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Effectiveness of Enteric Immunization in the Development of Secretory Immunoglobulin A Response and the Outcome of Infection with Respiratory Syncytial Virus

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Cotton rats were immunized via intranasal, intradermal, or enteric routes with respiratory syncytial virus (RSV) or a live recombinant vaccinia virus expressing the RSV F glycoprotein (vaccinia F). The animals were tested for the appearance of RSV-specific antibody responses in the serum, bronchoalveolar lavage, and nasal wash after immunization and for virus replication 4 days after intranasal challenge with RSV. RSV antibody response in the serum and respiratory tract was demonstrated in all immunization groups and was significantly increased after intranasal challenge with RSV. Immunoglobulin A (IgA) antibody response in bronchoalveolar lavage fluid after intranasal or enteric immunization was two- to threefold higher than that after intradermal immunization. Nasal-wash IgA antibody response was not significantly different among three immunization groups, although mean antibody titer was the highest in intranasal immunization group. Complete resistance to replication of RSV challenge was observed in the lungs of cotton rats immunized by the intranasal or enteric routes, whereas a low level of replication was detected in the lungs of rats immunized intradermally. Enteric or intradermal immunization conferred partial protection to the upper respiratory tract, but complete protection of the upper respiratory tract was observed in the intranasal immunization group. These observations suggest that while enteric immunization is quite effective in inducing antibody responses in the respiratory tract, the magnitude of antiviral immunity induced in the respiratory tract after intranasal immunization may be superior to that observed after enteric immunization.

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infection in infants and young children. Previous attempts to develop satisfactory inactivated or attenuated vaccines have not been successful (1, 3, 9, 10, 34). Recent studies have shown that the two envelope-associated glycoproteins of RSV, designated F and G, were responsible for inducing protective immunity against infection with RSV. For example, passive immunization of cotton rats and mice with monoclonal antibodies against either glycoprotein conferred protection to the lower respiratory tract against infection with RSV (30, 33). Moreover, animals immunized with the purified F or G glycoprotein developed complete pulmonary resistance to intranasal challenge with the virus (32). Recent progress in molecular studies of RSV has enabled us to clone cDNA copies of the mRNAs for both glycoproteins and construct live recombinant vaccinia viruses that express the RSV F or G glycoprotein. These recombinant viruses offer a new approach to RSV immunoprophylaxis, since rodents and monkeys immunized with them produced high-titer antibody response to RSV F and G glycoprotein and showed significant resistance to intranasal challenge with RSV (8, 22, 29). Unfortunately, immunization of chimpanzees with vaccinia virus-RSV recombinant viruses failed to induce a high level of RSV-specific antibody and resistance to RSV challenge (6).

It has been shown that antiviral antibodies in the respiratory tract are correlated better with resistance to respiratory virus infection than serum antibodies (12, 17, 19, 27). The bulk of local antibodies, which are primarily of the immunoglobulin A (IgA) isotype, are produced by plasma cells resident in the submucosa of secretory epithelium and in the glandular stroma. These IgA-secreting plasma cells can be induced in the respiratory tract by several routes. First, intranasal instillation of virus antigen induces the differentiation of locally present, antigen-specific B lymphocytes into IgA-secreting plasma cells. Second, enteric administration of antigens stimulates IgA precursor B cells in gut-associated lymphoid tissues such as Peyer's patches, which enter the blood stream and specifically migrate to the submucosa of the respiratory tract, where final differentiation into IgA-secreting plasma cells occurs. This migration of IgA plasma cell precursors to distant mucosal membranes has contributed to the concept of a common mucosal immune system (4, 7, 14). Third, parenteral injection with antigens can induce IgA-secreting plasma cells in the submucosa of the respiratory tract. Although the precise mechanism of this route is not known, it could involve transport of the antigen or migration of stimulated regulatory cells to the submucosae of the respiratory tract, where stimulation of the IgA-secreting cells occurs. In previous studies, comparative evaluation of immunization with vaccinia virus-RSV recombinant viruses via intradermal and intranasal routes has shown that intranasal immunization can provide complete protection in both the upper and lower respiratory tract. However, vaccinia virus-RSV recombinant viruses given intradermally provided incomplete protection in the upper respiratory tract (18, 22). In this study, the immunogenicity and effectiveness of the enteric and intradermal routes of immunization of cotton rats with vaccinia virus-RSV recombinant viruses were compared.

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MATERIALS AND METHODS

Cells and viruses. HEp-2 cells were purchased from the American Type Culture Collection (Rockville, Md.) and propagated in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (penicillin and streptomycin, 100 U/ml). RSV (strain A2) was obtained from G. W. Wertz (University of North Carolina at Chapel Hill) and propagated on HEp-2 cells. For animal experiments, virus was partially purified by polyethylene glycol precipitation followed by centrifugation in a 35 to 65% discontinuous sucrose gradient as described elsewhere (31). Live recombinant vaccinia viruses that expressed the RSV F or G glycoprotein (vaccinia F or vaccinia G) and parenteral vaccinia virus (strain WR) were grown on HEp-2 cells as described previously (22). For immunization, infected cell lysates were sonicated for 2 min and centrifuged, and the supernatant was layered over 35% sucrose in 10 mM Tris hydrochloride, pH 8.8. After centrifugation at 18,000 rpm in an SW27 rotor for 80 min at 4°C, the pelleted virus was resuspended in 10 mM Tris hydrochloride and stored at −70°C before use.

Animals. Male and female cotton rats (*Sigmodon hispidus*), weighing 125 to 150 g, were obtained from the Animal Laboratory, State University of New York at Buffalo, and used in all experiments.

Immunization and challenge. For enteric immunization, rats were anesthetized with Ketamine and Xylazine after overnight starvation, their abdomens were opened under aseptic conditions, and the duodenum was injected with 1.5×10^8 to 3.0×10^8 PFU of vaccinia F or vaccinia G through a 30-gauge needle. This procedure is referred to as enteric immunization. The abdomen was then closed with dual silk sutures, and the animals were housed separately and allowed to recover, with free access to food. For intradermal immunization, rats were anesthetized by inhalation of methoxyflurane and injected with 100 μl of vaccinia F (10^8 PFU). For intranasal immunization and virus challenge, 2.0×10^5 PFU of partially purified RSV was instilled into the noses of rats in 100 μl under methoxyflurane anesthesia.

Collection of samples. Blood was obtained from the retroorbital venous plexus at various times after immunization and/or virus challenge, and the serum was separated and stored at −20°C for the determination of antibody response to RSV and vaccinia viruses. Bronchoalveolar lavage and nasal wash samples were obtained 21 days after immunization and 7 days after virus challenge. Rats were killed by CO_2 asphyxiation, and the trachea and thoracic cavity were exposed by surgical procedure. A fine silk suture was tied around the trachea midway between the larynx and the bifurcation of the trachea. For bronchoalveolar lavage, 1 ml of phosphate-buffered saline (PBS) was gently injected into the trachea just below the suture, expanding the lungs. The lavage was pulled back into the syringe and reinjected into the lungs before final withdrawal. For nasal washings, animals were pinned into a support which was tilted to elevate the feet. PBS (1 ml) was then injected into the trachea above the suture and toward the nose. Nasal washings were collected in a tube as they exited the nares. Recovery from bronchoalveolar lavage averaged 0.6 to 0.7 ml; recovery from nasal washings averaged 0.8 to 0.9 ml. Both fluids were clarified by low-speed centrifugation and stored at −20°C for the determination of antibody titers by enzyme-linked immunosorbent assay (ELISA). Blood contamination in these fluids was estimated by Hemastix (TM Ames Division, Miles Inc., Elkhart, Ind.) before centrifugation.

For the vaccinia virus assay, the duodenum, small intestine, lung, and spleen were resected from the rats 1 to 4 days after immunization and homogenized in MEM containing 2% FCS and antibiotics. After repeated freeze-thawing, the homogenates were clarified by centrifugation and the supernatant fluid was stored at −70°C for plaque assay. For the RSV assay, lungs and nasal tissues (including turbinates) were collected 4 days after intranasal challenge with RSV after CO_2 asphyxiation. Both tissues were weighed and homogenized in 10 parts (wt/vol) of Hanks' balanced salt solution supplemented with 0.218 M sucrose, 4.4 mM glutamate, 3.8 mM KH_2PO_4, and 7.2 mM K_2HPO_4 and stored at −70°C until assayed.

Virus assay. Vaccinia virus and RSV were assayed by the plaque method on HEp-2 cells in 24-well microtiter plates. The overlay for plaque assay consisted of medium 199 supplemented with 2% FCS, antibiotics, and 1% methylcellulose. Plates were incubated for 6 or 7 days at 37°C. After the methylcellulose was removed, plaques were fixed with 10% formaldehyde and stained with 0.1% crystal violet.

Antibody assay. Serum antibody response to RSV and vaccinia virus was determined by a fluorescent-antibody technique. For RSV, HEp-2 cells were grown on multichambered slides (Nunc, Inc., Naperville, Ill.) and infected with the A2 strain of RSV at a multiplicity of infection of 1 PFU/cell. After 2 days of incubation, the slides were washed with PBS and used as an antigen (fluorescent antibody to membrane antigen method). HEp-2 cells prepared in a similar manner were used as a control antigen. Serially twofold-diluted sera were added to the RSV and control antigen and incubated for 1 h at 37°C. After being washed with PBS, the slides were incubated with fluorescein isothiocyanate-conjugated goat anti-rat IgG (whole molecule; Sigma, St. Louis, Mo.) for another 30 min at 37°C. Further washes were performed, and then the cells were examined with an Olympus fluorescent microscope.

For the vaccinia virus assay, HEp-2 cells were grown on coverslips in 12-well microtiter plates and infected with the WR strain of vaccinia virus at a multiplicity of infection of 3 PFU/cell. After 24 h of incubation, the coverslips were fixed with cold acetone and used as an antigen. The vaccinia virus antibody assay was performed as described above. The titer of antibody was taken as the reciprocal of the highest serum dilution showing positive fluorescence compared with that of control antigen. Serum neutralizing antibody response to RSV was performed by the plaque reduction method as described previously (5). Briefly, 0.15 ml of serial fourfold-diluted heat-inactivated sera was mixed with an equal volume of RSV (200 PFU/0.1 ml) and incubated at room temperature for 1 h. The mixtures (50 μl) were then inoculated on HEp-2 cells in 24-well microtiter plates and incubated for 6 or 7 days as described for the virus assay. The titer of neutralizing antibody was determined as the reciprocal of the serum dilution which produced a 60% reduction of RSV plaques. IgG and IgA antibody response to RSV in serum, bronchoalveolar lavage, and nasal wash samples was determined by ELISA. Partially purified RSV (10^8 to 10^9 PFU/ml; protein concentration, 0.5 to 1.0 mg/ml) was diluted 1:100 in carbonate buffer (pH 9.6), and 100 μl was coated onto polystyrene plates (Immulon 1; Dynatech Laboratories, Alexandria, Va.) overnight at 4°C. Uninfected HEp-2 cells treated in the same manner as virus-infected cells were also coated and served as a control. The plates were then washed three times with PBS containing 0.05% Tween 20 and incubated with PBS containing 1% bovine serum albumin at room temperature for 2 h. After being washed, the wells

were filled with 100 μl of twofold serial dilutions of samples in PBS containing 1% bovine serum albumin and Tween 20. The plates were incubated overnight at room temperature (2 h at 37°C for the detection of IgG in serum) and were again washed with PBS-Tween. For the detection of IgG, the wells were incubated for 2 h at 37°C with 100 μl of peroxidase-conjugated, affinity-purified goat anti-rat IgG (diluted 1:500; Cappel, West Chester, Pa.). For the detection of IgA, the wells were incubated with 100 μl of mouse monoclonal antibody anti-rat IgA (diluted 1:10,000–1:20,000; Serotic, Bicester, England) at 37°C for 2 h, then washed and incubated with 100 μl of peroxidase-conjugated, affinity-purified rat anti-mouse IgG (diluted 1:1,000–1:2,000; Pelfreeze, Rogers, Ark.) for another 2 h at 37°C. After being washed again, each well was developed for color with 200 μl of 0.04% *O*-phenylendiamine in citrate-phosphate buffer (pH 5.0), and the OD_{492} was determined with an automated microplate reader. The ELISA titers were calculated by the conventional positive-over-negative (P/N) method in which the endpoint was the highest dilution that gave a P/N ratio equal to or greater than 2. In such a calculation, the OD of an antigen-containing well (positive) is divided by the OD of the respective control well (negative). Tests to confirm the specificity of the antibodies for rat immunoglobulin isotypes were already checked and reported elsewhere (21).

Statistical analysis. Comparisons between experimental groups were evaluated by Student's *t* test.

RESULTS

Replication of recombinant vaccinia virus after enteric immunization. In order to determine whether recombinant vaccinia virus replicated in the gut after enteric immunization, vaccinia F (10^7 PFU) was injected into the stomach of lightly anesthetized rats by feeding tube. Infectious virus was not recovered from the gut during 1 week after immunization, whereas quite a high titer of virus (10^8 PFU) was demonstrated in the lungs of some rats 2 or 3 days after immunization. These results suggest that during or after injection, some amount of virus was regurgitated from the stomach, went into the lungs, and replicated there. Therefore, all enteric immunizations were carried out surgically in order to avoid the possibility of regurgitation.

Figure 1 shows the recovery of infectious virus from the intestine after enteric immunization with 1.5×10^8 PFU of vaccinia F. The virus titer was below the level of detection on day 1, peaked at 1.0×10^5 PFU on day 3, and then declined during the fourth day. Virus was not recovered from the lungs and spleens during 4 days after enteric immunization. This suggests that injection of recombinant vaccinia virus into the duodenum produces a mild infection. The replication of vaccinia F in the gut was further characterized by detecting serum antibody response to RSV after enteric immunization and comparing it with the antibody responses induced by intranasal immunization with RSV or intradermal immunization with vaccinia F (Fig. 2). At 7 days after immunization, only one of five rats in the enteric immunization group developed antibody to RSV, while all rats in the other two immunization groups developed antibody to RSV. At 14 days postimmunization, all rats in the enteric immunization group had antibody to RSV. However, the mean antibody titer in this group was twofold lower than that in the other two groups. Based on these results, a dose of 1.5×10^8 PFU was used for subsequent enteric immunization.

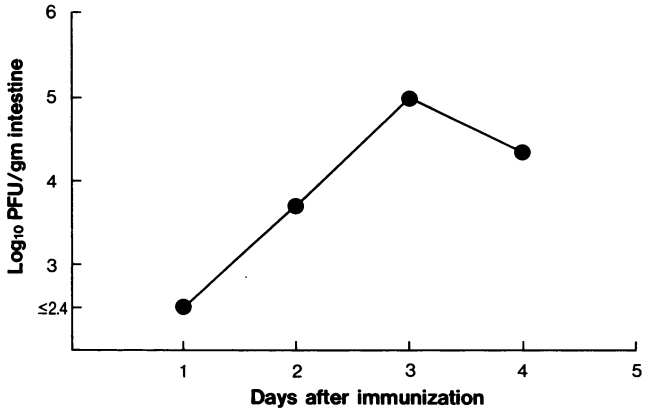

FIG. 1. Recovery of infectious vaccinia F in the intestine after enteric immunization. Each symbol represents mean $\log_{10}$ titers for three rats. The limit of detection in this assay was $10^{2.4}$ PFU/g of intestine. No virus could be detected on day 1.

Serum antibody response after infection with RSV or vaccinia virus-RSV recombinant virus. Sera were collected from all rats 21 days after immunization and tested for antibody response to RSV and vaccinia virus by the fluorescent-antibody technique (Table 1). Rats in the control group failed to develop an antibody response to either virus. All animals in the intranasal and intradermal immunization groups developed antibody to RSV. Seventeen rats in the enteric immunization group failed to develop antibody to RSV and vaccinia virus, indicating that these animals were probably not infected by the recombinant vaccinia virus.

Protective efficacy after immunization with RSV or vaccinia virus-RSV recombinant virus. Since serum neutralizing antibody to RSV has been shown to play an important role in the protection of the lower respiratory tract (23, 24), sera collected before challenge (day 21) were tested for neutralizing antibody response to RSV. As shown in Table 2, the neutralizing antibody titer induced by enteric immunization was significantly lower than that induced by intranasal immunization with RSV ($P < 0.001$) or intradermal immunization with vaccinia F ($P < 0.05$). However, complete protection of the lungs was observed in the enteric immunization group as well as the intranasal immunization group,

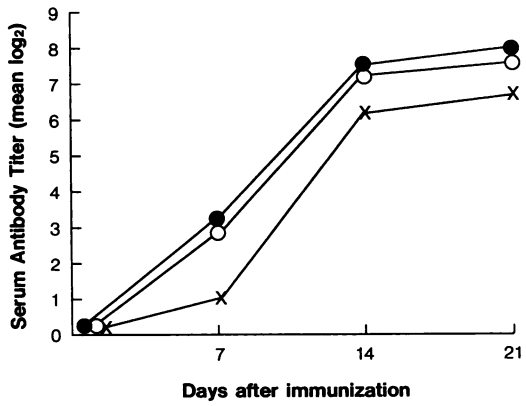

FIG. 2. Kinetics of serum IgG fluorescent-antibody response to RSV after intradermal (○) or enteric (×) immunization with vaccinia F or intranasal (●) immunization with RSV. Each symbol represents mean $\log_2$ titers for five or six rats.

TABLE 1. Serum IgG fluorescent-antibody titer 21 days after infection of cotton rats with RSV or vaccinia virus-RSV recombinant viruses

Virus used for immunization	Route	Dose (PFU)	No. of animals tested	Antibody response to:			
				RSV		Vaccinia virus	
				% of animals with rise in titer	Mean $\log_2$ titer ± SE	% of animals with rise in titer	Mean $\log_2$ titer ± SE
None			30	0	<2.0[a]	0	<3.0
RSV	Intranasal	2.0×10^5	25	100	8.4 ± 0.2	0	<3.0
Vaccinia F	Intradermal	1.0×10^8	30	100	7.1 ± 0.1	100	9.6 ± 0.1
	Enteric	1.5×10^8	30	93	6.6 ± 0.2[b]	93	8.3 ± 0.3
Vaccinia G	Enteric	1.5×10^8	20	50	6.4 ± 0.4	50	8.1 ± 0.6
		3.0×10^8	20	75	6.9 ± 0.4	75	7.4 ± 0.3

[a] Initial dilution was 1:4 in RSV assay and 1:8 in vaccinia virus assay.
[b] Mean $\log_2$ titers in rats with antibody response.

whereas a small amount of virus was recovered from 2 of 10 rats in the intradermal immunization group.

On the other hand, enteric and intradermal immunization conferred partial protection to the upper respiratory tract. Complete protection of the upper respiratory tract was observed only in the intranasal immunization group. Twelve of 20 rats which received enteric immunization with vaccinia G developed serum neutralizing antibodies to RSV. These antibody levels were two- to threefold lower than those induced by enteric immunization with vaccinia F. Complete protection of the lower respiratory tract was observed in one of five rats immunized with 1.5×10^8 PFU and in four of seven rats immunized with a dose of 3.0×10^8 PFU, although the mean neutralizing antibody titers were not significantly different between the two immunization groups. Partial protection of the upper respiratory tract was also observed in two groups.

The remaining eight rats (not shown in the table) did not develop serum neutralizing antibody to RSV. The mean virus titer recovered from those rats after intranasal challenge with RSV was 5.2 ± 0.1 PFU/g of tissue in the lungs and 4.8 ± 0.1 PFU/g of tissue in the nose. These titers were almost the same as those observed in the control group.

IgG and IgA antibody response to RSV in serum, bronchoalveolar lavage, and nasal wash samples. Samples were collected before (day 21) and after (day 28) intranasal challenge with RSV and tested for IgG and IgA antibody responses to RSV by ELISA. IgG antibody response in serum and bronchoalveolar lavage samples was observed in all immunization groups and increased two- to fourfold after intranasal challenge with RSV. Mean antibody titers of bronchoalveolar lavage IgG were highest in the intranasal immunization group and lowest in the enteric immunization group with vaccinia G. Nasal wash IgG antibody was not demonstrated in any immunization group except the intranasal immunization group challenged with RSV. However, it was demonstrated in all immunization groups after intranasal challenge with RSV (Table 3). IgA antibody response was observed in serum, bronchoalveolar lavage, and nasal wash samples in all immunization groups 21 days postimmunization and was two- to eightfold higher after intranasal challenge with RSV. Bronchoalveolar lavage IgA response in the vaccinia F enteric and intranasal immunization groups was two- to threefold greater than that in the intradermal immunization group, although this difference was not statistically significant. Nasal wash IgA response induced by intradermal or enteric immunization was not significantly different from that induced by intranasal immunization with RSV. Enteric immunization with vaccinia G developed significantly ($P < 0.001$) lower IgA antibody response in bronchoalveolar lavage and nasal wash than did the other three immunization protocols (Table 4). Bronchoalveolar lavage and nasal wash samples were checked with Hemastix for the presence of occult blood. The estimated blood contamination ranged

TABLE 2. Serum neutralizing antibody titer to RSV and protective efficacy after immunization of cotton rats with RSV or vaccinia virus-RSV recombinant viruses

Expt no.	Virus used for immunization	Route and dose (10^8 PFU)	No. of animals tested	Serum neutralizing antibody titer (mean $\log_2$ ± SE) on day 21	Virus replication 4 days after RSV challenge[a]			
					Nose		Lung	
					Virus recovered (% of animals)	Virus titer (mean $\log_{10}$ PFU/g ± SE)	Virus recovered (% of animals)	Virus titer (mean $\log_{10}$ PFU/g ± SE
1	None		10	<2.0[b]	100	4.8 ± 0.1	100	5.3 ± 0.1
	RSV	Intranasal	10	6.1 ± 0.2	0	≤1.9[c]	0	≤1.9
	Vaccinia F	Intradermal	10	5.8 ± 0.4	100	3.3 ± 0.2	20	1.9 ± 0[d]
		Enteric	10	4.3 ± 0.4	100	2.9 ± 0.2	0	≤1.9
2	None		6	<2.0[b]	100	4.7 ± 0.1	100	5.2 ± 0.1
	Vaccinia G	Enteric (1.5)	5	3.1 ± 0.5	100	4.2 ± 0.1	80	2.7 ± 0.3
		Enteric (3.0)	7	2.6 ± 0.3	100	3.8 ± 0.2	43	2.7 ± 0.4

[a] Cotton rats were challenged intranasally with 2.0×10^5 PFU of RSV on day 21 after immunization.
[b] Initial dilution was 1:4.
[c] The limit of virus detection was $10^{2.0}$ PFU/g of tissue.
[d] Samples with a virus titer below 2.0 were given a value of 1.9 for calculation of the mean.

TABLE 3. IgG antibody response to RSV in serum, bronchoalveolar lavage, and nasal wash samples[a]

Virus used for immunization	Route	Day 21 antibody titer (mean $\log_2$ ± SE) before challenge			Day 28 antibody titer (mean $\log_2$ ± SE) after challenge		
		Serum	Bronchoalveolar lavage	Nasal wash	Serum	Bronchoalveolar lavage	Nasal wash
None		≤3.0[b]	≤1.0	≤1.0	5.6 ± 0.2	1.2 ± 0.2[c]	≤1.0
RSV	Intranasal	12.3 ± 0.2	6.4 ± 0.2	2.0 ± 0.3	13.0 ± 0.4	7.6 ± 0.2	3.0 ± 0.3
Vaccinia F	Intradermal	11.6 ± 0.2	5.0 ± 0.5	≤1.0	12.8 ± 0.2	6.6 ± 0.2	5.2 ± 0.2
	Enteric	11.4 ± 0.5	5.2 ± 0.3	≤1.0	12.0 ± 0.3	6.6 ± 0.2	3.4 ± 0.2
Vaccinia G[d]	Enteric	10.0 ± 0.9	3.2 ± 0.2	≤1.0	12.8 ± 0.2	5.4 ± 0.5	2.4 ± 0.5

[a] Specimens were collected 7 days after RSV challenge, on day 28 after immunization. There were five to eight rats per group.
[b] Initial dilution was 1:16 in serum and 1:4 in bronchoalveolar lavage and nasal wash samples.
[c] Bronchoalveolar lavage and nasal wash specimens with a titer below 2.0 were assigned a titer of 1.0 for calculation of the mean.
[d] Two groups immunized with 1.5×10^8 or 3.0×10^8 PFU were combined to increase the number of seropositive rats.

between one part in $10^{5.0}$ and one part in $10^{6.0}$. These levels were too low to account for the observed respiratory antibodies, which therefore must have been locally synthesized and/or preferentially transported into respiratory secretions.

DISCUSSION

Working on the concept of a common mucosal immune system wherein IgA plasma cell precursors encountering viral antigens in the gut would migrate to various other mucosal membranes and secrete antiviral IgA antibodies, we attempted to use enteric immunization to induce antiviral immunity against viral infections in the respiratory tract in both animals (19) and humans (2, 28). This route is a convenient form of immunization and seems to be suitable for immunizing large populations. However, no enteric vaccines are being used to protect the respiratory tract against viral infections. One possible reason is that multiple administrations of large amounts of antigen are needed to induce a significant secretary IgA response in the respiratory tract. Therefore, several strategies, such as microencapsulation, incorporation in liposomes, and coupling to cholera toxin, have been considered for enhancing the mucosal immune response in the gut (25).

In this study, infection of the duodenum with vaccinia F could successfully induce anti-RSV antibody in the respiratory tract and conferred complete protection to the lungs and partial protection to the nasal tissues against intranasal challenge with RSV. These results suggest that a live recombinant vaccinia virus is a useful potential delivery vehicle for antigen for oral immunization. Furthermore, this approach offers the possibility that one administration of a polyvalent live vaccinia virus vector can protect various mucosal membranes from infection with several microorganisms.

Experimental infections of cotton rats with RSV have shown that serum neutralizing antibody plays an important role in the protection of lungs (23, 24). Enteric immunization with vaccinia F induced significantly lower neutralizing antibody levels than intradermal immunization with vaccinia F or intranasal immunization with RSV. However, protective efficacy in the lungs was similar among the three immunization groups with the exception of enteric immunization with vaccinia G. These results indicate that immunologic factors other than serum neutralizing antibody may also be involved in the mechanism of protection against RSV in the lungs. It has been shown that antiviral antibody in the respiratory tract is well correlated with the protection of the lungs in influenza virus (12) and Sendai virus (13, 19) infections. Many respiratory virus infections begin in the nasopharyx and spread into the lower respiratory tract. Thus, antiviral immunity in the respiratory tract should prevent the infection by interrupting the spread of virus.

In the present study, antiviral immunity in the bronchoalveolar lavage fluid was induced in all immunization groups and was significantly increased after intranasal challenge with RSV. These data are consistent with observations reported previously (29). However, the IgA antibody response induced by enteric or intranasal immunization was two- to threefold higher than that induced by intradermal immunization. The higher IgA response in the lower respiratory tract may have conferred complete protection to the lungs in the enteric immunization group. In addition, significantly lower bronchoalveolar lavage sample IgG and IgA antibodies induced by enteric immunization with vaccinia G may explain the incomplete protection of the lungs after intranasal challenge with RSV.

Previous studies on RSV infections in humans have demonstrated that nasal wash IgA antibodies play an important role in resistance to virus replication in the upper respiratory tract (15–17). In this study, nasal wash IgA antibodies were

TABLE 4. IgA antibody response to RSV in serum, bronchoalveolar lavage and nasal wash samples[a]

Virus used for immunization	Route	Day 21 antibody titer (mean $\log_2$ ± SE) before challenge			Day 28 antibody titer (mean $\log_2$ ± SE) after challenge		
		Serum	Bronchoalveolar lavage	Nasal wash	Serum	Bronchoalveolar lavage	Nasal wash
None		≤3.0	≤1.0	≤1.0	7.4 ± 0.2	1.6 ± 0.4	2.0 ± 0.4
RSV	Intranasal	12.2 ± 0.2	5.8 ± 0.2	4.2 ± 0.2	12.6 ± 0.2	6.2 ± 0.2	7.4 ± 0.2
Vaccinia F	Intradermal	12.2 ± 0.2	4.0 ± 0.8	3.5 ± 0.2	13.4 ± 0.2	5.2 ± 1.1	6.8 ± 0.2
	Enteric	12.8 ± 0.2	5.2 ± 0.6	3.6 ± 0.2	14.2 ± 0.2	6.4 ± 0.5	6.4 ± 0.2
Vaccinia G	Enteric	9.2 ± 0.7	2.6 ± 0.2	2.0 ± 0.6	11.8 ± 0.2	4.7 ± 0.6	5.2 ± 0.5

[a] See Table 3, footnotes *a*, *b*, *c*, and *d*.

demonstrated in all immunization groups. Mean antibody titers were the highest in the intranasal immunization group and lowest in the enteric group immunized with vaccinia G. Protective efficacy in the nasal tissues against virus challenge was complete in the intranasal immunization group, quite good after enteric or intradermal immunization with vaccinia F, and least good enteric immunization with vaccinia G. Thus, protective efficacy was well correlated with IgA antibody titers in nasal washes. However, since these IgA titers were not significantly different among the three immunization groups with the exception of enteric immunization with vaccinia G, nasal wash IgA antibody may not be the only immune factor responsible for protection in the upper respiratory tract.

Previous studies have demonstrated that cell-mediated cytotoxic activity could be induced in the respiratory tract of cotton rats after intranasal inoculation with RSV but not after parenteral immunization with it (11). The appearance of cell-mediated immunity on the surface of the mucosal membrane might have contributed complete protection to the upper respiratory tract. This possibility requires further investigation. A recent report has shown that intradermal immunization of mice with a recombinant vaccinia virus carrying the H2 influenza virus hemagglutinin gene protected only the lower respiratory tract, while intranasal immunization protected both the upper and lower respiratory tracts (26). Furthermore, intranasal administration of vaccinia virus-RSV recombinant viruses conferred complete protection to both lungs and nasal tissues (18). These observations and our results suggest that direct administration of virus antigen in the nasal tissues would be necessary to induce complete protective immunity in the upper respiratory tract. Alternatively, enteric immunization with vaccinia F followed by intranasal administration of live RSV may confer complete protection to both the upper and lower respiratory tract, because nasal wash IgG and IgA antibodies following enteric immunization were significantly boosted after intranasal challenge with RSV. This possibility is supported by the observation that enteric immunization combined with intranasal administration of killed virus resulted in protection of both the upper and lower respiratory tract from virus infection, although enteric or intranasal immunization alone could not induce protection (19). Such combined immunization might be more effective in inducing antiviral immunity in the respiratory tract than intranasal immunization alone. Finally, earlier studies on intestinal immunization with poliovirus vaccine found that it was inefficient in protecting the respiratory tract of human children against subsequent challenge with replicating poliovirus. These findings are consistent with the importance of immunization or infection of the respiratory tract itself to provide complete protection in the nose (20).

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