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Enhancement of Serum and Mucosal Immune Responses to a *Haemophilus influenzae* Type B Vaccine by Intranasal Delivery

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Intranasal (i.n.) vaccination is potentially the most direct method for conveying upper respiratory and mucosal immunity to respiratory pathogens. However, for unclear reasons, vaccines introduced into the nasal sinuses often have lower efficacy than vaccines administered by the more frequently used parenteral routes. We examined i.n. vaccination in a mouse immune-response model with a commonly used *Haemophilus influenzae* type B vaccine (Hibv) composed of the polyribosylribitol phosphate (PRP) capsule antigen conjugated to tetanus toxoid. Intranasal vaccination with Hibv using a Toll-like receptor 4 (TLR4) agonist as an adjuvant significantly increased the levels of IgA specific for the PRP capsule antigen in blood serum, saliva, and mucosal secretion specimens. In contrast, control mice vaccinated transdermally (t.d.) with Hibv did not produce significant levels of PRP-specific IgA in the blood serum and saliva, and anti-PRP IgG was increased only in serum. The i.n. and t.d. vaccinations resulted in equivalent bactericidal antibody responses in blood serum, suggesting that vaccine-derived IgG is protective against infection. Elevated levels of IgG specific for the tetanus toxoid carrier protein were measured in nasal sinuses and vaginal secretions in mice vaccinated by either the t.d. or i.n. route. Tissue culture studies confirmed that the nasopharynx-associated lymphoid tissue (NALT) was at least one of the sources of PRP-specific IgA and carrier-specific IgG within the nasal sinuses. We conclude that i.n. vaccination aided by a TLR4 agonist results in robust immune responses to both the carrier protein and bacterial polysaccharide components of the Hibv.

Haemophilus influenzae type b (Hib) vaccines (Hibv) are widely used in the pediatric population and require several intramuscular vaccination rounds of to achieve optimal efficacy. Children are significant reservoirs of Hib, with carriage rates ranging from 4.2% to 9% in the school-aged population (1, 2). Vaccination with the Hib vaccine significantly reduces Hib carriage in the pediatric population (3). Despite the widespread use and existence of the Hib vaccine, the annual burden of Hib infection continues to reach millions of cases worldwide and fatalities of several hundred thousand in children ≤5 years of age (4). Hib infection is spread by aerosolized droplets, with the nasal passages being the primary portal of entry, and can lead to meningitis, epiglottitis, pneumonia, cellulitis, and arthritis. The primary component of the Hib vaccine is polyribosylribitol phosphate (PRP), a ubiquitous polysaccharide of the bacterial outer wall that is delivered either with or without an aluminum-based adjuvant (5). Like all polysaccharides, the PRP moiety is poorly immunogenic (6), and conjugation to a protein carrier, such as tetanus toxoid (TT), is required to significantly increase vaccine immunogenicity (7). The internalization of PRP-protein conjugates by antigen-presenting cells (APC) followed by major histocompatibility complex (MHC) presentation (6, 8) is required for the stimulation of cytokine-secreting T cells, activation of polysaccharide-specific B cells, and Ig-isotype switching. A recent report on group B streptococcal polysaccharide coupled to a carrier protein suggested that carbohydrate-specific T helper cells recognize and respond to processed peptide glycoconjugates presented by MHC class II molecules (9, 10). In the case of the Hib vaccine, multiple parenteral vaccinations are required to induce a robust and long-lasting immunity, which is dominated by high levels of serum anti-PRP IgG and little or no mucosal antibody (11, 12).

We previously reported the efficacy of intranasal (i.n.) vaccination in a mouse model of staphylococcal toxic shock and the role played by the nasopharynx-associated lymphoid tissue (NALT) in the generation of local and systemic antibody responses (13). The NALTs are present at the base of the nasal sinuses above the hard palate throughout the lives of most mammals; in humans, they disappear at an early age only to be replaced by other nasopharyngeal lymphoid tissues (14). In the reported study, we used the protein-based recombinant staphylococcal enterotoxin B vaccine (STEBVax) to show that murine NALTs are essential for antibody responses to i.n. vaccination. A growing number of other reports have described the NALTs as highly responsive to aerosolized antigens and adjuvants, hence affecting local mucosal immune responses (15–20). Intranasal administration of polysaccharide antigens was previously demonstrated in small animal models to be a viable alternative to systemic vaccination. For example, mice vaccinated i.n. with group B streptococcus capsular polysaccharide conjugated to cholera toxin produced a strong and persistent capsule-specific IgA response that was broadly distributed in the mucosal surfaces and blood serum (21, 22). Similarly, the coadministration of cholera toxin or cytosine-phosphate-guanine (CpG)-containing immunostimulatory sequences with PRP conjugated to cross-reacting material (CRM_{197}) of diphtheria toxin significantly increased blood serum and mucosal anti-PRP IgG and IgA (23, 24) responses to i.n. vaccination. The relevance of mucosal immunity for controlling infection is also underscored by the observation that naturally oc-

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curring mucosal antibodies limit the nasopharyngeal colonization in mice by diverse *H. influenzae* strains (25). In the study described here, we examined protective immunity against nasally colonizing Hib and hypothesized that NALTs contribute to the efficacy of i.n. vaccination by leading to the induction of mucosal IgA in addition to the serum IgG that is usually generated by use of other routes of inoculation (26, 27). We specifically address associations between the NALTs and antibody responses to the polysaccharide-based Hib vaccine within the microenvironment of the upper respiratory tract and other mucosal sites.

MATERIALS AND METHODS

Reagents. The ActHIB *Haemophilus* b conjugate vaccine (tetanus toxoid conjugate) was purchased from Sanofi Pasteur SA (Swiftwater, PA). *H. influenzae* type B oligosaccharide-human serum albumin conjugate (HbO-HA antigen) was acquired from BEI Resources (Manassas, VA). Ultrapure *Escherichia coli* strain O111:B4 lipopolysaccharide (LPS) was purchased from InvivoGen (San Diego, CA) for use as a Toll-like receptor 4 (TLR4) agonist. Hib Rab strain was acquired from ATCC (Manassas, VA). Anti-Hib capsular polysaccharide serum was obtained from the CBER of the FDA (Rockville, MD).

Vaccination and sample collection. Female BALB/c mice (6 to 8 weeks old) were obtained from the National Cancer Institute (Frederick, MD). The mice were anesthetized with a ketamine-acepromazine-xylazine mixture given intraperitoneally and vaccinated three times in 2-week intervals either intranasally (i.n.) or transdermally (t.d.) with 5 μg of Hibv alone or with the TLR4 agonist (20 μg per dose). Blood was collected after each vaccination on days 14, 28, and 42. Blood was collected from the tail vein into a Microtainer serum separator (BD Biosciences, San Jose, CA), clotted, and centrifuged. The serum was transferred to a fresh tube for storage (−20°C) before testing. The same mice were used for the collection of salivary, nasal, and vaginal mucosa specimens. The saliva and nasal secretion specimens were collected 2 weeks after the third vaccination (i.e., day 42). Unless stated otherwise, all samples were individually analyzed. Saliva was collected by rinsing the area between the cheek and the tooth line of anesthetized mice with 20 μl of phosphate-buffered saline (PBS). The collected rinse (5 to 8 μl) was deposited in a fresh centrifuge tube containing 2× protease inhibitor and EDTA-free cocktail (Thermo Scientific, Rockford, IL) in 10 μl PBS. The nasal secretions were collected from euthanized (via CO_2 asphyxiation followed by cervical dislocation) mice by carefully introducing 30 μl PBS into the nostrils by a micropipette tip and collecting 10 to 20 μl of the rinse through the same micropipette tip. The collected nasal rinse was transferred to a fresh tube containing 2× protease inhibitors (Thermo Scientific) in 10 μl PBS. The vaginal secretion specimens were collected 2 weeks after the third vaccination (day 42) by inserting the tip of a pipette into the opening of the vulva of a euthanized mouse and rinsing with 50 μl of PBS. All rinses collected by pipette were transferred to a fresh tube containing 2× protease inhibitors (Thermo Scientific) in 10 μl PBS. All samples from mucosal secretions were stored frozen (−20°C) before analysis.

Antibody assays. Antibody responses to PRP were measured by an enzyme-linked immunosorbent assay (ELISA). Immulon 4 HBX 96-well plates (Thermo Scientific) were coated (90 min, 37°C) with PRP conjugated to human serum albumin (1 μg/ml in PBS). The plates were washed three times in PBS containing 0.1% Tween 20 and blocked (60 min, 22°C) with diluent/blocking solution (KPL, Gaithersburg, MD) containing a 3:10 dilution of 10% bovine serum albumin (BSA). The diluent/blocking solution was removed and replaced (70 to 100 μl) in the plates with sample dilutions (in Hanks' balanced salt solution [HBSS] with 1% human serum albumin, 10 mM HEPES [Sigma-Aldrich, St. Louis, MO] [pH 7.5]) of serum (100 μl) added in duplicate and incubated for 90 to 120 min (22°C), depending on the type of sample. The following sample dilutions were used: 1:7 for serum, saliva, nasal, and vaginal washes, and 1:4 for tissue culture supernatants. Isotype-specific standards (murine IgG and IgA) (Sigma-Aldrich, St. Louis, MO) were included in the ELISA plates to measure antibodies. The plates were washed three times (0.1% Tween 20, PBS buffer) and incubated (1 h, 22°C) with either 0.4 μg/ml goat anti-mouse IgG-horseradish peroxidase (HRP) (Immunopure; Pierce, Rockford, IL) or 0.35 μg/ml goat anti-mouse IgA-HRP (Immunopure; Pierce) to detect specific antibody isotypes. Bound antibodies were measured by the absorbance of accumulated peroxidase product at a 450-nm wavelength (PerkinElmer Victor3 V 1420 multilabel counter) using TMB Microwell peroxidase substrate followed by TMB stop solution (KPL, Gaithersburg, MD). Mouse anti-tetanus toxoid-specific IgG and IgA ELISA kits (Alpha Diagnostic International, San Antonio, TX) were used to measure the specific antibody responses to the carrier protein in biological samples diluted to the same factor as the anti-PRP ELISA. Bound antibodies were detected by absorbance at a 450-nm wavelength (Victor3 V 1420), and data were recorded in units/ml.

NALT cultures. NALTs were isolated from mice 2 weeks after the third vaccine dose (day 42). The isolation of palates for NALT cultures was performed as previously reported (19). The palates were transferred into 48-well plates (Costar, Cambridge, MA) containing 250 μl fresh prewarmed 10 mM HEPES, 10% fetal calf serum, RPMI 1640 containing 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μg/ml gentamicin (Sigma-Aldrich), and 1 μg/ml amphotericin B (Fungizone; Gibco-Invitrogen, Carlsbad, CA) and incubated at 37°C with 5% CO_2. Approximately 40% (100 μl) of the medium was removed for analysis from each NALT culture every 24 h, and the same volume of fresh medium was added back to the wells for continued culture. The collected medium samples were centrifuged to remove debris and stored at −20°C until assayed.

Hib preparation. Hib was reconstituted with brain heart infusion (BHI) broth containing 2% Fildes enrichment, spread on chocolate II agar plates (BD Biosciences), and incubated (12 h, 37°C, 5% CO_2 incubator). Ten colonies were transferred to 20 ml of BHI broth with 2% Fildes enrichment in a 50-ml glass vial and incubated (37°C, 5% CO_2 incubator) until the optical density at 600 nm (OD_{600}) reached 0.4 to 0.5. Glycerol (15% vol/vol) was added to the Hib cultures, and 0.5-ml aliquots were frozen at −70°C. Bacterial recovery was determined from the frozen vials by comparing the colony counts between 10-fold dilutions of unfrozen Hib and thawed Hib from 100 μl plated on chocolate II agar plates. The plates were incubated (12 h, 37°C) in candle jars with CO_2 gas packs (GasPak EZ CO_2; BD Biosciences), and the ratio of thawed bacterial colonies to unfrozen bacterial colonies was determined to be >0.8.

Bactericidal assay. Serum samples were collected 2 weeks after the third Hibv dose (day 42), pooled within each group, and used to measure bactericidal activity. The Hib bactericidal assay was performed as described elsewhere (28, 29). Briefly, mouse serum samples were diluted with Hanks' balanced buffer (containing Ca and Mg) and 2% Fildes enrichment (BD Biosciences). Ten microliters of each diluted serum sample was transferred in duplicate to a 96-well plate. Hib cells were thawed to prepare 1,000 CFU/20 μl in dilution buffer, and the Hib suspension was added (20 μl/well) to the 96-well plate and incubated for 15 min (37°C, 5% CO_2 incubator). Sterile rabbit complement (25 μl of serum from 3- to 4-week-old rabbits; Pel-Freez, Rogers, AR) and 25 μl of Hanks' buffer (as above) were added to each well and gently mixed. The plates were incubated for 60 min (37°C, 5% CO_2 incubator), and 5 μl of each mixture was spread on separate chocolate II agar plates (BD Biosciences). The bacteria were cultured for 12 h (37°C, 5% CO_2 incubator) and the bacterial colonies were enumerated. Bactericidal effects were calculated as the dilution of serum necessary to reduce ≥50% of the Hib CFU. Mouse gammaglobulin (100 μg/ml) (Jackson ImmunoResearch, West Grove, PA) was used as a negative control for the bactericidal assay, and anti-Hib capsular polysaccharide serum (CBER standard serum lot 1983) (70 μg/ml) was used as a positive control. The bactericidal assay was repeated three times using the same pooled samples.

Statistical analyses. Student's *t* test was used for the analysis of significance between the experimental groups.

Animal care and use. This research was conducted under an Institutional Animal Care and Use Committee (IACUC)-approved protocol in

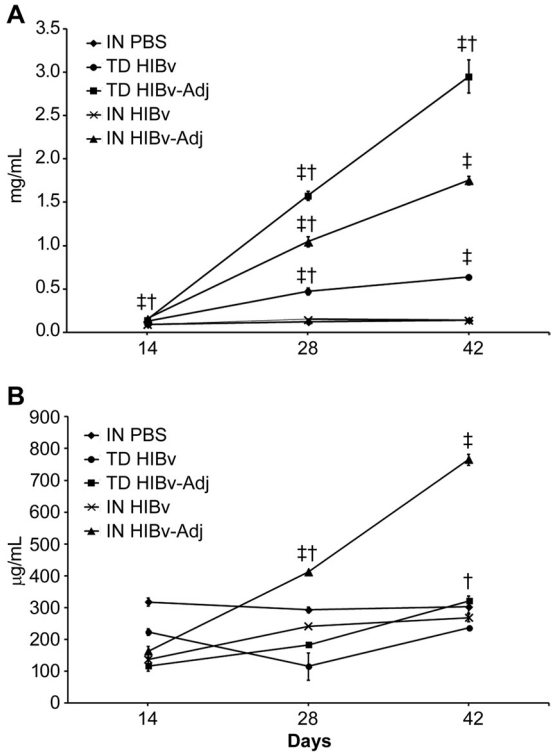

FIG 1 Blood serum polyribosylribitol phosphate (PRP)-specific IgG and IgA resulting from intranasal (i.n.) vaccination. Groups of female BALB/c mice ($n = 6$) were inoculated as described in Materials and Methods. The control group was given phosphate-buffered saline (PBS) by the i.n. route. Two weeks after each dose (days 14, 28, and 42), serum samples were analyzed for PRP-specific IgG (A) and IgA (B) by enzyme-linked immunosorbent assay (ELISA). † and ‡, statistically significant difference at P values of ≤0.05 and ≤0.01, respectively.

compliance with the Animal Welfare Act, PHS policy, and other federal statutes and regulations relating to animals and experiments involving animals.

The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International, and adheres to the principles stated in the *Guide for the Care and Use of Laboratory Animals*, 8th ed. (National Academies Press, Washington, DC).

RESULTS

Blood serum antibody responses to i.n. vaccination. We first examined the relationship between i.n. vaccination and blood serum antibody responses. Mice were given three doses of Hibv by the i.n. or transdermal (t.d.) route, alone or administered with a TLR4 agonist adjuvant (Hibv-Adj group). An i.n. control group received only PBS. Two weeks after each dose, serum samples were collected and analyzed by ELISA for PRP-specific IgG and IgA. t.d. vaccination generated levels of blood serum anti-PRP IgG responses (Fig. 1A) that were significantly higher than in the PBS controls on days 28 and 42 ($P \leq 0.01$). Anti-PRP IgG in the t.d. Hibv-Adj group was also significantly elevated compared to the i.n. Hibv-Adj group at days 28 and 42 ($P \leq 0.05$). The adjuvant significantly increased the levels of serum anti-PRP IgG in the t.d. group (days 28 and 42, $P \leq 0.01$) compared to t.d. vaccination without the adjuvant. The inclusion of the TLR4 agonist (Ultrapure LPS, *E. coli* strain 0111:B4) in the i.n. vaccinations (i.n. Hibv-Adj) was sufficient to generate anti-PRP IgG levels in the serum samples that were significantly ($P \leq 0.01$) above those measured in the i.n. Hib or the i.n. PBS groups at days 28 and 42. The i.n. Hibv-Adj group also generated significantly higher levels of anti-PRP IgG than the t.d. Hib group at days 28 ($P \leq 0.05$) and 42 ($P \leq 0.01$). We examined the generation of PRP-specific IgA in the serum samples and determined that only the Hibv-Adj group of those who had the i.n. vaccination (Fig. 1B) exhibited IgA levels significantly above background by days 28 ($P \leq 0.05$) and 42 ($P \leq 0.01$). These results indicate that while Hibv vaccination administered i.n. and t.d. with TLR4 agonist induced levels of anti-PRP IgG at days 28 and 42 that were significantly higher than those generated by the PBS group, only the i.n. vaccinated group presented significant levels of anti-PRP IgA at both time points.

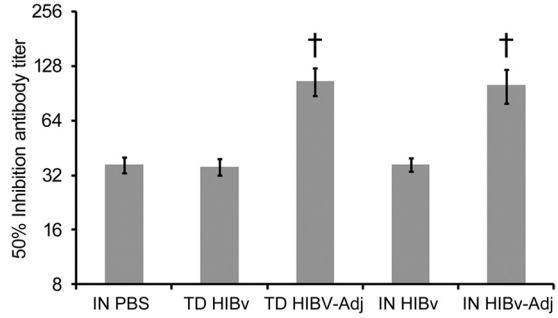

FIG 2 Bactericidal activity of blood serum PRP-specific IgG and IgA. Groups of female BALB/c mice ($n = 6$) were inoculated as described in Materials and Methods. The control group was given PBS by the i.n. route. Serum samples were collected 2 weeks after the last dose (day 42) and analyzed for number of CFU based on Hib bactericidal assays. The antibody titer resulting in 50% inhibition of CFU was determined by 2-fold dilutions of serum. The bactericidal assay was repeated three times using the same pooled samples. †, statistically significant difference at a P value of ≤0.05.

Bactericidal antibody responses to Hibv. In addition to measuring the total antigen-specific antibodies, we examined the antibody-dependent inhibition of Hib CFU in an *in vitro* assay of immunity to infection using serum samples collected 2 weeks after the third Hibv dose (day 42). Significant bactericidal antibody titers were observed with serum samples from both i.n. and t.d. vaccinations, but adjuvant was required for bactericidal antibodies regardless of the vaccination route (Fig. 2). Both PRP-specific IgG and IgA were present in serum samples from i.n. vaccinations, while only IgG responses were noted with serum samples from the t.d. group, suggesting that at least IgG was associated with serum bactericidal effects, although the role of IgA in bactericidal activity cannot be discounted.

Mucosal IgA levels from i.n. vaccination. Because Hib is a respiratory-tract pathogen, it was also important to assess the immune responses of the oral and upper respiratory mucosa. For this purpose, we measured PRP-specific IgG and PRP-specific IgA within the buccal cavity and nasal sinuses of vaccinated mice. In the saliva, only i.n. vaccination with Hibv-Adj induced significant levels of anti-PRP IgA ($P \leq 0.01$) above all other groups (Fig. 3A), while there were no significant responses for any group vaccinated without adjuvant. Little or no anti-PRP IgG was found in the saliva in any of the groups. Because the protective component of the Hibv is PRP conjugated to a protein carrier, we next sought to determine if humoral responses directed to the carrier tetanus toxin (TT) were similar to the anti-PRP response in the upper

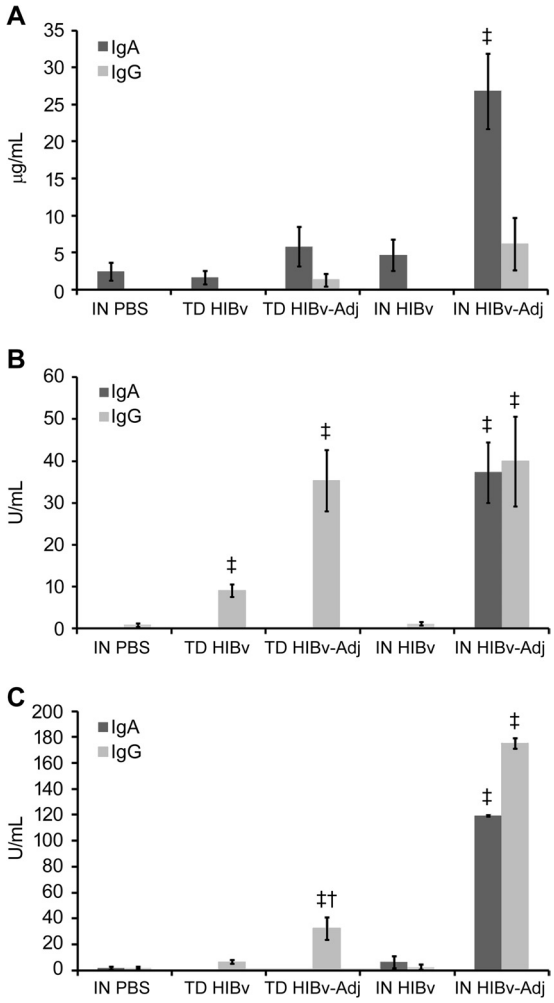

FIG 3 Salivary and nasal antibody responses to i.n. vaccination with Hibv. Groups of female BALB/c mice ($n = 6$) were inoculated as described in Materials and Methods. The control group was given PBS by the i.n. route. Two weeks after the last vaccination (day 42), saliva and nasal wash samples were collected and individually analyzed to measure the levels of saliva IgG and IgA specific for PRP (A) and tetanus toxoid (TT) carrier protein (B) and levels of nasal IgG and IgA specific for TT (C). The *y* axis represents arbitrary units in panels B and C. † and ‡, statistically significant difference at *P* values of ≤0.05 and ≤0.01, respectively.

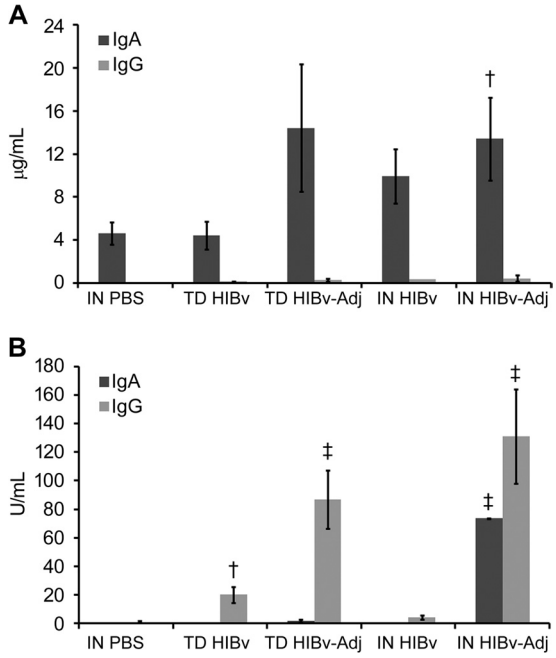

FIG 4 Antibody responses in distal mucosa sites resulting from i.n. vaccination. Groups of female BALB/c mice ($n = 6$) were inoculated as described in Materials and Methods. The control group was given PBS by the i.n. route. Two weeks after the last vaccination, vaginal wash samples were collected with PBS and analyzed to measure the levels of IgG and IgA specific for PRP (A) and tetanus toxoid (TT) carrier protein (B). The *y* axis represents arbitrary units in panel B. † and ‡, statistically significant difference at *P* values of ≤0.05 and ≤0.01, respectively.

airway. Figure 3B shows that in the saliva, as was the case with anti-PRP IgA, only the i.n. Hibv-Adj group exhibited significant levels of anti-TT IgA ($P \leq 0.01$). However, both i.n. and t.d. vaccinations resulted in high levels of anti-TT IgG (Fig. 3B). We found that the t.d. Hibv-Adj, t.d. Hibv, and i.n. Hibv-Adj groups all presented significantly higher levels of anti-TT IgG than the i.n. PBS group ($P \leq 0.01$). Further, inclusion of the adjuvant in the t.d. and i.n. Hibv formulations resulted in significantly higher levels of IgG directed against TT than the vaccination without adjuvant ($P \leq 0.01$). Although we did not detect anti-PRP antibodies in the nasal wash samples (data not shown), significantly higher (over the PBS and no-adjuvant groups) levels of IgG and IgA specific for TT were observed in the nasal sinuses ($P \leq 0.01$) as a result of i.n. vaccination with Hibv-Adj (Fig. 3C). The t.d. Hibv-Adj group also induced levels of TT-specific IgG for the t.d. Hibv-Adj group that were significantly greater than those of the i.n. PBS and t.d. Hibv groups ($P \leq 0.05$), but IgA responses to TT were not detected.

Because i.n. vaccination resulted not only in local mucosal humoral responses but also in systemic blood serum responses, we examined the possibility that antigen-specific IgG and IgA were also found in the vaginal mucosa, a distal mucosal site. As shown in Fig. 4A, significantly higher levels of Hibv-specific IgA antibodies in vaginal secretions were measured in the i.n. Hibv-Adj mouse group (over those in the PBS group; $P \leq 0.05$), consistent with the saliva and nasal secretion sample results (Fig. 3). In addition, while anti-TT IgG and IgA (Fig. 4B) were found to be significantly ($P \leq 0.01$) boosted in the vaginal wash specimens from the i.n. groups vaccinated with adjuvant, t.d. vaccination with Hibv generated modest but significant levels of anti-TT IgG in the vaginal mucosal secretions, both with adjuvant ($P \leq 0.01$) and without ($P \leq 0.05$). We concluded from these results that Hibv delivered through either t.d. or i.n. sites stimulated antibody responses in distal mucosal tissues.

NALTs produce PRP-specific IgA and carrier-specific IgG. Because Hibv-specific IgA appeared to result only from i.n. vaccination, whereas IgG levels were stimulated by both i.n. and t.d. vaccination routes, we sought to determine if the NALT was potentially a common source of B cells contributing to these immune responses. Vaccination with Hibv-Adj by the i.n. route induced levels of anti-PRP IgA (Fig. 5A) and anti-TT IgA (Fig. 5B) that were significantly higher than those of any other group ($P \leq 0.05$), while only NALT from the t.d. Hibv-Adj group secreted anti-PRP IgG (Fig. 5A) ($P \leq 0.05$). Interestingly, NALTs from the i.n. Hibv-

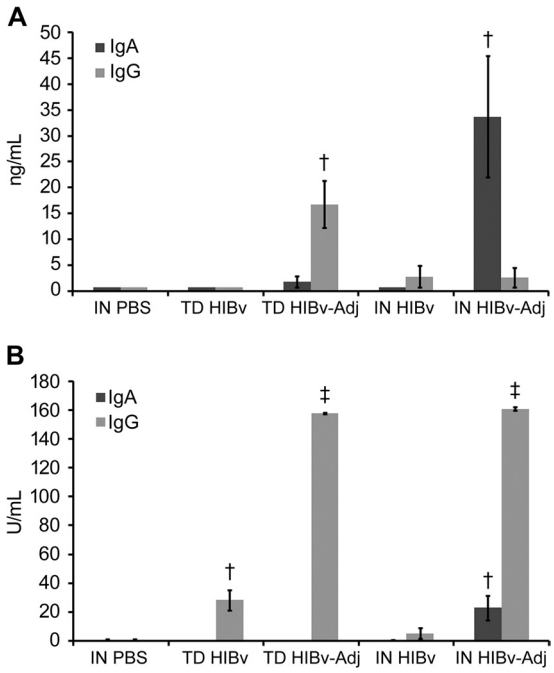

FIG 5 Secretion of PRP- and TT-specific IgG and IgA by cultured NALT. Groups of female BALB/c mice ($n = 6$) were inoculated as described in Materials and Methods. The control group was given PBS by the i.n. route. The mice were euthanized 2 weeks after the last dose and NALTs were removed, thoroughly washed, and placed in culture. After 24 h, the media were collected to measure the levels of IgG and IgA specific for PRP (A) and tetanus toxoid (TT) carrier protein (B). The *y* axis represents arbitrary units in panel B. †, statistically significant difference at a *P* value of ≤0.05.

Adj ($P \leq 0.01$), t.d. Hibv ($P \leq 0.05$), and t.d. Hibv-Adj ($P \leq 0.01$) groups secreted significant levels of anti-TT IgG in comparison to the PBS group. The adjuvant boosted IgA levels for i.n. delivery while also increasing IgG levels for both i.n. and t.d. delivery.

DISCUSSION

Our results show that i.n. delivery of the PRP-tetanus toxoid conjugate vaccine elicits humoral responses that are dominated by IgA directed toward both the polysaccharide and carrier protein. Antibodies specific for Hibv were detectable within the microenvironment of the upper respiratory tract and distal mucosal sites, and systemically in the blood. In contrast to i.n. vaccination, t.d. delivery of the Hibv elicited primarily blood serum IgG responses, while bactericidal Hib antibodies were detectable in the blood from mice vaccinated by either the i.n. or t.d. route. Measurements of antibody secretions, captured directly from the NALTs of vaccinated mice by using a tissue culture assay, suggested that the NALT plays a central role in the mucosal immune responses to i.n. vaccination, as the tissue culture results demonstrated that NALTs are a significant source of anti-PRP and anti-TT carrier antibodies. These results are relevant to the practice of Hib vaccination. Studies in small animal models and in infants have established clear correlations between Hib-specific antibodies of the serum and oral-nasal cavities and control of colonization or infection (3, 30–32). The rate of Hib vaccine failure among infants, although very low, has prompted some to question the quality of the antigen-specific antibodies generated after the typical three-dose intramuscular injections with the conjugated Hib vaccine (33, 34). Our data suggest that i.n. vaccination may be a reasonable alternative to the standard Hib injection, especially if combined with an adjuvant, such as the TLR4 agonist used in our study. Nonetheless, more work is needed to assess the impact of antigen-specific mucosal IgA in protection against infection.

The synergistic effects of adjuvants coupled with a Hib vaccine were reported previously for murine models (11, 23). Similarly, in our study, antibody responses to the Hibv in nasal sinuses or the buccal cavity required the TLR4 agonist, underscoring the effect of the adjuvant in the local immune environment. The data presented here further corroborate previous findings supporting the role of i.n. vaccination in activating humoral responses of the nasal-oral cavity. For example, we previously reported that the NALTs control specific kinds of immune responses within the nasal sinuses (13) and documented a contributing role of adjuvants in these responses. In the case of i.n. vaccinations, our results here suggest that an adjuvant is necessary to boost mucosal IgA responses to the PRP conjugate, as significant levels of anti-PRP IgA were only detected in the saliva as an outcome of i.n. vaccination with Hibv and adjuvant. While we were able to detect anti-carrier antibodies only in the nasal secretions of vaccinated mice, we could not conclude that anti-PRP antibodies were truly absent, as perhaps they were only beyond detectable limits due to low sample volumes. In further support for the role of anti-polysaccharide antibodies, we measured enhanced levels of IgA specific for PRP by using a more sensitive method to directly detect antibodies produced in NALTs collected from mice vaccinated i.n. with Hibv and adjuvant. Our observations suggest that NALT antibody responses to the protein carrier and PRP are not equal; for example, the IgG responses were highest to carrier protein while IgA responses were highest to PRP. According to a recently proposed model (10), the quantitative differences in antibody responses may be influenced by glycoconjugate density and the relative affinity of the peptide carrier for MHC molecules. However, the differences in isotype-specific responses are difficult to reconcile based solely on MHC presentation. Alternatively, i.n. vaccination may also enhance local immunity at distal mucosal sites, like vaginal mucosa (Fig. 4), by inducing NALT-derived blood plasma cells to home to distal sites (35–38). Although our results suggesting increased levels of IgA in vaginal secretions in response to t.d. vaccination with only low levels present in serum are intriguing, IgA only reached statistically significant levels in the i.n. plus adjuvant group.

In one important aspect, NALTs are specialized lymphoid clusters in the sinus mucosa composed of B cells undergoing isotype class switching (39). The IgA-committed B cells migrate from the NALT to diffuse mucosal tissue sites, including nasal passages (37, 38). The NALTs are ideally situated in the nasal passages above the hard palate of rodents and other mammals to access aerosolized antigens from pathogens, vaccines, or other sources that enter the respiratory tract (14, 40, 41). NALT organogenesis begins soon after birth and is dependent on several factors, including various chemokines, cytokines, and environmental cues (42–45). In humans, NALT-like structures are evident at a very young age but disappear by the age of 2 years, while equivalent organs, like the Waldeyer's ring, which also includes nasopharyngeal lymphoreticular tissues, persist throughout life (14). NALT-like structures in infants are morphologically distinct from the tonsils and adenoids but share common features with secondary lymphoid organs, such as lymphoid follicles (14). The architecture of the

NALT is structured like lymph nodes, organized into discrete compartments of immature B and T lymphocytes and antigen-presenting dendritic cells (46). While afferent lymphatic ducts conduct antigens to most lymph nodes, antigens are delivered to NALTs through the mucosal layer of the sinus air passages (47). NALTs also lack the characteristic germinal centers of the lymph nodes and Peyer's patches and are usually quiescent (46, 48). However, i.n. exposure to infectious agents, antigens, or i.n. vaccination results in the rapid expansion of germinal centers in the NALTs (19, 46), thus allowing these nasal lymphoid clusters to further contribute to overall immunity as a source of IgA-secreting plasma cells (16, 19). Regarding i.n. vaccination, the NALT follicle-associated epithelial (FAE) cell layer is heavily intercalated by M cells that internalize antigens and express TLRs (15, 20). The luminal surface of M cells expresses high levels of TLR4, and it has been shown that agonist binding to TLR4 in M cells, as well as other immunomodulatory peptides, increases antigen uptake from the mucosa and transport to the apical domain, as well as vaccine efficacy (20, 26, 49). M cells have a significant role in antigen retrieval from the mucosal surfaces of the air passages and transport across the epithelial layer to dendritic cells below (15). Further, it is likely that the adjuvant effects of the TLR4 agonist on i.n. vaccination are mediated by M cells intercalating the FAE cells that cover the NALTs (50, 51).

The detection of anti-PRP IgG secreted from blood plasma cells in the NALTs of t.d. vaccinated mice may be a result of migrating B cells or APCs, such as dendritic cells, homing to the nasal mucosa. We cannot rule out the possibility that some of the vaccine passed from the nasal sinuses into the digestive system to stimulate immune responses in distal sites (52). Nonetheless, the secretion of anti-PRP IgA into the oral-nasal cavities was only detected after i.n. vaccination in our study, and achieving high levels of antibody required the inclusion of a TLR4 agonist. The t.d. vaccinations also stimulated PRP-specific IgG in NALTs from mice that were coadministered adjuvant and Hibv. The results presented here confirm an association between NALT and antibody responses to the polysaccharide-based Hib vaccine within the microenvironment of the upper respiratory tract and other mucosal sites. Our observations are most relevant to considerations of alternative vaccination strategies for the prevention of Hib colonization and acute infections of the respiratory tract.

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