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# Intracellular neutralization of virus by immunoglobulin A antibodies

(mucosal immunity/host defense)

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ABSTRACT **IgA is thought to neutralize viruses at the epithelial surface of mucous membranes by preventing their attachment. Since IgA, a polymeric immunoglobulin, is transported through the lining of epithelial cells by the polymeric-immunoglobulin receptor and since viruses are obligate intracellular parasites, we hypothesized that IgA antibodies may also interfere with viral replication by binding to newly synthesized viral proteins within infected cells. Polarized monolayers of Madin–Darby canine kidney epithelial cells expressing the polymeric-immunoglobulin receptor were infected on the apical surface with Sendai virus. Anti-Sendai virus IgA monoclonal antibody delivered from the basolateral surface colocalized with viral protein within the cell, as documented by immunofluorescence. More importantly, anti-viral IgA reduced virus titers >1000-fold ($P < 0.0001$) in apical supernatants and >10-fold ($P < 0.0001$) in cell lysates from monolayers treated with anti-viral IgA compared with those treated with either anti-viral IgG or an irrelevant IgA monoclonal antibody. We believe that the differences in viral titers between cell layers treated with specific IgA, which enters the epithelial cell by binding to the polymeric-immunoglobulin receptor, and those treated with specific IgG, which does not enter the cells, or irrelevant IgA indicate that specific intracellular IgA antibodies can inhibit viral replication. Thus, in addition to the classical role of humoral antibodies in extracellular defense, IgA antibody may be able to neutralize microbial pathogens intracellularly, giving IgA a role in host defense that has traditionally been reserved for cell-mediated immunity.**

Secretory IgA, the predominant effector molecule of the mucosal immune system, forms the first line of immunologic defense against many microbial pathogens (1, 2). For example, resistance to viral infection correlates with the presence of specific IgA antibody in mucosal secretions (3, 4). Renegar and Small (5, 6) recently demonstrated that immunity to influenza virus could be abrogated by intranasal instillation of antibody to IgA but not by anti-IgG or anti-IgM, again consistent with IgA being the major mediator of virus neutralization.

The central role of secretory IgA, a polymeric immunoglobulin, in protecting the mucosal surface against viral infection relies on the existence of an active transepithelial transport mechanism for polymeric IgA that is not available to monomeric immunoglobulins like IgG (7, 8). Transport of IgA into mucosal secretions is mediated by the polymeric-immunoglobulin receptor, which is widely expressed on mucous membrane epithelial cells, including those of the respiratory tract (9, 10). Furthermore, the overwhelming majority of plasma cells in mucous membranes produces IgA. Present understanding of the mechanism(s) by which IgA neutralizes virus at an epithelial surface is, however, incomplete. Traditionally, IgA has been thought to neutralize extracellularly by coating virus, thus preventing attachment to the epithelium and internalization of virions into the cell (11). Recently, Kaetzel *et al.* (12) proposed another mechanism by which IgA may protect mucous membranes by demonstrating *in vitro* that complexes of IgA and antigen can be transported across epithelial cells from basal to apical surface. Hence, foreign substances that have breached the mucosal surface could be eliminated from the body by IgA-mediated transport back through the epithelium, a kind of excretory function for IgA.

Since IgA is transported through epithelial cells by the polymeric-immunoglobulin receptor, the external domain of which has been referred to as the secretory component, and since viruses are obligate intracellular parasites that replicate within cells, we considered a third possible host-defense function for IgA—namely, IgA may be able to complex with newly synthesized viral proteins within the infected epithelial cell, thereby preventing viral assembly and release. In this manner, IgA could abort viral infection.

Many viruses attach to the apical surface of epithelial cells and are internalized (13). Sendai virus, which contains negative-sense RNA, is a natural respiratory pathogen of rodents and is related to the parainfluenza viruses that infect humans. Once inside the cell, viral genetic material is transcribed and translated into constituent viral proteins on cytoplasmic ribosomes. The viral surface glycoproteins—namely, the hemagglutinin–neuraminadase (HN) and fusion (F) proteins—are transported through the endoplasmic reticulum and Golgi apparatus (13–15) and subsequently are inserted into the host cell's apical plasma membrane. If the transcytotic pathway of IgA were to intersect with the transport pathway of newly synthesized viral glycoproteins, viral-specific IgA could complex with viral proteins and inhibit virion assembly. We demonstrate here that during transcytosis, IgA antibodies colocalize with intracellular HN protein. Furthermore, cells treated with anti-HN IgA monoclonal antibody (mAb) produce much less virus than do cells treated with irrelevant IgA or with anti-HN IgG. Hence, we propose a new antiviral defense mechanism: intracellular IgA-mediated virus neutralization.

## MATERIALS AND METHODS

**Polarized Epithelial Cell Monolayers.** Madin–Darby canine kidney (MDCK) cells stably transfected with cDNA encoding

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Abbreviations: pIgR$^+$ and pIgR$^-$, phenotypes for expression of the polymeric-immunoglobulin receptor; MDCK, Madin–Darby canine kidney; HN, hemagglutinin–neuraminidase glycoprotein; mAb, monoclonal antibody; pfu, plaque-forming unit(s).

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the rabbit polymeric-immunoglobulin receptor (obtained from Keith Mostov, University of California, San Francisco) (16, 17) were cultured on nitrocellulose filters in microwell chambers (Millicell; Millipore) (12).

**Production and Transcytosis of Anti-Sendai HN mAbs.** IgA and IgG mAbs were produced and purified as described (18, 19). Ascites containing anti-viral mAbs IgA 380, IgA 37, or IgG 20 had similar plaque reduction–neutralization ($10^3$) and ELISA ($10^6$) titers, which are similar to serum IgG ELISA titers in infected convalescent mice.

Cellular uptake and transcytosis of anti-Sendai HN polymeric IgA was done as described (12). Briefly, MDCK cells expressing the polymeric-immunoglobulin receptor (pIgR$^+$ MDCK) or wild-type MDCK cells (pIgR$^-$ MDCK) were grown to confluence. $^{125}$I-labeled polymeric IgA or IgG (1.5 $\mu$g in 1.5 ml of HEPES-buffered minimal essential medium) was added to the lower compartment, and chambers were incubated at 0°C for 3 hr to allow binding of antibody to the basolateral cell surface. Monolayers were washed at 0°C and then warmed to 37°C, after which samples of apical medium (upper compartment) were collected at intervals. To determine total antibody transported, 200-$\mu$l aliquots of apical medium were precipitated with 10% $CCl_3COOH$, and radioactivity was measured.

**Infection and Intracellular Colocalization of IgA and HN Viral Protein.** Confluent pIgR$^+$ MDCK cell monolayers on nitrocellulose filters were infected with Sendai virus [1 plaque-forming unit (pfu) per cell] via the apical surface for 60 min at 37°C. After 8 hr, a 1:100 dilution of ascites containing anti-HN IgA was added to the lower compartment. Twenty-four hours after the addition of antibody, cells were detached with trypsin, cytocentrifuged onto glass slides, and fixed with acetone. Two-color immunofluorescence was used to detect HN glycoprotein and IgA simultaneously. The slides were incubated with biotin-labeled murine anti-HN IgG mAb (directed against a different epitope from the anti-HN antibody added to the cells in culture) in 1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 hr at room temperature. After the slides were washed in PBS, intracellular IgA was detected with fluorescein-labeled goat anti-murine IgA, and intracellular HN was detected with Texas red-conjugated streptavidin.

**Intracellular Neutralization of Virus by IgA.** Confluent pIgR$^+$ MDCK cells were infected with Sendai virus as above. Six hours later, a 1:10 dilution of ascites containing anti-Sendai HN IgA mAb 380, anti-Sendai HN IgG mAb 20, or MOPC-315, an irrelevant murine IgA, was added to the lower chamber. After an additional 4 hr, the basal surface of the cell layer was washed. In some experiments, both the apical and basolateral surfaces of the monolayers were treated with 0.25% trypsin/0.02% EDTA for 20 min at 37°C to remove any virus or antibody bound to the cell surface or filter. Preliminary experiments showed that this concentration of trypsin removed virtually all noninternalized virus. Soybean trypsin inhibitor (Sigma) (84 $\mu$g/ml of PBS) was added, and the monolayers were washed with PBS. Monolayers were then incubated for an additional 24 hr at 37°C, at which time the apical supernatants were removed. Cells were scraped off the filter into PBS and disrupted by freeze/thaw cycles. Cellular debris was removed from the lysate by centrifugation. The apical supernatants and cell lysates were tested for virus by plaque assay (20) in which samples were pretreated with 5 $\mu$g of trypsin to activate virus.

In some experiments, "conditioned" medium was produced by placing ascites containing anti-HN IgA mAb 380 below the uninfected monolayers and, after 4 hr, collecting the apical supernatants. Neutralization experiments were then performed as above with an added control group of infected monolayers treated with conditioned medium in the upper compartment.

**Statistical Analysis.** Comparisons among groups in each experiment were made by one-way analysis of variance with Fisher's protected *t* test.

## RESULTS

**Transport of Anti-Sendai IgA Across a Polarized Epithelial Cell Monolayer.** Polymeric IgA mAbs against Sendai HN were selectively transported across a monolayer of pIgR$^+$ MDCK cells, whereas in a separate experiment, anti-Sendai HN IgG mAbs were not transported (Fig. 1). No transport was seen in pIgR$^-$ MDCK cells, demonstrating that transport of pIgA is receptor-mediated. There was no significant difference in the transport of IgG across pIgR$^+$ or pIgR$^-$ MDCK monolayers, in accord with its inability to bind to the polymeric-immunoglobulin receptor (21, 22). In addition, apical culture supernatants from polarized monolayers incubated 4 hr with a 1:10 dilution of IgA ascites had an anti-viral ELISA titer of $\approx 10^{1.5}$, which is comparable to IgA antibody titers in mucosal secretions from immunized mice (23). No ELISA reactivity was detectable if monolayers were incubated with an IgG mAb.

**Intracellular Colocalization of IgA Antibody and HN Viral Protein.** By double immunofluorescence, anti-Sendai HN IgA colocalized with Sendai HN viral protein (Fig. 2). With the appropriate filters, compartments containing IgA appear green, those containing HN viral protein appear red, and those containing colocalized antibody and viral protein appear yellow. Anti-HN IgA antibody and HN viral protein colocalized to the same intracellular compartment (Fig. 2*D*), indicating that during transcytosis, IgA antibody contacted newly synthesized viral HN protein. In fact, every cell that stained for intracellular IgA also demonstrated HN protein in the same location. It is noteworthy that in uninfected control cultures, although polymeric IgA was transported (Fig. 1), it was not detected by immunofluorescence (Fig. 2*A*). Only in infected cells making viral protein was it possible to document significant amounts of accumulated virus-specific IgA by immunofluorescence; moreover, this IgA always colocalized with viral HN protein (Fig. 2 *B*, *C*, and *D*). In contrast, infected cells treated with MOPC-315, an irrelevant IgA, did

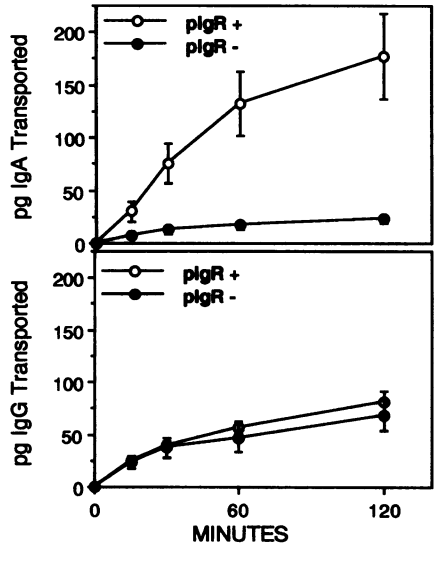


FIG. 1. Murine IgA, but not IgG, is transcytosed across pIgR$^+$ MDCK cell monolayers. $^{125}$I-labeled dimeric IgA (*Upper*) or $^{125}$I-labeled IgG (*Lower*) was presented at 0°C to the basolateral surface of pIgR$^+$ and pIgR$^-$ MDCK cell monolayers. Cultures were warmed to 37°C for various times, and transcytosed IgA or IgG was detected by measuring $CCl_3COOH$-precipitable radioactivity in the apical compartment.

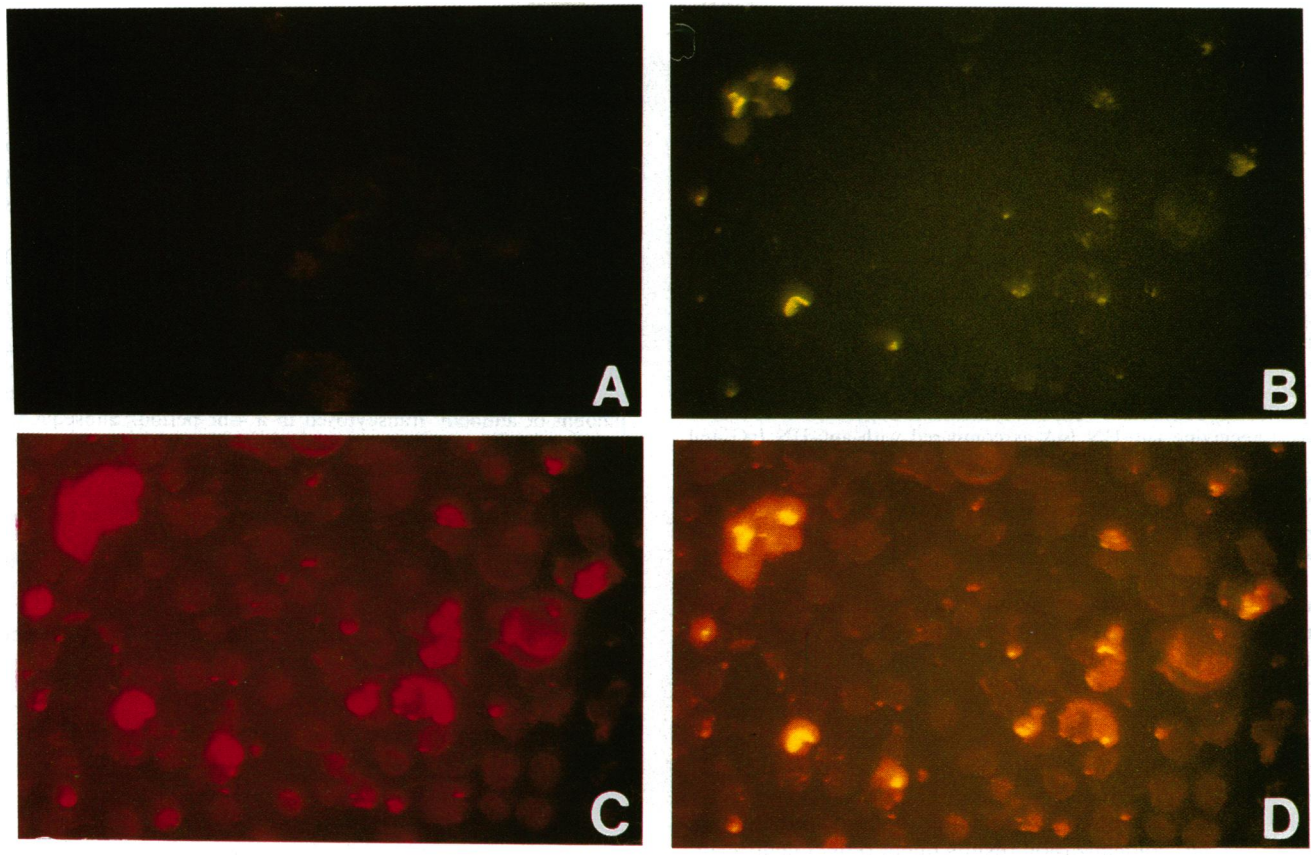


FIG. 2. Intracellular colocalization of anti-HN IgA and HN protein in pIgR$^+$ Sendai virus-infected MDCK cells. Anti-HN IgA was added to the basal surface of infected and uninfected cell monolayers. After 24 hr, the cells were trypsinized, attached to glass slides, and stained by two-color immunofluorescence for simultaneous detection of viral HN protein and IgA. Cellular compartments containing IgA appear green (*B*; single-color transmission), those containing HN protein appear red (*C*; single-color transmission), while those containing colocalized IgA antibody and HN protein appear yellow (*D*; two-color transmission). Uninfected cell monolayer controls (*A*) do not show either HN protein or anti-HN IgA. (×340.)

not demonstrate significant amounts of intracellular IgA by immunofluorescence (not shown), indicating that accumulation of IgA antibody is not attributable to a nonspecific interruption of IgA transport in infected cells. Collectively, these results show that in virus-infected cells, transport of specific IgA but not irrelevant IgA is impeded, resulting in its intracellular accumulation, whereas in uninfected cells, IgA rapidly moves through the cell and is undetectable by immunofluorescence.

**Intracellular Neutralization of Virus by IgA Antibody.** Experiments were performed to determine whether IgA antibody can neutralize virus within epithelial cells. In the first experiment, mean virus titers were reduced by >100 orders of magnitude in the apical supernatants and >1 order of magnitude in the cell lysates of monolayers treated with anti-HN IgA mAb 380 (Table 1). This result suggests that specific IgA antibody interacts with viral protein within the cell and, consequently, limits viral replication and egress. Conversely, cell monolayers treated with anti-HN IgG 20 or irrelevant IgA produced virus titers comparable to nonantibody-exposed controls. Since IgA, but not IgG, is capable of entering the epithelial cell by binding to the polymeric-immunoglobulin receptor (Fig. 1) and since both apical supernatant and cell lysate viral titers were reduced in the IgA antibody-treated cells compared with control cells, these data indicate that viral-specific IgA, during its transport across the epithelial cell, can inhibit viral replication. Similar results were obtained with two additional IgA anti-HN mAbs, 37 and 390 (18), demonstrating that IgA-mediated neutralization is not unique to one particular antibody (data not shown).

Additional experiments were performed to confirm that virus neutralization occurred within the cell and not extracellularly. After infection with virus and subsequent incubation with anti-HN IgA mAb 380, pIgR$^+$ MDCK cells were treated with trypsin to remove uninternalized virus and antibody. Mean virus titers in apical supernatants and cell lysates from such trypsin-treated cells were measured at 24 hr (Table 2). Virus titers were reduced ≈10 orders of magnitude ($P < 0.01$) in apical supernatants and 1 order of magnitude ($P \leq 0.01$) in lysates from cells treated with

Table 1. Treatment of Sendai virus-infected pIgR$^+$ MDCK cells with anti-HN IgA reduces intracellular and extracellular virus

| Antibody | Mean virus titer,* $\log_{10}$ pfu/ml | |
|---|---|---|
| | Apical supernatant | Cell lysate |
| Anti-HN IgA | 3.2 ± 0.6 | 4.3 ± 0.6 |
| Anti-HN IgG | 6.4 ± 0.1 | 5.6 ± 0.1 |
| Irrelevant IgA | 6.2 ± 0.1 | 5.3 ± 0.1 |

Anti-HN IgA (mAb 380) or IgG (mAb 20) or irrelevant IgA (MOPC-315) was added for 4 hr to the compartment below the infected monolayers. After 24 hr, virus titers were determined in both apical supernatants and cell lysates. A significant reduction in mean virus titers in both apical supernatants and disrupted cell lysates of pIgR$^+$ MDCK cell monolayers treated with anti-HN IgA as compared with anti-HN IgG or irrelevant IgA indicates intracellular neutralization of virus by specific IgA antibody.

*Virus titers (pfu/ml) in apical supernatants and cell lysates from cells exposed to anti-HN IgA are significantly reduced ($P \leq 0.0001$) compared with those from cells exposed to anti-HN IgG or irrelevant IgA. Data are means ± SD ($n = 4$).

Table 2. Neutralization of Sendai virus by anti-HN IgA occurs intracellularly

| Antibody | Mean virus titer,* $\log_{10}$ pfu/ml | |
|---|---|---|
| | Apical supernatant | Cell lysate |
| Anti-HN IgA | 3.9 | 4.5 |
| Anti-HN IgG | 5.7 | 5.4 |
| Irrelevant IgA | 5.7 | 5.3 |

Anti-HN IgA (mAb 380) or IgG (mAb 20) or irrelevant IgA (MOPC-315) was added to the compartment below the infected monolayers. After 4 hr, the apical and basal surfaces of the monolayers were treated with trypsin to remove surface-adsorbed antibody and virus. After a 24 hr incubation, virus titers were determined in both apical supernatants and cell lysates. Despite treatment of pIgR$^+$ MDCK cell monolayers with trypsin, mean virus titers were significantly reduced in both cell lysates and supernatants of monolayers receiving anti-HN IgA as compared with anti-HN IgG and MOPC-315 groups.

*Virus titers (pfu/ml) of apical supernatant and cell lysate in the anti-HN IgA 380 group are significantly reduced ($P = 0.01$) compared with anti-HN IgG 20 and irrelevant IgA groups. Data are means of two individual measurements.

anti-HN IgA mAb 380 compared with cells treated with either anti-HN IgG mAb 20 or irrelevant IgA. Since residual surface-adsorbed IgA and virus had been removed by trypsin, these results support the conclusion that IgA antibody interfered with viral replication within the cell. Since reduction of virus titers was observed in both cell supernatants and cell lysates, it is unlikely that neutralization occurred by antibody complexing completed virions after cell lysis.

To eliminate the possibility that the observed reduction in virus titer and colocalization results might reflect neutralization of newly synthesized and released virus in the apical supernatant by transported IgA with subsequent reendocytosis of the immune complexes into the cell, experiments were performed with "conditioned media." Conditioned media were produced by aspirating the apical supernatants from uninfected pIgR$^+$ MDCK monolayers that had transcytosed anti-HN IgA mAb 380 for 4 hr. Infected monolayers were then treated with anti-HN IgA mAb 380 or IgG mAb 20 in the basal compartment and/or conditioned medium in the apical compartment. The addition of conditioned medium containing specific IgA allowed a direct test of the possibility that virus was being neutralized after release at the apical surface. Since 10–12 hr are required for Sendai virus to complete one round of replication, antibody present in the apical supernatant at an earlier time would not be able to effect extracellular virus neutralization. Thus, infected cell layers were treated with conditioned media during different time intervals after infection. The experimental groups included: monolayers treated on the basal surface with anti-HN IgA (from 6 to 10 hr after infection) and subsequently with conditioned medium in the apical compartment (from 10 to 24 hr after infection) and monolayers treated apically with conditioned medium from 10 to 24 or from 6 to 10 hr after infection. One day after infection, apical supernatants and cell lysates were assayed for virus (Table 3).

Virus titers in supernatants and lysates of monolayers treated with anti-HN IgA via their basal surface (groups A and C) were reduced up to 100 orders of magnitude compared with monolayers treated with anti-HN IgG (group B) or conditioned medium alone (groups D and E). There were no significant differences in virus titers in both supernatants and cell lysates among the monolayers treated with anti-HN IgG (group B), conditioned medium only (groups D and E), or no antibody (group F). Therefore, only treating monolayers with IgA antibody from the basolateral surface for 4 hr caused a significant drop in virus titer; IgA antibody in conditioned medium applied to the apical surface was at best minimally

Table 3. Anti-HN IgA antibody neutralizes virus during transcytosis

| Group | Treatment | Mean virus titer,* $\log_{10}$ pfu/ml | |
|---|---|---|---|
| | | Apical supernatant | Cell lysate |
| A | Anti-HN IgA | 3.9 ± 0.8† | 1.7 ± 0.7† |
| B | Anti-HN IgG | 6.4 ± 0.0‡ | 4.6 ± 0.3‡ |
| C | Anti-HN IgA + CM (10–24 hr) | 3.5 ± 1.3† | 2.4 ± 0.8† |
| D | CM (10–24 hr) | 5.4 ± 0.2‡ | 3.7 ± 1.0‡ |
| E | CM (6–10 hr) | 6.0 ± 0.1‡ | 4.6 ± 0.2‡ |
| F | No antibody | 6.2 ± 0.2‡ | 4.6 ± 0.1‡ |

Conditioned medium (CM) was produced by adding anti-HN IgA mAb 380 below uninfected monolayers and, after 4 hr, collecting the apical supernatant. Hence, conditioned medium contained the amount of antibody transcytosed in a 4-hr period. Subsequently, infected monolayers were treated with anti-HN IgA (mAb 380) or IgG (mAb 20) via the basal surface and/or conditioned media via the apical surface for various time intervals after infection. Virus titers in supernatants and cell lysates were determined 24 hr after infection.

*Data are means ± SD ($n = 4$).

†Mean viral titers of groups A and C were significantly different from the other groups ($P \leq 0.01$) but were not significantly different from each other.

‡Mean viral titers in groups B, D, E, and F were not significantly different from one another.

effective in reducing virus titers. Hence, neutralization of virus by IgA appeared to be occurring within the cell and not during budding from the cell surface or after release.

## DISCUSSION

Secretory IgA plays a critical role in the defense of mucous membranes against foreign antigens and pathogens (1, 2). In keeping with the large area of the mucosal surfaces and the tissue density of mucosal IgA plasma cells, the quantity of IgA synthesized exceeds that of all of the body's other immunoglobulin classes combined (2, 24, 25). In addition to IgA's traditional barrier function and the potential to complex with and excrete antigens that have penetrated the mucosa (12), we have now shown that IgA can neutralize viruses (and presumably other pathogens) intracellularly.

The results of the conditioned media experiments along with the colocalization data tend to eliminate extracellular neutralization of virus by already transported IgA as the sole explanation for the reduction in virus titers seen with the anti-HN IgA-treated cells. Therefore, IgA appears capable of aborting a viral infection by binding to viral proteins within cells that bear the polymeric-immunoglobulin receptor. There are several stages in the viral life cycle during which this might occur: uncoating of the viral capsid, transcription and translation of the genome, posttranslational modification of viral proteins, and assembly and budding of progeny virions. We hypothesize that the virus life cycle is most vulnerable to interruption by intracellular IgA antibody during virion assembly, since the secretory and transcytotic pathways are most likely to intersect during viral protein maturation. All three mechanisms of antigen disposal by IgA (prevention of virus attachment by the formation of luminal immune complexes, transport of immune complexes from the lamina propria across the epithelium, and interruption of virus replication within the epithelial cell) could operate concurrently to maximize efficiency and limit the spread of virus.

Consistent with the proposed intracellular mechanism for virus neutralization, other investigators have demonstrated that the endocytic and exocytic pathways may intersect with resultant mixing of endocytic and exocytic vesicles (reviewed in ref. 26). For example, the toxin ricin enters cells by endocytosis and inactivates ribosomes. Youle and Colom-

batti (27) found that hybridoma cells producing anti-ricin antibodies were resistant to the toxic effects of ricin; their results suggest that the exocytic pathway of antibody and the endocytic pathway through which ricin enters meet within the cell. In human hepatoma cells, Stoorvogel *et al.* (28) documented a common intracellular localization of endocytosed transferrin and secretory albumin in the trans-Golgi reticulum. In a similar manner, endocytosed IgA complexed with the polymeric-immunoglobulin receptor may colocalize with viral proteins in the exocytic pathway, possibly within the trans-Golgi reticulum or in a distinct compartment common to the exocytic and transcytotic pathways.

The newly conceived role for IgA proposed here would have greatest utility in defending the mucosa against intracellular pathogens, including viruses. This capability thus represents a mechanism for recovery from viral infections in addition to the traditional role played by cell-mediated immunity. Indeed, recent studies have demonstrated that mice infected with influenza virus are able to recover in the absence of cytotoxic T cells (29–31). As suggested by our results, a possible explanation is that virus-specific IgA antibody interrupts viral replication within respiratory epithelial cells and promotes virus clearance and recovery.

In conclusion, our studies suggest an additional means by which IgA protects mucosal surfaces. As it moves through the mucosal epithelium on its way to the external secretions, IgA appears able to interact with intracellular antigens. As opposed to cell-mediated cytotoxicity, IgA-mediated intracellular virus neutralization might not destroy the infected cell. Finally, since IgA is inherently less inflammatory than IgG (32), it can limit virus spread while minimizing damage to epithelial cells, hence preserving the integrity of the mucosal surface.

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