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Role of IgA versus IgG in the Control of Influenza Viral Infection in the Murine Respiratory Tract¹

Kathryn B. Renegar,^{2*} Parker A. Small, Jr.,[†] Lou G. Boykins,[‡] and Peter F. Wright*

The roles of IgG and secretory IgA in the protection of the respiratory tract (RT) against influenza infection remain unclear. Passive immunization with Ab doses resulting in serum IgG anti-influenza virus Ab titers far in excess of those observed in immune mice has compounded the problem. We compared the effects of i.v. anti-influenza virus IgG and i.v. anti-influenza virus polymeric IgA (pIgA) mAb administered in amounts designed to replicate murine convalescent serum or nasal Ab titers, respectively. A serum anti-influenza virus IgG titer 2.5 times the normal convalescent serum anti-influenza virus IgG titer was required for detectable Ab transudation into nasal secretions, and a serum IgG titer 7 times normal was needed to lower nasal viral shedding by 98%. Anti-influenza virus pIgA at a nasal Ab titer comparable to that seen in convalescent mice eliminated nasal viral shedding. The RT of influenza-infected pIgA- or IgG-protected mice were studied by scanning electron microscopy. Only pIgA was found to prevent virally induced pathology in the upper RT, suggesting that IgG did not prevent viral infection of the nose, but neutralized newly replicated virus after infection had been initiated. In contrast, IgG, but not pIgA, was found to prevent viral pathology in the murine lung. Our results help to resolve the controversy of IgA- vs IgG-mediated protection of the RT; both Abs are important, with plasma IgG Ab serving as the back-up for secretory IgA-mediated protection in the nasal compartment, and IgG being the dominant Ab in protection of the lung. *The Journal of Immunology*, 2004, 173: 1978–1986.

In humans, secretion of IgA by the mucosal immune system accounts for ~70% of the body's total Ig production (1). B cells sensitized to intraluminal Ags within the Peyer's patches of the GALT and the draining lymph nodes of the BALT (2–4) home to the lamina propria of both intestinal and extraintestinal sites, such as the nose and trachea, where, upon re-exposure to the appropriate Ag, they become mature Ab-producing cells. Polymeric IgA (pIgA)³ produced by these lamina propria plasma cells is transported by the transmembrane polymeric Ig receptor (pIgR) from the basolateral to the apical surfaces of the mucosal epithelial cells where the receptor is cleaved, releasing the IgA molecule and an attached remnant of the pIgR, i.e., secretory component, into the mucociliary blanket (5, 6). Binding of pIgA to secretory component/pIgR requires the J chain, a small polypeptide that is produced in mucosal IgA-producing plasma cells and promotes the formation of pIgA and IgM (7). The secretory IgA (S-IgA) in the mucociliary blanket is believed to afford protection to mucosal surfaces by neutralizing or otherwise preventing the attachment of viruses, bacteria, and toxins to the mucosal epithelium (8). Studies to investigate this function have involved both the passive administration of pIgA by numerous routes, including topical application (9), i.v. injection (10, 11), and mAb release by

backpack hybridoma tumors (12), and the elimination of IgA from secretions by a variety of methods, including anti- α -chain nose drops (10), the IgA knockout (KO) mouse (13), the pIgR KO mouse (14), and the J chain KO mouse (15). Despite (or because of) the variety of reported studies, the relative roles of mucosal S-IgA and plasma IgG Ab in mucosal protection remain unclear.

The immune response to influenza virus provides a model in which the relative contributions of the cellular, humoral, and mucosal immune systems to antiviral immunity can be addressed. Recovery has long been believed to be mediated by the cellular immune system (16, 17), whereas prevention of lethal infection correlates with serum anti-influenza virus Ab titers (18–22). Plasma Ab is sufficient to prevent infection of the lung (15, 19), but does not appear to protect the upper respiratory tract of mice and ferrets against viral infection (16, 23, 24). Plasma influenza virus-specific IgG Ab is also unable to reduce viral shedding in the nasal secretions of normal immune mice with depressed mucosal IgA levels (25). Renegar and Small (10) showed that i.v. pIgA anti-influenza virus mAb could protect the murine nasopharynx from infection after intranasal (i.n.) challenge with influenza virus and that nasal immunity in influenza-convalescent mice could be abrogated by the administration of virus in anti- α -chain, but not in anti- γ - or anti- μ -chain, antiserum (11), suggesting that IgA is the principal mediator of influenza immunity in the murine nasopharynx. However, Mbawuike et al. (13), using the IgA KO mouse model, demonstrated decreases in both pulmonary and nasal viral replication in IgA-deficient immune mice, suggesting that IgA may not be not required for prevention of influenza virus infection in the murine nose.

Human studies showing the presence of IgA and IgG anti-viral Abs in nasal washes of influenza-immune individuals (26, 27) have suggested that live and inactivated influenza virus may activate different compartments of the immune system. Clements et al. (26), using a live, attenuated influenza virus vaccine administered i.n., found that protection correlated with local anti-hemagglutinin IgA Ab and with neuraminidase-inhibiting Ab in the serum,

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³ Abbreviations used in this paper: pIgA, polymeric IgA; EID₅₀, egg ID₅₀; EVND, egg viral neutralization dose; i.n., intranasal; KO, knockout; MID₅₀, mouse ID₅₀; NT, nasotracheal; pIgR, polymeric Ig receptor; RT, respiratory tract; S-IgA, secretory IgA.

whereas Wagner et al. (27) suggested that IgG was the major contributor to resistance in the nasal compartment of humans vaccinated i.m. with a commercial inactivated virus vaccine. They demonstrated that these Abs were not locally produced in the mucosa, but were derived from the plasma by a process of passive transudation along a concentration gradient and suggested that the magnitude of this gradient might have general implications for the protection of mucosal surfaces by systemic Ab. Recent work by Ito et al. (22) indicates that a similar gradient may exist in the murine lung, allowing IgG, but not pIgA, to move freely from the plasma into the serous fluid of the alveolar epithelia.

The goals of the studies presented in this paper were to determine whether the nasopharyngeal concentration gradient reported in Wagner's human studies is relevant to the control of nasal viral infection in mice as well, and whether serum anti-influenza virus IgG Ab or nasal anti-influenza virus IgA Ab at the titer observed in normal convalescent mice can prevent influenza infection of the respiratory tract (RT), as evidenced by both reduced nasal viral shedding and the lack of morphologic evidence of virally mediated damage to the RT. To accomplish these aims, the ability of increasing doses of i.v. pIgA or IgG₁ anti-influenza virus mAb to be transported or transuded into nasal secretions and to depress nasopharyngeal viral shedding was determined. Serum IgG anti-influenza virus Ab titers in passively immunized and normal convalescent mice were analyzed to determine whether the influenza-specific IgG Ab plasma to nasal secretion concentration gradient after natural infection is sufficient to allow protective transudation to occur. Furthermore, we hypothesized that although IgG might depress the shedding of virus into the nasotracheal (NT) lavages of influenza-infected mice, it would not prevent infection of the nasopharynx in the same manner as S-IgA. We hypothesized, rather, that plasma IgG Ab would leak into the nasopharyngeal secretions after viral replication had compromised the integrity of the ciliated epithelial cells lining the trachea and nasal passages and would neutralize free virus. Scanning electron microscopy was used to visualize the NT and bronchial mucosae to determine whether anti-influenza virus pIgA or IgG at normal convalescent mucosal or serum titers could prevent the pathologic changes associated with influenza infection of the murine RT and whether mucosal S-IgA and plasma IgG Ab control influenza infection in the same manner *in vivo*.

Materials and Methods

Mice

Four- to 6-wk-old female BALB/c mice for experimental protocols or retired breeder mice for ascites production were obtained from Charles River Breeding Laboratories (Wilmington, MA) and housed six to a cage in an American Association for Accreditation of Laboratory Animal Care-accredited animal facility. Experimental protocols were approved by an institutional animal care and use committee.

Monoclonal Abs

mAbs H37-66-1 (pIgA) and H36-1-2R6 (IgG₁), specific for the Sb epitope on the tip of the hemagglutinin of PR8 (A/PR8/34(H1N1)) influenza virus and generated as previously described (28), were gifts from Dr. W. Gerhard (Wistar Institute, Philadelphia, PA). Hybridomas were grown in BALB/c mice, and the ascites fluids were pooled and frozen. Ig concentrations were determined using an RID kit according to label instructions (ICN Immunobiologics, Lisle, IL). The pIgA pool contained 2200 μ g of influenza virus-specific pIgA/ml, whereas the IgG pool contained 7300 μ g of influenza virus-specific IgG₁/ml. These monoclonals have been well characterized *in vitro* and *in vivo*, and both are very effective in viral neutralization. Their relative neutralization abilities have been previously addressed in detail (29), and it is highly unlikely that their affinities for the viral Ag differ enough to significantly affect the outcome of the experiments we report in this study.

Antisera

Goat anti-mouse α -chain- and goat anti-mouse γ ₁-chain-specific Ab for use in ELISAs, were gifts from Dr. R. Asofsky (National Institute of Allergy and Infectious Diseases, Bethesda, MD). Affinity-purified goat anti-mouse γ -chain Ab (Sigma-Aldrich, St. Louis, MO.) was substituted for goat anti-mouse γ ₁-chain Ab for the determination of total IgG. Rabbit anti-goat IgG coupled to alkaline phosphatase was purchased from ICN Immunobiologics.

Virus

The A/PR8/34-Mt. Sinai (H1N1) influenza virus was a gift from Dr. W. Gerhard. This virus was subsequently passaged three times through mouse lungs and twice through embryonated eggs to generate a large pool of virus stock. The log₁₀ EID₅₀ (dose required to infect 50% of inoculated eggs) of the virus pool was 6.5, and the log₁₀ MID₅₀ (dose required to infect 50% of mouse noses) was 4.25. Virus used in scanning electron microscopy studies was passaged an additional time through specific pathogen-free eggs, pooled, and filtered through a 0.45- μ m pore size filter to generate a virus pool with a log₁₀ MID₅₀ of 5. This pool was then aliquoted and stored at -70°C.

Sample collection

Collection of sera and nasal washes for Ab titer determination and of virus-containing nasal wash samples was previously described (10). Mice were anesthetized via i.v. injection of pentobarbital solution (composition: 20 ml of sterile glycerol, 10 g of pentobarbital, 10 ml of absolute alcohol, and 80 ml of sterile water) to effect (0.1–0.2 ml) and were exsanguinated by transection of the abdominal aorta. Blood was collected in microcentrifuge tubes and allowed to clot, and the serum was retained for ELISA analysis. For the collection of nasal washings, a midline incision was made over the ventral aspect of the trachea slightly anterior to the thoracic inlet. The trachea was tied or clamped off at the thoracic inlet. For Ab titer determination, 400 μ l of PBS was slowly injected into the tracheal lumen cephalad to the obstruction using a 25-gauge B bevel needle attached to a tuberculin syringe. For virus detection studies, 1 ml of PBS was used. Because the serum anti-influenza virus Ab titers in passively immunized mice were so high that even low levels of blood contamination could invalidate nasal wash results, we monitored the amount of blood in the nasal washes using Bili-Labstix (Bayer, Elkhart, IN) as previously reported (10). Samples for Ab determination were frozen until assayed, whereas wash fluid for viral titer determination was immediately injected into fertile eggs.

Ab titer determination

Serum IgG₁ anti-influenza virus titers were determined by ELISA as previously described (10). PR8 influenza vaccine (gift from Dr. F. Brandon, Parke Davis, Rochester, MI) was dried onto 96-well, flat-bottom enzyme immunoassay microtiter trays (Linbro/TiterTek E.I.A. Microtitration plates; Flow Laboratories, McLean, VA). Unreacted sites were blocked with gelatin, then nasotracheal lavage or serum samples were added and incubated 1.5 h at room temperature. Trays were washed with PBS-Tween, and goat anti-mouse α - or γ -chain Ab was added and incubated 1.5 h at room temperature. The detection system was rabbit anti-goat IgG coupled to alkaline phosphatase (ICN Immunobiologics). Titers are reported relative to a monoclonal IgG₁ standard (7300 μ g of influenza virus-specific IgG₁/ml), with the standard being assigned a value of 100. For determination of total serum anti-influenza virus IgG, affinity-purified goat anti- γ -chain Ab was substituted for goat anti- γ ₁ in the ELISA. Blood contamination in NT lavages was determined by the Bililabstix method, and nasal Ab titers were standardized to titer per 50 μ g of protein, using a Bio-Rad protein assay (Hercules, CA) for the determination of nasal wash protein levels as previously described (10).

Viral titer determination

Nasal wash influenza virus titers were determined in eggs as previously described (10). Fresh nasal washes were kept on ice until all mice had been killed, then were serially diluted 10-fold in sterile PBS. One-tenth milliliter of each dilution was injected into each of three fertile eggs previously injected with 25,000 U of penicillin and 25 mg of streptomycin. After 3 days, allantoic fluid from the eggs was assayed for virus by hemagglutination. The EID₅₀ was calculated by the method of Reed and Muench (30).

Equivalent egg viral neutralization dose (EVND) determination

The relative neutralization titers of the anti-influenza virus mAbs were determined by comparing the ability of the monoclonals to neutralize the egg infectivity of PR8-Mt. Sinai influenza virus as previously described

(10). The amount of ascites fluid containing 5×10^5 50% EVND of each monoclonal was calculated to be 200 μ l (440 μ g Ab) for pIgA and 23 μ l (168 μ g Ab) for IgG₁.

Injection and protection protocols

Eight- to 10-wk-old female BALB/c mice were injected i.v. in the tail vein with varying amounts of either pIgA anti-influenza virus mAb or IgG₁ anti-influenza virus mAb. The injection volume was always 400 μ l even though the Ab content varied. Ascites fluids were spun in a clinical centrifuge before injection to preclude embolus formation. For virus shedding studies, at 4 h post-Ab injection, awake mice were challenged i.n. with 20 MID₅₀ of virus contained in a 20- μ l volume. This fluid was placed on the nares, and the mouse was restrained until the fluid had been inhaled. Care was taken to guarantee that the mouse inhaled the entire dose. Twenty-four hours after viral challenge the mice were anesthetized with pentobarbital, and their sera and nasal washes were harvested and processed for Ab determination (serum) or viral titer (nasal washes) as described above. For the transudation correlation experiments, mice were injected with IgG₁-containing ascites fluid and were killed at 4 h postinjection with no viral challenge.

Convalescent serum

Eight- to 10-wk-old female BALB/c mice were infected i.n. while awake with 200 MID₅₀ of PR8 influenza virus. Six weeks later they were killed by exsanguination after the injection of pentobarbital. Serum samples were stored at 4°C, and anti-influenza virus titers were obtained by ELISA as described above. We chose the 6 wk point for assessment of serum anti-influenza virus IgG titers because the murine serum Ab titer is still rising between 3 and 4 wk post-i.n. viral administration (25), but has reached its peak by 6–8 wk after viral infection (31).

Scanning electron microscopy

For scanning electron microscopic studies, mice (six per group) were injected i.v. in the tail vein with either 220 or 440 μ g of influenza virus-specific pIgA mAb or with either 168 or 730 μ g of influenza virus-specific IgG₁ mAb. Control mice (groups of three and six mice) received 440 μ g of ZF11-15 (a gift from Dr. Z. Moldoveanu, University of Alabama, Birmingham, AL), an IgA hybridoma that does not bind influenza virus. Four hours after Ab injection, the mice were briefly anesthetized with metofane, and the entire RT was infected by dropping 60 μ l of PBS containing 100 MID₅₀ of influenza virus onto the nares. One control group (six mice) received virus (positive controls for infection), and one control group (three mice) received PBS nose drops only (uninfected controls). Mice were killed by a ketamine-xylazine (50 mg/kg ketamine plus 50 mg/kg xylazine given i.p.) overdose (0.2–0.3 ml) at either 24 h (three mice from each IgA group and each IgG group, three mice from the positive infection control group, and the noninfected controls) or 48 h (remaining mice from each IgA group and each IgG group and the positive infection control group) postinfection, and lungs, trachea, and noses were removed, fixed in glutaraldehyde-cacodylate buffer (2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer), and stored at 4°C until processed. For processing, samples were postfixed with 1% osmium tetroxide; dehydrated with successive transfers to 70, 90, and 100% ethanol; and dried in a desiccator after treatment with hexamethyldisizane. The samples were then mounted and coated to a thickness of 80 nm with gold-palladium using a Hummer VII Sputtering System (Anatech, Alexandria, VA). Samples were viewed and photographed using the JEOL 840A (Peabody, MA) and XL30 Environmental Scanning Electron Microscope system (Philips Electronic Systems, Mahway, NJ).

Graphics

Figures were generated using CA-Crickett Graph III software (Computer Associates International, Islandia, NY) on an iBook (Apple Computer, Cupertino, CA). Photomicrographs were also processed on the iBook using Photoshop 7.0 software (Adobe Systems, San Jose, CA).

Results

Comparison of pIgA and IgG-mediated nasal protection

The first step in determining whether plasma IgG could reduce viral shedding in the murine nasopharynx was to assess the relative abilities of equivalent neutralizing doses of pIgA and IgG₁ anti-influenza virus mAb to reduce nasal viral shedding (Fig. 1 and Table I). Because pIgA is 7–10 times more effective than IgG in virus neutralization (29), equivalent neutralizing titers as determined in the egg (EVND) of the respective Igs were administered

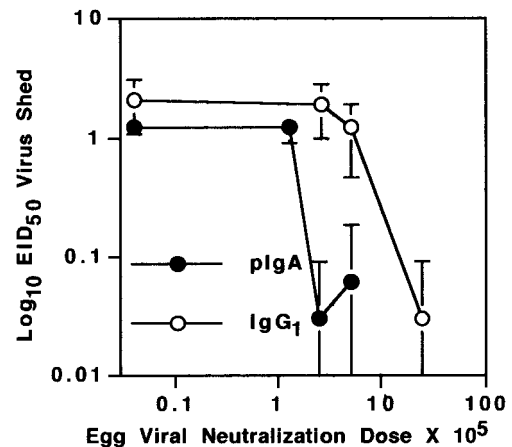


FIGURE 1. Virus shedding by mice injected with equivalent virus-neutralizing doses of pIgA and IgG anti-influenza virus mAbs. Mice were injected i.v. with varying EVND of pIgA (●) or IgG₁ (○) anti-influenza virus mAb and challenged i.n. with 20 MID₅₀ of PR8 influenza virus 4 h postinjection. Twenty-four hours later, mice were killed, and their nasotracheal lavages were assayed for viral shedding. No detectible viral shedding was defined as a log₁₀ EID₅₀ of 0.001. Each point represents a minimum of four mice.

to ensure that the injected Ab doses had similar neutralization potentials. Failure to make this dose adjustment can lead to aberrant experimental results. The injection of 2.6×10^5 EVND (220 μ g) of pIgA 4 h before viral challenge resulted in a 98% decrease in the amount of influenza virus present in nasal washes 24 h later, whereas 2.3×10^6 EVND of IgG₁ (730 μ g) was required to achieve a similar decrease in nasal wash virus titer. Injection of 5.2×10^5 EVND (168 μ g) of IgG had only a minimal effect on nasopharyngeal viral shedding (Fig. 1). Thus, i.v. pIgA is ~10 times more efficient than i.v. IgG in reducing viral shedding in the murine nasopharynx. This increased effectiveness is most likely a reflection of pIgR-mediated transport of pIgA into nasal secretions, resulting in enrichment of influenza-specific IgA relative to IgG in the nasopharynx. We demonstrated previously (10) that i.v. pIgA was selectively transported into nasal secretions relative to i.v. IgG₁, leading to a higher IgA than IgG nasal anti-influenza virus Ab titer despite a much higher IgG than IgA serum anti-influenza virus Ab titer.

Relationship between 24-h serum IgG titer and protection

The 24-h serum Ab titers of the mice injected with IgG₁ anti-influenza virus mAb in the experiment described above were determined and compared with the level of protection conferred by that Ab dose (Fig. 2 and Table I). The injection of 730 μ g of Ab was required to reduce the virus titer in the nasal washes by 98%. The injection of 168 μ g of IgG₁ had a minimal effect on the nasal wash viral titer. The serum Ab titer of mice injected i.v. with 730 μ g of Ab was 2.3 ± 0.8 (~168 μ g Ab/ml), whereas the serum Ab titer of mice injected with 168 μ g of Ab was 0.4 ± 0.16 (~29 μ g Ab/ml). Hence, a serum titer >0.4 is needed for protection from viral challenge.

To relate the serum anti-influenza virus IgG Ab titers obtained by passive immunization to the natural situation, serum anti-influenza virus IgG₁ and serum anti-influenza virus total IgG Ab titers relative to the mAb standard were determined in six convalescent mice 6 wk after influenza infection. The serum IgG₁ anti-influenza virus Ab titer was 0.0135 ± 0.0072 (~1 μ g Ab/ml), whereas the serum total IgG anti-influenza virus Ab titer was 0.32 ± 0.284 (~23 μ g Ab/ml). The serum anti-influenza virus IgG

Table I. Summary of protection parameters^a

mAb Class	mAb Dose (μg)	EVND	Nasal Virus	Nasal Pathology	Tracheal Pathology	Lung Pathology	4-h Nasal Anti-Influenza Virus Ab Titer	4-h Serum Anti-Influenza Virus Ab Titer
pIgA	440	5.2×10^5	No	No	No	Yes	$709 \pm 219 \times 10^{-5}$ (~ 156 ng of Ab/ml)	3.12 ± 3.07 (~ 69 μg of Ab/ml)
pIgA	220	2.6×10^5	No	No	No	Yes	$111 \pm 79 \times 10^{-5}$ ($\sim 24 \pm 17$ ng of Ab/ml)	2.93 ± 2.29 (~ 64 μg of Ab/ml)
IgG ₁	730	2.3×10^6	No	Yes	Yes	No	15×10^{-5} (~ 11 ng of Ab/ml)	2.3 ± 0.8 (~ 168 μg of Ab/ml)
IgG ₁	168	5.2×10^5	Yes	Yes	Yes	No	$\sim 1-2 \times 10^{-5}$ (< 1.5 ng of Ab/ml)	0.4 ± 0.16 (~ 29 μg of Ab/ml)

^a Values for normal convalescent immune mice. Total serum anti-influenza virus IgG, titer of 0.32 ± 0.28 or $\sim 23 \pm 20.7$ μg Ab/ml; nasal anti-influenza virus IgG, undetectable; nasal anti-influenza virus IgA, titer of $\sim 40 \times 10^{-5}$ or ~ 9 ng Ab/ml. Serum anti-influenza virus IgG titer must be > 1 for nasal wash IgG to be easily detectible.

titer in normal convalescent mice, then, is comparable to that shown to have no significant effect on nasal viral titers and is approximately one-seventh the serum anti-influenza virus IgG titer shown to reduce the viral titer by 98%.

There was minor blood contamination of the nasal washes in this protection study (levels ranging from a serum dilution of 10^{-5} – 10^{-6}). To rule out the possibility of postfacto neutralization of nasal wash virus by contaminating plasma Ab, the ability of a 10^{-5} dilution of serum from a mouse injected with 730 μg of IgG₁ to neutralize a $10^{3.5}$ EID₅₀ suspension of influenza virus was determined. The neutralizing abilities of pooled nasal washes from mice injected with either 730 μg of IgG₁ anti-influenza virus mAb or 220 μg of pIgA anti-influenza virus mAb were also determined. Influenza virus and Ab suspension (nasal wash pools or diluted serum) were vortexed, incubated on ice for 1 h, and assayed in eggs. Neither the diluted serum nor the Ab-containing nasal washes displayed any virus-neutralizing activity (data not shown). The lack of virus in the nasal washes of passively protected mice, then, was not due to postfacto neutralization. This is consistent with the work of Kris et al. (16), who demonstrated that decreased viral shedding in the ground lungs of influenza-immune mice was due to in vivo control of infection by protective plasma Ab and not to neutralization of virus present in the harvested sample by Ab released from the tissue during homogenization.

Relationship between 4-h serum and nasal wash IgG₁ titers

To determine the magnitude of the concentration gradient needed for plasma Ab to diffuse into nasal secretions, serum and nasal wash anti-influenza virus Ab titers were determined 4 h after uninfected mice were injected with varying amounts of IgG₁ anti-influenza virus mAb (Fig. 3). There was a high correlation (0.984) between serum and nasal wash IgG titers, suggesting transudation of IgG Ab from the plasma into nasal secretions. Linear regression analysis of the serum and nasal wash anti-influenza virus IgG Ab titers suggested that a serum titer > 0.84 (61 μg of IgG Ab/ml) was required before transudation of detectible IgG₁ anti-influenza virus Ab from the plasma into the nasal secretions occurred. In comparison, an anti-influenza virus serum pIgA mAb titer of ~ 1 (~ 20 μg IgA Ab/ml) resulted in a peak nasal secretion IgA titer of $\sim 40 \times 10^{-5}$ (10). We have previously shown (10) that the passively administered pIgA mAb found in nasal secretions after i.v. pIgA injection is most likely derived from pIgR-mediated transport and that its titer peaks at 4 h post-Ab injection, reflecting the short half-life of plasma pIgA in mice. In contrast, both the nasal and serum IgG₁ anti-influenza virus mAb titers were relatively constant over a 24-h period (10), with the nasal anti-influenza virus IgG₁ mAb titer remaining much lower than the peak pIgA nasal Ab titer despite the longer IgG half-life.

Histologic analysis of IgA- or IgG-mediated anti-influenza virus respiratory tract protection

To determine whether IgA or IgG prevented or suppressed viral infection, scanning electron microscopic studies of infected, Ab-injected murine RT were conducted. The Ab concentrations to be administered were determined from previous work with the monoclonal pools and with convalescent mice. Because 168 μg of IgG₁ anti-influenza virus mAb (5.2×10^5 EVND) resulted in a serum anti-influenza virus Ab titer comparable to that seen in influenza-convalescent mice and because 730 μg of IgG₁ anti-influenza virus mAb (2.3×10^6 EVND) reduced NT viral shedding by 98%, those Ab doses were chosen for the scanning electron microscopic study. A dose of 440 μg of pIgA anti-influenza virus mAb (5.2×10^5 EVND) was used in the previously reported protection studies (10), so its protective effects are well characterized. This dose produced a 4 h postinjection peak nasal lavage Ab titer of $709 \pm 219 \times 10^{-5}$ and an 8–24 h nasal lavage Ab titer ranging between ~ 30 and $\sim 60 \times 10^{-5}$ (12). The 220- μg pIgA (2.6×10^5 EVND) dose had also been shown to confer consistent protection against nasal influenza infection and produced a 4 h postinjection nasal lavage anti-influenza virus Ab titer of $111 \pm 79 \times 10^{-5}$, roughly comparable to our previously reported convalescent mouse IgA anti-influenza virus nasal wash Ab titer of $\sim 40 \times 10^{-5}$ (31). mAb was administered 4 h before viral challenge because we had previously determined that time point to be optimum for pIgA-mediated passive protection (10).

The respiratory mucosa of the normal murine nose, which is comprised of ciliated pseudostratified columnar epithelium anchored to basal cells and interspersed with Clara cells, microvillar cells, and mucous-secreting goblet cells, lines the paranasal (maxillary) sinuses and $\sim 60\%$ of the nasal cavity (32). In the noses of the uninfected mice in our study, the densely packed normal cilia did not adhere to each other (Fig. 4A) and tapered to a rounded tip (Fig. 4A, inset). Cellular debris was seen on the ciliated nasal epithelium in untreated influenza-infected mice (Fig. 4B); cilia were necrotic, as evidenced by the loss of distinct outlines, and appeared compressed and clumped with broken and knobbed tips (Fig. 4B, inset), consistent with the virally mediated ciliary agglutination reported by O'Neill et al. (33). As expected, injection of 168 μg of influenza virus-specific IgG₁ did not prevent the pathologic changes associated with viral infection (Fig. 4C). The outlines of the necrotic ciliated cells were indistinct, and areas of desquamation revealing the underlying basal cell layer were present (Fig. 4C, inset). A dose of 730 μg of influenza virus-specific IgG₁ mAb reduced the viral titer in nasotracheal lavages by 98%; however, this Ab dose did not prevent the characteristic changes associated

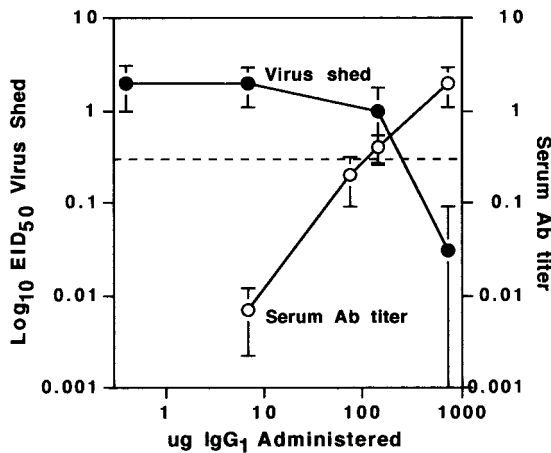


FIGURE 2. Serum IgG₁ levels and nasal viral shedding. Mice were injected i.v. with increasing doses of influenza virus-specific monoclonal IgG₁ Ab, then challenged i.n. at 4 h postinjection with 20 MID₅₀ of PR8 influenza virus. Twenty-four hours later, they were killed, and nasal wash viral shedding (●) and serum IgG₁ titer (○) were determined. No detectable virus shedding was defined as a log₁₀ EID₅₀ of 0.001. Each point represents a minimum of four mice. The broken line indicates the normal convalescent immune mouse serum anti-influenza virus IgG Ab titer.

with influenza viral infection (Fig. 4D). Areas of compressed, indistinct cells were present, and the cilia were bound in bundles with their tips adhering together (Fig. 4D, inset), an indication of viral infection (33). Injection of 220 μg of anti-influenza virus pIgA mAb, in contrast to IgG injection, protected the nasal mucosa from viral infection (Fig. 4E). Ciliary tufts were distinct, and ciliary tips (Fig. 4E, inset) were smoothly rounded and nonadherent. At 48 h postinfection, there was still no evidence of viral pathology in pIgA-injected mice (Fig. 4F), and the noses of mice injected with 730 μg of IgG₁ showed signs of recovery as well. Mice injected with 168 μg of IgG₁, however, showed signs of worsening infection, not recovery, at 48 h postinfection. Thus, IgG protection of the murine nose required more Ab than is normally present in the plasma of convalescent animals and appeared to be mediated by plasma Ab leaking into nasal secretions postinfection and neutralizing newly replicated virus, whereas pIgA appeared to prevent the onset of nasal infection.

The pattern of protection in the trachea (Fig. 5) mirrored that in the nose. The normal murine tracheal epithelium is comprised of ~52% Clara cells and 36% ciliated cells (34) (Fig. 5A). The cilia were long, with slightly tapered, rounded tips (Fig. 5A, inset). In uninfected animals, the smooth mucociliary blanket covered ~15% of the tracheal surface. In the influenza virus-infected trachea (Fig. 5, B and C), the mucociliary blanket appeared to be static and covered ~90% of the tracheal surface. Impressions of cilia could be observed in the mucous, and necrotic cilia were evident, especially at 48 h postinfection (Fig. 5C, inset). The mucociliary blanket developed areas with a moth-eaten appearance, which became more pronounced as the infection progressed. Presumably, the clumping and necrosis of the tracheal cilia produce ciliostasis, leading to impairment of the clearance mechanism (33). Injection of mice with 730 μg of IgG₁ anti-influenza virus mAb did not prevent stasis of the mucociliary blanket and development of the moth-eaten aspect (Fig. 5D); however, injection of 220 μg of pIgA maintained a normal mucociliary blanket and ciliary structure (Fig. 5E). As in the nose, the trachea of mice injected with 730 μg of influenza virus-specific IgG₁ showed evidence of resolution of viral infection 48 h postinfection; however, signs of past virus-induced pathology, i.e., debris and necrotic sloughed ciliated cells,

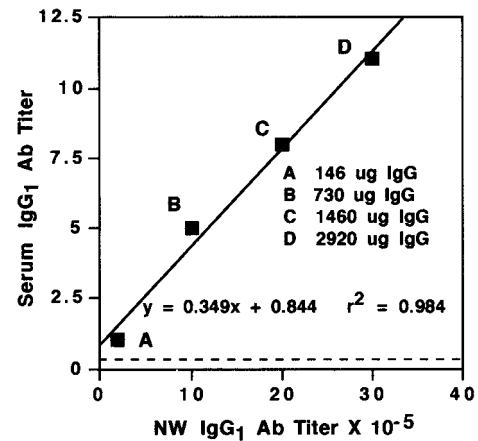


FIGURE 3. Relationship between serum and nasotracheal lavage IgG₁ titers. Mice were killed 4 h after i.v. injection of increasing amounts of IgG₁ anti-influenza virus mAb. Nasal wash and serum anti-influenza virus Ab titers were determined by ELISA. Each group contained a minimum of six mice. The linear regression was generated by CA-Crickett Graph III software. The broken line indicates the normal convalescent immune mouse serum anti-influenza virus IgG Ab titer.

were still evident (Fig. 5F). There is no indication that complement fixation by IgG₁ (35) contributes to the severity of either nasal or tracheal mucosal pathology, because the 48 h lesions in high dose (730 μg) IgG₁-injected mice were not as severe as those seen at 48 h in untreated infected mice.

In contrast to the nose and trachea, the bronchioles were protected by injection of IgG₁ anti-influenza virus mAb. The intrapulmonary bronchi of mice are, like the trachea, comprised of ~61% Clara cells and 36% ciliated cells (34). The cilia of these cells are distinct and are gently tapered to a rounded tip (Fig. 6A). In infected mice, mucous secretion increases, and the cilia become necrotic and trapped in the abundant mucous (Fig. 6B). Injection of the 168-μg dose of IgG₁ anti-influenza virus mAb, which was unable to protect the nose and trachea, prevented influenza virus-induced pathology in the bronchioles (Fig. 6C), whereas the 440-μg injection of influenza virus-specific pIgA, which protected the nose and trachea, had no effect on viral infection in the bronchioles (Fig. 6D). The 168-μg IgG₁ mAb dose, which prevented pulmonary pathology in the current study, is comparable to the 175-μg dose that Palladino et al. (36) found to produce serum IgG Ab titers equivalent to those in mice infected with influenza virus 4 wk previously and that cured pneumonia in SCID mice.

Discussion

We previously demonstrated that passively administered i.v. pIgA anti-influenza virus mAb could protect the murine nose against viral infection and that nasal immunity in the intact murine nose could be abrogated by the administration of anti-α-chain nose drops, but not by the administration of anti-γ or anti-μ-chain nose drops, suggesting that S-IgA was the major mediator of anti-influenza virus protection (10, 11). In contrast, Mbawuike et al. (13), using the IgA KO mouse, suggested that IgA was not necessary for nasal and pulmonary protection against influenza virus infection in mice immunized with influenza vaccine plus cholera toxin/cholera toxin B. Asahi et al. (14), however, reported that the blockade of transepithelial transport of pIgA in the pIgR KO mouse led to compromised nasal anti-influenza virus protection and the lack of IgA-dependent heterotypic cross-protection in the upper and lower RTs of vaccinated mice. A marked increase in

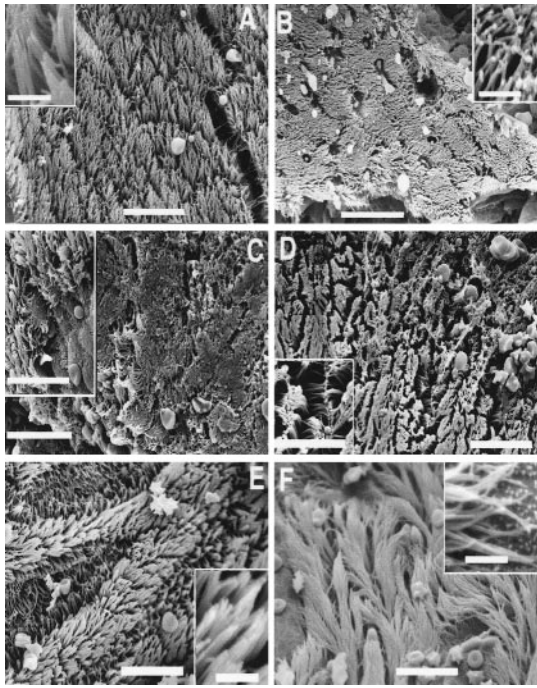


FIGURE 4. Effects of i.v. pIgA or IgG₁ anti-influenza virus mAb on nasal influenza infection. Mice were injected i.v. with one of the following: 220 µg of pIgA anti-influenza virus mAb, 440 µg of pIgA anti-influenza virus mAb, 168 µg of IgG₁ anti-influenza virus mAb, or 730 µg of IgG₁ anti-influenza virus mAb. Controls (both positive for viral effects and negative for uninfected noses) received 440 µg of ZF11-15 pIgA i.v. (pIgA that does not bind influenza virus). Four hours after Ab injection, sleeping mice were infected i.n. with 100 MID₅₀ influenza virus in 60 µl PBS (or PBS alone for the uninfected controls). Twenty-four hours after viral challenge, mice were killed, and respiratory tracts were removed and processed for scanning electron microscopic observation. *A*, Uninfected nose (magnification, ×1500; white bar, 20 µm); *inset*, healthy cilia (magnification, ×10,000; bar, 2 µm). *B*, Nose 24 h postinfection (magnification, ×1500; bar, 20 µm); *inset*, blunt-tipped cilia (magnification, ×10,000; bar, 2 µm). *C*, Nose 24 h postinfection, mouse injected with 168 µg of IgG₁ anti-influenza virus mAb (magnification, ×1500; bar, 20 µm); *inset*, desquamating cells (magnification, ×1500). *D*, Nose 24 h postinfection, mouse injected with 730 µg IgG₁ anti-influenza virus mAb (magnification, ×1500; bar, 20 µm); *inset*, clumped cilia (magnification, ×3500; bar, 10 µm). *E*, Nose 24 h postinfection, mouse injected with 220 µg pIgA anti-influenza virus mAb (magnification, ×1500; bar, 20 µm); *inset*, note healthy ciliary tips (magnification, ×10,000; bar, 2 µm). *F*, Nose 48 h postinfection, mouse injected with 440 µg pIgA anti-influenza virus mAb (magnification, ×1500; bar, 20 µm); *inset*, healthy cilia (magnification, ×3500; bar, 10 µm).

serum influenza virus-specific IgA concentration (~20 times normal) and a decrease in nasal IgA (0.3 times normal) was reported in these animals; however, the serum anti-influenza virus IgG level was unaltered compared with that in normal mice. As expected, plasma Ab mediated pulmonary protection in influenza virus immune J chain or pIgR KO mice challenged with homologous virus (14, 15). Serum total IgG levels were increased in pIgR KO mice, with evidence of undue triggering of systemic immunity and deterioration of epithelial barrier function (37).

In humans, decreased nasal viral shedding could be mediated by a plasma IgG transudate, provided the gradient between plasma and nasal IgG was great enough for diffusion to occur (27). In immune mice, however, the plasma IgG anti-influenza virus Ab level does not appear to be sufficient to provide protection against nasal viral infection, because the nasopharynxes of immune mice

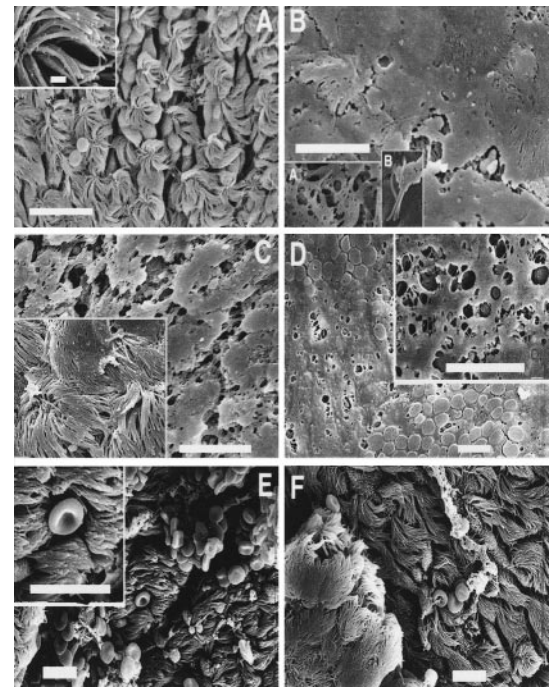


FIGURE 5. Effects of i.v. pIgA or IgG₁ anti-influenza virus mAb on tracheal influenza infection. Mice were injected i.v. with one of the following: 220 µg of pIgA anti-influenza virus mAb, 440 µg of pIgA anti-influenza virus mAb, 168 µg of IgG₁ anti-influenza virus mAb, or 730 µg of IgG₁ anti-influenza virus mAb. Controls (both positive for viral effects and negative for uninfected noses) received 440 µg of ZF11-15 pIgA i.v. (pIgA that does not bind influenza virus). Four hours after Ab injection, sleeping mice were infected i.n. with 100 MID₅₀ of influenza virus in 60 µl of PBS (or with PBS alone for the uninfected controls). Twenty-four hours after viral challenge, mice were killed, and respiratory tracts were removed and processed for scanning electron microscopy observation. *A*, Uninfected trachea (magnification, ×1500; white bar, 20 µm); *inset*, healthy cilia (magnification, ×10,000; bar, 2 µm). *B*, Trachea 24 h postinfection (magnification, ×3500; bar, 10 µm); *insets*: *A*, area of moth-eaten mucociliary blanket and *B*, desquamated cilia (magnification, ×3500). *C*, Trachea 48 h postinfection (magnification, ×3500; bar, 10 µm); *inset*, cilia matted in mucociliary blanket (magnification, ×3500). *D*, Trachea 24 h postinfection, mouse injected with 730 µg IgG₁ anti-influenza virus mAb (magnification, ×1500; bar, 10 µm); *inset*, moth-eaten mucociliary blanket (magnification, ×3500; bar, 10 µm). *E*, Trachea 24 h postinfection, mouse injected with 220 µg pIgA anti-influenza virus mAb (magnification, ×1500; bar, 10 µm); *inset*, note protected, healthy ciliary tips (magnification, ×3500; bar, 10 µm). *F*, Trachea 48 h postinfection, mouse injected with 730 µg IgG₁ anti-influenza virus mAb; note chunk of desquamated cilia (magnification, ×1500; bar, 10 µm).

fed total parenteral nutrition solution i.v. to depress the production of mucosal IgA, but leave the serum IgG anti-influenza virus Ab titer intact, were not protected against influenza viral infection (25). In support of the i.v. total parenteral nutrition findings, Ito et al. (22) reported that the influenza virus-specific Ab found in the serous fluid of alveolar epithelia of nasally vaccinated mice represented 90.8% of the total RT anti-influenza virus IgG, whereas the influenza virus-specific IgG in the nasal mucous represented only 5.8%. In contrast, the influenza virus-specific IgA found in the alveolar serous fluid represented only 3.9% of the total RT IgA, whereas the IgA found in nasal mucous represented 73.6% of the total RT IgA. This suggests that the amount of IgG found in normal influenza virus-immune murine nasal secretions is not adequate for anti-viral protection.

To validate this hypothesis and to determine whether plasma Ab could mediate murine nasopharyngeal immunity to influenza virus,

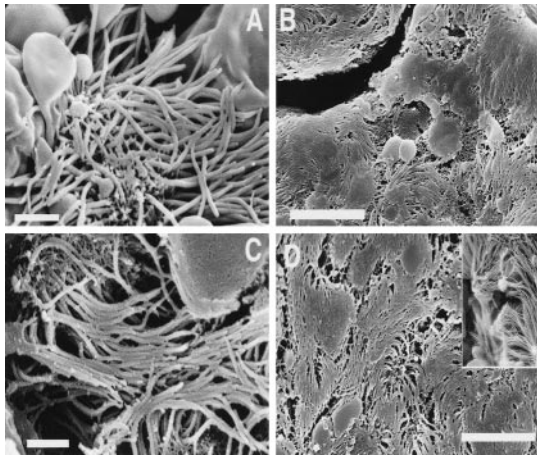


FIGURE 6. Effects of i.v. pIgA or IgG₁ anti-influenza virus mAb on pulmonary influenza virus infection. Mice were injected i.v. with one of the following: 220 µg of pIgA anti-influenza virus mAb, 440 µg of pIgA anti-influenza virus mAb, 168 µg of IgG₁ anti-influenza virus mAb, or 730 µg of IgG₁ anti-influenza virus mAb. Controls (both positive for viral effects and negative for uninfected noses) received 440 µg of ZF11-15 pIgA i.v. (pIgA that does not bind influenza virus). Four hours after Ab injection, sleeping mice were infected i.n. with 100 MID₅₀ influenza virus in 60 µl PBS (or PBS alone for the uninfected controls). Twenty-four hours after viral challenge, mice were killed, and respiratory tracts were removed and processed for scanning electron microscopic observation. *A*, Uninfected lung, ciliary tufts (magnification, ×10,000; white bar, 2 µm). *B*, Lung 48 h postinfection (magnification, ×3500; bar, 10 µm). *C*, Lung 48 h postinfection, mouse injected with 168 µg IgG₁ anti-influenza virus mAb (magnification, ×10,000; bar, 2 µm). *D*, Lung 48 h postinfection, mouse injected with 440 µg pIgA anti-influenza virus mAb (magnification, ×3500; bar, 10 µm); inset, clumped and blunted ciliary tips (magnification, ×3500).

we injected mice i.v. with anti-influenza virus IgG₁ mAb. Intravenous IgG₁ anti-influenza virus mAb was able to reduce the titer of virus in the nasal lavages of influenza virus-challenged mice by up to 98%; however, the serum titer of influenza virus-specific Ab required for this reduction was much greater than that seen in normal convalescent mice (see Table I and Fig. 2). In comparison, Wagner et al. (27) found that in human volunteers given inactivated influenza vaccine i.m., decreased viral shedding correlated with serum and nasal wash IgG titers. Using regression analysis, they showed that IgG Ab in nasal secretions both from volunteers given attenuated live virus i.n. and from volunteers given inactivated virus i.m. was a transudate from plasma and that an easily generated serum anti-hemagglutinin titer of ~1/350 was required before nasal wash IgG anti-hemagglutinin Ab became detectible. For detectible transudation of Ab into nasal secretions to occur in the intact murine nose, a serum anti-influenza virus IgG Ab titer >0.84 was required (obtained by solving the regression equation shown in Fig. 3 for the *y* intercept; $y = 0.349x + 0.844$). This titer is ~2.5 times the normal convalescent anti-influenza virus serum IgG Ab titer of 0.32, so it is likely that, in contrast to the human findings and assuming that IgG₁ is representative of all murine IgG subclasses, the amount of transudation that occurs in the nose of the normal immune mouse is minimal.

For a 98% reduction in NT viral shedding, the serum anti-influenza virus Ab titer had to be ~2.3 (Fig. 2), roughly 3 times the titer required for detectible nasal IgG transudation. The titer needed for IgG₁-mediated protection, then, was ~170 ($2.3 \div 0.0135$) times the serum IgG₁ anti-influenza virus titer seen in immune convalescent mice and 7 ($2.3 \div 0.32$) times the serum total IgG anti-influenza virus titer seen in convalescent mice. A

serum IgG titer of 0.4 ± 0.16 , comparable to the convalescent serum anti-influenza virus total IgG titer of 0.32, was shown to have a minimal effect on the amount of virus shed from the noses of passively immunized mice (Fig. 2). These findings, which are supported by the work of Ito et al. (22), suggest that although plasma IgG may make some small contribution to nasal immunity, the serum IgG titer in normal immune mice is not sufficient for transudated IgG to account for anti-influenza virus immunity in the intact murine nose.

An i.v. dose of 730 µg of IgG₁ anti-influenza virus mAb (producing 7 times the normal serum anti-influenza virus IgG titer) was able to reduce the titer of virus in the nasal secretions of influenza virus-challenged mice. Whether this represents neutralization of the original viral dose by transudated Ab present in the nasal secretions before viral challenge or neutralization of newly replicated virus by Ab leaking into the nasal secretions secondary to viral pathology cannot be determined from these data. Kris et al. (16) hypothesized that there is little protection from initial viral infection by IgG, but as mucosal epithelial cells are destroyed by virus replication, the epithelium becomes more permeable, so more IgG leaks into the secretions. This leads to neutralization of the replicating virus. If this occurs, then scanning electron microscopic studies of mice injected with anti-influenza virus mAb before viral challenge should reveal viral pathology in the RT (nose, trachea, or lungs) of mice injected with IgG₁, but not in the RT of mice injected with pIgA Ab.

We observed viral pathology in the nose (Fig. 4) and trachea (Fig. 5) of mice injected with either the dose of IgG₁ mAb producing serum anti-influenza virus IgG Ab titers comparable to those seen in convalescent mice (168-µg Ab dose) or the dose of IgG₁ mAb capable of reducing the nasal viral titer by 98% (730-µg mAb dose). Both pIgA doses (220 and 440 µg) protected the nose (Fig. 4) and trachea (Fig. 5) from infection, with little or no viral pathology evident. In the high dose IgG₁ (730 µg of Ab) mice, IgG did prevent an increase in the severity of nasal or tracheal infection between 24 and 48 h, suggesting that IgG neutralizes replicating virus, but does not prevent initial viral infection. These results explain and at least partially resolve the controversy over which Ab class (IgA or IgG) can protect the murine nose against influenza viral infection. The confusion arises because nasal viral shedding is not necessarily the best indicator for the role of Ab class, as IgG, which does not prevent viral infection of the murine nose, can eliminate viral shedding if plasma levels are high enough. If the experimental IgG dose is not carefully titrated to produce serum titers comparable to those seen in the normal immune animal, IgG may appear to provide protection of the nose in lieu of IgA when it actually does not do so. This criterion must be taken into consideration when evaluating reports of nasal protection without IgA or of nasal protection provided by the passive administration of IgG by backpack tumor or injection.

The question arises as to whether saturation of nasal tissue fluids with passively administered IgG was sufficient at 4 h post-Ab injection for a transudation gradient to develop between the plasma and the nasal mucous. We have previously studied the kinetics of transport of i.v. IgG₁ into the nasal secretions and found the injected material to be equilibrated with interstitial fluids by 2 h postinjection and the titer of IgG in nasal secretions to be relatively constant between 2 and 24 h postinjection (10). Hence, the nasal tissues were sufficiently saturated with IgG for a transudation gradient to develop and for potential protection of the nose and trachea by IgG. That this did not occur in animals with a serum IgG anti-influenza virus Ab titer comparable to that of normal convalescent mice indicates that such a gradient does not occur in the normal murine nose. Our results are supported by the finding of Ito

et al. (22) that only 5.8% of RT IgG Ab is present in the nasal mucous and only 3.7% of RT IgG Ab is present in the mucous of the trachea, bronchi, and bronchioles; therefore, it is not expected that the IgG initially present in the nose or trachea would be adequate to provide protection.

In contrast to the upper RT results, both doses of IgG₁ anti-influenza virus mAb were able to protect the lungs from viral infection (Fig. 6). The 168- μ g IgG₁ dose that was completely ineffective in nasal protection was effective in protecting the terminal bronchioles from viral infection, as evidenced by the lack of viral pathology (Fig. 6C), whereas even the high dose (440 μ g) of pIgA anti-influenza virus mAb was unable to provide protection in the lower RT. These findings are in agreement with those of Ito et al. (22) that most RT IgG is located in the serous fluid of the alveolar epithelia, whereas most RT IgA is located in the nasal mucous. We agree with their hypothesis that protection of the lower RT is due to transudation of IgG Ab from the plasma to the surface of the alveolar epithelia and that diffusion of Ab is probably along a concentration gradient. Because pIgR-bearing ciliated respiratory epithelium is not present in the alveoli, pIgA Ab is not transcytosed into the alveolar serous fluids; hence, i.v. pIgA does not protect the lower RT from viral infection. This explains, for example, why the lungs are protected in immune IgA, J chain, or pIgR KO mice (13–15) and why only IgG (not IgA or IgM) virus-neutralizing Abs can cure influenza virus pneumonia in SCID mice (36).

Our findings suggest that under normal conditions S-IgA may protect the murine nose by preventing viral infection, although if anti-influenza virus IgG Ab titers are sufficiently high, plasma Ab leaking through virally damaged nasal epithelium may neutralize newly replicated virus, thereby limiting nasal viral infection. Thus, both Ab classes may play an important role in control of mucosal viral infection and, given the proper circumstances, either one may reduce viral shedding in the mucosal secretions in the absence of the other. The reduced viral shedding reported in the NT lavages of IgA KO mice, for example, may be due either to neutralization of newly replicated virus by plasma Ab leaking through virally damaged nasal epithelium or to viral neutralization by the inordinately high level of mucosal anti-influenza virus IgG or IgM Ab that develops in these animals secondary to the blockage of the IgG to IgA class switch (13, 38).

Our current study suggests that in the genetically normal mouse, IgA is more important than IgG in the protection of the upper RT, whereas IgG is more important than IgA in the protection of the lungs. In humans, live i.n. vaccines stimulate the production of both nasal mucosal IgA and IgG Abs and plasma IgG, whereas inactivated vaccines given i.m. stimulate systemic IgG production, but not local mucosal IgA Ab (26, 27, 39). Both routes of vaccination lower nasal viral shedding after viral challenge (26). Our results indicate that in the nose there may be two lines of defense against influenza viral infection. If influenza virus-specific SIgA is present, then initial viral infection of the mucosal epithelial cells is prevented, and plasma Ab serves as back-up protection. If only plasma IgG is present, then infection of the nasal mucosa may occur, but its progression is limited by the second line of defense, i.e., leakage of plasma anti-influenza virus IgG through the virally damaged epithelium. This two-tier protection is in agreement with the suggestion by Clements et al. (26) that sufficient Ab in either plasma or nasal secretions is capable of conferring resistance and is borne out by the results of the 2-year multicenter efficacy trial of an intranasally administered, attenuated, trivalent, cold-adapted, live influenza virus vaccine recently concluded (40). It is, therefore, apparent that to be the most effective, vaccination to protect

mucosal surfaces should follow a protocol that elicits the production of both mucosal S-IgA and systemic IgG Abs.

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