Nasal Immunity to Staphylococcal Toxic Shock Is Controlled by the Nasopharynx-Associated Lymphoid Tissue

Stefan Fernandez,* Emily D. Cisney, Shannan I. Hall, and Robert G. Ulrich*

United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, Maryland 21702

Received 1 November 2010/Returned for modification 6 December 2010/Accepted 10 February 2011

The nasopharynx-associated lymphoid tissue (NALT) of humans and other mammals is associated with immunity against airborne infections, though it is generally considered to be a secondary component of the mucosa-associated lymphoid system. We found that protective immunity to a virulence factor of nasal mucosa-colonizing Staphylococcus aureus, staphylococcal enterotoxin B (SEB), requires a functional NALT. We examined the role of NALT using intranasal (IN) vaccination with a recombinant SEB vaccine (rSEBv) combined with an adjuvant in a mouse model of SEB-induced toxic shock. The rSEBv was rapidly internalized by NALT cells at the mucosal barrier, and transport into NALT was accelerated by inclusion of a Toll-like receptor 4 (TLR4) agonist. Vaccine-induced germinal centers of B cells formed within NALT, accompanied by elevated levels of IgA+ and IgG+ cells, and these were further increased by TLR4 activation. The NALT was the site of specific anti-rSEBv IgA and IgG production but was also influenced by intraperitoneal (IP) inoculation and perhaps other isolated lymphoid follicles observed within the nasal cavity. Vaccination by the IN route generated robust levels of anti-rSEBv IgA in saliva, nasal secretions, and blood compared to much lower levels after IP vaccination. IN vaccination also induced secretion of anti-rSEBv IgG in the blood and nasal secretions. Significantly, the efficacy of IN vaccination was dependent on NALT, as surgical removal resulted in greater sensitivity to IN challenge with wild-type SEB. Thus, protective immunity to SEB within the nasal sinuses was elicited by responses originating in NALT.

The Gram-positive bacterium *Staphylococcus aureus* is found on the skin and mucosa of healthy individuals and opportunistically causes a wide variety of infections (16, 19). Staphylococcal enterotoxins (SE) nonspecifically activate T lymphocytes by binding to and cross-linking major histocompatibility complex class II (MHC-II) proteins with T-cell antigen receptors, prompting deleterious immunological processes, from mild food poisoning to potentially life-threatening toxic shock syndrome (TSS). Symptoms of TSS include the sudden onset of fever, chills, vomiting, diarrhea, muscle aches, and rash. Rapid progression to severe and intractable hypotension and multiorgan failure may also occur, with a case fatality rate of 5%, as reported by the Centers for Disease Control and Prevention (CDC). In addition, staphylococcal enterotoxin B (SEB) is regulated by the CDC as a select agent because of its potential use as a biological weapon. We previously designed a recombinant SEB vaccine (rSEBv) that protects against lethal TSS in mice and rhesus macaques (44). Intranasal (IN) vaccination with rSEBv provides protection against wild-type (wt) SEB challenge in mice (30). The rSEBv was tested in combination with various adjuvants, including alum-based adjuvants and Toll-like receptor (TLR) agonists. Efficacy significantly increased if the vaccine was coadministered IN with a TLR4 agonist (30), suggesting that priming of nasopharyngeal immune components may contribute to immunity.

The nasopharynx-associated lymphoid tissue (NALT) is composed of a bell-shaped structure located in the nasal passages above the hard palate of rodents and other mammals (2, 7, 10). In mice, NALT organogenesis begins soon after birth and is dependent on several factors, including various chemokines and cytokines, as well as environmental cues (15, 17, 24, 35). In humans, NALT-like structures are evident at a very young age, but they disappear by the age of 2 years. The Waldeyer’s ring, which also includes nasopharyngeal lymphoid tissues, persists throughout life. The architecture of NALT is structured like lymph nodes, organized into discrete compartments of immature B and T lymphocytes and antigen-presenting dendritic cells (49). While afferent lymphatic ducts conduct antigens to most lymph nodes, antigens are delivered to NALT by the sinus passages above the hard palate of rodents and other mammals (2, 7, 10). In mice, NALT organogenesis begins soon after birth and is dependent on several factors, including various chemokines and cytokines, as well as environmental cues (15, 17, 24, 35). In humans, NALT-like structures are evident at a very young age, but they disappear by the age of 2 years. The Waldeyer’s ring, which also includes nasopharyngeal lymphoid tissues, persists throughout life. The architecture of NALT is structured like lymph nodes, organized into discrete compartments of immature B and T lymphocytes and antigen-presenting dendritic cells (49). While afferent lymphatic ducts conduct antigens to most lymph nodes, antigens are delivered to NALT by the sinus air passages (4). Furthermore, NALT lacks the characteristic germinal centers of lymph nodes or Peyer’s patches and is usually quiescent (18, 49). Germinal centers are rapidly expanded in NALT by IN exposure to infectious agents or antigens (49, 50). The follicle-associated epithelial cells (FAE) of the NALT are intercalated by M cells, responsible for antigen retrieval from the mucosal surfaces of the air passages and transport across the epithelial layer to dendritic cells below (33). An important feature of M cells present in the NALT is the abundance of TLR4 in their luminal location (43), which may explain the increased efficacy of the rSEBv vaccine when combined with TLR4 agonists (30). In addition to its functions as an antigen-
surveillance and processing organ, the NALT may further contribute to overall immunity as a source of IgA-secreting plasma cells (50, 51).

Though a growing number of reports have described the NALT as highly responsive to aerosolized antigens and adjuvants affecting local mucosal immune responses (23, 38, 50, 51), most conclude that the NALT alone is not essential for protection against infectious agents entering through the respiratory tract (3, 37, 47). We examined the role of NALT in protective immunity against virulence factors produced by nasal mucosa-colonizing bacteria. We hypothesized that the NALT contribution to the reported efficacy of intranasal rSEBv vaccination may stem from the induction of mucosal IgA in addition to the serum IgG1 and IgG2a usually generated by other routes of inoculation (30, 41). We showed that the murine NALT was the site of vaccine internalization, germinal center formation for SEB-specific IgA, and IgG secretion after IN vaccination, and furthermore, this process was time dependent and activated by TLR4 agonists. We also demonstrated that IN-vaccinated mice missing NALT were not protected against SEB-induced toxic shock, indicating that this organ is necessary for vaccine-derived immunity within the nasal passages.

MATERIALS AND METHODS

Mice and reagents. Female BALB/c mice (6 to 8 weeks old) were obtained from the National Cancer Institute (Frederick, MD). The rSEBv was produced under GMP conditions as previously reported (6). Endotoxin-free, wild-type (wt) SEB was supplied by Defense Science and Technology Laboratory (Salisbury, United Kingdom). Ultrapure Escherichia coli strain 0111:B4 lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA) and was used as a vaccine adjuvant. LPS from E. coli type 055:B5 (BD Dico TM, Franklin Lakes, NJ) was used in the mouse toxic shock model (described below) as reported previously (40).

Vaccination and sample collection. Anesthetized (IP with a mixture of ketamine-xecopramazine-xylazine) female BALB/c mice were vaccinated three times in 2-week intervals (unless otherwise noted) either IN at 10 μl volumes per dose, delivered in 5 μl per nare, or IP at 100 μl per dose with 20 μg of the rSEBv combined with an adjuvant (rSEBv-Adj) consisting of protease-treated and phenol-extracted LPS (InvivoGen; 20 μg per dose) that activates only the TLR4 pathway. Control mice received either the rSEBv alone (without adjuvant) or saline solution (phosphate-buffered saline [PBS]). In some experiments, 0.24 μg of the anti-TLR4 antibody (UT-41; eBioscience, San Diego, CA) was added to the IN vaccine before inoculation. Blood was collected from the tail vein into a microtainer serum separator (BD, San Jose, CA) and centrifuged. The serum was transferred to a fresh tube containing 10 μl PBS with 2% protease inhibitor cocktail set III (Calbiochem, San Diego, CA). Nasal lavage was collected by rinsing the area between the cheek and the tooth line of the mice and reagents. Female BALB/c mice (6 to 8 weeks old) were obtained from the National Cancer Institute (Frederick, MD). The rSEBv was produced under GMP conditions as previously reported (6). Endotoxin-free, wild-type (wt) SEB was supplied by Defense Science and Technology Laboratory (Salisbury, United Kingdom). Ultrapure Escherichia coli strain 0111:B4 lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA) and was used as a vaccine adjuvant. LPS from E. coli type 055:B5 (BD Dico TM, Franklin Lakes, NJ) was used in the mouse toxic shock model (described below) as reported previously (40).

Vaccination and sample collection. Anesthetized (IP with a mixture of ketamine-xecopramazine-xylazine) female BALB/c mice were vaccinated three times in 2-week intervals (unless otherwise noted) either IN at 10 μl volumes per dose, delivered in 5 μl per nare, or IP at 100 μl per dose with 20 μg of the rSEBv combined with an adjuvant (rSEBv-Adj) consisting of protease-treated and phenol-extracted LPS (InvivoGen; 20 μg per dose) that activates only the TLR4 pathway. Control mice received either the rSEBv alone (without adjuvant) or saline solution (phosphate-buffered saline [PBS]). In some experiments, 0.24 μg of the anti-TLR4 antibody (UT-41; eBioscience, San Diego, CA) was added to the IN vaccine before inoculation. Blood was collected from the tail vein into a microtainer serum separator (BD, San Jose, CA) and centrifuged. The serum was transferred to a fresh tube for storage (40).

Nasal lavage was collected from anesthetized mouse with 10 μl PBS with 2% protease inhibitor cocktail set III (Calbiochem, San Diego, CA). All palates were kept and processed separately. Once collected, the palates were washed 7 times with fresh medium and twice by placing them in prewarmed medium and incubating for 1 h. After being washed, the palates were transferred into fresh 48-well plates (Costar, Cambridge, MA) containing 250 μl fresh prewarmed medium and incubated at 37°C with 5% CO2. Every 24 h, approximately 40% (100 μl) of the medium was removed from each NALT and replaced with fresh medium. Collected media were centrifuged to remove cells and stored at −20°C until assayed.

rSEBv-specific IgG and IgA enzyme-linked immunosorbent assay (ELISA). Immunol 20H 96-well plates were coated (12 h at 4°C) with rSEBv (4 μg/ml in PBS). The plates were washed three times in 0.1% Tween 20 and PBS buffer and blocked (60 min at 37°C) with a 1:10 dilution of 10% bovine serum albumin (BSA) diluent/blocking solution (KPL, Gaithersburg, MD). Serum (diluted 1:20 in blocking buffer) and saliva and nasal lavage (diluted 1:10) samples were added (60 μl) to the plates and incubated at 37°C. Serum samples were incubated for 1 h, while saliva and nasal samples were incubated for 2 h. The plates were washed (three times in 0.1% Tween 20, PBS buffer) and incubated (1 h at 37°C) with either 0.4 μg/ml goat anti-mouse IgG-horseradish peroxidase (HRP) or 0.35 μg/ml goat anti-mouse IgA-HRP. Bound antibodies were detected by optical density (OD) absorbance (PerkinElmer Victor V 1420 multilabel counter) using TMB Microwell peroxidase substrate (KPL, Gaithersburg, MD).

Immunohistochemistry. Mouse heads were collected after euthanasia and fixed in 10% neutral buffered formalin (VAL Tech Diagnostics, Pittsburgh, PA) overnight. Specimens were decalcified in formic acid (12 h at room temperature), and cross sections including the nasal cavities were paraffin embedded and mounted onto microscope slides. Cross sections were deparaffinized and rehydrated with xylene and serial dilutions of ethanol. Antigen retrieval of the tissue sections and sections was accomplished using a microplate reader (97°C) Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0), heating (97°C) for 30 min, and then cooling (22°C) for 30 min. Sections were blocked (10% normal serum, 1% BSA, 1× Tris-buffered saline [TBS]; 2 h at 22°C) and then probed (overnight at 4°C) with rabbit anti-SEB IgG (Toxin Technology, Sarasota, FL) or biotinylated goat anti-mouse IgG (KPL, Gaithersburg, MD), biotinylated goat anti-mouse IgA (KPL), or biotinylated peanut agglutinin (PNA) (Vector Laboratories, Burlingame, CA) in 1× TBS with 1% BSA. Slides were incubated in 0.35% H2O2–1× TBS for 30 min at room temperature (RT) before addition of HRP-conjugated streptavidin (Abcam, Cambridge, MA). Slides were developed with DAB chromogen and DAB enhancer (Abcam) for 10 min at room temperature. Samples were counterstained with hematoxylin (Sigma–Aldrich, St. Louis, MO), followed by dehydration.

Statistical analyses. Student’s t test was used for analysis of data significance between experimental groups, with step-down Bonferroni adjustments included to compare mean time-to-death rates in the challenge experiments (SAS version 9.2). For all others, Student’s t test was used to establish the significance of the results.

RESULTS

Enhancement of vaccine transport across NALT epithelia by TLR4 activation. Because the nasal epithelial lining (follicle-associated epithelial cells [FAE]) is the primary barrier dividing the lymphoid tissue from the external environment, we first...
examined the movement of IN-delivered vaccine across these cells by immunohistochemistry (Fig. 1). As an adjuvant, we used an ultrapure form of LPS, which exclusively binds to TLR4 and acts similarly to synthetic TLR4 agonists (30). In mice inoculated with rSEBv combined with the TLR4 agonist (rSEBv-Adj) (Fig. 1A to F), vaccine uptake was detected across the FAE barrier as soon as 10 min (Fig. 1A) after inoculation and was continuously observed for at least 24 h (Fig. 1D). Uptake of rSEBv alone (Fig. 1G to L), did not take place until 60 min (Fig. 1H) after introduction and the uptake of vaccine was no longer observed by 24 h (Fig. 1J). The addition of an inhibitory, anti-TLR4 monoclonal antibody (12) to rSEBv-Adj (Fig. 1M to R) reduced the amount of observable vaccine in the NALT to only 1 h (Fig. 1M and N), with no rSEBv detected across the NALT by 4 h (Fig. 1O). Figure 1 also shows cross-sections of IN PBS-inoculated mice (UNT) at 10 and 60 min (Fig. 1S and T). These results indicated that vaccine transport across the NALT FAE was specifically enhanced by stimulation of TLR4 signaling.

Increased IgG$^+$ and IgA$^+$ cells within newly formed B-cell germinal centers as a result of vaccine transport assisted by TLR4 engagement. The increased rate of vaccine transport triggered by TLR4 stimulation suggested that the adjuvant might further enhance the production of antibody-producing cells within NALT. We again used immunohistochemical analyses of cross sections collected from NALT of vaccinated and control mice (Fig. 2). Germinal centers (PNA-positive [PNA$^+$] cells) of nonvaccinated animals (UNT) appeared as small and discrete areas in the NALT (Fig. 2A), expanding to include most of the NALT in IN-vaccinated mice after 2 weeks (Fig. 2B and C). Although germinal centers were enriched in the interior of NALT by IN vaccination, the increased formation did not appear to be dependent on adjuvant. Equally, IP vaccination resulted in increased PNA binding (Fig. 2D), suggesting a local effect after systemic vaccination. Similar results were detected 1 week after vaccination (data not shown). In contrast, we detected increased levels of B-cell differentiation induced in NALT of mice vaccinated IN with rSEBv-Adj, resulting in elevated amounts of IgG-producing (Fig. 2K) and IgA-producing (Fig. 2G) cells compared to those in control mice (Fig. 2I and E, respectively). Vaccination IP with rSEBv-Adj also caused differentiation of B cells into IgG$^+$ secretors (Fig. 2L), but minimal amounts of IgA$^+$ cells were detected (Fig. 2H). These results showed that the adjuvant not only increased vaccine transport into the NALT but also drove maturation of antibody-producing cells. Additionally, our data suggested that the IN route of vaccine delivery was critical to IgA$^+$ B-cell induction within the NALT.

Increased levels of rSEBv-specific secretory antibody due to nasal vaccination. We next examined the potential role of NALT in controlling circulating levels of antibodies. Mice were vaccinated with rSEBv-Adj either IN (10 μl) or IP (100 μl), while a control group received IN PBS. The vaccine was administered three separate times, 2 weeks apart. Saliva and

FIG. 1. Vaccine internalization across the NALT FAE layer was accelerated and sustained by TLR4 activation. Mice were IN inoculated with the following: rSEBv and adjuvant (rSEBv-Adj) (A to F), rSEBv only (rSEBv) (G to L), rSEBv with adjuvant and anti-TLR4 monoclonal antibody (rSEBv-Adj/a-TLR4) (M to R), or PBS only (UNT) (S and T). The mice were euthanized at the specified times and paraffin cross sections of the nasal cavities were prepared. Vaccine was localized (brown staining) across the FAE layer of the NALT using SEB-specific antibodies, and tissue was counterstained with hematoxylin. These images represent one of two similar experiments. Original magnification, ×40.
serum samples were collected 2 weeks after the last dose for analysis. Levels of anti-rSEBv IgA in saliva were significantly higher in the mice vaccinated IN with rSEBv-Adj (Fig. 3A) than in those in the other groups, while mice from the IP group failed to generate levels of anti-rSEBv IgA above those of the PBS control group. However, both IN and IP rSEBv-Adj vaccinations resulted in levels of anti-rSEBv IgG in the saliva which were significantly above those in the PBS group (Fig. 3A). The IN rSEBv-Adj vaccination also resulted in anti-rSEBv IgG and IgA levels in the blood that were significantly higher than those in the IN PBS group (Fig. 3B). IP vaccination did not result in higher levels of anti-rSEBv IgA. From these results, we concluded that IN vaccination was better for induction of IgA responses than IP delivery of rSEBv, though either route was sufficient for IgG responses.

**Nasal IgG and IgA originate in NALT.**
To corroborate the results obtained from the saliva and serum and to ascertain if the NALT was an important local contributor of vaccine-specific IgG and IgA, we next measured soluble anti-rSEBv IgA and IgG output from NALT collected from vaccinated mice. Mice were vaccinated IN or IP with rSEBv-Adj. One group was vaccinated IN with rSEBv without using the adjuvant (IN rSEBv), and a control group received IN saline (PBS). Approximately 2 weeks after the third dose, the mice were euthanized and NALTs were carefully collected and kept separated without disruption, repeatedly washed, and individually cultured. Medium was replenished (40%) daily. Supernatant from each cultured NALT was collected after the third day, and an ELISA was used to measure relative levels of anti-rSEBv IgA (Fig. 4A) and IgG (Fig. 4B). We determined that the NALT from IN vaccinations with rSEBv-Adj secreted levels of anti-rSEBv IgA that were significantly higher than those collected from the PBS controls or from the IN rSEBv group ($P < 0.01$). NALT from IN-vaccinated mice also secreted higher levels of IgA than NALT from the IP group, although with less statistical significance ($P = 0.075$). As was the case with IgA, NALT from mice vaccinated IN with rSEBv-Adj also secreted significantly higher levels of anti-rSEBv IgG than the PBS or IN rSEBv alone groups ($P < 0.01$). However, the NALT from the IP group secreted the highest levels of rSEBv-specific IgG ($P < 0.01$), corroborating the results obtained from the saliva samples (Fig. 3A). Collectively, data from NALT cultures were predictive of IgA and IgG levels found in mouse saliva and serum resulting from vaccination.

To further explore the relationship between NALT and IgA responses to IN vaccination, we surgically ablated NALT in a select group of animals. Ablation was accomplished through the mechanical disruption of the tissue after a small incision in the palates of anesthetized mice. Histological examinations of cross-sectioned nasal passages from mice euthanized at the end of the study confirmed the absence of NALT in each case (see Fig. S1 in the supplemental material). Control (sham) mice were treated identically except that NALTs were left in place. The no-NALT group and sham control mice were IN vaccinated (three doses applied in 2-week intervals) with rSEBv-Adj beginning 10 to 14 days after recovery from surgery. There were no weight discrepancies between the control mice and the mice undergoing surgeries (data not shown). Two
groups of normal (no surgery) mice were vaccinated IN either with rSEBv-Adj or with PBS. All mice were inoculated three times in 2-week intervals, and samples were collected for evaluation 2 weeks after the last vaccination. Results from this vaccination study indicated that mice lacking NALT (no NALT, IN rSEBv-Adj) secreted significantly lower levels of rSEBv-specific IgA, compared to the sham control (sham, IN rSEBv-Adj) and to the normal control (IN rSEBv-Adj) groups, in saliva (P < 0.05) (Fig. 5A), nasal passages (P < 0.01) (Fig. 5B), and blood (P < 0.01) (Fig. 5C). The levels of rSEBv-specific IgA secreted from the NALT-free, IN rSEBv-Adj group were not significantly different than those secreted by the PBS-inoculated normal mice in any of the samples analyzed. In the saliva samples, we found low levels of rSEBv-specific IgG after vaccination (Fig. 5A) in all the groups. However, in the nasal wash samples (Fig. 5B), we found significantly lower levels of IgG in the NALT-free, IN rSEBv-Adj group than in the sham control (sham, IN rSEBv-Adj) group (P < 0.05). There was no statistically significant difference after comparing results from the NALT-free, IN rSEBv-Adj group to the vaccinated normal mice (IN rSEBv-Adj), though the trend toward lower IgG secretion was evident. Similarly, removing the NALT failed to significantly reduce the levels of IgG secreted into the blood. This lack of reduction in secretion of rSEBv-specific IgG in the absence of NALT perhaps underscores the fact that other sites within the nasal cavities or upper respiratory tracts generate soluble IgG after IN inoculation that is sufficient to compensate. Our results indicate that after IN vaccination, the NALT may be the main contributor of rSEBv-specific IgA that is secreted locally and systemically as well as that of rSEBv-specific IgG secreted locally.

Essential role for NALT in protection against SEB-induced toxic shock. Our data to this point indicated that the murine NALT was a site for antigen uptake, germinal center formation, and generation of humoral immunity after IN exposure to antigens. Because SEB is commonly produced by nasal mucosa-colonizing S. aureus, we next addressed the contribution of NALT to protection from toxic shock, again using mice with surgically removed NALT. After complete recovery from the NALT ablation surgery (2 weeks), we separated the study mice into one group that was IN vaccinated with rSEBv-Adj and another that was IN inoculated with PBS only. We also included a control group comprised of normal mice treated IN with PBS. All the groups received three rounds of inoculations with rSEBv-Adj at 2-week intervals. Two weeks after the last inoculation, all
mice were IN challenged with wt SEB, followed 4 h later by an IP injection of LPS, as previously described (40). Tissue samples were collected at the end of the study to confirm the absence of NALT (see Fig. S2 in the supplemental material). Within the unvaccinated, normal group (IN PBS), 70% succumbed to fatal toxic shock after the wt SEB challenge, while nearly 70% of the surgery control group that were vaccinated survived (Fig. 6). Intranasal vaccination of NALT-free mice did not convey protection against toxic shock, and instead this group had the same survival rate as the unvaccinated (IN PBS) group (30%) and exhibited significantly \( P < 0.01 \) accelerated time to death compared to the surgery control group (sham, IN rSEBv-Adj). The NALT-free, vaccinated group showed slightly slower but significantly delayed \( P < 0.03 \) time of death compared to the control groups, suggesting an additional contribution to anti-SEB immunity that was not NALT dependent.

**DISCUSSION**

Our results indicate that the NALT is an essential contributor to systemic and local SEB-specific IgA and local IgG resulting from IN vaccination. Furthermore, our data demonstrate that the NALT is the site of the active uptake of vaccine introduced into the nasal sinuses and that this process is accelerated and sustained by TLR4 activation. The ultrapure form of LPS used as an adjuvant in our vaccination exclusively binds to TLR4 and mimics the activities of other synthetic TLR4 agonists currently under investigation (1, 30). NALT-dependent vaccination resulted in systemic as well as nasal humoral immunity, whereas only systemic antibody responses were obtained by IP vaccine delivery. We also established that NALT is the most important contributor of secreted IgG and IgA in saliva and blood and IgA in nasal secretions resulting from IN vaccination. Lastly, we showed that NALT is required for protection of mice from respiratory toxic shock caused by SEB, a virulence factor produced by many strains of colonizing *S. aureus*.

Lymphoid tissue in the mammalian nasopharyngeal area is organized into discrete sites, which in humans constitute the tonsils, adenoids (collectively termed Waldeyer’s ring), and other nasopharyngeal lymphoid follicles. The salivary glands also have immune faculties as they contain duct-associated lymphoid tissue and serve as hosts of IgA-secret-
ing plasma cells as well (31, 42). NALT-like structures are also present in young humans, but these disappear at an early age (10). Immune responses within the nasal sinuses provide IgA-dominated protection that carries over to the mucosa of the intestines and lower and higher respiratory cavities, as well as ocular tissues and auditory cavities (20, 25, 36). Furthermore, NALTs function as collection centers for sampling airborne antigens and debris from colonizing microorganisms (13, 29, 33). This collection process occurs mainly through M cells intercalating the follicular epithelial cells of the NALT (13, 29, 33). Exposure to these environmental antigens transported into NALT of naive mice leads to the differentiation of Th0 cells (22). Intranasal vaccination with viral or bacterial antigen preparations leads to a transient cell expansion characterized by an influx and maturation of CD11c+ dendritic cells, proliferation of CD45RB+ naive T cells, and selective secretion of Th1 and Th2 cytokines (21, 28, 34, 38, 50). Activation and expansion of T cells also results in Th17 differentiation within the NALT. Secretion of IL-17 is linked to bacterial clearance in the NALT and lungs of infected mice (46, 48). Within the NALT, most newly expanded DCs tend to segregate along T cells (14). Upregulation of Th1 cytokines marks the activation of CD4+ cells, necessary for maturation of B cells in the lymphoid organs (27). This process in turn drives expression of PNA receptors on B cells, characteristic of dark zones within the germinal center (6, 26, 45). Stimulation of NALT is characterized by the preferential secretion of soluble IgA, but also of IgG2 and IgG1 (11, 28, 38). It has been shown that in mice the NALT is the site of Ig isotype selection and generation of long-term immunity (28, 39).

Our data support the conclusion that the NALT provides local and systemic humoral immunity in a process that preferentially favors IN vaccinations and is highly sensitive to the presence of adjuvants. The local and systemic IgA and IgG responses of mice in our study elicited by IN vaccination with rSEBv required the use of the TLR4 agonist as an adjuvant. Although previous reports also suggested the importance of adjuvants to IN vaccination (11, 38), we describe the novel observation that a TLR4 agonist increased the rate of vaccine uptake into the NALT and prolonged the tissue half-life of antigen. The rate of antigen transport may thus represent the earliest step in which the adjuvant influences vaccine efficacy. This phenomenon is at least partially explained by TLR4 receptors present on the lumenal surfaces of M cells responsible for antigen uptake and transcytosis (43). Our histochemical analyses of NALT from vaccinated mice indicated that the generation of surface IgA- and IgG-positive cells was also dependent on inclusion of the adjuvant in the vaccine preparations. However, germinal center formation took place regardless of the adjuvant.

Our results demonstrated that NALT actively secreted specific IgA and IgG in response to a vaccine introduced into nasal passages. This was firmly established by measuring the specific antibody secretion into culture media of NALT obtained from IN-vaccinated mice. In contrast, IP vaccination resulted in only antigen-specific IgG and not IgA production by NALT. Plasma IgG leakage from the gingival spaces, as previously reported (5), or from other locations within the nasal passages (38), may explain the detection of specific IgG after IP vaccination. However, the sustained production of SEB-specific IgG from NALT cultures, as well as SEB-specific IgG present in the saliva after IP inoculation, suggests that both IgA- and IgG-secreting B cells are located within the NALT. Furthermore, the IgG plasma cells occurring in the NALT after IP vaccination may have originated from peripherally generated cells migrating to the NALT, homing through recognition of the unique set of adhesion molecules expressed in the high endothelial venules of NALT (9).

Vaccination of NALT-free mice by the IN route resulted in insignificant levels of SEB-specific IgA and IgG in the nasal passages, similar to levels found in unvaccinated mice (Fig. 5B), suggesting that NALTs are the most important contributors of humoral immunity to SEB in the nasal sinuses. Previously, Sekine et al. (38) demonstrated that the salivary glands and isolated lymphoid follicles may also be important contributors to nasal immunity. These tissues may contribute more to immunity within the buccal cavity, as our data showed that NALT ablations significantly reduced SEB-specific IgA, but not IgG, in the saliva and plasma. Furthermore, IN vaccination led to robust levels of antigen-specific IgG in the blood, comparable to IP inoculation. This observation indicates that introduction of vaccines into the nasal sinuses will result in local as well as systemic protection (51). Though we did not attempt to elucidate the origin of plasma IgG resulting from IN vaccination, it is possible that antigen may have entered the bloodstream after being absorbed through the nasal passages.

By surgically removing the NALT in young mice, the model used in our study facilitated the measurement of nasal contributions to local and systemic humoral immunity. We corroborated the success of the surgeries by cross-sectioning the nasal passages of each mouse postexperimentally to confirm the absence of NALT. Although labor-intensive, this method is preferable to the use of current transgenic mouse models lacking functional NALT, which we deemed unsuitable for our experiments. Current transgenic models are deficient in several important cytokines and chemokines that are essential to the development of various secondary lymphoid organs, including those associated with the common mucosal system. The transgenic models also lack important components involved in the onset of immunity (17, 35). Because the NALT-disrupted mice were not protected against SEB toxic shock by IN vaccination, this was most likely due to the absence of SEB-specific IgA in the nasal and oral cavities as well as in the blood. Any SEB-specific IgG circulating in the blood was not sufficient to prevent the lethal toxic shock. Our findings with NALT do not address the potential contributions of other disseminated lymphoid clusters and isolated lymphoid follicles, randomly located throughout the nasal cavities (7). It will be important to also elucidate the interactions between these seemingly isolated lymphoid foci of the nasal passages and the systemic immune response to respiratory pathogens.

ACKNOWLEDGMENTS

We thank Christine A. Mech and Gale A. Krienitz (USAMRIID) for their expert help with tissue processing, Larry K. Osby for his help in
REFERENCES

1. Alderson, M. R., P. McGowan, J. R. Baldridge, and P. Probst. 2006. TLR4 agonists as immunomodulatory agents. J. Endotoxin Res. 12:313–319.

2. Asanuma, H., et al. 1997. Isolation and characterization of mouse nasal-associated lymphoid tissue. J. Immunol. Methods 202:123–131.

3. Balmelli, C., S. Demotz, H. Acha-Orbea, P. De Grandi, and D. Nardelli-Haefliger. 2002. T-cell, lung, and tracheobronchial lymph node sites are the major sites where antigen-presenting cells are detected after nasal vaccination of mice with human papillomavirus type 16 virus-like particles. J. Virol. 76:12956–12962.

4. Bienenstock, J., and M. R. McDermott. 2005. Branchial- and nasal-associated lymphoid tissues. Immunol. Rev. 206:22–31.

5. Brandtzæg, P. 2007. Do salivary antibodies reliably reflect both mucosal and systemic immunity? Ann. N.Y. Acad. Sci. 1098:288–311.

6. Butcher, E. C., et al. 1982. Surface phenotype of Peyer’s patch germinal center cells: implications for the role of germinal centers in B cell differentiation. J. Immunol. 129:2696–2707.

7. Casteleyn, C., A. M. Broos, P. Simoons, and W. Van den Broeck. 2010. NALT (nasal cavity-associated lymphoid tissue) in the rabbit. Vet. Immunol. Immunopathol. 136:212–218.

8. Coffman, J. D., et al. 2002. Production and purification of a recombinant Staphylococcal enterotoxin B vaccine candidate expressed in Escherichia coli. Protein Expr. Purif. 24:302–312.

9. Csencsitz, K. L., M. A. Jutila, and D. W. Pascual. 2009. Induction of germinal center B cell differentiation of minor salivary glands and mucosal immunity. Immunology 127:178–180.

10. Debertin, A. S., et al. 2003. Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. Clin. Exp. Immunol. 134:503–507.

11. Etchart, N., et al. 2006. Intranasal immunization with inactivated RSV and bacterial adjuvants induces mucosal protection and abrogates eosinophilia upon challenge. Eur. J. Immunol. 36:1136–1144.

12. Fernandez, S., et al. 2007. Potential role for Toll-like receptor 4 in mediating Escherichia coli maltose-binding protein activation of dendritic cells. Infect. Immun. 75:5416–5420.

13. Fujimura, Y., T. Akiyasa, T. Harada, and K. Haruma. 2006. Uptake of microparticles into the epithelium of human nasopharyngeal lymph tissue. Med. Mol. Morphol. 39:181–186.

14. Fukunaga, T., et al. 2003. A combination of Flt3 ligand and CD40 ligation as nasal adjuvant elicits NALT dendritic cells for prolonged mucosal immunity. Vaccine 21:4849–4859.

15. Fukuyama, S., et al. 2006. Cutting edge: uniqueness of lymphoid chemokine requirement for the initiation and maturation of nasopharynx-associated lymphoid tissue organogenesis. J. Immunol. 177:4276–4280.

16. Gevaert, P., et al. 2005. Organization of secondary lymphoid tissue and local IgE formation to Staphylococcus aureus enterotoxin B in nasal polyp tissue. Allergy 60:71–79.

17. Harmsen, A., et al. 2002. Cutting edge: organogenesis of nasal-associated lymphoid tissue (NALT) occurs independently of lymphotixin-alpha (LT alpha) and retinoic acid receptor-related orphan receptor-gamma, but the organization of NALT is LT alpha dependent. J. Immunol. 168:986–990.

18. Heritage, P. L., B. J. Underdown, A. L. Arsenault, D. P. Snider, and M. R. McDermott. 1997. Comparison of murine nasal-associated lymphoid tissue and Peyer’s patches. Am. J. Respir. Crit. Care Med. 155:1256–1262.

19. Hari, U., et al. 1999. Airway exposure to bacterial superantigen (SEB) induces lymphocyte-dependent airway inflammation associated with increased airway responsiveness—a model for non-allergic asthma. Eur. J. Immunol. 29:1021–1031.

20. Hiro, T., T. Jiao, Z. Chen, C. Van Waes, and X. X. Gu. 2006. Kinetics of mouse allergy antibodies and lymphocyte responses during intranasal vaccination with a lipopolysaccharide-based conjugate vaccine. Immunol. Lett. 107:131–139.

21. Hiro, T., S. Kodama, M. Moriyama, T. Kawano, and M. Suzuki. 2009. The role of Toll-like receptor 4 in eliciting acquired immune responses against nonontypeable Haemophilus influenzae following intranasal immunization with outer membrane protein. Int. J. Pediatr. Otorhinolaryngol. 73:1657–1665.

22. Hiro, T., et al. 1998. Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. Eur. J. Immunol. 28:3346–3353.

23. Hou, Y., W. G. Hu, T. Hirano, and X. X. Gu. 2002. A new intra-NALT route elicits mucosal and systemic immunity against Moraxella catarrhalis in a mouse challenge model. Vaccine 20:2375–2381.

24. Kiyono, H., and S. Fukuyama. 2004. NALT–versus Peyer’s-patch-mediated mucosal immunity. Nat. Rev. Immunol. 4:699–710.

25. Kodama, S., S. Suenaiga, T. Hirano, M. Suzuki, and G. Mogi. 2000. Induction of specific immunoglobulin A and IgG2a immune responses to P6 outer membrane protein of nontypeable Haemophilus influenzae in middle ear mucosa by intranasal immunization. Infect. Immun. 68:2294–2300.

26. Kosco, M. H., A. K. Szakal, and J. G. Tew. 1988. In vivo obtained antigen presented by germline center B cells to T cells in vitro. J. Immunol. 140: 359–360.

27. Lahvis, G. P., and J. Cerny. 1997. Induction of germinal center B cell markers in vitro by activated CD4+ T lymphocytes: the role of CD40 ligand, soluble factors, and B cell antigen receptor cross-linking. J. Immunol. 159: 107–115.

28. Liang, B., L. Hyland, and S. Houd. 2001. Nasal-associated lymphoid tissue is a site of long-term virus-specific antibody production following respiratory virus infection of mice. J. Virol. 75:5416–5420.

29. Nair, P. N., and H. E. Schroeder. 1986. Duct-associated lymphoid tissue (DALT) of minor salivary glands and mucosal immunity. Immunology 57:171–180.

30. National Research Council. 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.

31. Petukhova, G., et al. 2008. Synthetic Toll-like receptor 4 agonist enhances vaccine efficacy in an experimental model of toxic shock syndrome. Clin. Vaccine Immunol. 14:1499–1504.

32. Richards, C. M., A. T. Aman, T. R. Hirst, T. J. Hill, and N. A. Williams. 2001. Protective mucosal immunity to oral herpes simplex virus type 1 infection in mice by using Escherichia coli heat-labile enterotoxin B subunit as an adjuvant. J. Virol. 75:1604–1617.

33. Sabirov, A., and D. W. Metzger. 2008. Intranasal immunization of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue. J. Immunol. 175:4904–4913.

34. Sabirov, A. G., A. Garza, R. G. Ulrich, and J. W. Boles. 2001. Mucosal vaccination with recombinantly attenuated staphylococcal enterotoxin B and protection in a murine model. Infect. Immun. 69:2031–2036.

35. Stool, M. W., N. E. Evenhuis, F. G. Kroese, and N. A. Bos. 2008. Rat salivary gland reveals a more restricted IgA repertoire than ileum. Mol. Immunol. 45:719–727.

36. Tyrer, P. R., A. R. Foewell, A. W. Cripps, M. A. Apicella, and J. M. Kyd. 2006. Microparticle pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. Infect. Immun. 74:625–631.

37. Ulrich, R. G., M. Olson, and S. Bavari. 1996. Bacterial superantigenic vaccines, p. 135–141. In E. N. F. Brown, D. Burton, and J. Mekalanos (ed.), Vaccines 9: molecular approaches to the control of infectious diseases. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

38. van Kempen, M. J., G. T. Rijkers, and P. B. Van Cauwenberge. 2000. The nasal-associated lymphoid tissue or cervical lymph nodes. J. Immunol. 167:1319–1326.

39. Wang, B., et al. 2010. Induction of TGF-beta1 and TGF-beta1-dependent Th1 differentiation by group A streptococcal infection. Proc. Natl. Acad. Sci. U. S. A. 107:5937–5942.

40. Wiley, J. A., M. P. Tighe, and A. G. Harmsen. 2005. Upper respiratory tract resistance to influenza infection is not prevented by the absence of either nasal-associated lymphoid tissue or cervical lymph nodes. J. Immunol. 175:3186–3196.
48. Ye, P., et al. 2001. Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. Am. J. Respir. Cell Mol. Biol. 25:335–340.

49. Zuercher, A. W., and J. J. Cebra. 2002. Structural and functional differences between putative mucosal inductive sites of the rat. Eur. J. Immunol. 32: 3191–3196.

50. Zuercher, A. W., S. E. Coffin, M. C. Thurnheer, P. Fundova, and J. J. Cebra. 2002. Nasal-associated lymphoid tissue is a mucosal inductive site for virus-specific humoral and cellular immune responses. J. Immunol. 168:1796–1803.

51. Zuercher, A. W., et al. 2006. Intranasal immunisation with conjugate vaccine protects mice from systemic and respiratory tract infection with *Pseudomonas aeruginosa*. Vaccine 24:4333–4342.