JOURNAL OF VIROLOGY, Jan. 1997, p. 832–838 Vol. 71, No. 1
0022-538X/97/$04.00+0
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Protection of Mice from Respiratory Sendai Virus Infections by Recombinant Vaccinia Viruses

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Received 25 July 1996/Accepted 27 September 1996

Mechanisms of protection of mice from Sendai virus, which is exclusively pneumotropic and causes a typical respiratory disease, by immunization with recombinant vaccinia viruses (RVVs) were investigated. Although the RVV carrying a hemagglutinin-neuraminidase gene of Sendai virus (Vac-HN) propagated in the noses and lungs of mice by either intranasal (i.n.) or intraperitoneal (i.p.) inoculation, no vaccinia virus antigens were detected in the mucosal layer of upper and lower airways of the i.p.-inoculated mice. The mice immunized i.n. with Vac-HN or Vac-F (the RVV carrying a fusion protein gene of Sendai virus) demonstrated the strong resistance to Sendai virus challenge both in the lung and in the nose, whereas the i.p.-immunized mice showed almost no resistance in the nose but showed a partial resistance in the lung. Titration of Sendai virus-specific antibodies in the nasal wash (NW), bronchoalveolar lavage (BAL), and serum collected from the Vac-F-immunized mice showed that the NW from the i.n.-immunized mice contained immunoglobulin A (IgA) antibodies but no IgG and the BAL from the mice contained both IgA and IgG antibodies. On the other hand, neither IgA nor IgG antibodies were detected in the NW from the i.p.-immunized mice and only IgG antibodies were detected in the BAL, although both i.n.- and i.p.-immunized mice exhibited similar levels of serum IgG, IgA, and neutralizing antibodies. The resistance to Sendai virus in the noses of i.n.-immunized mice could be abrogated by the intranasal instillation of anti-mouse IgA but not of anti-IgG antiserum, while the resistance in the lung was not significantly abrogated by such treatments. These results demonstrate that IgA is a major mediator for the immunity against Sendai virus induced by the RVVs and IgG is a supplementary one, especially in the lung, and that the RVV should be intranasally inoculated to induce an efficient mucosal immunity even if it has a pantropic nature.

Respiratory virus infections begin in the nasopharynx, and, depending on the nature of the virus and virus-host interactions, infections may either be limited to the upper airways or move down the tracheobronchial tree and into the lung. The most severe complications of respiratory virus infections, such as bronchitis, bronchiolitis, croup, and pneumonia, occur when the infection is allowed to proceed beyond the upper airways (14). For controlling some severe respiratory viral infections, spreading of the infection from the upper airways to the lower respiratory tract must be interrupted. Virus shedding from the upper airways must be suppressed for preventing infections among humans. Thus, efficacy of respiratory virus vaccines and their mechanisms for protection should be studied from this point of view.

Recently recombinant vaccinia viruses (RVVs) carrying a surface glycoprotein gene of respiratory viruses, such as human influenza virus, respiratory syncytial virus, human parainfluenza virus type 3, and Sendai virus (HVJ), have been attempted as live vaccines for induction of respiratory local immunity in experimental animals by various immunization routes and demonstrated a potential usefulness in immunoprophylaxis for such respiratory viral diseases (4, 17, 23, 26–29, 36). Mechanisms of protection, however, have not been investigated precisely in these studies. Since vaccinia virus causes a systemic infection (2, 7, 37), a possibility is present that it may exert protective ability by a mechanism different from that of the usual respiratory virus live vaccines.

The mucosal surface of the respiratory tract is usually only the site where respiratory viruses infect and cause a disease. In resistance to reinfection with respiratory viruses, mucosal immunity plays an important role in the upper and lower respiratory tracts. Secretory immunoglobulin A (S-IgA) antibody has been long presumed to be the most likely mediator of respiratory mucosal immunity based on the studies that show a positive correlation between protection and specific IgA antibody titers in the respiratory secretions (3, 5, 12, 14, 16, 25, 32). A direct causal relationship between them has been recently established in influenza virus infections by a protection abrogation experiment using anti-IgA antiserum (21, 22).

Sendai virus, a prototype of the paramyxovirus family, of which natural hosts are mice, rats, hamsters, and guinea pigs, causes a typical respiratory infection and has been used as a model for respiratory virus infections. The virus has two envelope glycoproteins named HN and F. The former has hemagglutinin and neuraminidase activities and is responsible for virus attachment to the host cell receptor (24, 34). The latter possesses fusion and hemolytic activities and mediates virus entry by fusing the viral envelope with the plasma membrane of host cells (6, 24). We have recently constructed RVVs expressing each of the Sendai virus structural proteins and shown that growth of Sendai virus is almost completely suppressed in the lung of mice immunized with the RVVs carrying either the HN (Vac-HN) or the F (Vac-F) gene of Sendai virus (23).

In the present study, we investigated protective efficacy of Vac-HN and Vac-F against Sendai virus infections and mediators for immunity in the upper and lower respiratory tracts of

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mice with respect to immunization routes, intranasal (i.n.) and intraperitoneal (i.p.), to elucidate mechanisms of the protection by the RVVs. The overall results obtained show that specific S-IgA antibodies are produced in the mucosal layer of the upper and lower respiratory tract only in the i.n.-immunized mice and function as a major mediator for the mucosal immunity, and that serum IgG antibodies mediate the resistance mainly in the lower respiratory tract of the i.n.- and i.p.-immunized mice.

HEp-2 and LLC-MK_2 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% newborn calf serum. The RVVs Vac-HN and Vac-F, which were constructed previously in our laboratory (23), and the RVV carrying the HN gene of Newcastle disease virus, Vac-HN(NDV), were propagated in HEp-2 cells. Vac-HN(NDV) is a generous gift from Y. Nagai, Institute of Medical Science, the University of Tokyo (15). Infectivity of the RVVs was assayed by the plaque method with LLC-MK_2 cell monolayers. The Hamamatsu strain of Sendai virus, which is a wild-type strain freshly isolated from a laboratory infection and propagates well in the mouse lung (9), was grown in 10-day-old embryonated chicken eggs and used as a challenge virus. Infectivity of Sendai virus was measured by the immunofluorescent cell-counting method using LLC-MK_2 (8) and expressed as cell-infecting units (CIU).

Specific-pathogen-free, 6-week-old male mice of the ICR/Crj(CD-1) strain were purchased from Charles River Japan Inc. They were kept under bioclean conditions at 22°C and 55% humidity throughout the experiments. Each mouse was inoculated i.n. with 2×10^6 PFU or i.p. with 5×10^7 PFU of each RVV under mild anesthetization with ether. In the challenge experiments, 21 days after immunization with the RVV, mice were challenged i.n. with 25 μl (10^3 CIU) of Sendai virus. Infectivities of RVV or Sendai virus in the lung and in the nasal turbinate were assayed as described previously (9). Briefly, the lung and nasal turbinate, which were collected from a mouse separately, were homogenized in 1 ml of MEM and centrifuged at $1{,}500 \times g$ for 20 min, and infectivity in the supernatant was measured. For assay of infectivity of the RVV in the blood, heparinized blood was collected from the femoral artery and centrifuged at low speed, and infectivity in the supernatants was measured.

For collection of bronchoalveolar lavages (BALs) and nasal washes (NWs), mice were bled from the femoral artery and sacrificed by cervical dislocation under anesthetization with ether. Collection of BALs and NWs from mice were performed as described previously (31, 32). Briefly, the trachea was exposed surgically and, for BAL collection, the oral portion of trachea was clamped, a 1-ml syringe equipped with a 22-gauge needle was inserted into the trachea, 1 ml of phosphate-buffered saline (PBS) was injected into the peripheral airways, and three cycles of aspiration and injection were repeated. For NW collection, a 1-ml syringe was inserted into the trachea toward the nares of the mouse, and then 1 ml of PBS was injected slowly into the trachea and the fluid which flowed out from the nares was collected. The BALs and NWs were centrifuged at $8{,}000 \times g$ for 10 min, and hemoglobin in 10 μl of the supernatants was quantitated by using tetramethylbenzidine-hydrogen peroxide (10) to determine the level of blood contamination. The contamination was expressed as a percentage of hemoglobin in the same volume of blood assayed after freeze-thawing. Only samples with a contamination level lower than 0.1% were used for anti-Sendai virus antibody assay.

Sendai virus-specific IgG and IgA antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Purified virions of the Hamamatsu strain of Sendai virus were disrupted with 2% Triton X-100 and 1 M KCl, diluted in the coating buffer (0.05 M $NaHCO_3/Na_2CO_3$, pH 9.6) to 100 μg of protein per ml, and dispensed to a 96-well ELISA plate at 100 μl/well. After allowing to absorb overnight at 4°C, the wells were washed with PBS–0.05% Tween 20 (Tween-PBS) and blocked for preventing nonspecific binding by incubation with 4% bovine serum albumin in Tween-PBS for 2 h at room temperature. A 100-μl aliquot of samples was serially twofold diluted with PBS containing 10% heat-inactivated newborn calf serum in the plate and allowed to react for 1 h at 37°C. The plate was then washed, and 100 μl of goat anti-mouse IgG(γ) antiserum (1:200) (KPL Inc., Gaithersburg, Md.) for IgG ELISA or rabbit anti-mouse IgA(α) antiserum (1:400) (Bethyl laboratories, Inc., Montgomery, Tex.) for IgA ELISA was added and the plate was incubated for 1 h at 37°C. The plate was then washed, and 100 μl of rabbit horseradish peroxidase-labelled anti-goat IgG antibody (1:3,200) for IgG ELISA or goat horseradish peroxidase-labelled anti-rabbit IgG (1:6,400) (Organon Teknika-Cappel, Durham, N.C.) for IgA ELISA was added and the plate was incubated for 1 h at 37°C. After washing, 0.5 mg of *o*-phenylendiamine per ml and 0.02% H_2O_2 in 0.05 M citrate buffer, pH 5.0, were added and allowed to react at room temperature for 10 min, and the reaction was stopped with 100 μl of 4 N H_2SO_4. Colorimetric conversion of the substrate was measured in a microplate spectrophotometer at 492 nm (Easy Reader EAR400; SLT-Labinstruments, Grodig, Austria). Titers of the samples were calculated from endpoint dilutions showing optical densities at 492 nm that were higher than the optical density at 492 nm + 3× standard deviations of the blank well. Sendai virus-specific neutralizing antibodies were measured as described previously (23).

Immunohistochemical staining of the lungs and noses of mice was carried out as follows. Tissue samples of the lung and nose were fixed with 10% formalin and embedded in paraffin. Nose samples were treated with 10% formic acid–10% formalin (1:1) for 24 h to be decalcified before fixation in 10% formalin. Tissue sections (5 μm thick) were made and mounted on a glass slide. The sections were deparaffinized with xylene and dehydrated in a graded alcohol series. For immunoperoxidase staining of vaccinia virus antigens, tissue sections were treated with 0.5% periodic acid for 10 min to block endogenous peroxidase activity. After treatment with normal goat serum (1:40) for 10 min, the tissue sections were incubated for 20 min in anti-vaccinia virus rabbit antiserum (1:40) and then for another 20 min in horseradish peroxidase-labelled anti-rabbit IgG antibody (goat) (Organon Teknika-Cappel, Durham, N.C.). The sections were incubated for 3 min in 0.05 M Tris-HCl, pH 7.6, containing 0.2 mg of diaminobenzidine per ml and 0.003% H_2O_2. The reaction was stopped by immersing in distilled water, and the sections were then counterstained with 1% methyl green, dehydrated, and coverslipped. Washing at each step was carried out with PBS, and the antibodies used were diluted with PBS containing 1% bovine serum albumin.

Protection abrogation experiments by anti-IgA(α) or anti-IgG(γ) were done as follows. Mice were immunized i.n. with Vac-F as described above, and 3 weeks later they received i.n. 25 μl of 1:5 diluted goat anti-mouse IgA(α) or IgG(γ) antiserum (KPL Inc. Gaithersburg, Md.) or PBS. Thirty minutes later they were challenged i.n. with 10^6 CIU of Sendai virus and subsequently received 25 μl of the same antiserum or PBS 5, 11, and 17 h after challenge infection. The i.n. instillations of the antisera were performed under mild anesthetization with ether. Twenty-five hours after initial viral challenge, mice were sacrificed and infectivities in the nose and lung were assayed.

Replication of Vac-HN in mice. First, replication of a recombinant vaccinia virus inoculated i.p. was compared with that

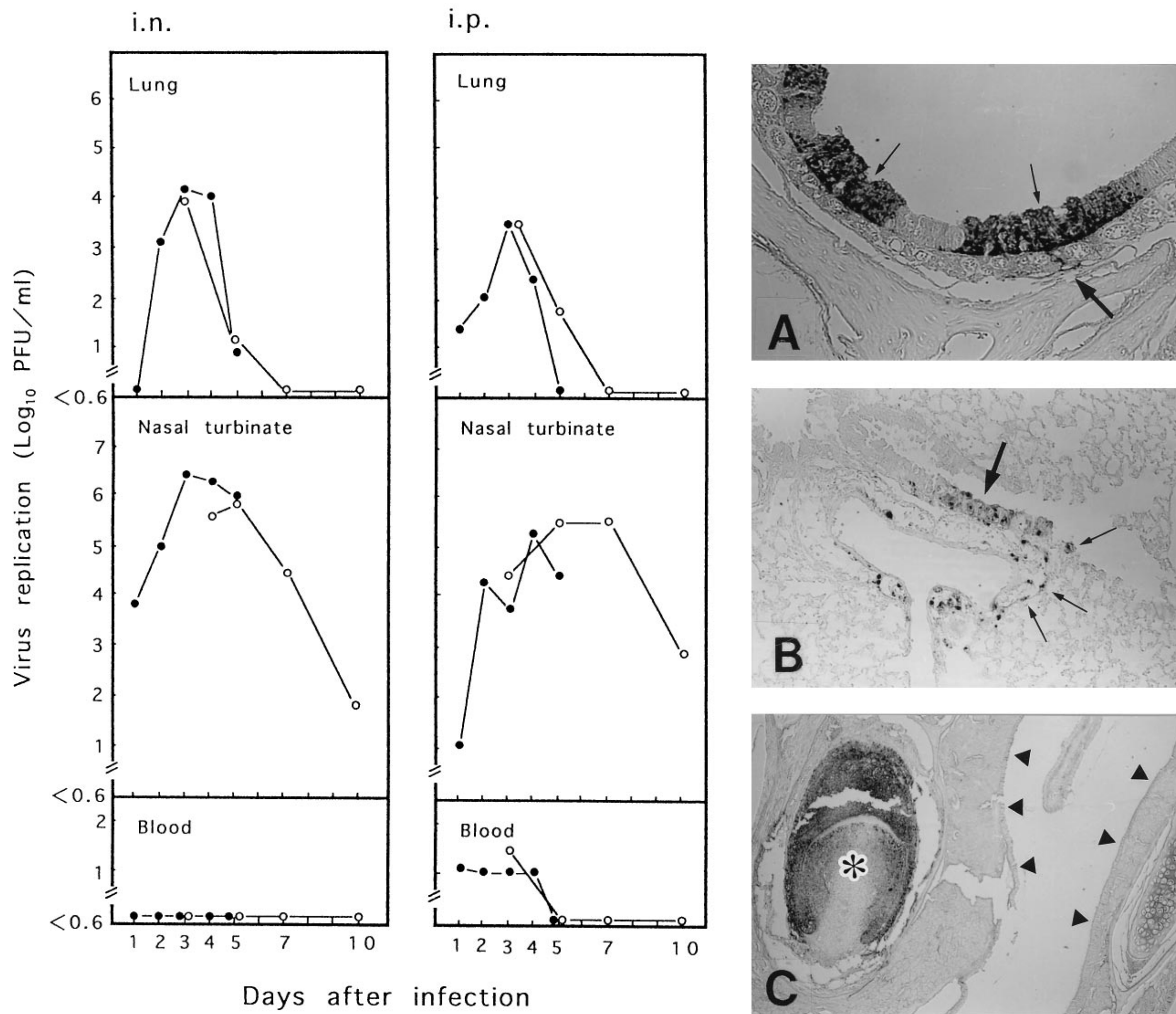

FIG. 1. Replication of Vac-HN in mice. Six-week-old male ICR/Crj (CD-1) mice were inoculated i.n. with 2×10^6 PFU or i.p. with 5×10^7 PFU of Vac-HN per mouse. The graph on the left side shows replication kinetics of Vac-HN in mice. Two mice of each group were sacrificed various days after inoculation and examined for viral replication in the lung, nasal turbinate, and blood. Open and closed circles indicate the results obtained from two independent experiments. Each plot is the mean result for the two mice. The pictures on the right side show immunoperoxidase staining of vaccinia virus antigens in the nose and lung of mice inoculated with Vac-HN. Immunoperoxidase staining of viral antigens was done with anti-vaccinia virus rabbit antiserum. (A) The nose section on day 3 after i.n. inoculation. Large amounts of viral antigens were observed in the mucosal epitherial cells (→) and lower but significant amounts of the antigens were also observed in the lamina propria (➜) of nasal turbinate. Magnification ×100. (B) The lung section on day 4 after i.n. inoculation. Viral antigens were observed in the bronchiolar epitherial cells (➜) and in the alveolar cells (→). Magnification, ×100. (C) The nose section on day 4 after i.p. inoculation. Viral antigens were observed only in the tooth germ (*) and not in the epithelial cells (▼) of nasal turbinate. Magnification, ×40.

inoculated i.n. in mice, since vaccinia virus has a pantropic nature. Mice were inoculated i.n. or i.p. with Vac-HN, and virus infectivities in the lung, nasal turbinate, and blood were assayed various days after inoculation. As shown in Fig. 1, the virus inoculated i.n. grew well both in the lung and in the nasal turbinate, but no infectivity was detected in the blood throughout the experimental period. In the i.p.-inoculated mice, virus infectivities were detected in the blood from day 1 to day 4 after inoculation, and the virus propagated well in the lung and in the nasal turbinate, although the peak virus titers in both sites were slightly lower than those in the i.n.-inoculated mice. To clarify replication sites of Vac-HN in the respiratory tract, mice inoculated i.n. or i.p. with Vac-HN were further examined for vaccinia virus antigens various days after inoculation by immunoperoxidase staining using anti-vaccinia virus antiserum. In the mice inoculated i.n., Vac-HN antigens were detected from day 2 in epithelial cells of the mucosal layer of nasal turbinate and became the most prominent in staining density and positive cell number on day 3 (Fig. 1A). The viral antigens were also observed in the lamina propria mucosa. The antigens decreased thereafter and were not detected on day 10. In the lung the viral antigens were observed on day 4 in epithelial cells of the bronchiolus, capillary artery, and alveolus (Fig. 1B). On the other hand, in the i.p.-inoculated mice, large amounts of Vac-HN antigens were detected exclusively in the tooth germ on days 3 and 4 (Fig. 1C) and disappeared by day 10. No viral antigens were detected in the mucosal layer of nasal turbinate (Fig. 1C) and in the lung throughout the experimental period. These results indicate that, although Vac-HN appears to propagate in the nose and lung of mice

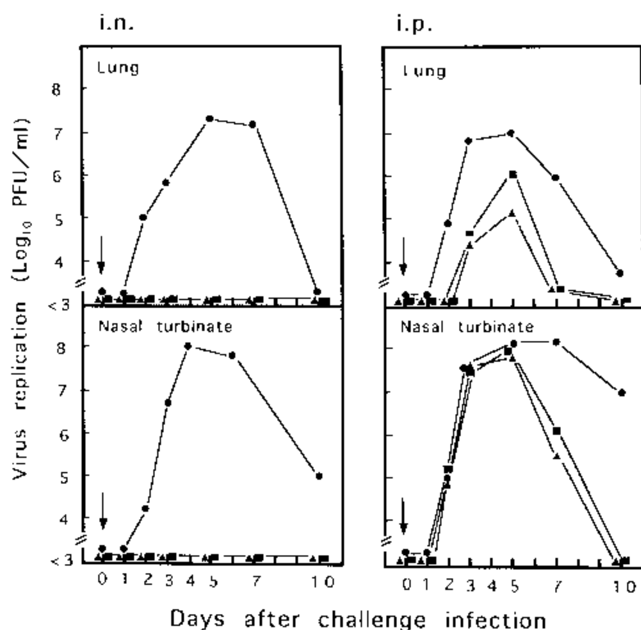

FIG. 2. Protection of mice from Sendai virus infections by immunization with the recombinant vaccinia viruses. Mice were inoculated with each of Vac-HN (■), Vac-F (▲), and Vac-HN (NDV) (●) at input doses of 2×10^6 PFU/mouse for i.n. inoculation and 5×10^7 PFU/mouse for i.p. inoculation. The mice were challenged i.n. with 10^3 CIU of the Hamamatsu strain of Sendai virus per mouse 21 days after immunization with the RVVs (↓). Two mice of each group were sacrificed various days after challenge infection and examined for Sendai virus replication in the lung and in the nasal turbinate. Each plot is the mean result for the two mice.

inoculated i.n. and i.p., the precise replication sites are different from each other depending on its inoculation route.

Protective efficacy of the RVVs against Sendai virus infections in mice with respect to their immunization routes. To compare protective efficacy of the RVVs against Sendai virus infections with respect to their immunization routes, mice were inoculated i.n. or i.p. with each of Vac-HN, Vac-F, and Vac-HN(NDV), and the mice were challenged with Sendai virus 21 days after immunization. Two mice of each group were sacrificed various days after challenge infection, and infectivities of Sendai virus in the lung and in the nasal turbinate were measured (Fig. 2). As shown in the control mice immunized i.n. or i.p. with Vac-HN(NDV), the challenge virus grew well both in the lung and in the nasal turbinate, reaching a maximum on day 5 and decreasing significantly by day 10. However, the mice immunized i.n. with Vac-HN or Vac-F did not exhibit replication of the challenge virus in both sites. On the other hand, in the mice immunized i.p. with Vac-HN or Vac-F, replication of the challenge virus in the lung was significantly suppressed, but the suppression was not so complete as was observed in the i.n.-immunized mice. In the nasal turbinate of the same mice, the challenge virus propagated until day 5 as well as in the control mice, but the virus titers on the days 7 and 10 appeared to be lower than those in the control mice. The partial suppression of challenge virus growth observed in the lung of i.p.-immunized mice is not considered to be due to in vitro neutralization of virus infectivity by contaminated blood during material preparation, because almost no reduction of the infectivity was observed when lung homogenates of the mice immunized i.p. with Vac-HN or Vac-F were mixed in vitro with known amounts of infectious Sendai virus (data not shown). These results indicate that both Vac-HN and Vac-F can induce strong resistance to Sendai virus infections when mice were immunized i.n., but that they can hardly induce the resistance in the mice immunized i.p., especially in the nose, in spite of growth of Vac-HN in the nose and lung.

Primary and secondary antibody responses against Sendai virus in the serum and respiratory secretions of mice immunized i.n. or i.p. with Vac-F. To elucidate mechanisms of the difference in protection by the immunization routes, primary and secondary antibody responses to Sendai virus F protein were examined. Mice were immunized i.n. or i.p. with Vac-F, and one group of the mice were then challenged with Sendai virus 21 days after immunization. The mice were sacrificed before and 5 days after challenge infection; the serum, NW, and BAL were collected, and blood contamination of each NW and BAL sample was evaluated by quantitating hemoglobin. Table 1 shows IgG and IgA antibody titers to Sendai virus F protein assayed by ELISA and Sendai virus-neutralizing antibody titers in the i.n.-immunized mice before challenge infection. High titers of IgG and neutralizing antibodies (2,100 ± 1,370 and 126 ± 25.3, respectively) were detected in the sera from all of the mice examined, and lower amounts of IgA antibodies (8.0 ± 4.2) were detected in 8 of the 10 mice. In the BAL samples low but significant amounts of IgG and IgA antibodies (21.0 ± 14.5 and 24.0 ± 26.3, respectively) were detected in 9 and 6 of the 10 mice, respectively. In contrast, no IgG antibody was detected in the NWs from all of the 10 mice, while significant amounts of IgA antibodies (10.0 ± 9.4) were present in 6 of the 10 mice. It is clear that the IgG and IgA antibodies detected in the BAL and NW were not from the

TABLE 1. Primary and secondary antibody responses to Sendai virus in respiratory secretions and serum of immunized mice[a]

Mouse group	Antibody titer (mean ± SD)						
	BAL		NW		Serum		
	IgG	IgA	IgG	IgA	IgG	IgA	NT
i.n. with Vac-F							
Before challenge	21.0 ± 14.5	24.0 ± 26.3	<10	10.0 ± 9.4	2,100 ± 1,370	8.0 ± 4.2	126 ± 25.3
After challenge	30.0 ± 32.4	31.1 ± 38.9	<10	29.0 ± 21.8	1,800 ± 1,229	13.0 ± 4.8	162 ± 56.2
i.p. with Vac-F							
Before challenge	21.3 ± 8.3	<10	<10	<10	2,555 ± 1,130	7.8 ± 4.4	147 ± 39.9
After challenge	8.8 ± 8.3	<10	<10	<10	3,000 ± 2,345	8.9 ± 3.3	230 ± 73.9
i.n. with Vac-HN(NDV) before challenge	<10	<10	<10	<10	<10	<10	<10

[a] Ten mice of each group were immunized i.n. or i.p. with Vac-F or i.n. with Vac-HN(NDV), and one group of the mice immunized with Vac-F were then challenged with Sendai virus 21 days after immunization. The mice were sacrificed before and 5 days after challenge infection, and serum, NW, and BAL samples were collected separately and assayed for Sendai virus-specific IgG, IgA and neutralizing (NT) antibodies. Only BAL and NW samples with a blood contamination level less than 0.1% were used for ELISA.

TABLE 2. Abrogation of protection to Sendai virus in the noses and lungs of immunized mice[a]

Mouse treatment (no. of mice)	Infectivity (log_{10}CIU/ml)	
	Nose	Lung
Expt 1		
Nonimmunized + PBS (4)	7.06 ± 0.17	4.94 ± 1.45
Nonimmunized + anti-IgA(α) (5)	7.38 ± 0.42	4.06 ± 1.68
Nonimmunized + anti-IgG(γ) (5)	7.25 ± 0.16	5.11 ± 0.75
VacF immunized + PBS (4)	4.09 ± 0.98	2.67 ± 0.33
VacF immunized + anti-IgA(α) (5)	5.66 ± 0.80*[b]	4.02 ± 1.37
VacF immunized + anti-IgG(γ) (5)	3.35 ± 0.47	2.72 ± 0.74
Expt 2		
Nonimmunized + PBS (10)	6.61 ± 0.14	4.79 ± 1.43
VacF immunized + PBS (10)	2.55 ± 0.68	1.54 ± 0.52
VacF immunized + anti-IgA(α) (10)	4.40 ± 0.20**[b]	2.55 ± 1.34

[a] Mice immunized i.n. with Vac-F as described in the legend to Fig. 2 or nonimmunized mice were given nose drops of anti-mouse IgA(α) antiserum, IgG(γ) antiserum, or PBS 30 min before and 5, 11, and 17 h after challenge with 25 μl (10^6 CIU) of Sendai virus, and infectivities in the nose and lung were measured 25 h after challenge infection. The infectivities are expressed as the mean ± SD.

[b] Significant abrogation of protection at $P < 0.05(*)$ and $P < 0.01(**)$ in Mann-Whitney *U* test.

blood by contamination during material preparation, since blood contamination in each sample was less than 0.1%. It is also clear that these antibodies detected are Sendai virus-specific as shown by the absence of antibodies in the samples from the mice immunized with Vac-HN(NDV). When the i.n.-immunized mice were challenged with Sendai virus, the titers of IgA antibody in the NW became higher 5 days after challenge infection in all of the 10 mice (29.0 ± 21.8), but IgG antibodies were not detected in any of the mice even after challenge infection (Table 1). Increase of the antibody titers in the BAL and serum after challenge appeared to be not as significant as that in the NW. On the other hand, as shown also in Table 1, in the i.p.-immunized mice before challenge, significant amounts of IgG (21.3 ± 8.3) but no IgA antibodies were detected in the BAL samples, and neither IgG nor IgA antibodies were detected in the NWs, although the serum antibody titers (IgG, 2,555 ± 1,130; IgA, 7.8 ± 4.4; neutralization, 147 ± 39.9) were at levels similar to those of the i.n.-immunized mice. Essentially the same results were obtained in the i.p.-immunized mice after challenge with Sendai virus as those before challenge (Table 1). These results suggest that the strong protection against Sendai virus infection observed in the lung and nose of mice immunized i.n. with Vac-HN or Vac-F is due to both IgA and IgG antibodies detected in the bronchoalveolar secretions and due to the IgA antibody in the nasal secretions, respectively. In addition, the results also suggest that the failure of protection in the nose and the partial protection in the lung observed in the i.p.-immunized mice are due to the absence of IgA and IgG antibodies in the nasal secretions and due to the presence of IgG antibody in the bronchoalveolar secretions, respectively.

Protection abrogation by anti-IgA(α) or anti-IgG(γ). To confirm that IgA was the mediator of local immunity against Sendai virus, IgA-mediated protection was abolished by the method of Renegar and Small (20, 21). Mice immunized i.n. with Vac-F or nonimmunized mice were given nose drops of anti-mouse IgA(α) antiserum, anti-mouse IgG(γ) antiserum, or PBS 30 min before and 5, 11, and 17 h after challenge with 10^6 CIU of Sendai virus, and infectivities in the nose and lung were assayed 25 h after challenge infection. Table 2 represents data for the nose and the lung from two experiments. In both experiments, the mice immunized with Vac-F were protected against Sendai virus challenge in the nose and lung as compared with the nonimmunized mice treated with PBS, anti-IgA(α), or anti-IgG(γ). In contrast, when the Vac-F-immunized mice were treated with anti-IgA(α), the amount of virus produced in the nose was significantly greater than that produced in the protected control mice ($P < 0.05$, experiment 1; $P < 0.01$, experiment 2). Treatment of the immunized mice with anti-IgG(γ), however, showed no effect on the protection to Sendai virus, although preincubation in vitro with the anti-mouse IgG(γ) antiserum (1:20) could completely eliminate the Sendai virus-neutralizing ability of the Vac-F-immunized mouse serum (1:50), which has a virus neutralization titer of 3.3, an ELISA IgG titer of 80, and an IgA titer of 0.2. On the other hand, when the Vac-F-immunized mice were treated with anti-IgA(α), the virus amounts detected in the lung appeared to increase, but the increase was statistically not significant in both experiments. Treatment of the immunized mice with anti-IgG(γ) showed no effect on the protection in the lung. These results indicate that IgA antibody is a real mediator of the local immunity against Sendai virus in the noses of mice i.n. immunized with Vac-F.

The present study was undertaken to clarify mechanisms of protection of mice from Sendai virus infection, a typical respiratory local infection, by recombinant vaccinia viruses. Depending on the immunization route, i.n. or i.p., with Vac-HN or Vac-F, a marked difference was seen in induction of the resistance to Sendai virus challenge in spite of its pantropic nature. Vac-HN inoculated i.p. appeared to spread via blood and proliferate in the nose and lung, but the precise replication sites were different between the mice inoculated i.n. and i.p.; in contrast to the i.n.-inoculated mice, no replication of Vac-HN was detected in the mucosal layer of nose (its replication was limited exclusively in the tooth germ [Fig. 1C]) and lung of the mice inoculated i.p., although the replication site could not be localized in the lung of the mice. The difference in protection observed in the present study is considered to be due to the difference in replication of vaccinia virus in the respiratory mucosal layer.

Intranasally immunized mice with Vac-HN or Vac-F demonstrated the strong resistance to Sendai virus challenge both in the nose and in the lung, whereas the i.p.-immunized mice showed almost no resistance in the nose but partial resistance in the lung. The present results that the NW from the i.n.-immunized mice contained only IgA antibodies and that the resistance was significantly abrogated by the i.n. instillation of anti-IgA(α) but not by that of anti-IgG(γ) antiserum indicate that the mediator responsible for the strong resistance in the nose of i.n.-immunized mice is anti-Sendai virus IgA antibody but not IgG. On the other hand, the mediator for the resistance in the lungs of i.n.-immunized mice could be both IgA and IgG antibodies, which were detected in the BAL samples. Statistically significant abrogation of the resistance was not observed in the lung by the i.n. instillation of anti-IgA(α) or anti-IgG(γ) antiserum, suggesting that IgG antibodies, in addition to IgA, are involved in the resistance, although the possibility cannot be ruled out that the amount (25 μl) of antiserum instilled might be too small to reach the lung sufficiently and resulted in insufficient protection abrogation. This was supported, however, by the data obtained from the i.p.-immunized mice, in which only IgG antibodies were detected in the BAL and neither IgA nor IgG antibodies were detected in the NW. A partial resistance observed in the lung of i.p.-immunized mice is considered to be due to the IgG antibodies detected in the BAL, and the poor resistance observed in nose tissue could be owing to lack of IgA and IgG antibodies in the NW. Further-

more, the reduction of challenge virus growth in the nose at the late stage in the mice immunized i.p. with Vac-HN or Vac-F (Fig. 2) is probably due to cytotoxic T lymphocytes against HN or F, because neither IgA nor IgG antibodies were detected in the NW even 5 days after challenge infection, and the reduction was also observed in the mice immunized with the vaccinia viruses expressing Sendai virus internal proteins as described previously (23).

Anti-Sendai virus IgA antibodies detected in the NW and BAL from the mice immunized i.n. with Vac-F could be produced in the respiratory mucosal layer and actively transported into the respiratory secretions from the following reasons: (i) although the serum IgA antibody titers were at similar levels between the i.n.- and i.p.-immunized mice, no IgA antibodies were detected both in the NW and in the BAL from the i.p.-immunized mice, and (ii) IgA-positive cells in the mucosal layer of nasal turbinate, which were detected by immunoperoxidase staining using anti-mouse IgA(α) antibodies, increased significantly 5 days after Sendai virus challenge in the mice immunized i.n. with Vac-HN or Vac-F but not in the i.p.-immunized mice. These IgA-positive cells increased appeared to be Sendai virus specific, because the increase was not observed in the mice immunized i.n. with Vac-HN(NDV) even 5 days after Sendai virus challenge infection (unpublished data). On the other hand, no detection of IgG antibodies in the NW from the i.n.-immunized mice even 5 days after Sendai virus challenge implies that the IgG antibodies detected in the BAL samples from the i.n.- and i.p.-immunized mice may be derived from the serum by a process of passive transudation but not produced in the respiratory mucosal layer. This view is consistent with the previous studies suggesting that protective effect of serum antibody on respiratory virus infections differs between the different levels of respiratory tract (1, 18–20, 31, 33, 35).

The overall results of the present study indicate that the S-IgA antibody is a major mediator for the protection of mice against Sendai virus infection by recombinant vaccinia viruses and that replication of the vaccinia virus in the respiratory mucosal layer is required for induction of the efficient mucosal immunity, which is accomplished only by i.n. inoculation with the vaccinia virus. The present study confirms and extends the previous works on the role of S-IgA antibodies in protection against Sendai virus infection (11, 14) and influenza virus infection (12, 21, 22). Although the previous studies using RVVs demonstrated some protective effects against various respiratory viral infections by immunization routes (intradermal, i.p., or intravenous) other than the i.n. route, the protection observed would be caused by exuded serum antibodies, and the protection was in fact insufficient in the upper airways as compared with the lower respiratory tract (4, 17, 27, 28). Thus, when RVV vaccines are used for immunoprophylaxis of respiratory viral diseases, they should be inoculated via a nasal or oral route to elicit a mucosal immunity and to prevent spreading of infections among humans, even if RVVs have a pantropic nature. For this purpose, vaccinia virus vectors safer than the WR strain are desired, although one such virus, the host range-restricted and highly attenuated MVA strain of vaccinia virus, was not so effective in mice against a lethal influenza virus challenge by immunization via the nasal route (30). Otherwise, enteric immunization with RVVs might be an effective and safe method for prevention of viral respiratory diseases, as suggested by Meitin et al. (13).

This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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