MECHANISM OF NEUTRALIZATION OF INFLUENZA VIRUS BY SECRETORY IgA IS DIFFERENT FROM THAT OF MONOMERIC IgA OR IgG

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A significant proportion of the protection mediated by the immune system against reinfection with influenza virus is due to the presence of neutralizing antibody directed against the hemagglutinin (HA)1 of influenza virus (1-5). However, little is understood about the mechanism by which interaction of neutralizing antibody with virus results in loss of infectivity. The consensus of opinion has been that neutralizing antibody prevents entry of virus into susceptible cells by sterically blocking the association of virus with cellular receptors, but this has only been demonstrated with reovirus (6). In other instances, attachment of poliovirus neutralized by polyclonal (7) and some monoclonal antibodies (8) is unaffected and infectivity is lost, probably because the virus is unable to uncoat. Vesicular stomatitis virus, similarly neutralized, still attaches to host cells (9) but, in this case, attachment is thought to be mediated by a novel receptor. Work carried out in this laboratory on type A influenza viruses has shown that avian and human strains, neutralized by polyclonal or monoclonal immunoglobulin G (IgG) directed against the HA, attached to a variety of different cell types (human foreskin fibroblasts, L cells, chick embryo fibroblasts [CEF] cells, and baby hamster kidney 21 [BHK-21] cells) at temperatures from 4 to 37°C with kinetics indistinguishable from those of nonneutralized virus (10-12). Moreover, the kinetics of internalization of neutralized virus, its subsequent uncoating, and the transport of virion RNA to the nuclei of CEF and BHK-21 cells were indistinguishable from nonneutralized virus. From this it has been inferred (12) that loss of infectivity results from the inhibition of some subsequent stage of infection. Thus, instead of a general theory of neutralization, it seems that different viruses are neutralized by different mechanisms (13).

In addition to IgG, the predominant antibody in hyperimmune or convalescent serum, IgA and IgM also have neutralizing ability (14), and secretory IgA (sIgA) in respiratory tract secretions is believed (15, 16) to play a major role in the resistance to influenza virus infection. However, there are no data on the mechanisms by which these classes of antibody inactivate infectivity. In this study

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1 Abbreviations used in this paper: BPL, beta propiolactone; CEF, chick embryo fibroblast; HA, hemagglutinin; HAU, hemagglutination unit; HI, hemagglutination inhibition; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PFU, plaque-forming unit; SC, secretory component; sIgA, secretory immunoglobulin A.
we have investigated how scIgA obtained from rat bile neutralizes influenza virus. scIgA reduced, but did not abolish, the attachment of virus to BHK-21 cells, and prevented it from being internalized. However, neutralizing monomeric IgA derived from scIgA by differential reduction did not inhibit attachment or internalization; in this respect, its action was indistinguishable from that of IgG (10, 11).

Materials and Methods

Viruses and Cells. The avian strain of influenza virus A/fowl plague virus/Rostock/34 (H7N1) (FPV/R) was grown in the allantoic cavity of embryonated hen eggs. 35P (Amersham International plc, Amersham, England)-labeled influenza virus was prepared (12) in roller bottles containing 2 × 10^6 primary CEF cells (17). Infectivity of the virus was determined by plaque assay on CEF cell monolayers under 0.9% agar in 199 medium containing 5% newborn calf serum, and expressed as plaque-forming units (PFU)/ml; hemagglutination was assayed using chicken erythrocytes. 35P-labeled virus was precipitated from tissue culture fluid on ice with 60% saturated (NH4)2SO4 and purified by sucrose velocity centrifugation (18), except that no unlabeled carrier was added. PFU/HA ratios of different preparations varied from 10^4.5 to 10^5.5 but attachment and internalization of virus by BHK-21 cells were unaffected by this variation (data not shown).

BHK-21 cell monolayers were cultivated in roller bottles in Glasgow minimum essential medium (Gibco-BRL Ltd., Paisley, Scotland) supplemented with 100 U penicillin, 100 μg streptomycin sulphate, and 0.295 mg tryptose phosphate per milliliter and 5% voi/vol newborn calf serum (Gibco-BRL Ltd.). When confluent, cells were removed by trypsinization (0.05% wt/vol trypsin, 0.02% wt/vol versene in phosphate-buffered saline [PBS]) and reseeded in roller bottles or in 51-mm diam Nunclon plastic petri dishes (Gibco-BRL Ltd.) at a concentration of 6 × 10^6 cells per dish.

Antisera. Antiserum to the HA of FPV/R was raised in rabbits by inoculation with A/FPV/Dutch/27 (H7N7) (FPV/D), which has the HA antigen closely related to that of FPV/R and an antigenically unrelated NA. 10^4 hemagglutination units (HAU) of purified FPV/D were injected intravenously at days 1 and 22 and serum was obtained by bleeding at day 28. A murine neutralizing monoclonal IgG (HC2) to FPV/R HA was kindly provided by J. J. Skehel (National Institute for Medical Research, London). As a control, we used another monoclonal antibody (171/7), which neutralized X49 influenza virus (H3N2) but not FPV/R.

Rabbit and mouse Ig were purified by affinity chromatography on a column of protein A–Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO) and stored at −70°C. Hemagglutination inhibition (HI) titrations were performed as described by Webster and Laver (19).

Preparation of scIgA. Purified FPV/D in PBS was inactivated with 0.0025% beta propiolactone (BPL) (Sigma Chemical Co.) for 8 h at 4°C, after which BPL was renewed and the mixture left for 24 h. The virus suspensions were dialyzed against PBS, and, when tested in embryonated eggs, contained no residual infectivity.

Inactivated virus (7 × 10^6 HAU/rat) was injected into the Peyer's patches of inbred male Wistar rats (Wistar/PC61), 10 wk old and weighing ~200 g (20). Rats were confined in Bollman restraining cages (21) while bile was collected over days 7–9 postinoculation, by cannulating the common bile duct. After removing bile salts by dialysis against 0.01 M Tris-HCl buffer and 0.15 M sodium chloride (pH 7.4), the bile was concentrated on an Amicon B15 filter (Amicon Corp., Lexington, MA). IgG was removed by passing the bile concentrate seven times down a protein A–Sepharose column to which scIgA does not bind (22). Material was stored at −70°C.

scIgA was purified using an affinity column constructed by coupling goat anti-rat α chain antibody (Nordic Immunological Laboratories Ltd., Maidenhead, Berkshire, United Kingdom) to cyanogen bromide–activated Sepharose (Sigma Chemical Co.). 2 ml of bile concentrate was passed through the column in 0.01 M Tris-HCl, 0.15 M sodium chloride (pH 8). scIgA was eluted from the column with 0.1 M ammonium hydroxide and 0.05 M
diethylamine (pH 11.5). Eluted samples were neutralized with HCl, dialyzed overnight against 1 mM Tris-HCl, 0.05 M sodium chloride (pH 7.4), and then lyophilyzed. Purity of sIgA was monitored by polyacrylamide gel electrophoresis (PAGE) (23) and by immunodiffusion against anti-α chain antibody.

Preparation of Monomeric IgA. Monomeric IgA was prepared from purified sIgA by differential reduction with 0.01 M β-mercaptoethanol (BME) (Sigma Chemical Co.) in 0.3 M Tris-HCl (pH 8) for 1 h at 25°C. After dialyzing overnight against 1 mM Tris-HCl, 0.015 M sodium chloride (pH 7.4), IgA was separated from the secretory component (SC) and J chain by affinity chromatography on an anti-α chain column as described above. The SC and J chain emerged in the void volume and IgA monomers were eluted as before and lyophilyzed. IgA was characterized by PAGE and by immunodiffusion against anti-α chain antibody.

Attachment of Neutralized and Nonneutralized 35P-labeled FPV/R to BHK-21 Cells. The attachment of nonneutralized and neutralized 35P-labeled FPV/R to BHK-21 cells was measured as described previously (10, 11). Virus was first incubated for 1 h at 25°C with neutralizing Ig, bile concentrate, or an equivalent mass of Ig from preimmune sources. 100-μl volumes of virus-Ig mixtures were inoculated onto monolayers of BHK-21 cells in plastic petri dishes, held on ice to give a temperature at the monolayer surface of 4°C, unless otherwise stated. This temperature prevents virus-directed macromolecular synthesis (24). At the required times monolayers were rinsed three times with cold PBS and removed by scraping. Radioactivity associated with cells and washes was determined by binding to Whatman DE81 filter discs, which were washed successively in sodium dihydrogen orthophosphate (three times), water, and ethanol. This represents 35P-labeled virion RNA (lipids and proteins do not bind).

Assay for the Uptake of Virion RNA into Nuclei. Cells were fractionated by removing cytoplasm from cell monolayers by incubating for two successive periods of 5 min, in 0.5% Nonidet P-40 (NP-40) (BDH Pharmaceuticals Ltd., London) in 0.25 M sucrose, 0.01 M Tris-HCl (pH 7.4), 1 mM MgCl2 (11, 25) on ice. Nuclei remained attached to the petri dish and were scraped into the same buffer without NP-40, rinsed vigorously on a vortex mixer, and then pelleted at 150 g for 5 min at 4°C. The supernatant was pooled with the cytoplasmic fraction. This method gives a high yield of nuclei that have a cytoplasmic contamination of only 5% (11). Uptake of 35P-virion RNA into nuclei was measured by inoculating monolayers with nonneutralized or neutralized virus and fractionating cells at intervals. Radioactivity was collected as before using Whatman DE81 filters. This procedure provides a measure of the early stages of infection, including uncoating and transport to the nucleus of the uncoated virion RNA (25).

Proteinase K Removal of 35P-labeled Virus Attached to the Cell Surface. Preliminary experiments showed that incubation with 2 μg/ml proteinase K (sp act, 20 Anson units/g; BDH Pharmaceuticals Ltd.) for 60 min at 4°C, removed 85% of the 125I-labeled concanavalin A bound to BHK-21 cells. The same concentration of proteinase K also removed infectious virus from BHK-21 cells, as measured by a reduction in the number of plaques compared with nontreated cells. To determine the percentage of virus attached to BHK-21 cells, 35P-labeled virus-antibody mixtures were incubated with monolayers for 2 h, and cells were then rinsed three times with cold PBS and incubated at 4°C with 500 μl proteinase K. After 60 min, the enzyme solution was removed and pooled with two subsequent washes of 250 μl PBS. The cells were then fractionated into nuclei and cytoplasm and radioactivity determined as before.

Results

Preliminary Detection and Characterization of Antiviral Activity in Bile. Bile concentrate from immunized rats had a titer of 1,800 HI units/ml, which was unaffected by the removal of IgG by protein A-Sepharose chromatography. The presence of IgA was confirmed by double immunodiffusion in agar with anti-rat IgA. No IgM was detected by immunodiffusion. Bile concentrate neutralized 98.5% of 10^7 PFU FPV/R (H7N1) but failed to neutralize another strain of...
influenza virus, A/WSN (H1N1). Bile concentrate from nonimmunized rats had no antiviral activity.

Our previous studies (11) showed that FPV/R attaches to BHK-21 cells at 4°C and that the rate of attachment was unaffected by neutralization with polyclonal or monoclonal IgG directed to the HA, even in saturating amounts (unpublished data). However, virus that was neutralized with immune bile concentrate did not attach in significant amounts to BHK-21 cells (Fig. 1). To be certain which constituent of the crude bile concentrate was responsible for the neutralization, we purified scIgA as described below.

**Purification of scIgA and Preparation of Monomeric IgA.** Purified scIgA obtained using an anti-α chain affinity column retained antiviral activity without loss. By nonreducing PAGE (Fig. 2), scIgA was shown to consist of a single protein species (Fig. 2 b) of ~400,000 mol wt, as expected (26). By differential reduction with 0.01 M BME, scIgA was separated into its major components, monomeric IgA (160,000 mol wt) and the secretory component (70,000 mol wt) (Fig. 2c). Monomeric IgA was isolated by using an affinity column to which rat anti-α chain antibody was coupled (Fig. 2a). 94 μg of scIgA yielded 46.8 μg of IgA. The HI titer per microgram of IgA was never <80% of the titer of the scIgA from which it was derived. J chain (15,000 mol wt; 26) was not detected on the gels. The neutralization activities of bile concentrate, scIgA, and IgA are shown in Table I. All failed to neutralize WSN.

**Mechanism of Neutralization by scIgA.** The attachment of virus neutralized with purified scIgA is compared in Fig. 3 with virus neutralized with IgG. Preimmune scIgA did not restrict the attachment of virus to the BHK-21 cell monolayers, but attachment of virus neutralized by scIgA (to 96.5%) remained at the background level. Virus neutralized by IgG, on the other hand, attached to cells normally, as previously reported (11).

**Mechanism of Neutralization by IgA.** To investigate whether the difference in

![Figure 1](image-url)"
TABLE I

Neutralization of FPV/R Influenza Virus by Bile Concentrate, Purified scIgA, and Monomeric IgA

| Immune samples          | Percentage neutralization* of: | FPV/R | WSN |
|-------------------------|---------------------------------|-------|-----|
|                         | $1 \times 10^7$ PFU              |       |     |
| Bile concentrate        | 98.5                            | 0     |     |
| Purified scIgA          | 96.5                            | 0     |     |
| Purified monomeric IgA  | 98.5                            | 0     |     |

* Relative to virus incubated with the appropriate control (bile concentrate, purified scIgA, and monomeric scIgA) obtained from nonimmunized rats. These gave negligible neutralization.

the ability of neutralized virus to attach to cells was related to the structure of the neutralizing Ig, we determined the attachment of virus neutralized with IgA monomers prepared from scIgA. Fig. 4 shows that monomeric IgA did not inhibit this process. Thus, inhibition of attachment by scIgA appears related to its dimeric structure and not to any intrinsic property of the Ig a heavy chain itself.

Association of Virion RNA from Neutralized Virus with the Nucleus. After attachment to the cell, virus is internalized and uncoated; the virus envelope components remain in the cytoplasm while the transcriptase complex of virion RNA and associated proteins enter the nucleus (24). By determining the proportion of cell-associated virion RNA that has entered nuclei, we have shown (10, 11) that these early stages of infection are unchanged after the virus has been neutralized with IgG. Similar experiments on the small fraction of virus neutralized by scIgA that attaches to cells (Fig. 5) show that virion RNA remained in
FIGURE 3. Attachment of nonneutralized $^{32}$P-labeled FPV/R and virus neutralized with sIgA to BHK-21 cell monolayers. Virus was first incubated for 1 h at 25°C with neutralizing sIgA (nSIgA), neutralizing IgG (nlgG), preimmune sIgA (pSIgA), or preimmune IgG (pIgG). Immune sIgA and IgG neutralized virus by 96.5 and 99.5%, respectively. The subsequent procedure is described in Fig. 1, except that each 100 μl inoculated onto the cell sheet contained initially $1.2 \times 10^7$ PFU, 36 HAU, and $1.2 \times 10^5$ cpm.

FIGURE 4. Attachment of nonneutralized $^{32}$P-labeled FPV/R and virus neutralized with monomeric IgA to BHK-21 cell monolayers. Virus was first incubated for 1 h at 25°C with neutralizing IgA (nIgA), neutralizing IgG (nlgG), preimmune IgA (pIgA), or preimmune IgG (pIgG). Immune IgA and IgG neutralized virus by 98.5 and 99.9%, respectively. The subsequent procedure is described in Fig. 1, except that each 100 μl inoculated onto the cell sheets contained initially $1.3 \times 10^7$ PFU, 47 HAU, and $2 \times 10^5$ cpm.

the cytoplasmic fraction whereas that from virus neutralized by IgG entered the nucleus, as previously described. It was therefore of particular interest to determine if monomeric IgA, which had no effect on attachment of neutralized virus (see above), prevented internalization like sIgA or behaved like IgG. Fig. 6 shows that RNA from virus neutralized by monomeric IgA accumulates in the nucleus at the same rate as RNA from nonneutralized virus.

*Attachment of Nonneutralized and Neutralized $^{32}$P-labeled FPV/R to BHK-21 Cell Monolayers at 25 and 37°C.* Although it is experimentally convenient to examine the early stages of infection at 4°C (since this temperature prevents virus macromolecular synthesis), it is possible that virus may behave differently at
NEUTRALIZATION OF INFLUENZA VIRUS BY sIgA, IgA, AND IgG

FIGURE 5. Association of nonneutralized and neutralized 32P-labeled FPV/R with cytoplasmic and nuclear fractions of BHK cells. Virus was neutralized with neutralizing sIgA (nslgA) or neutralizing IgG (nlgG) and inoculated as previously described in Fig. 1. nslgA and nlgG neutralized virus by 96.5 and 99.5%, respectively. Each 100 μl inoculated contained initially 1.2 x 10^7 PFU, 36 HAU, and 1.2 x 10^5 cpm. At the intervals indicated nuclear and cytoplasmic fractions were prepared.

FIGURE 6. Kinetics of migration of virion RNA from cytoplasm to nucleus in cells inoculated with virus neutralized by monomeric IgA and nonneutralized virus. Virus was neutralized and inoculated as previously described in Fig. 1. Immune IgA neutralized virus by 98.5%. Each 100 μl inoculated initially contained 1.3 x 10^7 PFU, 47 HAU, and 1.2 x 10^5 cpm. At the intervals indicated nuclear and cytoplasmic fractions were prepared.

physiological temperatures. Fig. 7 shows that, although sIgA almost completely prevented attachment of neutralized virus at 4°C, attachment at 25 and 37°C was merely reduced by half. However, unlike virus neutralized by IgA (see above) or IgG (12), the majority of virion RNA remained in the cytoplasmic fraction (Fig. 8) and <5% RNA was taken up into the nuclei.
Because the cytoplasmic fraction includes the plasma membrane, we could not tell if virus was attached to the outside of the cell or had been internalized. The question was resolved by treating with proteinase K cells inoculated with virus neutralized by scIgA. This released the majority of virus attaching at 25 and 37°C (58 and 68.2%, respectively), demonstrating that the virus had not been internalized but remained attached to the outside of the cell. Treatment of cells
that were inoculated with virus treated with preimmune scIgA released no more than 8% cell-associated virus (Fig. 9).

Discussion

Studies of local immunity directed against infections of mucosal surfaces have been hindered in the past because of the problem of obtaining sufficient amounts of secretory IgA, the predominant Ig in external body fluids (27, 28). We demonstrate here that this can be overcome by using bile from appropriately immunized rats. Such antibody was neutralizing in both the crude and purified form, and could discriminate between different strains of type A influenza virus (Table I). scIgA comprises ~70% of the total Ig in bile and about half the protein content (29). As a source of scIgA, bile has certain advantages over lung or nasal washings, which produce scIgA in a diluted form (16, 30), often contaminated with various cellular products, including other Igs and lymphokines (15, 16, 30). Further, immunochemical analysis has established (29, 30, 31) that scIgA from bile and respiratory secretions is identical. It has also been shown (32) that scIgA, stimulated by immunization of the gastrointestinal tract of human volunteers with inactivated influenza virus, is established in respiratory secretions.

In this study our interest has been to determine the mechanism of neutralization by scIgA, since, in the respiratory tract, neutralizing scIgA is a major determinant of resistance to reinfection with influenza and other respiratory viruses (15, 16, 33, 34). We also sought to compare this mechanism with the mechanism of neutralization by IgG. Previous work (11) showed that FPV/R neutralized by IgG attaches to and penetrates BHK-21 cells, at temperatures from 4 to 37°C, at the same rate as nonneutralized virus. In contrast, the results of this study show that neutralizing scIgA almost completely inhibits attachment
at 4°C but acts far less effectively at 25 and 37°C. Despite attaching to cells, however, virus that was neutralized by scIgA was not internalized. Thus, neutralization by scIgA and IgG are qualitatively different phenomena. The nature of this difference was investigated by preparing IgA monomers from scIgA. IgA neutralized with comparable efficiency but did not affect attachment and penetration, which were indistinguishable from those effected by virus incubated with nonneutralizing IgA.

The blocking of attachment of influenza virus by scIgA recalls earlier work with bacterial systems, where the attachment to cells of Vibrio cholerae (35), Streptococcus sp. (36), and Escherichia coli (37) were inhibited by scIgA. However, the parallel is less than perfect, since half the neutralized influenza virus did attach to cells; the reason why this virus is noninfectious is yet unknown.

It seems likely that scIgA acts sterically to prevent attachment of neutralized virus to cells at 4°C. Increased thermal agitation is probably the reason why virus that was neutralized by scIgA attached to cells more efficiently at higher temperatures but, since this did not lead to internalization of the virus, such attachment may not have been correctly oriented. To expand this interpretation, we plan to determine the conformation of scIgA when it is bound to virus. Since scIgA comprises two Ig units linked by the SC and the J chain (28), scIgA may bind either in a “hair pin” conformation, with both Ig units attached to the virus, or through only one of the units. The latter interpretation would approximately double the steric hindrance between cell and virus; it is supported by the failure of monomeric IgA to prevent attachment. However, the SC may also contribute to steric hindrance.

Since we detected no difference in the early stages of infection between virus neutralized by IgA or by IgG and infectious virus, it may be that IgA and IgG have the same mechanism of neutralization. Previously (12, 13), we suggested that neutralization by IgG results from an effect on virion transcriptase, arising from conformational changes triggered by the binding of neutralizing antibody to the virus hemagglutinin, that is conveyed across the envelope to the transcriptase complex.

Summary

We have found that bile is a useful source of secretory IgA (scIgA) which can specifically neutralize influenza virus infectivity. Using purified scIgA, we compared the mechanism of neutralization with that mediated by IgA monomers (prepared from scIgA by differential reduction) and IgG. At 4°C, scIgA prevented the attachment of neutralized virus, while neither monomeric IgA nor IgG had any affect on this process or on the subsequent stages of infection by which virion RNA accumulates in nuclei. At 25 and 37°C, scIgA permitted the attachment of approximately half the neutralized virus, but the virus was not internalized. Clearly, the neutralization depends on the character of the antibody used. scIgA may act by steric hindrance (with attachment or penetration, depending on temperature), whereas IgA and IgG neutralize infectivity at a stage subsequent to accumulation of the virus genome in the nucleus.

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