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# Protection Against Influenza Virus Infection in Polymeric Ig Receptor Knockout Mice Immunized Intranasally with Adjuvant-Combined Vaccines

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The role of secretory IgA in conferring cross-protective immunity was examined in polymeric (p)IgR knockout (KO) mice immunized intranasally with different inactivated vaccines prepared from A/PR/8/34 (H1N1), A/Yamagata/120/86 (H1N1), A/Beijing/262/95 (H1N1), and B/Ibaraki/2/85 viruses and infected with the A/PR/8/34 virus in the upper respiratory tract (RT)-restricting volume. In wild-type mice, immunization with A/PR/8/34 or its variant (A/Yamagata/120/86 and A/Beijing/262/95) vaccines conferred complete protection or partial cross-protection against infection, while the B-type virus vaccine failed to provide protection. The protection or cross-protection was accompanied by an increase in the nasal A/PR/8/34 hemagglutinin-reactive IgA concentration, which was estimated to be >30 times the serum IgA concentration and much higher than the nasal IgG concentration. In contrast, the blockade of transepithelial transport of dimeric IgA in pIgR-KO mice reduced the degree of protection or cross-protection, in parallel with the marked increase in serum IgA concentration and the decrease in nasal IgA concentration (~20 and 0.3 times those in wild-type mice, respectively). The degree of the reduction of protection or cross-protection was moderately reversed by the low but non-negligible level of nasal IgA, transudates from the accumulated serum IgA. These results, together with the absence of the IgA-dependent cross-protection in the lower RT and the unaltered level of nasal or serum IgG in wild-type and pIgR-KO mice, confirm that the actively secreted IgA plays an important role in cross-protection against variant virus infection in the upper RT, which cannot be substituted by serum IgG. *The Journal of Immunology*, 2002, 168: 2930–2938.

The mucosal immune system is the first immunological barrier against most pathogens that invade the body via the mucosal surface (1). Secretory IgA (S-IgA)<sup>2</sup> Abs are major effector molecules in this system, and their roles as the first defense line against influenza viruses in the respiratory mucosa have been studied extensively (2–4). The influenza virus causes an annual epidemic of influenza by altering the antigenic properties of its surface hemagglutinin (HA), which is involved in the initiation of infection (5). To control influenza, inactivated vaccines are parenterally administered to induce serum anti-HA IgG Abs, which are highly protective against homologous virus infection but are less effective against heterologous virus infection (5, 6). In contrast, a large number of studies have shown that the mucosal immunity acquired by natural infection, which is largely due to S-IgA

in the respiratory tract (RT), is more effective and more cross-protective against virus infections than the systemic immunity induced by parenteral vaccines in humans (7–10) and mice (3, 11). Based on these findings, S-IgA induction in the RT has been advocated to improve the protective efficacy of inactivated influenza vaccines (2, 3, 12). In this regard, we have demonstrated that intranasal (i.n.) immunization of mice with an inactivated vaccine together with cholera toxin B subunits (CTB) containing a trace amount of the holotoxin (CTB\*) induces not only S-IgA against HA, which provides strong cross-protection against variant virus infection within the same subtype in the upper RT, but also serum IgG, which provides weak cross-protection against the variant virus infection in the lower RT (13–16). These findings support the concept that mucosal IgA is primarily involved in protection against infection in the upper RT, whereas serum IgG, which diffuses into the lower RT, is predominantly involved in the protection in the lower RT (17–19).

The direct biological role of S-IgA in protection against virus infection has also been demonstrated in a number of studies (3, 4, 20–25). Anti-influenza S-IgA purified from the RTs of mice immunized with HA molecules protected nonimmune mice from the virus infection, when administered i.n. (21, 22). Anti-HA-specific monoclonal polymeric (p)IgA injected i.v. was transported more efficiently into the nasal surface than were monomeric IgA or IgG1 (23). Treatment of mice, which were immunized with the live virus, with anti-IgA, but not anti-IgG and anti-IgM, abrogated the protection (24). In addition, monoclonal pIgA and S-IgA are several times more effective than monoclonal monomeric IgA in terms of hemagglutination inhibition (HI) and virus neutralization (NT) activities (25). These results suggest that S-IgA is a major mediator

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<sup>2</sup> Abbreviations used in this paper: S-IgA, secretory IgA; p, polymeric; KO, knockout; A/PR8, A/PR/8/34; A/Yamagata, A/Yamagata/120/86; A/Beijing, A/Beijing/262/95; B/Ibaraki, B/Ibaraki/2/85; RT, respiratory tract; HA, hemagglutinin; CT, cholera toxin; CTB, CT B subunit; CTB\*, CTB containing a trace amount of CT; i.n., intranasal(y); dIgA, dimeric IgA; HI, hemagglutination inhibition; NT, virus neutralization; SC, secretory component.

of nasal immunity and has inherently greater antiviral activity due to its polymeric nature.

It has been proposed that S-IgA Ab is a dimeric IgA (dIgA) that is produced by IgA-producing B cells in the lamina propria and secreted across the mucosal epithelial cells to the mucosal surface. Thus, dIgA binds to a pIgR on the basolateral surface and is transported to the apical side of the epithelial cells, where the secretory component (SC), the extracellular region of pIgR cleaved by a specific protease, remains bound to the dIgA, resulting in the formation of S-IgA (26). The involvement of pIgR in the transepithelial transport of dIgA has been verified by many investigators (27–29), and data showing that pIgR binds to dIgA via the J chain have also been reported (30). Recently, to further define the physiological roles of the J chain, IgA or pIgR, KO mice with a disruption of each gene, have been generated (31–35).

Mbawuikwe et al. (33) demonstrated that in IgA knockout (KO) mice, i.n. and i.p. immunizations with a CTB\*-combined vaccine provided protection against a lethal dose of homologous viruses, in parallel with the induction of both mucosal and serum anti-HA IgG and IgM Abs, suggesting that IgA is not required for preventing influenza virus infection. The IgA-KO mouse seems to be an ideal model for individuals with selective IgA deficiency whose IgG and IgM levels are either normal or elevated (36–38). Thus, the finding that IgA is not necessary for mucosal protection in IgA-KO mice challenges the notion that S-IgA is responsible for protection against virus infection in the RT. However, in the previous experiments, the important role of S-IgA Abs in providing cross-protection against heterologous virus infection in the upper RT was not examined. The ability of S-IgA to provide cross-protection can be determined by analyzing Ab and virus titers in the nasal wash of mice immunized i.n. with a CTB\*-combined vaccine and challenged by a RT infection with heterologous viruses (14).

Shimada et al. (34) have established a mouse strain lacking exon 2 of the *pIgR* gene and demonstrated that in pIgR-KO mice both intestinal and hepatic transcytoses of dIgA were severely blocked, resulting in the massive accumulation of dIgA in the serum; this suggests that dIgA is mainly transported by pIgR on the epithelial cells of the intestine and hepatocytes. Johansen et al. (35) have also established a mouse strain lacking exon 3 of the *pIgR* gene and demonstrated similar results. Under these circumstances, the superior ability of S-IgA over IgG in preventing influenza in the upper RT remains to be clarified in vaccine-immunized pIgR-KO mice.

In this study, the protective roles of S-IgA against influenza were examined in pIgR-KO mice immunized i.n. with different CTB\*-combined inactivated vaccines and challenged by a RT infection with the virus. In addition, to delineate the transport of dIgA across the mucosal membrane in the upper RT, the nasal anti-HA IgA and IgG Ab concentrations were determined by estimating the nasal mucus volume. The results confirm that S-IgA plays important roles not only in protection against upper RT infection by homologous viruses, which can be substituted by serum IgG, but also cross-protection against upper RT infection by variant viruses, which cannot be substituted by serum IgG.

## Materials and Methods

### *pIgR-KO mice*

pIgR-transgenic KO mice were generated as previously described (34). Heterozygous mice were generated by mating male chimeric mice and female BALB/c mice, and F<sub>1</sub> mice were backcrossed with BALB/c mice eight times (N8). Some experiments were conducted using littermates of the N8 or N9 generation. Additional experiments were conducted by intercrossing pIgR-KO mice with homozygous mice from the N10 generation.

Screening for mice with a disruption at exon 2 of the *pIgR* gene in littermates of a cross between heterozygous mice was conducted by PCR analysis of genomic DNAs extracted from each mouse tail using a DNeasy

Tissue kit (Qiagen, Tokyo, Japan). Sense primer I-1 (5'-GACCCCTTAC CAGACTCCTGGTCTT), antisense primer E2-1 (5'-ATTCTCATACAA GAAGCCAACCTGA) of the wild-type genome, and antisense primer Neo-1 (5'-AAGTATCCATCATGGCTGATGCAAT) of the gene-targeting region were used in PCR. PCR products were separated by agarose gel electrophoresis (Fig. 1A).

### Western blot analysis

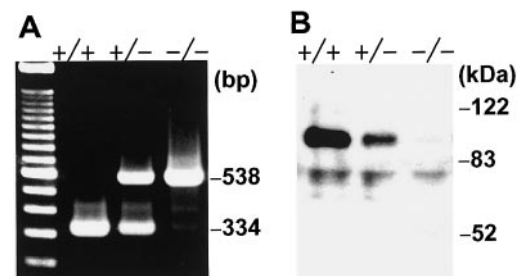
Nasal wash samples were collected from the decapitated heads of wild-type (pIgR<sup>+/+</sup>), heterozygous (pIgR<sup>+/-</sup>), and homozygous (pIgR<sup>-/-</sup>) BALB/c mice as described previously (15, 34). After SDS-PAGE was conducted, proteins were blotted onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) using the wet blotting system (Bio-Rad, Hercules, CA). To detect pIgR and SC proteins, the membrane was incubated with rabbit anti-mouse SC IgG (3 μg/ml) (34) and then treated with peroxidase-conjugated anti-rabbit Igs (Amersham, Arlington Heights, IL). Thereafter, the membrane was soaked in ECL reagents (Pierce, Rockford, IL). The SC protein was detected as a single band of ~100 kDa in the nasal wash samples from pIgR<sup>+/-</sup> and pIgR<sup>+/+</sup> mice, but it was not detected in the nasal wash of pIgR<sup>-/-</sup> mice (Fig. 1B). A band of ~70 kDa for the nasal wash was regarded as nonspecific staining, because the band was detected using both rabbit anti-mouse SC IgG and normal rabbit serum (34).

### HA vaccines and influenza viruses

HA vaccines (split-product virus vaccines) were prepared from influenza viruses A/PR/8/34 (A/PR8; H1N1), A/Yamagata/120/86 (A/Yamagata; H1N1), A/Beijing/262/95 (A/Beijing; H1N1), and B/Ibaraki/2/85 (B/Ibaraki) according to the method of Davenport et al. (39) at the Kitasato Institute (Saitama, Japan). The HA vaccine was produced from the viruses, grown in the allantoic cavity of 10- to 11-day fertile chicken eggs, highly purified, and disintegrated with ether. The vaccine contained all the proteins from the virus particle, including HA molecules as the major component (~30% of the total protein). These viruses, with the exception of A/PR8, which have been used to prepare vaccines in Japan, are immunologically different from one another (15). The A/Yamagata (H1N1) or A/Beijing (H1N1) vaccine-immunized mice contained a serum HI Ab that is cross-reactive to the A/PR8 virus, with titers of 1/128 or <1/128. The B/Ibaraki vaccine-immunized mice contained no serum HI Ab that is cross-reactive to A/PR8 virus (titers of <1/128). The A/PR8 virus was passaged 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day fertile chicken eggs.

### Immunization

Wild-type (pIgR<sup>+/+</sup>) and homozygous (pIgR<sup>-/-</sup>) female and male BALB/c mice, 6–8 wk old, were used in all experiments. Groups of five to six mice for each experiment were anesthetized by i.p. injection of amobarbital sodium (0.25 ml of a 1 μg/ml solution). Then they were immunized by dropping 1 μl of PBS containing the required concentration of



**FIGURE 1.** A, PCR products from genomic DNAs that were extracted from tails of littermates of a cross between heterozygous (pIgR<sup>+/-</sup>) mice and separated by agarose gel electrophoresis. PCR was performed using a sense primer, an antisense primer of the wild-type genome, and an antisense primer of the gene-targeting region. pIgR<sup>+/+</sup> and pIgR<sup>-/-</sup> mice provided 334- and 538-bp bands, respectively, and pIgR<sup>+/-</sup> mice provided both 334- and 538-bp bands. B, Western blot analysis of SC proteins from nasal washes from littermates of a cross between heterozygous (pIgR<sup>+/-</sup>) mice. Nasal wash samples were separated by reducing SDS-PAGE, and the separated proteins were blotted onto a polyvinylidene difluoride membrane and probed with rabbit anti-mouse SC IgG Ab. Detection was conducted using HRP-labeled anti-rabbit Igs and then with chemiluminescence reagents.



a CTB\*-combined HA vaccine into each nostril (15). In a single-dose regimen, the mice were immunized with the HA vaccine (5  $\mu\text{g}/2 \mu\text{l}$ ) prepared from A/PR8, A/Yamagata, A/Beijing, or B/Ibaraki virus, together with CTB\* (1  $\mu\text{g}/2 \mu\text{l}$  CTB from Sigma-Aldrich (St. Louis, MO) supplemented with 0.2% cholera toxin (CT; Sigma-Aldrich)) (16). In a two-dose regimen, the mice immunized primarily with the adjuvant-combined HA vaccine were boosted with the HA vaccine (5  $\mu\text{g}/2 \mu\text{l}$ ) alone 4 wk later.

### Infection

Two conditions for virus infection were used (14, 40, 41). Under one of these conditions, each mouse was anesthetized and then infected by i.n. dropping 20  $\mu\text{l}$  of PBS containing an A/PR8 virus suspension with  $10^{4.1}$  EID<sub>50</sub> per mouse. This procedure induced a total RT infection that caused virus shedding from the nose and lungs and ultimately death from viral pneumonia  $\sim 7$  days later. The virus inoculum was  $20 \times \text{LD}_{50}$ , where LD<sub>50</sub> was determined by the total respiratory infection method. The nasal and lung wash virus titers on day 3 after infection were used as indices of protection in the upper and lower RTs of immunized mice, respectively. Under the second infection condition, which was mainly used in the present experiments, anesthetized mice were infected by dropping 1  $\mu\text{l}$  of PBS containing an A/PR8 virus suspension with  $10^{4.1}$  EID<sub>50</sub> into each nostril (a total of 2  $\mu\text{l}$  per mouse). The nose-restricted volume (2  $\mu\text{l}$ ) of the virus suspension induced nose-localized infection that began in the nasal mucosa and spread to the lungs in 3–7 days but was not lethal. The nasal wash virus titers, the peak of which was attained 3 days after infection, were used as indices of protection in the upper RTs of the immunized mice.

### Specimens

Mice were anesthetized and then bled from the heart with a syringe. Serum was separated from blood by centrifugation and used for Ab titration. After bleeding, the bronchoalveolar wash was collected by washing the excised trachea and lungs twice with 2 ml of PBS containing 0.1% BSA, as described previously (40). The nasal wash was collected by washing the nasal cavity of the excised head three times with the same 1 ml of PBS containing 0.1% BSA. The nasal wash removed  $\sim 80\%$  of nasal mucus-associated Abs after three washes and  $\sim 95\%$  after seven washes, as determined by ELISA for Ab titers of nasal washes from the immunized mice. The nasal or bronchoalveolar wash was used for Ab and virus titrations after removing cellular debris by centrifugation. The physical manipulation of the nose to collect secretions at the luminal surface caused leakage of blood from the lamina propria. Blood contamination of the nasal wash was estimated to be  $0.003 \pm 0.002\%$  (mean  $\pm$  SD) by counting the RBCs and comparing the count with that of RBCs ( $\sim 8 \times 10^9$  cells/ml) from 10 specimens.

### ELISA

The levels of IgA and IgG Abs against HA molecules purified from the A/PR8 viruses were determined by ELISA as described previously (15). Briefly, ELISA was conducted sequentially from the solid phase (EIA plate; Costar, Cambridge, MA) with a ladder of reagents consisting of the following: first, HA molecules purified from the A/PR8 virus according to the procedure of Phelan et al. (42); second, nasal wash, bronchoalveolar wash or serum; third, goat anti-mouse IgA Ab ( $\alpha$ -chain specific; Amersham) or goat anti-mouse IgG Ab ( $\gamma$ -chain-specific; Amersham) conjugated with biotin; fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies, Rockville, MD); and, finally, *p*-nitrophenylphosphate. The chromogen produced was measured for absorbance at 405 nm with a SJeia Autoreader (model er-8000; Sanko Junyaku, Tokyo, Japan). A 2-fold serial dilution of purified HA-specific IgA or HA-specific monoclonal IgG (160 ng/ml) was used as a standard. The Ab concentration of an unknown specimen was determined from the standard regression curve constructed for each assay with the programmed SJeia Autoreader. The chromogen produced was measured for absorbance at 405 nm using an ELISA reader.

The approximate amount of anti-A/PR8 IgM Abs was also determined indirectly by ELISA using the purified HA-specific monoclonal IgG as the standard, after purification of IgM in nasal wash and serum samples by passing them through an affinity column conjugated with goat anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA), because a reliable standard for the IgM assay was not obtained. Briefly, ELISA was conducted sequentially from the solid phase with a ladder of reagents consisting of the following: first, purified HA molecules; second, nasal wash or serum IgM; third, goat anti-mouse  $\kappa$ -chain-specific Ab conjugated with biotin (BioSource International, Camarillo, CA), which could detect  $>95\%$  of IgM in mice (43); fourth, streptavidin conjugated with alkaline phosphatase (Life Technologies); and, finally, *p*-nitrophenylphosphate.

The level of albumin in the nasal wash, bronchoalveolar wash, or serum was also determined by ELISA using purified mouse albumin (Biogenesis, Poole, U.K.) as the standard (35). Briefly, ELISA plates were coated with

goat anti-mouse albumin IgG (Bethyl Laboratories, Montgomery, TX) and then incubated with nasal wash, bronchoalveolar wash, or serum specimens after blocking the coated plate with 1% BSA in PBS. The bound albumin was detected by the sequential addition of goat anti-mouse albumin Ab conjugated with alkaline phosphatase (Bethyl Laboratories) and *p*-nitrophenylphosphate.

### Virus titrations

Serial 10-fold dilutions of the nasal or bronchoalveolar wash were prepared, and 0.2-ml aliquots of each dilution were added to Madin-Darby canine kidney cells in a six-well plate. After 1 h of adsorption, each well in the plate was overlaid with 2 ml of agar medium according to Tobita et al. (44, 45). After 2 days of incubation in a CO<sub>2</sub> incubator, the plaques were counted. The virus titer was expressed in PFU per milliliter. The virus titer of each experimental group was represented by the mean  $\pm$  SD of the virus titers per milliliter of specimens from five mice in each group.

### Estimation of mucus volume in the nasal cavity of mice

The nasal wash concentrations of anti-A/PR8 HA IgA and IgG Abs per milliliter of mucus volume, which covers the surface of the nasal cavity of BALB/c mice (10-wk-old female), were estimated as follows. First, the surface area of the nasal cavity, which is covered with squamous, columnar, and olfactory epithelia, was estimated. Briefly, serial tissue sections (each 3- $\mu\text{m}$  thick) from the upper jaw, which were fixed with 4% formaldehyde in PBS and decalcified with EDTA before embedding in paraffin, were prepared and stained with H&E (46). The circumference of the nasal cavity was measured based on photographs of each of the 104 tissue sections that were taken at 110- $\mu\text{m}$  intervals of the serial sections from the nostrils to the posterior nasopharynx pore. The total surface area of the nasal cavity was estimated by integration using the trapezoidal rule for the surface area of 104 section blocks calculated based on both the circumference of the nasal cavity and the thickness of the tissue blocks (113  $\mu\text{m}$ ). The circumference was calculated using software PIXS 2000 Ver 2.0 (Inotech, Hiroshima, Japan). Next, the mucus volume was estimated by assuming that the thickness of the mucus layer, including both the mucus blanket (4- to 8- $\mu\text{m}$  thickness) and the periciliary space (cilia, 5–7  $\mu\text{m}$  long), was 0.01 mm (47, 48). Table I shows that the surface area of the nasal cavity is  $\sim 300 \text{ mm}^2$  and the mucus volume is  $\sim 3 \text{ mm}^3$  when the thickness of the mucus layer is 0.01 mm.

### Statistical analyses

Differences in virus titers of groups between wild-type and pIgR-KO mice were compared by ANOVA. Comparisons between experimental groups were performed by the Student *t* test. Values of *p* < 0.05 were considered significant unless otherwise indicated.

## Results

### Ab responses and protection against total RT infection by A/PR8 viruses in vaccinated wild-type mice

Experiments were designed to confirm that the cross-protection against variant influenza virus infection in the upper RT is dependent primarily on S-IgA Abs and is much stronger than that in the lower RT (14, 15). BALB/c mice were immunized i.n. with different CTB\*-combined, inactivated vaccines prepared from A/PR8 (H1N1), A/Yamagata (H1N1), A/Beijing (H1N1), and B/Ibaraki viruses. Four weeks after immunization, the mice were challenged by the total RT infection method with an A/PR8 virus suspension

Table I. Estimation of mucus volume that covers the surface of the nasal cavity of BALB/c mice (10 wk old, female)

Nasal Cavity	Surface Area (mm <sup>2</sup> )	Mucus Volume (mm <sup>3</sup> ) <sup>a</sup>
Squamous cell epithelium	30.5	0.3
Columnar epithelium	146.7	1.5
Olfactory epithelium	122.6	1.2
Total	299.8	3.0

<sup>a</sup> The mucus volume that covers the surface of the nasal cavity was calculated by assuming that the thickness of the mucus blanket including periciliary space was 0.01 mm.

(20  $\mu$ l). Three days later, nasal wash, lung wash, and serum specimens were obtained for anti-A/PR8 HA IgA and IgG titration by ELISA and for virus titration as an index of protection. Fig. 2 shows that i.n. immunization with the A/PR8 vaccine and its variant (A/Yamagata or A/Beijing) vaccine conferred complete protection and partial cross-protection, respectively, against A/PR8 virus infection in the upper RT. The protection was accompanied by the induction of A/PR8 HA-reactive IgA with low IgG levels in the nasal wash, while the cross-protection was accompanied by induction of lower levels of A/PR8 HA cross-reactive IgA in the nasal wash. In contrast, the immunization conferred complete protection against homologous virus infection but negligible cross-protection against variant virus infection in the lower RT. The protection was accompanied by the induction of high levels of A/PR8 HA-reactive IgG in the lung wash. Thus, cross-protection against variant influenza virus infection, which depends primarily on anti-A/PR8 HA-reactive IgA, was detected in the upper RT. To clarify the roles of S-IgA Abs in cross-protection against variant virus infection in the upper RT, the nonlethal upper RT infection method using an A/PR8 virus suspension (2  $\mu$ l) was used in subsequent experiments.

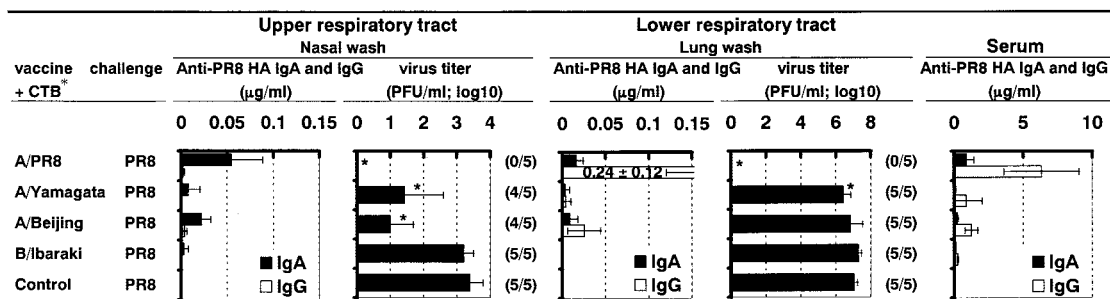
#### Ab responses and protection against upper RT infection by A/PR8 viruses in vaccinated pIgR-KO mice

The roles of S-IgA in providing cross-protection against variant virus infection in the upper RT were examined in pIgR-KO mice immunized i.n. with different CTB\*-combined, inactivated A/PR8 (H1N1), A/Yamagata (H1N1), A/Beijing (H1N1), and B/Ibaraki virus vaccines. Four weeks after immunization, the mice were challenged by the upper RT infection with the PR8 virus. Three days later, nasal wash and serum specimens were obtained. First, anti-A/PR8 HA Ab and albumin levels in the nasal wash and serum were compared between pIgR-KO mice immunized with CTB\*-combined A/PR8 vaccine and wild-type mice immunized under the same conditions. Table II shows that the blockade of transepithelial transport of dIgA in pIgR-KO mice caused a marked increase in the serum IgA concentration, leaving serum IgG and IgM levels almost unaltered. Contrary to the accumulation of serum IgA, the blockade of transepithelial transport of dIgA caused a decrease in the IgA level in the nasal wash, leaving the

nasal wash IgG level almost unaltered. In both wild-type and pIgR-KO mice, serum IgM was a minor Ab component at less than one-tenth of serum IgG. Therefore, in the subsequent experiments, A/PR8 HA-reactive IgA and IgG were assayed as Ab components involved in protective immunity. Table II also shows that there was no significant difference between wild-type and pIgR-KO mice in the albumin level in the nasal wash, which was an index of the leakage of blood from the lamina propria by physical manipulation of the nose to collect secretions, or the disruption of the pIgR gene. The nasal wash albumin level was 0.01–0.02% of the serum, which corresponded to the blood contamination estimated by counting the RBCs in the nasal wash (see *Materials and Methods*). Thus, the leakage of blood in the nose was not enhanced by the disruption of the pIgR gene in pIgR-KO mice. In addition, the leakage of blood did not affect the concentrations of IgA and IgG.

Next, A/PR8 HA-reactive IgA and IgG titers in the nasal wash and serum, and the nasal wash virus titers in the vaccinated pIgR-KO mice were compared with those in the immunized wild-type mice (Fig. 3). In the wild type-mice, the immunization with the A/PR8 vaccine conferred complete protection against the A/PR8 virus challenge and was accompanied by a predominant A/PR8 HA-reactive IgA Ab titer in the nasal wash. The immunization with A/Yamagata or A/Beijing vaccine conferred or tended to confer partial cross-protection against the A/PR8 virus challenge, when the specific protection afforded by the respective influenza A vaccines was statistically compared with the nonspecific protection afforded by the B/Ibaraki vaccine (40, 49). The cross-protection was accompanied by relatively low A/PR8 HA-reactive IgA titers in the nasal wash.

In the pIgR-KO mice, the immunization with the A/PR8 vaccine conferred incomplete protection against the A/PR8 virus challenge, in which low nasal wash virus titers were detected in four of the five mice in the group (Fig. 3). The incomplete protection was accompanied by a decrease in nasal wash anti-A/PR8 HA IgA titer (approximately one-fourth of that for the wild-type mice) and by a marked increase in serum anti-A/PR8 HA IgA titer (~20 times higher than that in the wild-type mice). The immunization with the A/Yamagata vaccine reduced cross-protection against the A/PR8 virus challenge to a greater degree than that observed in wild-type



**FIGURE 2.** HA-reactive Ab responses and protection against influenza virus infection in wild-type BALB/c mice immunized i.n. with different CTB\*-combined inactivated vaccines, prepared from A/PR8 (H1N1), A/Yamagata (H1N1), A/Beijing (H1N1), and B/Ibaraki viruses, and challenged by a total RT infection with an A/PR8 virus suspension (20  $\mu$ l) 4 wk later. Three days after the challenge, nasal wash, lung wash, and serum specimens from each group of five mice were obtained for anti-A/PR8 HA IgA and IgG Ab titration and for virus titration as an index of protection. Each bar represents the mean A/PR8 HA-reactive IgA or IgG ELISA titer  $\pm$  SD in nasal wash, lung wash, and serum or the mean virus titers (PFU per milliliter)  $\pm$  SD for nasal and lung washes. Mice with virus titers below the limit of detection (<5 PFU/ml) were considered to have cleared the infection, and a value of 0 was assigned for the purpose of statistical analysis. As the nonspecific protection afforded by the CTB\*-combined influenza B vaccine can often be involved in this type of experiment (40, 49), the specific protection afforded by the respective CTB\*-combined influenza A vaccine was statistically compared with the CTB\*-combined B/Ibaraki vaccine-immunized mice. ★, The significant difference from the B/Ibaraki vaccine-immunized mice ( $p < 0.05$ ) in mean values among the groups within the nasal wash virus titer or within the lung wash virus titer. The values shown in parentheses are the numbers of mice that failed to clear nasal viruses/total mice.

Table II. Characterization of nasal and serum anti-A/PR8 HA IgA, IgG, and IgM Ab and albumin levels in wild-type and pIgR-KO BALB/c mice immunized i.n. with adjuvant-combined A/PR8 vaccine

Mice Immunized with PR8 Vaccine + CTB*	A/PR8 HA-Reactive Abs						Albumin	
	Nasal wash (ng/ml)			Serum (μg/ml)			Nasal wash (μg/ml)	Serum (mg/ml)
	IgA	IgG	IgM <sup>a</sup>	IgA	IgG	IgM <sup>a</sup>		
Wild-type	54.4 ± 33.9	2.2 ± 1.5	<10	0.9 ± 0.5	6.3 ± 2.7	0.3 ± 0.1	1.7 ± 0.6	9.6 ± 3.2
pIgR-KO	8.4 ± 7.4	1.0 ± 0.9	<10	16.4 ± 3.4	3.3 ± 2.4	0.3 ± 0.1	1.9 ± 0.8	9.5 ± 3.0

<sup>a</sup> Assayed for IgM fractions purified from serum and nasal wash samples by goat anti-mouse IgM-conjugated affinity column. IgM Ab titer of <10 ng/ml was below the limit of detection.

mice, and this difference in the degree of protection between wild-type and pIgR-KO mice was statistically significant (*t* test). The immunization with the A/Beijing vaccine also tended to reduce partial cross-protection to a greater degree than that observed in the wild-type mice (statistically not significant in *t* test). The difference in the degree of protection and cross-protection between the wild-type and pIgR-KO mice was statistically significant when three groups immunized with H1N1-subtype vaccines were compared between the wild-type and pIgR-KO mice by analysis of variance ( $p < 0.006$ ). The reduction of the ability to provide cross-protection in pIgR-KO mice immunized with A/Yamagata and A/Beijing vaccines was accompanied by the reduction of nasal wash anti-A/PR8 HA IgA titers, when compared with those in the wild-type mice. For the B/Ibaraki vaccine group, there was no difference in the influenza-types nonspecific protection between the wild-type and pIgR-KO mice. These results indicate that the degree of protection or cross-protection provided by immunization with A/PR8 or other variant virus vaccines within the same subtype (H1N1) was reduced in pIgR-KO mice, when compared with that in the wild-type mice, in parallel with the reduction of nasal wash A/PR8 HA-reactive IgA Ab titers in the pIgR-KO mice. In addition, the presence of a non-negligible level of A/PR8 HA-reactive IgA Abs in the nasal wash of pIgR-KO mice suggests that they are involved in compensation for the reduction in the degree of protection or cross-protection.

#### Characterization of A/PR8 HA-reactive IgA and IgG Abs in upper RT of the vaccinated pIgR-KO mice

To investigate in detail the transport of dIgA across the mucosal membrane of the upper RT, the nasal wash A/PR8 HA-reactive IgA or IgG titer, shown in Fig. 3, was further represented by the Ab concentration in the nasal mucus and compared with the concentration of the serum Ab. The IgA and IgG concentrations in the nasal mucus were determined as follows. The mucus volume was estimated to be ~3 mm<sup>3</sup> by multiplying the surface area of the nasal cavity (299.8 mm<sup>2</sup>) by the thickness of the mucus blanket including the periciliary space (0.01 mm) (Table I). The nasal wash specimens that were collected from the immunized mice by washing three times were estimated to contain ~80% of all the mucus-associated Abs. Thus, the anti-A/PR8 HA IgA and IgG concentrations in the nasal mucus (micrograms per milliliter of mucus volume) were calculated by multiplying each nasal wash-ELISA Ab titer (micrograms per milliliter) in Fig. 3 by 333 (1000/3 mm<sup>3</sup>) and 1.25 (100/80%), respectively.

Table III shows the characteristics of nasal and serum A/PR8 HA-reactive Abs in the wild-type and pIgR-KO mice immunized i.n. with the A/PR8 vaccine. In the wild-type mice, the concentration of anti-A/PR8 HA IgA in the nasal mucus was ~30 times higher than that of IgG. The nasal mucus IgA concentration was 40 times higher than the serum IgA concentration, which suggests that

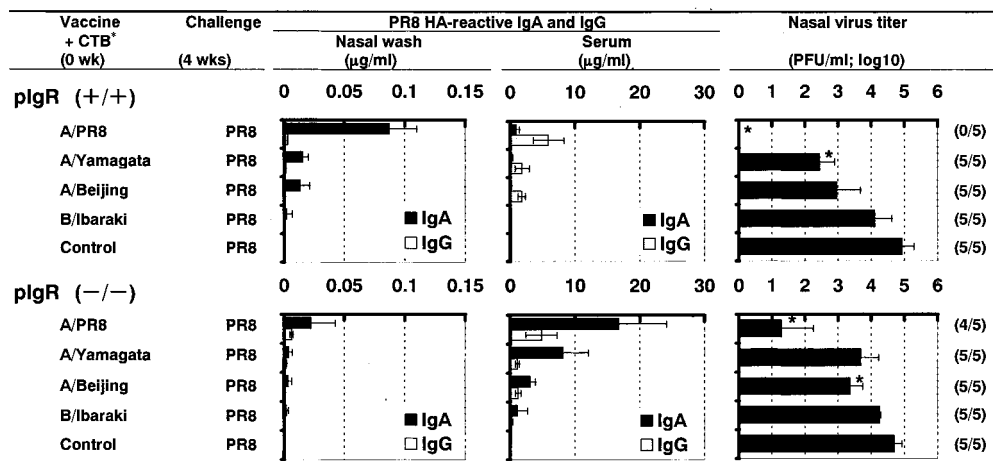


FIGURE 3. HA-reactive Ab responses and protection against virus infection in wild-type and pIgR-KO mice immunized i.n. with CTB\*-combined different inactivated vaccines and challenged by an upper RT infection with an A/PR8 virus suspension (2 μl) 4 wk later. Three days after the challenge, nasal wash and serum specimens from each group of five mice were obtained for anti-A/PR8 HA IgA and IgG Ab titration and for virus titration as an index of protection. Each bar represents the mean A/PR8 HA-reactive IgA or IgG Ab titer ± SD in nasal wash or serum, or the mean virus titers (PFU per milliliter) ± SD in nasal wash. ★, The significant difference from the B/Ibaraki vaccine-immunized mice ( $p < 0.05$ ) in mean values among the groups within the wild-type mice or within the pIgR-KO mice. For other details, see Fig. 2.



Table III. Characterization of nasal and serum anti-A/PR8 HA IgA and IgG Abs in wild-type and pIgR-KO BALB/c mice immunized i.n. with adjuvant-combined A/PR8 vaccine

Immunization	Mice <sup>a</sup>	A/PR8 HA-Reactive Abs (μg/ml)							
		Nasal mucus <sup>b</sup>			Serum			Nasal mucus/ serum	
		IgA	IgG	IgA/IgG	IgA	IgG	IgA/IgG	IgA	IgG
PR8 vaccine + CTB*	Wt	36.2 ± 9.7	1.2 ± 0.2	30	1.0 ± 0.6	5.9 ± 2.4	0.2	37	0.2
	KO	9.9 ± 6.0	0.9 ± 0.8	11	16.8 ± 7.3	4.8 ± 2.4	3.5	0.6	0.2
	KO/Wt	0.3	0.7		17	0.8			
A/Yamagata vaccine + CTB*	Wt	6.2 ± 2.1	0.6 ± 0.1	10	0.2 ± 0.1	1.9 ± 1.1	0.1	26	0.3
	KO	1.7 ± 1.2	0.8 ± 0.2	2.1	8.1 ± 3.9	1.1 ± 0.3	7.1	0.2	0.7
	KO/Wt	0.3	1.4		34	0.6			

<sup>a</sup> Wt, wild type; KO, pIgR-KO.

<sup>b</sup> The concentration of anti-A/PR8 HA Abs in the nasal mucus was calculated by assuming that the mucus volume of nasal cavity was 3 μl based on the measured value in Table I, and that the nasal wash removed all the mucus-associated Abs in the nasal tract. Thus, the concentration was calculated by multiplying each nasal wash ELISA Ab titer (measured in micrograms per milliliter) in Fig. 3 by 333 (1000/3 mm<sup>3</sup>) and 1.25 (100/80%).

IgA was secreted actively by pIgR-mediated transcytosis across the mucosal membrane. In contrast, the IgG was the major component in the serum and serum IgA concentration was approximately one-fifth of the serum IgG concentration. The nasal mucus IgG concentration was approximately one-fifth of the serum IgG concentration.

In the pIgR-KO mice immunized with the A/PR8 vaccine, a higher concentration of the A/PR8 HA-reactive IgA in the serum indicated the blockade of the pIgR-mediated transcytosis of pIgA. It was approximately four times higher than the serum IgG concentration and ~20 times higher than the serum IgA concentration in the wild-type mice. The accumulation of serum IgA resulted in the appearance of a small quantity of nasal mucus IgA via pathways other than pIgR-mediated transcytosis. The nasal mucus IgA concentration of was ~10 times higher than the nasal mucus IgG concentration and approximately one-fourth of the nasal IgA concentration of the wild-type mice. This low but non-negligible level of nasal mucus IgA seemed to be sufficient to provide partial protection against A/PR8 virus infection, because the reduction in the degree of protection, accompanied by the reduction in the nasal IgA level, was only partial (Fig. 3). These results, together with the data that the IgG levels in the nasal mucus and serum of the pIgR-KO mice were almost the same as those of the wild-type mice, confirm that the A/PR8 HA-reactive IgA in the nasal mucus is essential for providing complete protection against homologous virus infection.

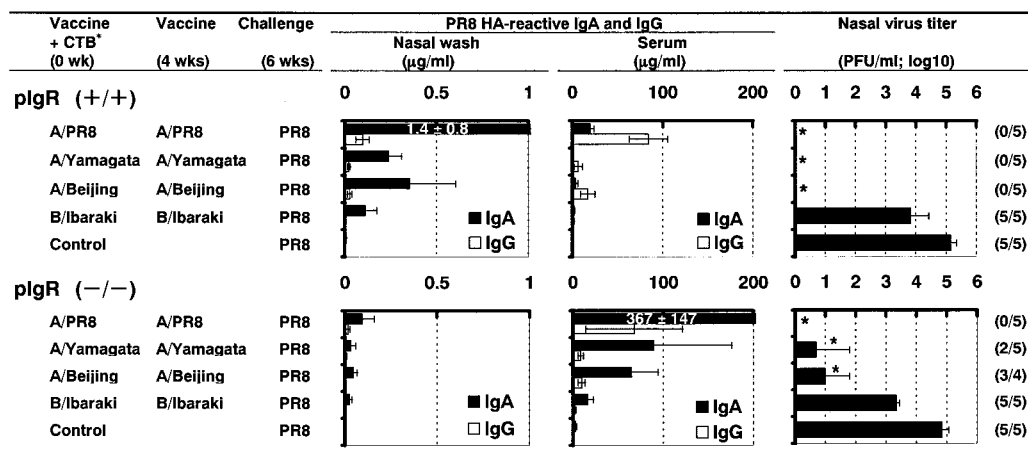
The characteristics of the nasal and serum anti-A/Yamagata IgA and IgG, which were cross-reactive to A/PR8 HA molecules, were also examined in the A/Yamagata vaccine-immunized wild-type and pIgR-KO mice (Table III). The levels of A/PR8 HA-reactive IgA and IgG in the A/Yamagata vaccine-immunized wild-type and pIgR-KO mice corresponded to the reduced levels of those in the A/PR8 vaccine-immunized wild-type and pIgR-KO mice, respectively. Thus, the degree of cross-protection seemed to be dependent on the nasal IgA concentration. These results suggest that the nasal A/PR8 HA-reactive IgA in the A/Yamagata vaccine-immunized mice plays an essential role in providing cross-protection against A/PR8 virus infection.

#### Ab responses and protection against upper respiratory infection by A/PR8 viruses in pIgR-KO mice vaccinated in a two-dose regimen

To further confirm the superior role of S-IgA to IgG in the upper RT in providing protection against influenza, A/PR8 HA-reactive

Ab responses and protection against A/PR8 virus infection were compared between the wild-type and pIgR-KO mice immunized i.n. with various vaccines (5 μg) together with CTB\* (1 μg) and boosted with each vaccine alone 4 wk later (5 μg). Two weeks after the second immunization, the immunized mice were challenged by upper RT infection with the A/PR8 virus, and 3 days later nasal wash and serum specimens were collected for virus and Ab titration. Fig. 4 shows A/PR8 HA-reactive IgA and IgG responses in both the nasal wash and serum, and nasal wash virus titers of the immunized wild-type and pIgR-KO mice. The nasal and serum A/PR8 HA-reactive Ab titers in wild-type and pIgR-KO mice immunized in a two-dose regimen were ≥10 times those in the wild-type and pIgR-KO mice immunized in a single-dose regimen (Fig. 3). In the wild-type mice, the immunization with the A/PR8 virus vaccine in a two-dose regimen conferred complete protection against the A/PR8 virus challenge and was accompanied by the highest nasal wash anti-A/PR8 HA IgA titer. The immunization with the A/Yamagata and A/Beijing vaccines conferred complete cross-protection against the A/PR8 virus challenge and was accompanied by a relatively high level of nasal anti-A/PR8 HA IgA, which was >20 times higher than that in mice immunized in a single-dose regimen. Thus, the degree of protection or cross-protection correlated with the level of anti-A/PR8 HA IgA.

In the pIgR-KO mice, the immunization with the A/PR8 vaccine conferred complete protection against the A/PR8 virus challenge, in parallel with the induction of a relatively high level of nasal wash anti-A/PR8 HA IgA. The immunization with the A/Yamagata or A/Beijing vaccine conferred partial cross-protection against the challenge, in which two of five or three of four mice, respectively, failed to clear nasal viruses. The difference was statistically significant when three groups immunized with the H1N1-subtype vaccines were compared between the wild-type and pIgR-KO mice by analysis of variance ( $p < 0.006$ ). The reduction of the degree of cross-protection in the immunized pIgR-KO mice against the A/PR8 virus challenge, when compared with the wild-type mice, was accompanied by the reduction of nasal wash anti-A/PR8 HA IgA titers. Thus, the reduced degree of cross-protection in the immunized pIgR-KO mice, shown in a single-dose regimen (Fig. 3), was reproduced in a two-dose regimen. This further confirms that A/PR8 HA-reactive IgA in the nasal mucus is essential for providing protection or cross-protection against homologous or heterologous virus infection in the upper RT.



**FIGURE 4.** HA-reactive Ab responses and protection against virus infection in wild-type and pIgR-KO mice which received a primary i.n. immunization with CTB (1 μg)-combined vaccines (5 μg) and, 4 wk later, a secondary i.n. immunization with the respective vaccines (5 μg). Two weeks after the second immunization, mice were challenged by upper RT infection with an A/PR8 virus suspension; 3 days after the challenge, nasal wash and serum specimens were obtained to measure Ab titers and virus titers. For other details, see Fig. 3.

## Discussion

In the present experiments, the role of IgA in providing protection or cross-protection against homologous or heterologous influenza virus infection in the upper RT was compared between the wild-type and pIgR-KO mice immunized i.n. with inactivated vaccines. It has already been shown that the pIgR-KO mice, generated by the disruption of exon 2 of the *pIgR* gene, have the following characteristics (34): the pIgR and SC proteins are not detected in the extracts of small-intestinal epithelial cells, bile, or small-intestinal fluids. A marked increase in IgA concentration and unaltered levels of IgM, IgG, and IgE (at the age of 9–10 wk) are observed in the serum, whereas IgA is reduced, though not up to negligible levels, in the bile, feces, and intestinal contents of pIgR-KO mice. In the present experiments, it was further shown that no SC proteins were detected in the nasal wash (Fig. 1B) and that a marked increase in anti-A/PR8 HA IgA Ab level and unaltered levels of the IgM and IgG Abs were observed in the serum with reduced but not negligible IgA levels in the nasal wash (Tables II and III). These data demonstrate that the transepithelial transport of dIgA by pIgR is severely blocked in the epithelial cells of the respiratory and digestive tracts of the pIgR-KO mice. In addition, the data showing that the IgM was not a major serum Ab component with an unaltered level between the wild-type and pIgR-KO mice (Table II) suggest that the role of pIgM in providing protection in the upper RT is minimal. Table II also shows that the level of albumin was not altered in the nasal wash of pIgR-KO mice (Table II). In this regard, Johansen et al. (35) showed that pIgR-KO mice had elevated levels of albumin in the saliva and feces, suggesting that deterioration of their epithelial barrier function in the absence of S-IgA (and S-IgM) results in leakage of serum proteins. The discrepancy between our results and theirs remains to be clarified, although it may be due to the difference in the strains of pIgR-KO mice used.

The characteristics of nasal IgA in the immunized mice were first analyzed in this study by estimating the IgA and IgG Ab concentrations in the mucus that covered the surface of the nasal cavity (Tables I and III). The estimation of mucus volume seemed to be very useful, because it enabled us to understand directly the relationship of Ab concentration in serum and mucus and to illustrate the transport of Abs from serum to nasal secretion. The concentration of anti-A/PR8 HA IgA in the nasal mucus of the wild-type mice immunized with CTB\*-combined A/PR8 vaccine was

~30 times higher than that of IgG, whereas the concentration of the nasal IgA in the immunized pIgR-KO mice was ~10 times higher than that of IgG. This result confirms that IgA is a major Ab component in the upper RT. Also, as the nasal IgA concentration in the wild-type mice was ~40 times higher than the serum IgA concentration, it was evident that IgA is secreted actively across the mucosal membrane. In contrast, as the nasal IgA concentration in the pIgR-KO mice was lower than the serum IgA concentration (about one-half), it was evident that the transepithelial transport of dIgA was blocked in pIgR-KO mice. These results reinforce the idea that nasal anti-A/PR8 HA IgA plays a major role in providing protection or cross-protection against influenza virus infection in the upper RT.

This study showed that in the pIgR-KO mice immunized i.n. with A/PR8 and variant H1N1 virus vaccines the degree of protection or cross-protection against A/PR8 virus infection in the upper RT was reduced, compared with that in the wild-type mice, in parallel with a decrease in nasal anti-A/PR8 HA IgA titer (Figs. 3 and 4). The results indicate that S-IgA is associated with the mechanisms of protection or cross-protection against influenza virus infection in the upper RT. In contrast, Mbawuike et al. (33) demonstrated that IgA is not required for the protection in IgA-KO mice immunized i.n. with a CTB\*-combined vaccine and challenged with a lethal dose of homologous virus. However, in their experiments, the role of IgA in the cross-protection against heterologous virus infection in the upper RT, which cannot be conferred by serum IgG, remained to be examined. Thus, the present results imply that the IgA-KO mice are less protected than the wild-type mice against a variant influenza virus infection in the upper RT. We cannot exclude the possibility that other protective mechanisms that are not the primary means of protection in the upper RT of wild-type mice, such as elevated levels of IgM and IgG, may become active in congenitally S-IgA-deficient mice. In this regard, Renegar et al. (50, 51) have demonstrated that genetically normal immune mice, given chemically defined total parenteral nutrition by the i.v. route, results in loss of nasal anti-influenza immunity with a significant drop in influenza-specific S-IgA titer in the upper RT compared with chow-fed mice, suggesting that the impairment of mucosal immunity in genetically normal mice cannot be substituted by serum IgG for the role of S-IgA in the protective immunity against influenza virus infection.



This study also showed that in the pIgR-KO mice the blockade of transepithelial transport of dIgA by pIgR resulted in the accumulation of IgA in the serum and the reduction of nasal mucus IgA titer (Table III). However, the level of nasal IgA was not negligible, and was ~10 times higher than that of nasal mucus IgG Abs. This result suggests that pathways other than pIgR-mediated transcytosis, such as asialoglycoprotein receptor-mediated endocytosis (2, 52), CD89 (Fc $\alpha$ R)-mediated binding (53), or intercellular diffusion from the mucosal tissue (serum) to the mucosal surface are involved in the IgA transport. With respect to IgG Abs, Wagner et al. (54) have shown that anti-HA IgG in the nasal wash is a transudate from serum according to the concentration gradient of the IgG. The non-negligible nasal wash IgA level in the pIgR-KO mice, transudates from the accumulated serum IgA, probably provided compensation for the reduction of the degree of protection or cross-protection against influenza virus infection in the upper RT (Figs. 3 and 4) (2).

In this study, i.n. immunizations with the A/PR8 (H1N1) vaccine and its variant (A/Yamagata or A/Beijing) vaccines conferred complete protection and a partial cross-protection against the A/PR8 virus infection in the upper RT of wild-type mice, respectively, and was accompanied by the induction of predominant S-IgA reactive to A/PR8 HA in the nasal wash (Fig. 2 and 3). In addition, in mice immunized in a two-dose regimen, the immunization with the variant vaccines conferred complete cross-protection against the A/PR8 virus infection, in parallel with the induction of the higher cross-reactive S-IgA Ab responses (Fig. 4). Moreover, we previously showed that almost complete cross-protection by variant virus vaccines within the H3N2 subtype and the challenge virus (H3N2) was induced with the induction of the cross-reactive S-IgA in the upper RT (14). Recently, Tumpey et al. (49) demonstrated that complete cross-protection by the H3N2 vaccine and the H5N1 (or H1N1) virus was induced with the subtype cross-reactive IgA and IgG in the RT of mice immunized three times at weekly intervals with adjuvant-combined H3N2 vaccine and challenged with lethal H5N1 (or nonlethal H1N1) virus. These results suggest that the degree of cross-protection depends on the concentration of cross-reactive IgA or IgG Abs, which increases with the increase in the dose of the vaccine, the frequency of vaccination, and the antigenic similarity between virus strain used for nasal vaccination and challenge virus strain. Under the immunization conditions described above, the nasal IgA was detected by ELISA but failed to neutralize the A/PR8 virus in vitro. The data regarding NT activities were not included in the results, because the protection or cross-protection in the upper RT did not correlate with NT activities of nasal IgA. This finding, as also reported by other investigators (49, 55), suggests that anti-HA IgA Abs act through other in vivo mechanisms to neutralize viruses and/or to enhance clearance of virus-infected cells.

A slight but significant reduction in nasal virus titers was observed in wild-type and pIgR-KO mice immunized with the B/Ibaraki vaccine and challenged with an immunologically unrelated influenza A (A/PR8) virus (Figs. 2–4). We and others (40, 49) have already shown that a partial nonspecific reduction of virus titers was observed in mice immunized i.n. with CTB<sup>\*</sup>, or *Escherichia coli* heat-labile toxin-combined B-type virus Ags and challenged with A/PR8 or H5N1 virus. Moreover, we have demonstrated that CTB<sup>\*</sup> can confer the ability to reduce the virus replication nonspecifically, when given i.n. into mice, suggesting that the effector cells involved in innate immunity, such as NK or  $\gamma\delta$  T cells, could be stimulated by i.n. immunization with CT or *Escherichia coli* heat-labile toxin derivatives as an adjuvant for nasal influenza vaccine (56–61). The restimulation of NK or  $\gamma\delta$  T cells could result in partial reduction of virus replication observed

after viral challenge. It has also been observed that the number of NK and  $\gamma\delta$  T cells increases in the lung tissue of mice following infection with influenza virus (59) and that  $\gamma\delta$  T cells proliferate nonspecifically in response to virus-infected cells (60, 61).

The development of a mucosal vaccine that stimulates cross-protection against variant viruses, including viruses with pandemic potential, would be strategically important for improving the efficacy of currently inactivated vaccines that induce high levels of serum antiviral IgG Abs to provide protection against homologous viral infection. The i.n. immunization procedure using adjuvant-combined inactivated vaccines was useful, because mucosal IgA Abs that are capable of providing cross-protection against a variant virus infection in the upper RT could be induced by this procedure (Figs. 3 and 4) (14, 62, 63). Field trials will be required to confirm the effectiveness of the adjuvant-combined vaccine in preventing or in attenuating illness without negative side effects, and also to develop an effective nasal influenza vaccine in the near future.

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