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Intracellular Neutralization of Influenza Virus by Immunoglobulin A Anti-Hemagglutinin Monoclonal Antibodies

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Traditionally, immunoglobulin A (IgA) was thought to neutralize virus by forming complexes with viral attachment proteins, blocking attachment of virions to host epithelial cells. Recently we have proposed an intracellular action for dimeric IgA, which is actively transported through epithelial cells by the polymeric immunoglobulin receptor (pIgR), in that it may be able to bind to newly synthesized viral proteins within the cell, preventing viral assembly. To this effect, we have previously demonstrated that IgA monoclonal antibodies against Sendai virus, a parainfluenza virus, colocalize with the viral hemagglutinin-neuraminidase protein within infected epithelial cells and reduce intracellular viral titers. Here we determine whether IgA can interact with influenza virus hemagglutinin (HA) protein within epithelial cells. Polarized monolayers of Madin-Darby canine kidney epithelial cells expressing the pIgR were infected on their apical surfaces with influenza virus A/Puerto Rico/8-Mount Sinai. Polymeric IgA anti-HA, but not IgG anti-HA, delivered to the basolateral surface colocalized with HA protein within the cell by immunofluorescence. Compared with those of controls, viral titers were reduced in the supernatants and cell lysates from monolayers treated with anti-HA IgA but not with anti-HA IgG. Furthermore, the addition of anti-IgA antibodies to supernatants did not interfere with the neutralizing activity of IgA placed in the basal chamber, indicating that IgA was acting within the cell and not in the extracellular medium to interrupt viral replication. Thus, these studies provide additional support for the concept that IgA can inhibit replication of microbial pathogens intracellularly.

The mucosal immune system and its predominant effector, secretory immunoglobulin A (IgA), provide the initial immunologic barriers against most pathogens that invade the body at a mucosal surface (9, 14). This is especially true for viruses, since resistance to infection has been strongly correlated with the presence of specific IgA antibody in mucosal secretions (10, 15). Consistent with IgA's role as the major mediator of virus neutralization at a mucosal surface, studies by Renegar and Small (18) recently demonstrated that resistance to influenza virus could be abrogated by intranasally instilling antibody to IgA but not to IgG or IgM.

The prominence of IgA as the major mucosal antibody results in part from active transepithelial transport of polymeric immunoglobulins like IgA that is unavailable to monomeric antibodies like IgG (1, 4, 5, 8, 21). Transport of IgA is mediated by the polymeric immunoglobulin receptor (pIgR), which is expressed on the basolateral surfaces of epithelial cells lining secretory mucosae (1, 4, 5, 8, 21). In addition, the majority of plasma cells in the lamina propria beneath epithelial surfaces are committed to IgA production. Despite its central role in defense of mucosal surfaces, our understanding of IgA's mode of neutralization of viruses remains incomplete.

Traditionally, neutralization of virus by IgA is thought to result from binding of antibody in mucosal secretions to virion attachment proteins, thereby preventing adherence to epithelial cells. Recent work by Armstrong and Dimmock suggests that the mechanism of neutralization may vary according to the number of extracellular immunoglobulin molecules per virion (2). At high concentrations, anti-hemagglutinin (HA) polymeric IgA inhibited attachment of influenza virions to tracheal epithelial monolayers. At lower concentrations of IgA, although attachment of virions occurred, fusion activity was inhibited and/or transcription of the influenza virus genome was prevented (2).

In addition to the functions displayed by antibody which has been transported into mucosal secretions, IgA has two other potential modes and sites of action in mucous membranes (7, 11, 12). First, IgA in the lamina propria beneath the epithelium may be able to complex with and transport antigens that have breached the mucosal barrier across epithelial cells (7, 12). In this manner, IgA could effectively eliminate from the body foreign substances which have penetrated the mucosa via an IgA-mediated transport shuttle back through the epithelium.

As another possible site of mucosal antibody action, IgA, as it is transported through the epithelial cell by the pIgR, may be able to interact with intracellular pathogens such as viruses, preventing replication (11, 12). As obligate intracellular parasites, many viruses gain access to the body at mucosal surfaces. After attachment and internalization of the virion at the apical surfaces of the lining epithelial cells, the viral genome is transcribed and translated into constituent viral proteins. If the transcytotic pathway of IgA should intersect with sites of viral protein synthesis or aggregation, specific IgA could complex with viral proteins and inhibit assembly of progeny virions. To this effect, we have demonstrated in a polarized epithelial monolayer system infected in vitro with Sendai virus that IgA anti-hemagglutinin-neuraminidase (HN) antibodies colocalize with HN viral protein within the cells and that monolayers treated with IgA anti-HN produce much less virus than do cells treated with irrelevant IgA or with IgG anti-HN (11). On the basis of our previous work (11), we hypothesize that during transcytosis IgA can abort a viral infection within the host epithelial cells. To extend our investigations and to document that this novel mechanism of neutralization is applicable to other kinds of viruses, we studied the ability of IgA anti-HA

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TABLE 1. Characteristics of anti-HA MAbs

Clone	ELISA titer (log_{10})	Neutralization titer (log_{10})	Hemagglutination inhibition titer (log_{10})	% Polymeric IgA
IgA 59	6.5	5.5	4.0	50
IgA 119	5.5	4.5	3.4	29
IgA 340	6.0	4.5	3.4	28
IgG 134	6.0	5.0	4.0	NA[a]
IgG 316	5.5	5.0	4.0	NA

[a] NA, not applicable.

monoclonal antibodies (MAbs) to interrupt influenza virus replication within epithelial cells. The life cycle of influenza virus is significantly different from that of Sendai virus and hence, may render influenza virus replication more or less susceptible to intracellular antibody-mediated disruption. In addition, experiments using an anti-IgA antibody control further confirm that IgA antibody is capable of acting within epithelial cells in addition to the extracellular secretions (see Table 3).

Virus culture. Influenza virus A/Puerto Rico/8-Mount Sinai was provided by Walter Gerhard, the Wistar Institute, Philadelphia, Pa. Influenza virus for immunization was grown in fertilized chicken eggs and concentrated and purified by differential centrifugation (3). Virus was quantified in a plaque assay on Madin-Darby canine kidney (MDCK) cells (3, 6) and, when desired, was inactivated with 0.05% β-propiolactone plus 6 min of UV irradiation 20 cm from a germicidal lamp.

Production and characterization of anti-influenza virus HA MAbs. IgA and IgG anti-influenza virus MAbs were produced by a mucosal immunization protocol as previously described (13). Briefly, BALB/c mice were immunized intragastrically four times over an 8-week period, the first three times with 0.5 mg of inactivated influenza virus plus 10 μg of cholera toxin (List Biological Laboratories, Inc., Campbell, Calif.). For the last immunization, cholera toxin was omitted and, in addition to intragastric virus administration, mice also received an intravenous booster immunization with 30 μg of inactivated virus. Three days later, mice were sacrificed and their splenic lymphocytes were hybridized to SP2/0 murine myeloma cells (13). Clones were screened for secretion of IgA and IgG anti-influenza virus antibody by an enzyme-linked immunosorbent assay (ELISA) (adapted from reference 13). After multiple subclonings, stable IgA secretors were injected intraperitoneally into pristane-primed BALB/c mice and the ascitic fluid was harvested and clarified. The antigenic specificities of the MAbs were confirmed by a Western immunoblotting technique (13). The biologic activities of the MAbs were characterized by determining an ELISA titer, neutralization titer, and hemagglutination inhibition activity (Table 1) (6).

Intracellular colocalization of IgA and HA viral protein. MDCK cells stably transfected with cDNA encoding the rabbit pIgR (obtained from Keith Mostov, University of California, San Francisco) (16) were cultured on nitrocellulose filters in microwell chambers (Millicell; Millipore, Bedford, Mass.) (11). Confluent $pIgR^+$ MDCK cell monolayer filters were infected with influenza virus (1 PFU per cell) via the apical surface for 60 min at 37°C. After 8 h, a 1:100 dilution of ascites containing equivalent ELISA titers of either IgA or IgG anti-HA MAbs was added to the lower compartment. Twenty-four hours after the addition of antibody, cells were detached with trypsin (0.25% in 0.02% EDTA) (JRH Biosciences, Lenexa, Kans.), cytocentrifuged onto glass slides, and fixed with acetone. Two-color immunofluorescence was used to detect HA glycoprotein and IgA simultaneously. The slides were incubated with fluorescein-labeled goat anti-murine IgA or IgG (Southern Biotechnology Associates, Inc., Birmingham, Ala.) and after extensive washing with PBS, biotin-labeled murine IgG anti-HA MAb (directed against a different epitope from the anti-HA antibody added to the cells in culture) in 1% bovine serum albumin in phosphate-buffered saline (PBS) was added for 1 h at room temperature. After the slides were washed in PBS, HA protein was detected with Texas red-conjugated streptavidin (Fisher Biotech, Pittsburgh, Pa.).

IgA anti-influenza virus HA MAbs colocalized with HA viral proteins as documented by two-color immunofluorescence by which identical microscopic fields were viewed through separate filters that descriminated the appropriate wavelengths (Fig. 1). Compartments containing IgA antibodies were green, while those containing HA proteins were red. In double exposures, cellular sites in which both IgA anti-HA MAbs and HA proteins were present appeared yellow. These observations are consistent with the hypothesis that during epithelial transcytosis, specific IgA antibody can interact with newly synthesized viral HA protein. In contrast, infected monolayers treated with specific IgG did not demonstrate intracellular antibody, since IgG is not transported through the epithelium. Influenza virus-infected cells treated with irrelevant IgAs, including IgA anti-Sendai virus HN and IgA anti-dinitrophenol, did not stain for the presence of antibody (not shown), indicating that accumulation of intracellular antibody was due to combination with viral protein and not a result of nonspecific interference of IgA transport by the viral infection. In addition, uninfected monolayers treated with specific IgA did not demonstrate intracellular aggregation of antibody (not shown). Collectively, these studies document that in cells infected with virus, transport of specific IgA but not irrelevant IgA is impeded, resulting in intracellular accumulation only of specific IgA.

Intracellular neutralization of virus by IgA. Experiments were designed to document that IgA anti-HA MAbs could interact with intracellular HA proteins within infected epithelial cells, thereby reducing viral titers (Tables 2 and 3). Confluent MDCK cells expressing the pIgR were infected with influenza virus as described above. Six hours later, a 1:10 dilution of ascites containing equivalent ELISA titers of IgA anti-influenza virus HA MAbs, IgG anti-influenza virus HA MAbs, MOPC-315, an irrelevant murine IgA, or IgA anti-Sendai virus HN MAbs (clone 380) was added to the lower chamber. In some experiments, anti-murine IgA, in an amount that was predetermined to effectively inhibit specific IgA from binding to and neutralizing virus as documented in ELISA and plaque reduction assays, was added to the apical chamber of some groups. After an additional 4 h, the specific IgA was removed from the basal chamber and the basal surface of the cell layer was washed. Monolayers were then incubated for an additional 24 h at 37°C, at which time the apical supernatants were removed. Cells were scraped off the filters into PBS and disrupted by three successive 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 (3, 6, 11) in which samples were pretreated with 5 μg of trypsin (Gibco, Grand Island, N.Y.) to activate virus. Comparisons among groups in each experiment were made by one-way analysis of variance with Fisher's protected t test.

In the first experiment (Table 2), mean virus titers were significantly ($P < 0.01$) reduced by approximately 10-fold in both the supernatants and cell lysates of polarized epithelial monolayers treated with IgA anti-HA (clone 119) compared with those from monolayers receiving IgG anti-HA (clone 316) and virus controls. There was a slight reduction of virus titers

FIG. 1. Intracellular colocalization of IgA anti-HA and HA proteins in influenza virus-infected MDCK cells that express the pIgR. Identical fields were examined through separate filters that descriminate for the appropriate wavelengths. Single exposures for IgA (A) or IgG (D) reveal intracellular accumulation only of IgA. Single exposures for HA viral protein reveal comparable accumulations and successful infection of both cells treated with specific IgA (B) and IgG (E) in the basal medium. In double exposures (C and F), cellular sites containing antibody appear green, those containing HA protein but no antibody appear red, while those containing colocalized antibody and HA protein appear yellow. Note, as a result of intense staining of viral HA proteins, an orange cellular hue is apparent in the single exposure for IgG detection (D).

in the supernatant but not in the cell lysate of monolayers treated with IgG anti-HA compared with those of controls. Since there was no difference in virus titers of cell lysates from monolayers receiving IgG compared with those of virus controls, the reduction in titer in the supernatants may be a result of some neutralization by a small amount of IgG antibody, passively diffusing across the monolayers into the apical chamber.

Similar results were obtained with two additional IgA anti-HA MAbs (clones 59 and 340), demonstrating that IgA-mediated neutralization is not unique to one particular antibody (Table 2). In an additional experiment, in contrast to IgA anti-HA (clone 59), IgA anti-Sendai virus HN did not reduce influenza virus titers in either the apical supernatants or cell lysates when compared with those of virus controls (data not shown). This observation suggests that in order to prevent viral synthesis and assembly within the epithelium, polymeric IgA must be specific for viral protein.

In order to determine whether part of the observed reduction of virus titers could be due to neutralization of virus in the

TABLE 2. IgA anti-HA reduces influenza virus titers in MDCK cells

Antibody (clone no.)	Mean virus titer ($\log_{10}$)[a]	
	Supernatant	Cell lysate
Expt 1		
IgA anti-HA (119)	4.00 ± 0.18[b]	3.70 ± 0.16[b]
IgG anti-HA (316)	4.88 ± 0.16[c]	4.76 ± 0.10
Irrelevant IgA	5.33 ± 0.19	4.88 ± 0.14
No antibody	5.49 ± 0.28	4.70 ± 0.28
Expt 2		
IgA anti-HA (59)	4.29 ± 0.08[d]	2.97 ± 0.03[e]
IgA anti-HA (340)	4.77 ± 0.20[d]	3.62 ± 0.37
IgG anti-HA (134)	5.32 ± 0.11	3.57 ± 0.11
No antibody	5.31 ± 0.06	4.17 ± 0.14

[a] Mean represents four individual measurements ± standard deviation.
[b] $P \leq 0.001$ versus groups treated with IgG anti-HA, irrelevant IgA, or no antibody.
[c] $P \leq 0.05$ versus groups receiving irrelevant IgA or no antibody.
[d] $P \leq 0.001$ versus groups receiving IgG anti-HA or no antibody.
[e] $P \leq 0.01$ versus groups treated with anti-HA (clone 340), IgG anti-HA, or no antibody.

TABLE 3. Anti-IgA does not interfere with influenza virus neutralization by IgA anti-HA

Antibody (clone no.)	Anti-IgA	Mean virus titer ($\log_{10}$)[a]	
		Supernatant	Cell lysate
IgA anti-HA (59)	No	4.76 ± .19[b,c]	3.48 ± .08[c,d]
IgA anti-HA (59)	Yes	4.16 ± .05[b,e]	2.93 ± .36[b,e]
IgG anti-HA (316)	No	6.27 ± .36	4.02 ± .07
No antibody	No	6.18 ± .05	4.17 ± .13

[a] Mean represents four individual measurements ± standard deviation.
[b] $P \leq 0.0001$ versus monolayers treated with IgG.
[c] $P < 0.001$ versus monolayers receiving no antibody.
[d] $P \leq 0.01$ versus monolayers treated with IgG.
[e] $P < 0.0001$ versus monolayers receiving no antibody.

supernatant by transported antibody, goat anti-murine IgA was added to the apical chamber above the monolayer to complex with any transcytosed IgA anti-HA (Table 3). Ten times as much anti-IgA as IgA was needed to be able to compete with binding of virus by antibody in both a competitive ELISA and a plaque reduction assay (data not shown). Therefore, 10 times as much anti-IgA was added to the apical supernatant as IgA anti-HA was deposited in the basal chamber. As shown in Table 3, mean virus titers were significantly reduced up to 100-fold in the supernatants and cell lysates from monolayers treated with IgA anti-HA compared with those from monolayers receiving IgG anti-HA ($P < 0.0001$ to 0.01) or from virus controls ($P < 0.0001$ to 0.001). The addition of anti-IgA to the apical chamber did not alleviate the ability of specific IgA to interrupt viral replication when deposited in the basal chamber. These results confirm that IgA anti-HA introduced into the basal medium is interferring with virus synthesis and, hence, infectivity within the cells and that IgA is not neutralizing virus within the apical supernatant after transcytosis.

When one considers the large surface area of the mucous membranes, the critical role that secretory IgA plays in the body's defense against foreign antigens and pathogens becomes evident. The magnitude of IgA's role is suggested by the fact that the rate of synthesis of IgA by the body far exceeds that of all of the other immunoglobulin classes combined (9, 14). Although traditional thinking had limited mucosal IgA's immune activity to extracellular antigens in mucosal secretions, our work expands the territory of IgA's role in host defense to include intracellular pathogens in the special case of mucosal epithelia that are transporting IgA.

The results of the anti-IgA experiment (Table 3), along with the colocalization data, eliminate the possibility that the observed reduction in virus titers from monolayers treated with IgA anti-HA MAbs is a result of neutralization of virus by antibody transported into the apical medium. From these and previous studies (11), specific IgA thus appears capable of preventing viral replication by binding to viral proteins being synthesized within infected cells that express the pIgR and are capable of internalizing and transforming IgA.

While all IgA anti-HA MAb neutralized virus, some IgA MAb were more efficient than others at reducing virus titers within cell lysates and supernatants. Since only polymeric IgA binds to the pIgR and is transcytosed, the degree to which a specific IgA MAb reduces viral titers may be due in part to the relative ratio of polymeric versus monomeric antibody secreted by the hybridoma cells. However, the design of the current experiments provided for delivery of excess of antibody to the basolateral surface of the monolayer, such that the amount of polymeric IgA would not be rate limiting. Therefore, the percentage of polymeric IgA contained within specific MAb preparations is probably not sufficient to account for differences in the efficiency with which a given MAb interrupts viral replication. Another reason for this observation may be that certain epitopes on the HA protein to which a specific MAb binds are more critical to virus assembly and infectivity than others. Finally, the intrinsic affinity of a particular IgA antibody for the viral protein is another plausible explanation for the difference in neutralizing capacity of the various IgA anti-HA MAbs.

The in vivo significance and degree to which polymeric IgA fuctions within mucosal epithelial cells in a living creature remain to be elucidated. Although the local concentration of IgA at a mucosal surface has not been measured directly, during active viral infection a sufficient amount of viral specific antibody may be present in the lamina propria to efficiently interrupt viral synthesis within the epithelium. In fact, a proportionately smaller quantity of specific IgA working intracellularly may be more effective in reducing viral titers and controlling viral spread than a larger amount of extracellular antibody neutralizing released viral progeny. In reality, intracellular and extracellular IgA probably act synergistically to erradicate viral infection from a mucosal surface.

Mucosal surfaces such as those in the gastrointestinal and respiratory tracts form an interface between the external environment and the internal milieu of the body. The barrier function of a mucosal surface is vital to the health of an individual. The characteristics and functions of mucosal IgA appear ideally designed to maintain the integrity of the mucosal epithelium while defending the body against foreign pathogens and antigens. The ability of IgA to abort virus replication within epithelial cells provides a mechanism for the elimination of intracellular pathogens while potentially preserving the epithelium. In contrast, recovery from viral infection by traditional mechanisms necessitates the destruction of virally infected cells by cytotoxic T cells, resulting in denudation of the epithelium, which could compromise its barrier function. Furthermore, IgA is only a weak activator of complement (17, 19, 20) compared with the other major classes of antibody, including IgG and IgM. This property should tend to minimize any maladaptive local inflammatory response. For these reasons IgA appears ideally suited to protect the epithelium while preserving its barrier nature.

In summary, these results confirm our hypothesis in a second viral system and support a new role for IgA antibody whereby it can prevent virus replication within mucosal epithelial cells. The capacity of IgA to act within such cells along with its more traditional extracellular functions supports the rationale for developing more effective strategies of mucosal immunization to protect against pathogens that gain entry to the body and produce disease at mucosal surfaces.

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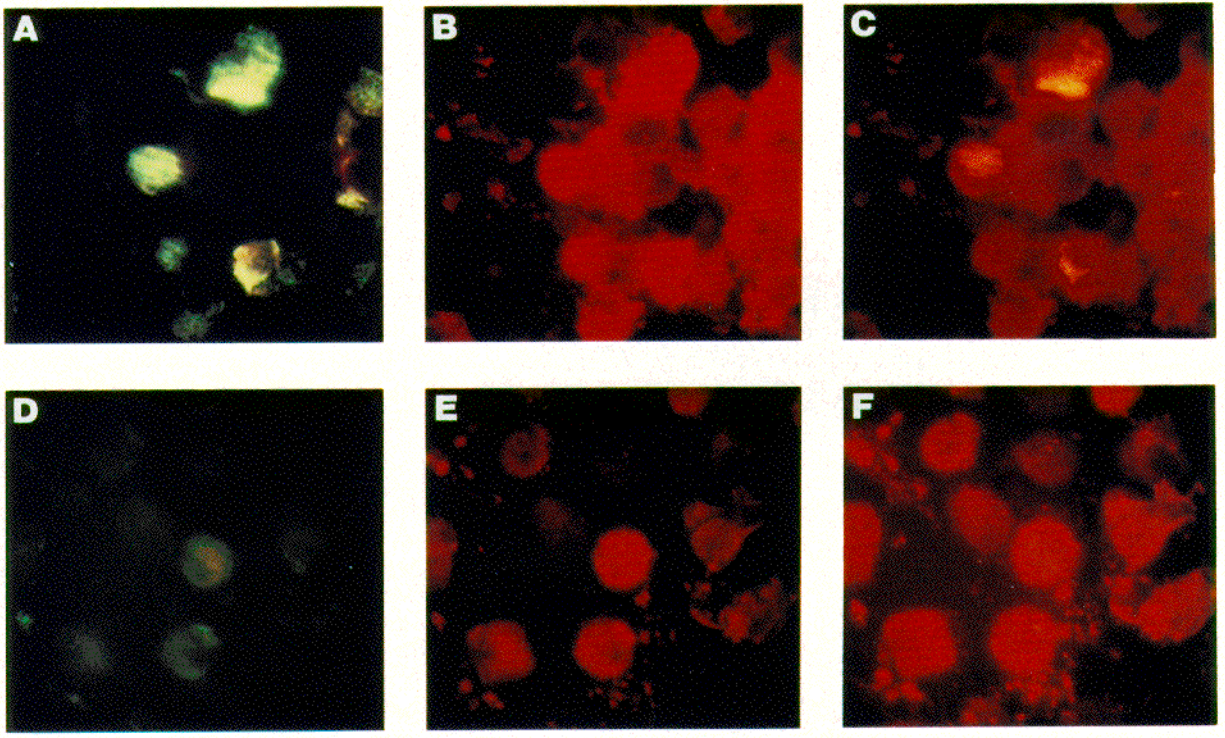
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