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Mucosal Immunity to Influenza Without IgA: An IgA Knockout Mouse Model

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IgA knockout mice (IgA \(^{-/-}\)) were generated by gene targeting and were used to determine the role of IgA in protection against mucosal infection by influenza and the value of immunization for preferential induction of secretory IgA. Aerosol challenge of naïve IgA \(^{-/-}\) mice and their wild-type IgA \(^{+/+}\) littermates with sublethal and lethal doses of influenza virus resulted in similar levels of pulmonary virus infection and mortality. Intranasal and i.p. immunization with influenza vaccine plus cholera toxin/ cholera toxin B induced significant mucosal and serum influenza hemagglutinin-specific IgA Abs in IgA \(^{+/+}\) (but not IgA \(^{-/-}\)) mice as well as IgG and IgM Abs in both IgA \(^{-/-}\) and IgA \(^{+/+}\) mice; both exhibited similar levels of pulmonary and nasal virus replication and mortality following a lethal influenza virus challenge. Monoclonal anti-hemagglutinin IgG1, IgG2a, IgM, and polymeric IgA Abs were equally effective in preventing influenza virus infection in IgA \(^{-/-}\) mice. These results indicate that IgA is not required for prevention of influenza virus infection and disease. Indeed, while mucosal immunization for selective induction of IgA against influenza may constitute a useful approach for control of influenza and other respiratory viral infections, strategies that stimulate other Igs in addition may be more desirable. *The Journal of Immunology, 1999, 162: 2530–2537.*

The mucosal immune system represents the first line of immunological defense against pathogens encountering the mucosal surfaces of the body. IgA is the primary Ig isotype induced at the mucosal surface (1, 2). Secretory IgA (S-IgA) \(^{1}\) in mucosal secretions provides protection against bacterial (3–6) and viral (7–16) pathogens and neutralizes microbial toxins (17, 18). S-IgA binds protein Ags, thus limiting their absorption, and helps to prevent allergies and other hypersensitivity reactions (19–21). Selective IgA deficiency in humans is quite common and occurs at a rate of about 1 case/500–700 people (22, 23). Most of these individuals are asymptomatic, but some individuals suffer from recurrent respiratory, gastrointestinal, and urogenital infections. Since IgG and IgM levels may be normal or elevated, the suggestion that S-IgA is essential for mucosal protection remains controversial (24, 25). The heterogeneity of IgA deficiency (23) and the fact that approximately 25% of IgA-deficient individuals may have unsuspected IgG subclass deficiencies (26) make it difficult to ascertain the precise role of IgA in mucosal pathogenesis.

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\(^{1}\)Abbreviations used in this paper: S-IgA, secretory IgA; CT, cholera toxin holoenzyme; CFB, cholera toxin B subunit; HA, hemagglutinin; pIgA, polymeric IgA; mIgA, monomeric IgA; KO, knockout; ES, embryonic stem; i.n., intranasal; SV, subunit vaccine; SPA, small particle aerosol; MDInd, minimum 50% infectious dose; MDCK, Madin-Darby canine kidney; GMT, geometric mean titer.

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IgA (mIgA) or IgG1 (7, 8). Mice injected with plgA were significantly protected against influenza virus challenge compared with those injected with IgG1. It was further shown that treatment with anti-IgA Abs of immune mice previously infected with influenza virus, but not anti-IgG1 or anti-IgM Abs, abrogated the IgA-mediated protection. In related studies, Gerhard and co-workers have shown that i.p. injection of SCID mice with plgA and IgM prevented infection with influenza virus but did not cure mice previously infected with virus (42). In contrast, IgG1, IgG2a, IgG2b, and IgG3 injections in mice resulted in a cure of influenza virus infection, even when injection was performed 7 days after virus infection. These and other studies (40, 41) suggest that IgA (and IgM) function primarily to prevent virus infection, while IgG cures virus infection.

Transgenic IgA−/− knockout (KO) mice were previously generated by gene targeting in which deletion of the entire IgA switch region and the 5′ half of the constant region occurred (43). In the present study these selective IgA-deficient animals were used to determine the role played by IgA in protection against influenza virus infection and disease. In addition, the value of preferential induction of S-IgA Abs by influenza vaccination was determined.

Materials and Methods
IgA KO mice
IgA transgenic KO mice were generated as previously described (43). Briefly, a targeting vector that produced a deletion of the entire IgA switch region as well as the first two Cε exons and part of the third Cε exon as previously described (45) was introduced by electroporation (44) into an Iα2 embryonic stem (ES) cell that was previously generated by us to produce an Iα exon KO mouse (45, 46). Successfully targeted ES cell clones were introduced into blastocystos, and chimeric mice were generated. Male chimeric mice with a high degree of chimerism were mated to female C57BL/6 mice by standard methods. Agouti offspring, which inherited either a wild-type or a mutated allele from the targeted ES cells, were phenotyped for expression of IgA in serum by ELISA (31, 47, 48). F1 heterozygous mice expressing the targeted ES cell allele were interbred to produce homozygotes.

Immunization of wild-type and IgA-deficient mice with influenza virus vaccine
Homozygous IgA−/− mice derived by inbreeding of F1 heterozygotes, and wild-type nontargeted littermates (IgA+/+) were immunized by intranasal (i.n.) or i.p. inoculations, 3 wk apart, using 2 tissue culture infectious doses of influenza virus at room temperature for 1 h. The serum-virus mixture was then transferred to the MDCK monolayer plates and incubated at 37°C. Trypsin was added at 2 μg/ml of trypsin after 24 h. The level of influenza virus-specific neutralizing Ab was determined by the inhibition of virus-induced hemagglutination of chicken RBC 5 days later.

Neutralizing Ab tests
Influenza virus-specific neutralizing Ab was determined as previously described (53). Briefly, MDCK cells were allowed to adhere in microtiter wells at 37°C for 4–6 h before aspiration of medium. Serial dilutions of sera or mAbs in 0.05 ml of serum-free MEM were incubated with 100 50% tissue culture infectious doses of influenza virus at room temperature for 1 h. The serum-virus mixture was then transferred to the MDCK monolayer plates and incubated at 37°C. Trypsin was added at 2 μg/ml of trypsin after 24 h. The plates were then washed three times with PBS and blocked with 1% BSA in PBS for 2 h at 37°C. Plates were again washed with PBS/0.25% Tween, and dilutions of serum or secretions to be tested were added to wells and incubated at 37°C for 2 h. Next, the plates were washed with PBS/Tween and incubated with appropriately diluted alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA (5 μg/ml) in PBS and left overnight at 4°C. The plates were then washed, substrate (p-nitrophenylphosphate in diethanolamine) was added, and the plates were allowed to develop, then were read in an ELISA reader. Ab titers were determined by end-point titration (i.e., the final dilution at which the OD value remained 2 SDs above the mean value of control samples). The Ag-specific IgM, IgG, or IgA titers were expressed as a ratio of the titer of specific Ab to total IgM, IgG, or IgA, respectively, that was present in serum or secretions to correct for varying amounts of total Ig in secretions or sera of individual mice and for variations in the amount of secretions collected.

ELISA for IFN-γ and IL-4
Secretions and serum were analyzed for Ag specificity and isotype (IgM, IgG, or IgA) using Ag-coated ELISA plates and isotype-specific anti-Ig Abs, similar to previously described methods (48, 54). In brief, ELISA grade polystyrene microtiter plates were coated with Ag (2.5 μg/ml) or goat anti-mouse IgM, IgG, or IgA (5 μg/ml) in PBS and left overnight at 4°C. The plates were then washed three times with PBS and blocked with 1% BSA in PBS for 2 h at 37°C. Plates were again washed with PBS/0.25% Tween, and dilutions of serum or secretions to be tested were added to wells and incubated at 37°C for 2 h. Next, the plates were washed with PBS/Tween and incubated with appropriately diluted alkaline phosphatase-conjugated goat anti-mouse IgM, IgG, or IgA. Finally, the plates were washed, substrate (p-nitrophenylphosphate in diethanolamine) was added, and the plates were allowed to develop, then were read in an ELISA reader. Ab titers were determined by end-point titration (i.e., the final dilution at which the OD value remained 2 SDs above the mean value of control samples). The Ag-specific IgM, IgG, or IgA titers were expressed as a ratio of the titer of specific Ab to total IgM, IgG, or IgA, respectively, that was present in serum or secretions to correct for varying amounts of total Ig in secretions or sera of individual mice and for variations in the amount of secretions collected.
FIGURE 1. Susceptibility of naive IgA KO and normal mice to influenza virus infection. A, Naive IgA\(^{-/-}\) KO and wild-type IgA\(^{+/+}\) mice (16–20 wk old) were infected with 0.1 LD\(_{50}\) of A/Taiwan/1/86 virus by SPA. On days 4 and 15, two to four mice per group were killed by CO\(_2\) inhalation. The lungs were harvested, and the levels of virus replication were determined. The mean ± SEM of virus titer (GMT log\(_{10}\)) for two experiments (five to eight mice per group) are presented. B, Mortality among naive mice that were infected with 3 LD\(_{50}\) of virus by aerosol. The data represent the percent cumulative mortality for four mice per group (B).

To assess the role of CD8\(^{+}\) cells in these mice, splenic lymphocytes were harvested from IgA\(^{-/-}\) knockout and wild-type IgA\(^{+/+}\) mice (16–20 wk old) 15 days after priming with influenza A/Taiwan/1/86 with 0.1 LD\(_{50}\) by aerosol. The cells were stimulated or not with influenza A/Taiwan/1/86 (H1N1) virus in vitro for 6 days. The percent lysis of EL-4 cells (H-2\(^b\)) target cells infected with influenza A/Taiwan or A/Beijing/89 (H3N2) was determined in a 4-h chromium release assay. Both IgA\(^{-/-}\) and IgA\(^{+/+}\) mice exhibited similar levels of influenza A virus-specific CTL against EL-4 cells infected with the homologous A/H1N1 virus and against cross-reactive A/Beijing (H3N2) virus (data not shown). No activity was exhibited against uninfected target cells or by unstimulated effector cells. A similar pattern of response was obtained from lymphocytes harvested 8 days after priming (data not shown). Analysis of IFN-\(\gamma\) (Th1) and IL-4 (Th2) secretion by ELISA and mRNA expression for the same cytokines showed similar patterns among IgA\(^{-/-}\) and IgA\(^{+/+}\) mice (data not shown).

Requirement for induction of IgA for protection of mucosal sites

To determine whether the induction of Ag-specific IgA at mucosal sites is required for protection against influenza virus infection, two experiments were performed. Firstly, IgA\(^{-/-}\) and IgA\(^{+/+}\) mice were immunized and boosted 3 wk later by coadministration of 2 \(\mu\)g of influenza A/Taiwan/1/86 virus SV and CTB/CT (20 ng/2 \(\mu\)g) i.n. or i.p., a regimen previously shown to induce mucosal IgA (50). Two weeks after the second inoculation, three or four mice per group were bled for serum, then killed, and the nasopharynx was washed. Serum and mucosal influenza A/Taiwan-specific IgA, IgG, and IgM Abs were determined. Influenza SV plus CTB/CT induced significant influenza HA-specific IgA in the nasal wash of IgA\(^{+/+}\) (but not IgA\(^{-/-}\) ) mice and IgG and IgM Abs in both IgA\(^{-/-}\) and IgA\(^{+/+}\) mice (Table I). As expected, i.n. immunization stimulated a significantly higher level of IgA in the nasal wash specimen than i.p. immunization among IgA\(^{+/+}\) mice. Interestingly, IgA\(^{-/-}\) mice may have compensated for IgA deficiency by producing a markedly higher level of IgG than IgA\(^{+/+}\) mice (\(p = 0.09\)) in mucosal sites as well as in systemic sites after i.n. immunization (24, 25). The higher than expected IgG levels in IgA\(^{+/+}\) mice following i.p. immunization are most likely due to one mouse that exhibited an extremely high level of IgG Ab compared with others in the same group.

Three weeks after the second injection, the remaining mice were challenged with 3 LD\(_{50}\) of A/Taiwan virus by SPA. On days 4 and...

Results

Susceptibility of naive IgA\(^{-/-}\) mice to influenza virus infection and mortality

To determine whether IgA\(^{-/-}\) deficiency rendered these mice intrinsically more susceptible to influenza virus infection and disease, two experiments were performed. First, naive IgA\(^{-/-}\) and IgA\(^{+/+}\) littermates (16–20 wk old) were infected with a sublethal (0.1 LD\(_{50}\)) dose of A/Taiwan/1/86 virus by SPA. Four and fifteen days later, four mice per group were killed by CO\(_2\) inhalation. The lungs were harvested, and the levels of virus replication were determined. Influenza A virus replicated to the same peak levels on day 4 in both groups of mice and declined at identical rates to similar titers 15 days later (Fig. 1A). In the second experiment mice were infected with a lethal (3.0 LD\(_{50}\)) dose of virus by SPA. Cumulative mortality was recorded for 14 days. Both groups of mice were killed by the lethal influenza virus infection at the same rate (Fig. 1B). These results suggest that IgA\(^{-/-}\) KO mice were not more susceptible to influenza virus infection and mortality than normal IgA\(^{+/+}\) littermates. They also cleared virus infection normally.

Four times. Substrate (one or two tablets of \(\rho\)-nitrophenol phosphate per 10 ml of diethanolamine buffer, pH 9.8) was then added, and color was allowed to develop for 10–120 min. Absorbance in each well was read at a wavelength of 405 nm using a Molecular Devices automatic microplate reader (Menlo Park, CA). The data were collected in a SOFTmax data reduction software. Murine recombinant IFN-\(\gamma\) and IL-4 were used to generate standard curves. The amount of cytokine in the test samples was extrapolated from the standard curves and expressed as picograms per milliliter of cytokine.

Generation of secondary CTL response and \(^{51}\)Cr release assay for CTL

Influenza A virus-specific CTL were generated by stimulating splenic lymphocytes with virus-infected autologous cells for 6 days. The effector cells so generated were assayed for cytotoxicity against virus-infected target cells in a 4-h chromium release assay as previously described (51, 53).

Statistical analysis

Differences in cumulative mortality among different groups were determined by the \(\chi^2\) test using TRUE EPISTAT statistical software (Epistat Services, Richardson, TX). Comparisons of geometric mean titer (GMT) for virus and Ab titers, mean cytokine levels (picograms per milliliter), and stimulation indexes between groups were made with a two-tailed Student’s \(t\) test procedure in ANOVA using the Statistical Analysis System (SAS Institute, Cary, NC).

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Three weeks after the second injection, the remaining mice were challenged with 3 LD\(_{50}\) of A/Taiwan virus by SPA. On days 4 and...
Table I. Nasal wash (NW) and serum influenza HA-specific Abs in mice immunized with influenza A/Taiwan subunit vaccine (SV)*

<table>
<thead>
<tr>
<th>Mice</th>
<th>n</th>
<th>Vaccine</th>
<th>NW IgA</th>
<th>Serum IgA</th>
<th>NW IgG</th>
<th>Serum IgG</th>
<th>NW IgM</th>
<th>Serum IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA−/−</td>
<td>3</td>
<td>CTB/CT, i.n.</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IgA+/+</td>
<td>5</td>
<td>CTB/CT + SV, i.n.</td>
<td>ND</td>
<td>ND</td>
<td>2,048 ± 0</td>
<td>32,768 ± 0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IgA−/−</td>
<td>4</td>
<td>CTB/CT, i.n.</td>
<td>ND</td>
<td>ND</td>
<td>1,184 ± 505</td>
<td>25,088 ± 768</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
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</tr>
<tr>
<td>IgA−/−</td>
<td>3</td>
<td>CTB/CT + SV, i.p.</td>
<td>0.00</td>
<td>0.00</td>
<td>853 ± 615</td>
<td>8192 ± 0</td>
<td>85 ± 85</td>
<td>64 ± 32</td>
</tr>
<tr>
<td>IgA+/+</td>
<td>3</td>
<td>CTB/CT + SV, i.p.</td>
<td>12 ± 4</td>
<td>96 ± 32</td>
<td>1,365 ± 683</td>
<td>176,128 ± 165,936</td>
<td>106 ± 77</td>
<td>192 ± 32</td>
</tr>
</tbody>
</table>

* Mice were immunized by 2 i.n. or i.p. inoculations, 3 wk apart, using 2 μg of influenza A/Taiwan/1/86 (H1N1) SV with CTB/CT (2 μg/20 ng) as adjuvants (in 20 μl PBS), followed by light anesthesia with metofane. Two weeks after the second inoculation, three to five mice per group were bled for serum and then killed, and the nasopharynx was washed. Serum and mucosal IgA, IgG, and IgM Abs to A/Taiwan/1/86 virus were determined by ELISA.

The Journal of Immunology

FIGURE 2. Protective efficacy of influenza SV plus CTB/CT against pulmonary influenza virus replication and mortality in immunized IgA−/− and IgA+/+ mice. IgA−−/− KO and wild-type IgA+/+ mice (16–20 wk old) immunized with SV+ CTB/CT were challenged with 3LD50 of A/Taiwan/1/86 virus by SPA as described in Table I. On days 4 and 8 mice were killed by CO2 inhalation, and lungs were harvested for virus titration. An asterisk indicates that the virus titer is significantly lower than that in mice that received only CTB/CT (p < 0.05; four to six mice per group). Mortality was observed in the remaining seven or eight mice per group for 21 days.

8 post-virus challenge, a subset of mice was killed for pulmonary virus measurements. The remaining mice were observed for mortality for 21 days. Both IgA−− and IgA+/+ mice immunized i.n. with influenza SV with CTB/CT exhibited significant reductions in lung virus on day 8 compared with mice that received CTB/CT alone (Fig. 2A). Influenza SV and CTB/CT stimulated significant protection from influenza mortality among both IgA−− and IgA+/+ mice compared with CTB/CT alone (Fig. 2B and C). Both IgA−− and IgA+/+ mice immunized i.p. with influenza SV and CTB/CT also exhibited significant reductions in lung virus replication on days 4 and 8. Both immunized IgA−− and IgA+/+ mice were completely protected from death (Fig. 2B and C). Unimmunized control (CTB/CT) IgA−− mice exhibited a higher level of mortality (88% vs 63%) and virus replication than IgA+/+ control mice. However, the differences were not significant.

In the second experiment IgA−− and IgA+/+ mice were immunized with influenza SV as described above and then challenged with a nonlethal (50 MID50) dose of A/Taiwan/1/86 virus by SPA. Twenty-four and forty-eight hours later four mice per group were killed by CO2 inhalation. Lungs, nasal turbinates, and nasal washes were harvested, and the levels of virus replication were determined. The low dose virus challenge allowed for assessment of the requirement of IgA for prevention of infection in the nasal passages as well as in the lung. HA-specific IgA Abs were again induced in IgA+/+, but not in IgA−−, mice, while IgG and IgM Abs were induced in both (data not shown). The number of infected mice was similar in the two groups following challenge with a nonlethal (50 MID50) dose of influenza virus by aerosol (data not shown). Twenty-four hours following virus challenge the level of virus in the lungs of IgA+/+ mice was significantly reduced among immunized mice compared with that in unimmunized controls (p < 0.0001), but was only slightly reduced in IgA−− mice (Fig. 3). Virus replication in the nasal turbinates was unchanged in both IgA−− and IgA+/+ mice, while nasal wash virus was barely detectable at 24 h. Forty-eight hours following virus challenge, the level of virus in the lungs of both IgA−− and IgA+/+ mice was significantly reduced among immunized mice compared with that in unimmunized controls (p < 0.0001). Viral replication was also significantly reduced in the nasal turbinates of immunized IgA−− and IgA+/+ mice (p < 0.01) and IgA+/+ (p < 0.001) mice. Interestingly, influenza vaccination reduced nasal wash virus to undetectable levels in both IgA−− (p < 0.01)
and IgA subtypes (p, 0.1) compared with that in unimmunized mice. Immunization with influenza vaccine resulted in a higher level of virus reduction among IgA+/+ mice compared with IgA-/- mice. A tendency toward a higher level of virus among unimmunized IgA-/- mice compared with IgA+/+ mice may explain this discrepancy.

Adoptive transfer of Igs

To directly determine the Ig classes or subclasses that could mediate protection against local influenza virus challenge, naive IgA-/- mice were administered i.p. mAbs containing 4-log10 virus-neutralizing units of activity against influenza A/PR/8/34 virus as previously described by Gerhard and co-workers (42). Four hours later, treated mice were challenged with 50 MID50 of influenza A/PR/8/34 virus by small particle aerosol. Figure 4 shows the distribution of virus-neutralizing activity of transferred mAb in three representative mice sacrificed 4 h after transfer. Serum from mice administered influenza virus-specific pIgA, mIgA, IgM, or IgG1 mAb exhibited significant neutralizing activity against influenza A/PR/8/34 virus. In addition, all four mAbs, except mIgA, had transduced or transcytosed to the lungs and nasal turbinates, while only pIgA and IgM exhibited some activity in the nasal passages. Significant serum virus-neutralizing activity was also detected at 24 and 48 h postchallenge for all mAbs except IgM, which declined significantly, possibly due to its short half-life (42). None was detected in the nasal wash specimens.

Twenty-four hours following influenza virus challenge pIgA, mIgA, IgM, and IgG1 were all effective in preventing virus infection as indicated by reduced lung virus titers (p < 0.05; Fig. 5A). Administration of IgA-/- mice with IgG2a mAb also resulted in significant reduction of pulmonary virus (p < 0.05) 24 h following virus challenge (Fig. 5B). In addition, pIgA, IgG1, and IgG2a prevented further replication of virus (curing of virus infection), as indicated by further reduction of virus titer determined 48 h after virus challenge. It is noteworthy that only pIgA and IgG2a exhibited any efficacy in reducing virus titers in the nasal turbinates and passages (nasal wash).

Discussion

An IgA KO mouse (IgA-/-) has been generated by gene targeting and was used to determine the role of IgA in protection against mucosal infection and the value of immunization for preferential induction of S-IgA. The IgA-/- mice are totally deficient in IgA Igs and produce increased levels of IgG and IgM, possibly as a compensatory mechanism, as shown for IgA-deficient humans (23, 24). These mice respond normally to T and B cell mitogens and express normal levels of IFN-γ and IL-4 and virus-specific CD8+ CTL activity, indicative of their immunocompetence.

The importance of IgA Ab in susceptibility to influenza virus infection and disease and in preventing influenza infection was determined using three approaches. The first approach tested the hypothesis that if IgA plays an important role in the infectivity of the upper respiratory tract with influenza virus, then naive IgA-/- mice were administered i.p. mAbs containing 4-log10 virus-neutralizing units of activity against influenza A/PR/8/34 virus as previously described by Gerhard and co-workers (42). Four hours later, treated mice were challenged with 50 MID50 of influenza A/PR/8/34 virus by small particle aerosol. Figure 4 shows the distribution of virus-neutralizing activity of transferred mAb in three representative mice sacrificed 4 h after transfer. Serum from mice administered influenza virus-specific pIgA, mIgA, IgM, or IgG1 mAb exhibited significant neutralizing activity against influenza A/PR/8/34 virus. In addition, all four mAbs, except mIgA, had transduced or transcytosed to the lungs and nasal turbinates, while only pIgA and IgM exhibited some activity in the nasal passages. Significant serum virus-neutralizing activity was also detected at 24 and 48 h postchallenge for all mAbs except IgM, which declined significantly, possibly due to its short half-life (42). None was detected in the nasal wash specimens.

Twenty-four hours following influenza virus challenge pIgA, mIgA, IgM, and IgG1 were all effective in preventing virus infection as indicated by reduced lung virus titers (p < 0.05; Fig. 5A). Administration of IgA-/- mice with IgG2a mAb also resulted in significant reduction of pulmonary virus (p < 0.05) 24 h following virus challenge (Fig. 5B). In addition, pIgA, IgG1, and IgG2a prevented further replication of virus (curing of virus infection), as indicated by further reduction of virus titer determined 48 h after virus challenge. It is noteworthy that only pIgA and IgG2a exhibited any efficacy in reducing virus titers in the nasal turbinates and passages (nasal wash).
results were further confirmed by challenging IgA−/− mice and their heterozygous normal IgA+/− littermates infected with a sublethal dose (0.1 LD50) of influenza A/Taiwan/1/86 (H1N1) virus by SPA. In addition, IgA deficiency did not render these mice more susceptible to severe influenza disease and mortality because the levels of cumulative mortality among IgA−/− KO and wild-type IgA+/− mice infected with a lethal dose (3.0 LD50) of A/Taiwan/1/86 virus by SPA were similar. The results reported above also suggest that IgA does not play a role in attenuation of influenza disease. Recent LD50 titration in both groups of mice reveal similar susceptibility to influenza mortality (data not shown).

The second approach tested the hypothesis that if selective induction of Ag-specific IgA at mucosal sites is required for protection against influenza virus infection, then mucosal immunization with influenza vaccines will be more effective in IgA−/− mice. To determine whether the induction of virus-specific IgA Ab was essential for protection against influenza infection and disease, IgA−/− along with wild-type control nontargeted littermate IgA+/− mice were immunized with influenza SV with a combination of CTB/CT, a regimen that stimulated significant mucosal and serum IgA, IgG, and IgM Abs in normal mice and IgG and IgM in IgA−/− mice. Following challenge with a lethal (3 LD50) dose of influenza virus by small particle aerosol, the levels of virus replication in the nasopharynx, nasal turbinate, and lung were similar. In addition, the protective efficacy of the influenza vaccine-CTB/CT regimen was similar among IgA−/− and IgA+/− mice. These results suggest that IgA was not essential for preventing viral infection, a reduction in the severity of disease, or both. These results were further confirmed by challenging IgA−/− and IgA+/− mice immunized with influenza SV as described above with 50 MID50 of influenza A/Taiwan by aerosol to test the effect of immunization on a less overwhelming virus load. Again, the levels of virus in the lung, nasal turbinate, and nasal cavity determined 24 and 48 h later showed that the influenza vaccine-CTB/CT regimen prevented virus infection to the same extent in IgA−/− and IgA+/− mice. Furthermore, the results showed that while virus replication increased in control mice that received CTB/CT 48 h following challenge, influenza vaccine-CTB/CT regimen either prevented a further increase in virus titer or completely suppressed their replication in the nasal passages. No differences were demonstrated between IgA−/− and IgA+/− mice.

The final approach was to determine definitively whether passive immunization with influenza-specific pIgA was more effective than other isotypes for prevention of influenza infection in IgA−/− mice. Challenge of IgA−/− mice passively transferred with influenza A/PR/8/43 virus HA-specific mAb of pIgA, mlgA, IgG1, IgG2a, or IgM before challenge with A/PR/8/34 virus demonstrated that IgG1, IgG2a, and IgM as well as pIgA isotypes were all effective in preventing virus infection to varying degrees in the lungs, nasal turbinate, and nasal passages. More importantly, IgG2a and to some extent IgG1 exhibited a similar virus-curing capacity as pIgA by preventing further virus replication, particularly in the lung.

The present results are in partial agreement with those of other studies (7, 8, 42); namely, 1) mAbs of the pIgA, mlgA, IgM, IgG1, and IgG2a isotypes were effective in preventing (or at least reducing) influenza virus infection; and 2) pIgA, but not mlgA, is most efficiently transcytosed to the mucosal surfaces, where they prevent or reduce virus infection. The present data suggest that in addition to IgG1 and IgG2a, pIgA was somewhat effective in "curing" virus infection. The present results are in agreement with two previous studies that demonstrated that passively transferred pIgA (but not mlgA) prevented mucosal influenza virus infections (7, 8, 42); these results also show, however, that IgG1 could protect against mucosal influenza infection in contrast to one of the studies (7, 8). The difference in results may be due to the different modes of infection employed in the two studies. In those studies, mice were infected by small volume i.n. inoculation, which deposited virus initially in the nasal passages (7, 8), while in the present study, as in that by Palladino et al. (42), mice were infected by aerosolization, which deposited virus in both the lungs and nasal passages. Nonetheless, the passive transfer results, in which neutralizing Ab activity was detected in the lungs and nasal passages, suggest that IgG1 and IgG2a were equally as effective as pIgA in preventing virus infection and inhibiting replication in those compartments. Our results are further supported by a previous study showing that passive immunization with purified influenza HA-specific IgG was as effective as IgA in preventing homotypic influenza virus infection in normal mouse respiratory tract even though IgA was more effective against heterotypic challenge (40).

This study has presented very compelling evidence that IgA may not be required for prevention of influenza virus infection and disease. This is supported by the fact that not all IgA-deficient subjects suffer from persistent respiratory infections (22, 23). A recent study showed that J chain KO mice were readily protected...
from influenza virus infection, indicating that IgA receptor-mediated transport is not required (56) as suggested by other studies. Similar results were obtained in showing that IgA was not required for protection against Helicobacter pylori infection in the gut (57).

The present results have strong implications regarding strategies for stimulating selective induction of IgA in the mucosa. Since the i.p. immunization in the present studies was as or more effective than i.n. immunization in protecting the mucosa, and IgG2a protected equally well as IgA, one can argue that efforts should be directed at optimizing well-established systemic immunization strategies that induce both mucosal and systemic Ab responses, some of which can transudate and confer protection at the mucosal surfaces. Such an approach may include increasing the dose of Ags used for i.m. immunization. One recent study clearly showed that a ninefold increase in the dose of influenza virus vaccine was very effective in stimulating strong systemic as well as mucosal IgG and IgA Ab responses in young volunteers (58). Influenza virus infection affects the upper respiratory (where IgA predominates) and IgA Ab responses in young volunteers (58). Influenza virus infection affects the upper respiratory (where IgA predominates) and lower respiratory (where IgG predominates) tracts, and MHC class I-restricted CD8+ cytotoxic T cells have been demonstrated to also mediate the clearance of viral infection. In principle, therefore, vaccination strategies that stimulate multiple Ab isotypes rather than being targeted exclusively to one isotype and that also induce CD8+ CTL responses represent the best approach for control of influenza virus infection and disease.

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References

region in mice leads to IgA deficiency with alterations in expression of other immunoglobulin isotypes. J. Immunol. 162:2521.


