Induction of Mucosal B-Cell Memory by Intranasal Immunization of Mice with Respiratory Syncytial Virus

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The capacity of live or inactivated respiratory syncytial virus (RSV) to induce B-cell memory in respiratory-associated lymphoid tissues of mice was examined. Eight weeks after primary inoculation with either live or inactivated RSV, adult BALB/c mice were challenged with 4 × 10^9 PFU of RSV. Protection from viral shedding and mucosal production of RSV-specific antibodies were examined at various time points after challenge. We found that primary immunization with live, but not inactivated, RSV induced complete and durable protection upon challenge within the upper and lower respiratory tract. Also, primary immunization with live, but not inactivated, RSV enhanced the production of mucosal RSV-specific immunoglobulin A (IgA) upon challenge. Secondary mucosal IgA responses were characterized by (i) the early production of mucosal IgA by B cells that reside in organized nasal-associated lymphoid tissues, cervical lymph nodes, and bronchial lymph nodes, and (ii) the subsequent production of RSV-specific IgA by mucosal effector tissues, such as the tracheal lamina propria and lung. These findings suggest that primary infection of mice with live RSV might induce mucosal IgA-committed memory B cells. A greater understanding of the characteristics of RSA-specific mucosal memory B cells may facilitate the development of an RSV vaccine.

MATERIALS AND METHODS

Mice. Conventionally reared 6-week-old BALB/c female mice (Taconic Breeding Laboratories, Germantown, N.Y.) were housed in microisolator cages. Mice inoculated with RSV were housed in a separate HEPA-filtered isolation unit. Prior to inoculation, sera from mice did not contain RSV-specific antibodies, as determined by enzyme-linked immunosorbent assay (ELISA). Virus. Human RSV strain Long (American Type Culture Collection, Manassas, Va.) was grown in Hep-2 cells (American Type Culture Collection). Supernatant fluids were clarified and titrated for infectivity by plaque assay as previously described (15). RSV was inactivated by incubation at 56°C for 30 min. Inactivated virus contained <10 PFU/ml.

Immunization of mice. Groups of five adult BALB/c mice were lightly anesthetized with ketamine (NLS Animal Health, Baltimore, Md.) and xylazine (NLS Animal Health). Mice were inoculated intranasally (i.n.) with 20 μl containing 9 × 10^9 PFU of RSV or comparable quantities of inactivated RSV (IRSV). Inoculations were performed with a micropipettor by repeated placement of small volumes of inoculum on nares until the entire volume had been inhaled. Control mice (five per group) were inoculated i.n. with 20 μl of Hep-2 cell medium (Eagle's minimum essential medium [BioWhittaker, North Brunswick, N.J.], 10% fetal bovine serum [FBS; BioWhittaker], 1% HEPES [Gibco, Rockville, Md.], 1% L-glutamine [Gibco], 1% MEM essential vitamins [Gibco], penicillin G at 14 U/ml, and streptomycin at 14 μg/ml [Gibco]).

Challenge of mice. Eight or 59 weeks after primary inoculation, five mice per group per time point were anesthetized as above and challenged i.n. with 20 μl containing 4 × 10^6 PFU of RSV strain Long.

Lymphoid organ cultures. To assess the production of RSV-specific antibodies by RALT, lymphoid organ cultures were established at various time points after challenge using a modification of previously published methods (1, 12). In brief, under sterile conditions organized nasal-associated lymphoid tissues (NALT) (as previously described [11]), cervical lymph nodes (CLN), and bronchial lymph nodes (BLN) were isolated. Following perfusion of the right cardiac ventricle with 3 ml of sterile phosphate-buffered saline (PBS), the right upper lobe of the lung was harvested. An approximately 4-mm tracheal segment, including the tracheal bifurcation, was isolated from each animal. Sublingual glands (SL), submandibular glands (SM), parotid glands (P), and palatine salivary glands (PSG) were harvested (3). All tissues were washed in Iscove’s medium (CELLgro) containing 10% FBS and 0.1% gentamicin. Under a dissecting microscope (30× magnification), fat and connective tissue were removed from NALT, thymus, salivary glands, and lymph nodes. Four equivalent fragments were dissected from the harvested lung tissue. Each lung fragment, tracheal segment, BLN, CLN, SL, SM, P, PSG, or NALT was placed in an individual well of a 48-well plate (Costar Scientific, Braintree, Mass.) containing 0.5 ml of medium (Kennett’s HY medium [Gibco], 100 μg of streptomycin [JRJ, Lenexa, Kans.]/ml, 50 μg of gentamicin [Gibco]/ml, and 0.25 μg of amphotericin B [Fungizone; JRJ/ml]). Samples were incubated at 37°C in a humidified atmosphere of 95%
O2 and 5% CO2 for 5 days. Supernatant fluids were collected and tested for the presence of RSV-specific and total immunoglobulins by ELISA. Prior studies have demonstrated that the antibodies detected in lymphoid organ culture fluids are due to active production of antibodies by cultured tissues and not due to the passive transudation of serum-derived antibodies (2). We calculated the ratio of virus-specific to total antibodies detected in supernatant fluids to adjust for discrepancies in tissue size and viability.

Collection of mucosal secretions. An intravenous catheter with a 22-gauge 1-in. needle was inserted into the trachea, and 0.1 ml of sterile PBS was injected and retrieved. This bronchoalveolar lavage (BAL) specimen was collected and added to 0.9 ml of viral freezing medium (Eagle's minimum essential medium, 5% FBS, 100 mM MgSO4, and 50 mM HEPES; pH 7.5). After decapitation and removal of the lower jaw, an intravenous catheter was inserted into the posterior nasopharynx. A 0.1 ml volume of sterile PBS was flushed through the nasal passage and collected from the nares into a 1.2-ml cryogenic vial containing 0.9 ml of viral freezing medium. BAL fluids and nasal wash samples were tested for the presence of RSV-specific and total immunoglobulins by ELISA, and virus titers were determined.

Isolation of lung tissue for viral quantification. A 0.5-ml volume of viral freezing medium was added to a 1.2-ml cryogenic vial and then weighed. After sacrifice, the right cardiac ventricle was perfused with 3 ml of sterile PBS. The left lobe of the lung was placed in a 1.2-ml cryogenic vial containing 0.5 ml of viral freezing medium and stored in liquid nitrogen.

Preparation of lung tissue for viral quantification. Lung samples were quickly thawed and weighed. For processing of the lung tissue, a sterile 7-ml Pyrex tissue grinder (Fisher Scientific) was used. One sample and 500 μl of Hep-2 medium were added to each grinder and mechanically homogenized. Samples were then diluted for viral quantification.

Quantification of infectious RSV. BAL, nasal washes, and processed lung samples were tested to determine the quantities of infectious RSV in each sample. Viral titrations were prepared in triplicate using subconfluent monolayers of Hep-2 cells in 12-well plates (Falcon) (15). The viral titers were determined by calculating the mean number of PFU in each set of triplicate wells. A positive control sample of known titer was plated with each assay.

Detection of RSV-specific and total immunoglobulins by ELISA. Supernatant fluids, mucosal secretions, and sera were tested for the presence of RSV-specific total IgA and IgG antibodies. For detection of RSV-specific immunoglobulins, alternating wells of 96-well plates (Costar, Cambridge, Mass.) were coated with 105 BCH4 (a persistently RSV-infected fibroblast cell line) or BC (parental cell line) cells in 100-μl volumes (7). After overnight incubation in a humid chamber, plates were washed five times with PBS-0.05% Tween 20 (Sigma, St. Louis, Mo.), blocked with 300 μl of PBS containing 2% FBS-0.05% Tween (FBS-T), and incubated for 1 h at room temperature (RT). Wells were washed as described above, and 50 μl of supernatant fluid, mucosal secretions, or sera diluted in FBS-T was added to both positive and negative wells and incubated at RT for 1 h. After wells were washed, 50 μl of horseradish peroxidase-conjugated goat anti-mouse IgA or IgG (Southern Biotechnology Associates, Birmingham, Ala.) diluted 1:2,000 in FBS-T was added to the wells. Plates were incubated at RT for 1 h, and then wells were washed as described above. Equal volumes of TMB peroxidase substrate and peroxidase solution B (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) were mixed, and 50 μl of this solution was added to each well. Plates were incubated for 5 min at RT, then 50 μl of 50% o-phosphoric acid (Fisher Scientific) was added to each well and, using a 450-nm filter on a microplate ELISA reader (Dynex Technologies, Chantilly, Va.), the optical density (OD) of each well was determined. Samples were considered positive if the mean OD value for RSV- or anti-immunoglobulin-coated wells was both ≥0.1 OD units and ≥2-fold the OD value for the corresponding negative control well. Quantities of total immunoglobulins were determined as previously described (1). Quantities of virus-specific and total immunoglobulins were determined using an isotype-specific standard curve that was constructed for each assay based on serial dilutions of purified mouse IgA and IgG (Sigma). Using the standard curve equations, threshold mean values were determined. A tissue sample was considered nontoxic if no antibodies were detected in supernatant fluids by total immunoglobulin ELISA.

Statistical analysis. Continuous variables were expressed as means and were compared by use of the Student t test. Linear regression was used to control for time and compare the quantity of virus for the three groups. A two-tailed P value of <0.05 was considered statistically significant. Stata statistical software (Stata 8.0; Stata Corp.) was used for all calculations.

RESULTS

Live, but not inactivated, RSV induced protection against challenge within the upper and lower respiratory tract. To evaluate the capacity of live RSV strain Long to induce protection against challenge, five mice per group were inoculated i.n. with 9 × 104 PFU of RSV or comparable quantities of iRSV. Mice were challenged 8 or 59 weeks later with 4 × 104 PFU of RSV. Immunization with live RSV induced durable protection against RSV challenge that persisted over 1 year after primary immunization. Nasal wash fluids, BAL, and lung samples of animals primarily immunized with live RSV did not contain detectable quantities of virus 0 to 9 days after challenge. However, mice inoculated with iRSV were not protected against challenge. Comparable quantities of RSV were recovered from nasal wash fluids, BAL, and lung samples (P > 0.05) 3 and 6 days after challenge of animals previously immunized with either iRSV or Hep-2 medium (Fig. 1).

Primary immunization with live, not inactivated, RSV enhanced production of RSV-specific IgA by B cells that were resident in the inductive tissues of RALT. To evaluate the production of RSV-specific immunoglobulins by inductive tissues of RALT, lymphoid cultures were established 0, 3, 6, and 9 days after challenge of previously immunized mice. RSV-specific IgA was not detected in supernatant fluids from lymphoid cultures 8 weeks after primary immunization of mice with either live or inactivated RSV. However, 3 days after challenge of mice previously immunized with live RSV, virus-specific IgA was produced by cells within the inductive tissues of RALT, including NALT, CLN, and BLN. Because no antibodies were detected in two lung fragments from one mouse sacrificed 3 days after challenge, only the results of two lung fragments from this animal were included in the analysis. Primary immunization with iRSV did not enhance production of RSV-specific IgA by B cells resident in inductive sites upon challenge (Fig. 2A).

RSV-specific IgG was detected in fluids from lymphoid cultures 8 weeks after immunization of mice with live RSV. After challenge of mice previously immunized with live RSV, virus-specific IgG production by B cells within the inductive tissues of RALT persisted, but was not enhanced. Primary immunization with iRSV did not enhance production of RSV-specific IgG by B cells resident in inductive sites upon challenge (Fig. 3A).

Primary immunization with live, not inactivated, RSV enhanced production of RSV-specific IgA by B cells resident in the effector tissues of the lower respiratory tract. To evaluate the production of RSV-specific immunoglobulins by effector tissues of RALT, lymphoid cultures were established from five mice per time point on days 0, 3, 6, and 9 after challenge of previously immunized mice. Six or 9 days after challenge, RSV-specific IgA was produced by B cells within the effector tissues of the lower respiratory tract, including the tracheal lamina propria and lungs harvested from mice previously immunized with live RSV (Fig. 2B). In addition, RSV-specific IgA was detected in BAL fluids 6 days after challenge of mice previously immunized with live RSV (Fig. 4). However, no RSV-specific IgA was produced by putative effector tissues within the upper respiratory tract, including SL, SM, P, and PSG, after challenge of mice previously
immunized with live or inactivated RSV (data not shown). Primary immunization with iRSV did not enhance production of RSV-specific IgA by B cells resident in effector sites upon challenge (Fig. 2B).

RSV-specific IgG was detected in fluids from lymphoid cultures and mucosal washes 8 weeks after immunization of mice with live RSV. After challenge of mice previously immunized with live RSV, production of virus-specific IgG by effector tissues of RALT persisted but was not enhanced (Fig. 3B).

RSV-specific IgG was produced by potential effector tissues of the upper respiratory tract, including SL, SM, P, and PSG, upon challenge of mice previously immunized with live RSV (data not shown). In contrast, enhanced secretion of RSV-specific IgG was detected in BAL fluids 3 days after challenge of mice previously immunized with live RSV (Fig. 4). Primary immunization with iRSV did not enhance production of RSV-specific IgG by B cells resident in effector sites upon challenge (Fig. 3B).

FIG. 1. Eight and 59 weeks after primary i.n. immunization with live RSV, iRSV, or medium, five adult BALB/c mice per group per time point were challenged i.n. with $20 \mu l$ containing $4 \times 10^5$ PFU of RSV. Nasal washes, BAL fluid, and processed lung samples were tested to determine the quantities of PFU of RSV in each sample. Viral titrations were determined by plaque assay. Geometric means were calculated. *, $P < 0.05$ compared to quantities of virus detected in samples from control mice; all other differences were not statistically significant ($P > 0.05$).
Primary immunization with live, not inactivated, RSV induced durable secondary mucosal humoral immune responses. At 59 weeks after primary immunization with live RSV, secondary mucosal RSV-specific IgA responses were 7- to 10-fold less than those observed at 8 weeks (Fig. 5). RSV-specific IgG was produced by all tissues tested 59 weeks after primary immunization with live RSV. However, RSV-specific IgG production was six- to eightfold less than that observed 8 weeks after primary inoculation (Fig. 6). No primary or secondary mucosal immune responses were observed in mice 1 year after primary immunization with iRSV.

**DISCUSSION**

Our studies of secondary mucosal RSV-specific humoral immune responses in mice revealed that virus-specific IgA was
first produced upon challenge by B cells resident within the inductive tissues of RALT. RSV-specific IgA was subsequently produced by the effector tissues of the lower airway, such as the tracheal lamina propria and lung. We hypothesize that IgA-committed memory B cells might be located in organized NALT, CLN, and BLN. Upon reexposure, RSV-specific memory B cells might be induced to undergo activation and expansion, resulting in the production of virus-specific mucosal IgA within 3 days of challenge. We observed that RSV-specific IgA production of NALT, CLN, or BLN decreased within 6 days of challenge. In contrast, production of RSV-specific IgA by the tracheal lamina propria or lung was first detected 6 to 9 days after challenge. These observations are consistent with work by others suggesting that antigen-specific memory B (2, 13, 19) or

![Graphs showing percentage of RSV-specific IgG antibodies](image)

FIG. 3. Eight weeks after primary i.n. immunization with live RSV, iRSV, or medium, five adult BALB/c mice per group per time point were challenged i.n. with 20 μl containing 4 × 10⁵ PFU of RSV. Inductive tissues (NALT, CLN, and BLN) (A) and effector tissues (SM, tracheal lamina propria, and lung) (B) were isolated and cultured. The quantities of RSV-specific IgG produced were determined by ELISA 0, 3, 6, and 9 days after challenge. Data represent the arithmetic mean and standard error of the mean of the percentage of RSV-specific IgG compared to the total IgG produced. *, P < 0.05 compared to the percentage of RSV-specific IgG in samples from control mice; all other differences were not statistically significant (P > 0.05).
T (10, 14) cells might reside within the inductive tissues of the mucosal lymphoid system. In addition, our findings suggest that mucosal RSV-specific, IgA-committed memory B cells might be stimulated to undergo differentiation and expansion upon reexposure to RSV, with subsequent migration from mucosal inductive to effector tissues, as has been previously demonstrated within the gut (18). However, our findings of antigen-specific mucosal IgA memory responses in mice appear to be inconsistent with the short-lived protection from reinfection experienced by humans. This inconsistency might reflect fundamental differences in the pathophysiology of murine, compared to human, mucosal immunity.

The relative contribution of RSV-specific IgA, compared to that of IgG, to protective immunity remains unclear. We found that complete protection of both the upper and lower airway was correlated with the presence of RSV-specific IgG in mucosal secretions at the time of challenge. While our studies demonstrated the active production of RSV-specific IgG by the effector tissues of the lower respiratory tract, the origins of mucosal RSV-specific IgG detected in nasal wash fluids remain unclear. Liang and coworkers found that the diffuse NALT of rodents was a site of prolonged production of antigen-specific antibodies (11). Although we found RSV-specific IgG was produced by SM B cells, we do not know whether antibodies produced by these tissues contribute to the protection of the nasal mucosa. In contrast to our findings, other investigators have demonstrated a critical role of antigen-specific IgA in RSV immunity. For example, Weltzin and colleagues found that mucosal IgA, if present at the time of challenge, could protect against upper and lower respiratory tract infection (16, 17). However, Fisher et al. demonstrated that mucosal RSV-specific IgA and IgG induced equivalent protection against viral replication (6). Our data suggest that RSV IgG, or another mucosal immu-

![Graph A](image1.png)

**Fig. 4.** Eight weeks after primary i.n. immunization with live RSV, iRSV, or medium, five adult BALB/c mice per group per time point were challenged i.n. with 20 μl containing 4 × 10^5 PFU of RSV. Nasal washes, BAL fluid, and processed lung samples were tested to determine the quantities of RSV-specific IgA (A) or RSV-specific IgG (B) produced by ELISA. Graphed data represent the arithmetic mean and standard error of the mean of the percentage of RSV-specific antibodies compared to the total antibodies produced. Tables report the mean quantities of RSV-specific antibodies versus total antibodies.
nologic effector function present at the time of challenge, may mediate protection against viral replication within both the upper and lower airway. Similarly, investigators have found that parenteral administration of virus-specific IgG may reduce the titer of RSV in the respiratory secretions of infants infected with RSV. Other possible mediators of protection include cytotoxic T lymphocytes, antiviral cytokines, and innate immune factors.

In contrast to other investigators (8), we found that i.n. inoculation of mice with live RSV induced complete and durable protection against challenge. These observed differences might be related to our use of either a low challenge dose or a different viral strain than was used by some other investigators. Interestingly, we found that protection persisted for over 1 year in the absence of ongoing production of RSV-specific IgA by mucosal lymphoid tissues of the upper and lower airways. Conversely, RSV-specific IgG production persisted for 59 weeks after primary immunization with live RSV. Because epidemiological studies have demonstrated that repeated infections with RSV are common, our findings suggest that the murine model used for these
studies is not a successful model for further study of human immune responses to primary RSV infections.

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