion must therefore be a property of polymeric IgA and presumably depends on cross-linking of the virus surface units. We

TABLE 4. Infectivity and distribution of M1 and NP antigens of nonneutralized and IgA-neutralized virus induced to fuse at the plasma membrane^a

Presence or absence of IgA	Treatment inducing PM fusion ^b	Distribution of antigen ^c		Infectivity (%)
		Cytoplasm	Nucleus	
—	None	M1	NP	100
—	Medium (pH 5.4)	M1, NP	(NP)	14
—	PEG (pH 7)	M1, NP	(NP)	11
—	PEG (pH 5.4)	M1	NP	92
+	None	M1, NP		0
+	Medium (pH 5.4)	M1, NP		0
+	PEG (pH 7)	M1, NP		0
+	PEG (pH 5.4)	M1	NP	0

^a Data are from Fig. 7 and 8 and Table 3.

^b PM, plasma membrane.

^c By immunofluorescence; parentheses indicate that reduced amounts of antigen were present.

have seen IgA molecules lying along the virion surface spanning up to three HA spikes. IgA molecules are flexible, so the antibody may be able to engage an HA spike with each of its four binding sites, but exactly how IgA inhibits fusion remains unestablished.

Our data here and those of others allow us to conclude that type A influenza virus is neutralized by polymeric IgA by three different mechanisms, depending on the number of immunoglobulin molecules per virion. Firstly, with high concentrations of IgA, the majority of virus is prevented from attaching to cells; this holds for both sIgA (39) and polymeric monoclonal IgA which lacks the secretory component (28 and the present report). Secondly, a proportion of neutralized virus which attaches to cells is not internalized; this is shown by its removal from the cell surface by protease (39) or neuraminidase (28). Lastly, by using a low but still neutralizing dilution of IgA which allows maximum attachment of virus, we have demonstrated, in the present study, that this neutralized virus is internalized but is unable to carry out the fusion event which is necessary for uncoating. All of these mechanisms have the common property of acting on virus which is already noninfectious (see below). However, each mechanism may be significant; for example, virus neutralized by low concentrations of IgA which is internalized by the cell may be proteolytically processed and presented by major histocompatibility complex proteins. This remains undetermined.

IgA neutralization is not reversed by using PEG to induce virus entry into the cell. PEG is a commonly used fusogenic agent, and neutral PEG has been used to overcome IgG neutralization of herpes simplex virus type 1 (10, 11), a virus which normally enters the cell by fusing with the plasma membrane at neutral pH. Figure 6 shows that influenza virus introduced into the cell by neutral PEG fusion was only 50% infectious. Acidified PEG gave efficient entry, with maximum infectivity in 1 min, whereas under the same conditions, neutral PEG gave only 23% infectivity. Acidified medium was as effective as acidified PEG but required longer exposure (3 to 4 min), presumably because it induced normal fusion via the HA. These data are supported by immunofluorescence observations, according to which the most efficient transport of NP into the nucleus was achieved by using acidified PEG. The simultaneous presence of M1 in the cytoplasm shows definitively that secondary uncoating has taken place. We conclude, therefore, that for this to occur, not only does the virus have to fuse with the cell, but acidification is also required. M1 is intimately associated with the lipid bilayer, and part of the molecule is exposed on the surface of the intact virion and is able to react with monoclonal antibody (20). Furthermore, antibodies to purified M1 neutralize A/PR/8 and fix complement (31). It is conceivable, therefore, that acid treatment of the virion surface changes M1 in a way which permits secondary uncoating. The apparent lack of requirement of acidification for infectivity shown after treatment of virus-inoculated cells with neutral PEG is probably illusory, since EM study showed some virus entering by endocytosis (data not shown). This virus would then be acidified in the normal way and would be the infectious fraction; virus which is fused with the plasma membrane at neutral pH may in fact not be infectious.

There is in addition a cellular dimension, since influenza virus A/FPV/R entered MDCK cells after fusion to the plasma membrane at acidic pH but was not infectious (26). However, A/WSN fused to MDCK cells (40) and A/PR/8 fused to MDCK cells in the same way were infectious (2a).

TABLE 5. Neutralization by a low concentration of IgA

Immuno- globulin	Inoculation condition	Entry	Uncoating ^a			Infectivity
			1	2	3	
None	Medium (pH 7)	+	+	—	+	+
	PEG (pH 7)	+	+	± (?) ^b	±	±
	PEG (pH 5.4)	+	+	+	+	+
IgA	Medium (pH 7)	+	—	—	—	—
	PEG (pH 7)	+	+	—	—	—
	PEG (pH 5.4)	+	+	+	—	—

^a 1, primary uncoating: removal of viral envelope and associated proteins to produce a viral core; 2, secondary uncoating: removal of M1 from the viral core; 3, tertiary uncoating: activation of the RNP to allow transcription and eventual formation of infectious progeny. +, the event proceeds normally; ±, partially; —, not at all.

^b Half the virus may fuse with the plasma membrane, not undergo secondary uncoating, and be noninfectious; the other half may enter normally through endocytosis (see Discussion).

Despite obtaining high levels of infectivity after fusion of nonneutralized virus with the plasma membrane, no infectivity was obtained by applying the same treatment to IgA-neutralized virus even though it had undergone secondary uncoating and was in the correct cellular compartment. This experiment was conducted to test the proposal that IgA-neutralized virus lost infectivity because fusion was inhibited (Fig. 5). We conclude that although fusion activity of neutralized virus is inhibited, the virus has lost infectivity because of inhibition of another viral function which operates later in the infectious process. We suggest that there may be a tertiary level of uncoating, without which there is no transcription.

What might the mechanism be by which neutralization comes about? The data presented in this paper show that efficient infectious entry of virus into cells via the plasma membrane has two requirements: (i) fusion of viral and cellular membranes and (ii) acidification of the virus particle. These events would normally take place within the endosome and would be followed by secondary uncoating (removal of the M1 shell) in the cytoplasm. The data in this report lead us to postulate that there is another event, which activates the RNP-polymerase complex, and that this is inhibited by neutralization (Table 5). We emphasize that this conclusion applies only to the neutralization conditions defined in this report; other neutralization mechanisms operate under other conditions.

One possibility is that M2, a recently described minor component of the virion (19, 42), acts as a proton channel into the virion, as it does under other circumstances (13). There are estimated to be 54 amino acids of M2 internal to the transmembrane region (42), which are more than enough to span the 4-nm monomolecular layer of capsid formed by M1 (32). Data suggest that at most there is sufficient M2 (42) to form several proton channels, each consisting of four molecules (16, 38). We suggest that binding of IgA to adjacent HA spikes might, through conformational or associational alterations, lead to the nonfunctioning of the M2 channel, so that no protons would be conducted to the interior of the virion. The data presented here suggest that acidification of the virion exterior is a necessary prerequisite for secondary uncoating and acidification of the NP within the core might also be the cause of changes which permit transcription of virion RNA when the RNP reaches the

nucleus. One possibility is that acidification alters the conformation of the RNP to a relaxed structure, which is compatible with transcription. Alternatively, it is possible that there is incomplete removal of the M1 protein from the RNP of neutralized virus since M1 and RNP are known to associate and the link is acid labile (43). In vitro addition of M1 to RNP at 20% of the proportion in the virion inhibits polymerase activity (43). Such an amount might not affect entry of RNP into the nucleus and might not be detectable in the nucleus of acidic PEG-treated cells. Either mechanism might be related to the M2-mediated inhibition of influenza virus replication by low doses of amantadine (22), which has been shown elsewhere to inhibit virion transcription (14). We report that under conditions of low-dose amantadine inhibition, virions undergo secondary uncoating, with NP locating in the nucleus and M1 remaining in the cytoplasm (2a). Thus, there is a nice parallel between the action of amantadine and that of the type of neutralization described in this report.

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REFERENCES

- Armstrong, S. J., and R. D. Barry. 1974. The topography of RNA synthesis in cells infected with fowl plague virus. *J. Gen. Virol.* **24**:535-547.
- Armstrong, S. J., and R. D. Barry. 1975. The detection of virus-induced RNA synthesis in the nuclei of cells infected with influenza virus, p. 491-499. *In* B. W. J. Mahy and R. D. Barry (ed.), *Negative strand viruses*, vol. 1. Academic Press, New York.
- Armstrong, S. J., and N. J. Dimmock. Unpublished data.
- Armstrong, S. J., M. C. Outlaw, and N. J. Dimmock. 1990. Morphological studies of the neutralization of influenza virus by IgM. *J. Gen. Virol.* **71**:2313-2319.
- Bukrinskaya, A. G., N. K. Vorkunova, R. Kornilayeva, R. A. Narmanbotova, and G. K. Vorkunova. 1982. Influenza virus uncoating in infected cells and effect of rimantadine. *J. Gen. Virol.* **60**:49-59.
- Bukrinskaya, A. G., N. K. Vorkunova, and R. A. Narmanbotova. 1980. Rimantidine hydrochloride blocks the second step of influenza virus uncoating. *Arch. Virol.* **66**:275-282.
- Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard. 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). *Cell* **31**:417-427.
- Dourmashkin, R. R., and D. A. J. Tyrrell. 1974. Electron microscopic observations on the entry of influenza virus into susceptible cells. *J. Gen. Virol.* **24**:129-141.
- Dourmashkin, R. R., G. Virella, and R. M. E. Parkhouse. 1971. Electron microscopy of human and mouse myeloma serum IgA. *J. Mol. Biol.* **56**:207-208.
- Feinstein, A., E. A. Munn, and N. E. Richardson. 1971. The three dimensional conformation of M and A globin molecules. *Ann. N.Y. Acad. Sci.* **190**:104-121.
- Fuller, A. O., R. E. Santos, and P. G. Spear. 1989. Neutralizing antibodies specific for glycoprotein H of herpes simplex virus permit viral attachment to cells but prevent penetration. *J. Virol.* **63**:3435-3443.
- Fuller, A. O., and P. G. Spear. 1987. Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion-cell fusion at the cell surface. *Proc. Natl. Acad. Sci. USA* **84**:5454-5458.
- Hall, J. G., and E. Andrew. 1980. Biliglobin: a new look at IgA. *Immunol. Today* **1**:100-104.
- Hay, A. J. 1989. The mechanism of action of amantadine and rimantadine against influenza viruses, p. 361-367. *In* A. L. Notkins and M. B. A. Oldstone (ed.), *Concepts in viral pathogenesis*, vol. 3. Springer-Verlag, New York.
- Hay, A. J., and M. C. Zambon. 1984. Multiple actions of amantadine against influenza viruses, p. 301-315. *In* Y. Becker (ed.), *Antiviral drugs and interferon: the molecular basis of their activity*. Martinus Nijhoff Publishing, Boston.
- Herz, C., E. Stavnezer, R. M. Krug, and T. Gurney. 1981. Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus. *Cell* **26**:254-256.
- Holsinger, L. J., and R. A. Lamb. 1991. Influenza virus M2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology* **183**:32-43.
- Hudson, J. B., J. Flawith, and N. J. Dimmock. 1978. Early events in influenza virus infection. III. The formation of a nucleoplasmic ribonucleoprotein complex from the input virion. *Virology* **86**:167-176.
- Jackson, D. A., A. J. Caton, S. J. McCready, and P. R. Cook. 1982. Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature (London)* **296**:366-368.
- Jackson, D. C., X.-L. Tang, K. G. Murti, R. G. Webster, G. W. Tregear, and W. J. Bean. 1991. Electron microscopic evidence for the association of M2 protein with the influenza virion. *Arch. Virol.* **118**:199-207.
- Joassin, L., C. Vincenzotto, J.-M. Cloes, M. Bouchet, and M. Reginster. 1987. Monoclonal antibodies detect M-protein epitopes on the surface of influenza virions. *Arch. Virol.* **95**:183-195.
- Kelly, D. C., and N. J. Dimmock. 1974. Fowl plague virus replication in mammalian cell-erythrocyte heterokaryons: studies concerning the actinomycin D and ultra-violet sensitive phase in influenza virus replication. *Virology* **61**:210-222.
- Kendal, A. P., and H. D. Klenk. 1991. Amantadine inhibits an early, M2 protein-dependent event in the replication cycle of avian influenza (H7) viruses. *Arch. Virol.* **119**:265-273.
- Kilbourne, E. D. 1987. *Influenza*. Plenum Medical Book Co., New York.
- Krug, R. M. 1985. The role of RNA priming in viral and trypanosomal mRNA synthesis. *Cell* **41**:651-652.
- Martin, K., and A. Helenius. 1991. Transport of incoming influenza virus nucleocapsids into the nucleus. *J. Virol.* **65**:232-244.
- Matlin, K. S., H. Reggio, A. Helenius, and K. Simon. 1981. Infectious entry of influenza virus in a canine kidney cell line. *J. Cell Biol.* **91**:601-613.
- Morser, M. J., S. I. T. Kennedy, and D. C. Burke. 1973. Virus-specified polypeptides in cells infected with Semliki Forest virus. *J. Gen. Virol.* **21**:19-29.
- Outlaw, M. C., and N. J. Dimmock. 1990. Mechanisms of neutralization of influenza virus on mouse tracheal epithelial cells by mouse monoclonal polymeric IgA and polyclonal IgM directed against the viral hemagglutinin. *J. Gen. Virol.* **71**:69-76.
- Patterson, S., and J. S. Oxford. 1986. Early interactions between animal viruses and the host cell: relevance to viral vaccines. *Vaccine* **4**:78-89.
- Patterson, S., J. S. Oxford, and R. R. Dourmashkin. 1979. Studies on the mechanism of influenza virus entry into cells. *J. Gen. Virol.* **43**:223-229.
- Reginster, M., L. Joassin, and P. Fontaine-Delcambe. 1979. Ligands for antibody to M-protein are exposed at the surface of influenza virions: effect of proteolytic treatment on their activity. *J. Gen. Virol.* **45**:283-289.
- Ruigrok, R. W. H., L. Calder, and S. A. Wharton. 1989. Electron microscopy of the influenza virus submembrane structure. *Virology* **173**:311-316.
- Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B₂) in virion infectivity. *J. Virol.* **29**:1149-1158.
- Shapiro, G. I., T. Gurney, and R. M. Krug. 1987. Influenza virus gene expression: control mechanisms at early and late times of infection in nuclear-cytoplasmic transportation of virus-specific

- RNAs. *J. Virol.* **61**:764–773.
35. **Skehel, J. J.** 1973. Early polypeptide synthesis in influenza virus-infected cells. *Virology* **56**:394–399.
36. **Staudt, L. M., and W. Gerhard.** 1983. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. I. Significant variation in repertoire expression between individual mice. *J. Exp. Med.* **157**:687–704.
37. **Stephenson, J. R., and N. J. Dimmock.** 1975. Early events in influenza virus multiplication. I. Location and fate of the input RNA. *Virology* **65**:77–86.
38. **Sugrue, R. J., and A. J. Hay.** 1991. Structural characteristics of the M2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology* **180**:617–624.
39. **Taylor, H. P., and N. J. Dimmock.** 1985. Mechanism of neutralization of influenza virus by secretory IgA is different from that of monomeric IgA or IgG. *J. Exp. Med.* **161**:198–209.
40. **Yoshimura, A., K. Kuroda, K. Kawasaki, S. Yamashina, T. Maeda, and S.-I. Ohnishi.** 1982. Infectious cell entry mechanism of influenza virus. *J. Virol.* **43**:284–293.
41. **Yoshimura, A., and S. I. Ohnishi.** 1984. Uncoating of influenza virus in endosomes. *J. Virol.* **51**:497–504.
42. **Zebedee, S. L., and R. A. Lamb.** 1988. Influenza A virus M2 protein: monoclonal antibody restriction of virus growth and detection of M2 in virions. *J. Virol.* **62**:2762–2772.
43. **Zhirnov, O. P.** 1992. Isolation of matrix protein from influenza viruses by acid-dependent extraction with nonionic detergent. *Virology* **186**:324–330.