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Intranasal Immunization Strategy To Impede Pilin-Mediated Binding of *Pseudomonas aeruginosa* to Airway Epithelial Cells

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Prevention of pulmonary *Pseudomonas aeruginosa* infections represents a critical unmet medical need for cystic fibrosis (CF) patients. We have examined the tenet that a mucosal immunization approach can reduce interactions of a piliated form of this opportunistic pathogen with respiratory epithelial cells. Vaccinations were performed using ntPEpilinPAK, a protein chimera composed of a nontoxic form of *P. aeruginosa* exotoxin A (ntPE), where the C-terminal loop amino acid sequence of the PAK strain pilin protein was inserted in place of the ntPE Ib domain. Intranasal (i.n.) immunization of BALB/c mice with ntPEpilinPAK generated both serum and saliva immune responses. A series of in vitro studies showed that diluted samples of saliva obtained from immunized mice reduced pilin-dependent *P. aeruginosa* binding to polarized human tracheal epithelial cells, protected human pulmonary epithelial cells from cytotoxic actions associated with bacterial challenge, and reduced exotoxin A toxicity. Overall, i.n. administration of ntPEpilinPAK induced mucosal and systemic immune responses that may be beneficial for blocking early stage adhesion and/or infection events of epithelial cell-*P. aeruginosa* interactions at oropharyngeal surfaces.

Onset of pulmonary *Pseudomonas aeruginosa* infection is a major predictor of morbidity and mortality in cystic fibrosis (CF) patients (19). Strains of *P. aeruginosa* initially isolated from the lungs of CF children are environmental (47), nonmucoid, and antibiotic sensitive (5). Environmental strains of *P. aeruginosa* are generally piliated, expressing type IV pili as fibers extending from their surface (38). These pili are composed of the protein pilin, organized as a homopolymer, that has a C-terminal disulfide-bonded loop (DSL) capable of mediating bacterial adherence to epithelial cells (49). It is interesting that although each pilin monomer of an individual type IV pilus theoretically has an epithelial cell adherence site, only those at the pilus tip appear to function as "binders" (33), consistent with crystal structure information for this protein (10). A comprehensive study examining pilin DSL sequences identified six major groupings for the isolates analyzed. Group I, having 17-amino-acid DSL sequences, were most prevalent in environmental and CF patient isolates, while group II isolates containing 12 DSL amino acids were more common in non-CF strains (31).

Pilin-mediated binding through DSL sequences to epithelial cell surfaces has been proposed to occur via interactions with the common cell surface glycosphingolipids asialo-G_{M1} and asialo-G_{M2}, both containing the common disaccharide 4-*O*-(2-acetomido-2-deoxy-β-D-galatopyranosyl)-D-galactose (reviewed in reference 23). Enrichment of these asialo-sugar structures in the plasma membrane of epithelial cells in vitro increases *P. aeruginosa* binding (9), and epithelial cells from CF patients express abnormally high levels of asialo sugars (56). Purified pilin has been shown to inhibit *P. aeruginosa* binding to epithelial cells (28), as does a synthetic, oxidized peptide resembling the C-terminal loop of the protein (49). Although a reduction in adherence of *P. aeruginosa* to CF epithelium using an anti-asialo-G_{M1} antibody has also been demonstrated (14), others have questioned the relevance of asialo-G_{M1} as a major cellular receptor for clinical isolates of *P. aeruginosa* (57). Regardless, reduction in *P. aeruginosa* type IV pili interactions with epithelial cell surfaces has been proposed to lower infection frequencies in CF patients. A previous approach for active immunization against the DSL of *P. aeruginosa* pilin has been described previously (64).

Actions of cell-associated as well as secreted products of *P. aeruginosa* have long been known to contribute to the virulence of this pathogen (53). Microarray analysis of mRNA abundance in a human respiratory epithelial cell line, A549, demonstrated that a *P. aeruginosa* pilin-mediated host-pathogen interaction stimulated the expression of several host proteins involved in chemotaxis and inflammation as well as transcription factors known to drive these events (26). Additionally, asialo-G_{M1}-mediated binding of *P. aeruginosa* induces cytotoxic events in epithelial cells (9). Exotoxin A (PE), considered to be the most potent secreted virulence factor associated with *P. aeruginosa* infection (37), is directly toxic to cells due to its ability to ADP-ribosylate elongation factor 2, an activity that induces cell death in antigen-presenting cells (APCs) such as macrophage (54) and dendritic cells (13). Additionally, PE can

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stimulate fluid absorption from distal airspaces of the lung (52), disrupt neutrophil function (41), inhibit gamma interferon (IFN-γ) synthesis to suppress specific and nonspecific defense mechanisms associated with bacterial clearance (39), stimulate macrophage-based tumor necrosis factor (TNF)-mediated apoptosis (58), and impede the restoration of epithelial tight junction structures damaged by the actions of neutrophil-derived elastase (2). Neutralizing antibodies to PE have been shown to not only promote uptake and killing of *P. aeruginosa* by neutrophils (11) but also be beneficial in clearing *P. aeruginosa* infections (18, 59). Antibodies to PE can also reduce the binding of both piliated and nonpiliated *P. aeruginosa* to tracheal cells in vitro (42), and several studies have described strategies to generate neutralizing immune responses to PE as a method of reducing the clinical impact of this virulence factor (8, 20, 25, 50).

In the present studies we have examined immune responses to a chimera protein composed of *P. aeruginosa* exotoxin A (ntPE) with the pilin DSL from the PAK strain of this bacterium inserted in place of the Ib domain of ntPE. The chimera protein ntPEpilinPAK is similar to a chimera containing a shorter pilin loop sequence that was administered to rabbits by subcutaneous injection (25). Here we show that intranasal (i.n.) administration of ntPEpilinPAK can induce both mucosal and systemic immunity. Further, saliva obtained from i.n.-immunized animals blocked pilin-mediated binding of *P. aeruginosa*, impeded cytotoxicity associated with pilin-mediated *P. aeruginosa* interaction with epithelial cells, and reduced toxicity associated with PE. In summary, these results provide support for a mucosal vaccination approach targeted at disrupting pilin-based epithelial interactions for the prophylactic prevention of respiratory *P. aeruginosa* infections in CF patients.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and pilin isolation. *P. aeruginosa*, PAK strain (ATCC 53308; Manassas, VA), was grown overnight at 37°C in Luria-Bertani broth (Difco, Becton Dickinson, Franklin Lakes, N.J.) in flasks swirled at 75 rpm in a rotary shaker to an optical density at 600 nm of 0.6 (~1×10^9 CFU per ml). For use in adhesion studies, bacteria were pelleted at ~$700 \times g$ for 15 min at 4°C and suspended in antibiotic-free Ham's F-12 media at desired concentrations. For pilin protein isolation, bacteria were pelleted at ~$700 \times g$ for 15 min at 4°C, suspended in isotonic phosphate-buffer saline (PBS), and vortexed aggressively six times for 15 s with 10-s rests on ice. Bacteria were removed by centrifugation at $12,000 \times g$ for 30 min, and the supernatant containing sheared pili was dialyzed overnight against 20 mM Tris-HCl, 1 mM EDTA (pH 8.0). Pilin protein was purified by sequential column chromatography using Q Sepharose HP and Sephadex 200 (Amersham Biosciences, Upsala, Sweden). Protein purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining to be >95%.

Oligoduplex formation and plasmid construction. The ntPEpilinPAK construct was generated by a multistep process. A 78-bp DNA oligonucleotide duplex encoding the desired 24 amino acids of the PAK strain of *P. aeruginosa* (sense, 5′-GACTAGTACTGCAGCTGATGGTCTCTGGAAGTGCACCAGTGATCAGGATGAGCAGTTTATTCCGAAAGGTTGCTCTAAGCAGGGCCCGG-3′, and antisense, 5′-CCGGGCCCTGCTTAGAGCAACCTTTCGGAATAAACTGCTCATCCTGATCACTGGTGCACTTCCAGAGACCATCAGCTGCAGTACTAGTC-3′) was digested with SpeI and ApaI and gel purified (QIAGEN, Valencia, CA). A DNA fragment of PE encoding amino acids 1 to 360 was generated by PCR using pPE64pstΔ553 (25) as a template. The PCR fragment was digested with HindIII and SpeI and gel purified (QIAGEN). The two purified fragments (the pilin oligoduplex and PCR fragment) were ligated into the HindIII-ApaI site of pPE64pstΔ553. Incorporation of this DNA resulted in the destruction of the original PstI restriction site and introduction of a unique SpeI site. The final construct, termed pPilinovax-A, and the correct orientation of the insert were verified by restriction enzyme digestion and sequencing (data not shown). A toxic form of the chimera, termed PEpilinPAK, was constructed by ligating the pilin oligoduplex and PCR fragment into the HindIII-ApaI site of pPE64-PstI. The resulting plasmid, pPEpilinPAK, was verified as described above.

Protein expression and purification and biotin modification. *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA) were transformed with ntPEpilinPAK, PEpilinPAK, ntPE, or PE plasmid by heat shock (1 min at 42°C). Transformed cells, selected on antibiotic-containing media, were isolated and grown in Luria-Bertani broth (Difco). Protein expression was induced by addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG). Two hours following IPTG induction, cells were harvested by centrifugation at $5,000 \times g$ for 10 min at 4°C. Inclusion bodies were isolated following cell lysis, and proteins were solubilized in 6 M guanidine HCl and 2 mM EDTA (pH 8.0) plus 65 mM dithiothreitol. Following refolding and purification, as previously described (25), proteins were stored in PBS (pH 7.4) lacking Ca^{2+} and Mg^{2+} at −80°C. Some purified ntPEpilinPAK was biotinylated using a 20-fold molar excess of sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce Chemical Co., Rockford, IL) at 4°C for 2 h. Prior to use, biotinylated ntPEpilinPAK was repurified (to >95% purity) by size-exclusion chromatography using a Superdex 200 column (Amersham).

Chimera protein characterization. ntPEpilinPAK was assessed by size-exclusion chromatography using a ZORBAX GF-450 column (Agilent, Palo Alto, CA) and demonstrated to be >95% monomeric (data not shown). Isoelectric focusing analysis showed ntPEpilinPAK to have the anticipated isoelectric point of ~5.1 (data not shown). Total amino acid analysis and N-terminal sequencing of ntPEpilinPAK demonstrated the desired composition (data not shown). The ntPEpilinPAK material used in these studies contained 6.5 ng host cell protein/mg ntPEpilinPAK, <2 pg host cell DNA/mg ntPEpilinPAK, and ~6.3 endotoxin units of endotoxin/mg ntPEpilinPAK (data not shown). Proper folding of PEpilinPAK was verified functionally using a protein synthesis inhibition assay (48).

Measurement of ntPEpilinPAK binding to asialo-G_{M1}. Plastic 96-well plates (NUNC COVALINK NH F8) were coated with 100 ng/well of either asialo-G_{M1} or monosialo-G_{M1} (1 mg/ml stock in methanol) or exposed to an equal volume (100 μl) of carrier solvent (methanol) overnight at 4°C. Plates were blocked with 200 μl/well of blocking buffer (PBS-Tween 20–0.5% bovine serum albumin) at room temperature (RT) for 1 h. After washing four times with 300 μl, various concentrations of ntPEpilinPAK or ntPE or carrier buffer (PBS) were added in a 100-μl volume and allowed to incubate for 3 h at RT. After washing as described before, 1 ng of the monoclonal antibody M40-1 (which binds amino acids 300 to 310 of ntPE) was added and allowed to incubate for 1 h at RT. After washing as described before, bound ntPE or ntPEpilinPAK was complexed with an anti-mouse polyclonal horseradish peroxidase (HRP) conjugate. The relative quantity of protein-antibody complexes in each well was determined by the level of color produced by the conversion of TMB (3,3′,5,5′tetramethylbenzidine).

Immunizations. BALB/c mice (Charles River Laboratories, Wilmington, MA) 6 to 8 weeks old at initial dosing (8 mice per group) were used in these studies. Mice in all groups received four total exposures on a schedule of 0, 7, 14, and 28 days. Serum and saliva samples were collected on day 35. Intranasal (i.n.) administration was performed under light anesthesia with isoflurane. Using a positive displacement pipette, 40 μl of ntPEpilinPAK (20 μl/nares) in PBS was administered so that mice received 1, 10, or 100 μg per dose. The use of isoflurane for i.n. administration results in suppression of the swallowing reflex and facilitates preferential delivery to the trachea rather than the esophagus (27). Negative control animals received an equal volume of carrier buffer (PBS) by i.n. instillation. As a positive control, mice received a subcutaneous (s.c.) injection of 10 μg ntPEpilinPAK in a regimen of complete (first exposure) and incomplete (three subsequent exposures) Freund's adjuvant. All animal studies were approved by the Institutional Animal Care and Use Committee of Children's Hospital Oakland Research Institute, where these studies were performed.

Cellular distribution of CD91 and biotinylated ntPEpilinPAK. Nasal tissue from nonimmunized mice were collected following exposure to biotin-labeled ntPEpilinPAK in PBS or following exposure to PBS alone. Tissue sample sections (5 μm thick) were mounted on superfrost plus slides (Erie Scientific Company, Portsmouth, NH) and allowed to air dry prior to fixation with acetone. Mounted sections were rinsed with PBS, blocked with Peroxidase I (Biocare, Walnut Creek, CA), rinsed in PBS, and blocked with casein protein (Biocare) according to the manufacturer's instructions. Following a PBS bath, a 1:20 dilution of rabbit polyclonal anti-CD91 antibody (3) was applied to each slide. Following application of a prediluted goat anti-rabbit secondary immunoglobulin G (IgG) antibody (Biocare), streptavidin-horseradish peroxidase was applied. Approximately 5 min of development of the chromogen 3-amino-9-ethylcarba-

zole was performed, and the reaction product was viewed by light microscopy visualization for hematoxylin (Biocare) staining.

Antibodies and synthetic peptides. The mouse monoclonal antibody 1D10, produced at A&G Pharmaceutical, Inc. (Columbia, Maryland), was shown by enzyme-linked immunosorbent assay (ELISA) to react with ntPEpilinPAK but not ntPE (data not shown). 1D10 bound selectively (data not shown) to pilin PAK peptide (biotin-KCTSDQDEQFIPKGCSK-NH_2) but not to a scrambled (control) peptide (biotin-KCDDFKQGTQEPISCSK-NH_2), both being manufactured at SynPep (Dublin, CA). Horseradish peroxidase (HRP)-conjugated goat antibodies raised against mouse serum IgG or against mouse serum IgA were purchased from Pierce Chemical Company (Rockford, IL) and Kirkegaard & Perry Laboratories (Gaithersburg, Maryland), respectively.

Assessment of antibody responses. Mouse saliva (typically 50 to 100 μl) was collected from the buccal cavity over an ~10-min period using a positive displacement pipette. Hypersalivation in animals was induced by an intraperitoneal injection of 0.1 mg pilocarpine (13). Serum samples (~100 μl) were obtained from blood collected from periorbital bleeds or by cardiac puncture. Serum and saliva samples were then aliquoted in 10-μl volumes and stored at −70°C until analysis. Antibodies against ntPEpilinPAK were detected by enzyme-linked immunosorbent assay (ELISA). Costar 9018 EIA/RIA 96-well plates were coated overnight with 0.6 μg/well of ntPEpilinPAK in 0.2 M $NaHCO_3$-Na_2CO_3, pH 9.4. Each 96-well plate was washed four times with PBS containing 0.05% Tween 20–0.01% thimerosal (wash buffer) and blocked for 1 h with PBS-Tween 20 containing 0.5% bovine serum albumin–0.01% thimerosal (assay buffer). Serum and saliva samples were diluted with assay buffer, loaded onto a 96-well plate, and incubated for 2 h for serum IgG and overnight for saliva and serum IgA. Each 96-well plate was then washed four times with wash buffer; horseradish peroxidase (HRP)-conjugated goat anti-mouse serum IgG or serum IgA was added and incubated for 1 and 4 h, respectively. All incubation and coating steps were performed at RT on a shaker at 4 rpm in accordance to the specified time; plates were covered using parafilm. TMB (3,3′,5,5′tetramethylbenzidine), a substrate for HRP, was used to quantify bound antibody at 450 nm. Specific immune responses against pilin PAK peptide were assessed by coating each plate overnight with 1 μg/well of streptavidin. Each plate was then blocked with assay buffer for 1 h, and 1 μg/well of biotin-pilin PAK and biotin-scrambled (control) peptide were added and incubated for 1 h. The remainder of the ELISA procedure is the same as that for the assay used to recognize ntPEpilinPAK.

Quantification of bacterial adherence. A549 (ATCC CCL-185) cells were maintained in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 2.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Prior to seeding onto chamber slides or electrode arrays for assays, A549 cells were washed to remove residual antibiotic and transferred to antibiotic-free Ham's F-12 medium. Once seeded, cells were grown to near-confluent densities, ~1×10^5 cells per chamber, in Lab-Tek II 8-chamber slides (Nunc) in antibiotic-free medium. Cells were exposed to ~5×10^6 *P. aeruginosa* PAK strain bacteria, premixed with test samples or control media, for 2 h at 37°C and 5% CO_2. Three washes with Hanks' balanced salt solution were performed to remove unbound bacteria prior to fixation for 1 h in 3.7% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2. Fixed cells and associated bacteria were stained with 10% Giemsa stain for 10 min. After washing to remove excess Giemsa stain, the number of bacteria associated with 50 cells in each well was determined by counting under light microscopy at 1,000× magnification. A total of 100 cells were counted for each experimental group. All experiments were replicated several times to verify reproducibility, but only one set, having all internal controls for that data set, is presented. *P. aeruginosa* adhesion studies were also performed using primary cultures of human tracheal epithelium (HTE) grown as confluent, polarized sheets as previously described (69). HTE sheets were used from 5 to 10 days postplating on 12-mm opaque inserts (0.45 μm pore size; Corning, Acton, MA) coated with human placental collagen after verification that *trans*-epithelial resistance values of >100 Ω · cm^2 and a *trans*-epithelial potential difference of >5 mV had been achieved using a "chopstick voltmeter" (Millicell ERS; Millipore, St. Louis, MO).

Quantitative real-time PCR. Bacterial pellets obtained from HTE-PAK or A549-PAK incubation supernatants by centrifugation at 5,000 × *g* for 5 min were saved at −70°C until further processing. Differential displays of mRNAs for PAK pilin was determined using an Applied Biosystems 7300 Real Time PCR system (Foster City, CA) following isolation of total RNA from bacteria using the RNeasy Protect Mini kit (QIAGEN). Total RNA was used to generate cDNA for oligo(dT) oligodeoxynucleotide primer (T12-18) following the protocol for Omniscript Reverse Transcriptase (QIAGEN). The following primers were designed using Primer Express software (Applied Biosystems) and synthesized by Operon (Alameda, CA): PAK pilin forward, AGGTACAGAGGACGCTACTAAGAA AGA; PAK pilin reverse, TCAGCAGGATCGGGTTTGA. Equal amounts of cDNA were used in duplicates and amplified with SYBR Green I Master Mix (Applied Biosystems). Thermal cycling parameters were as follows: activation for 10 min at 95°C and 40 cycles of PCR (melting for 15 s at 95°C and annealing-extension for 1 min at 60°C). A standard curve was constructed with a dilution curve (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) of total RNA from PAK for PAK pilin. A "no template" control was included with each PCR.

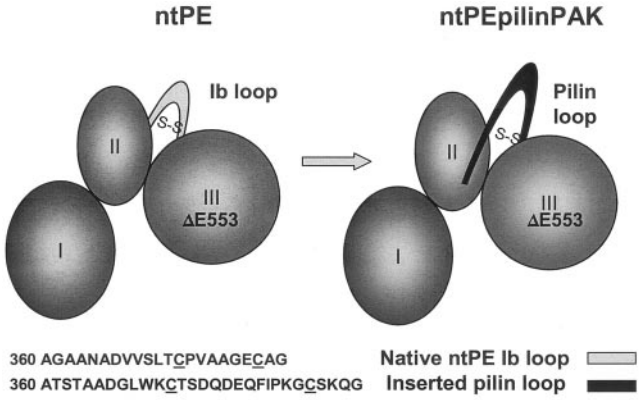

FIG. 1. Cartoon of the protein chimera ntPEpilinPAK. The gene encoding *P. aeruginosa* exotoxin A (PE) was modified to delete one codon, removing a glutamic acid at position 553 (ΔE553), to produce a nontoxic form of the enzyme (ntPE). The Ib site of ntPE was restricted to remove 20 amino acids and was replaced with an oligonucleotide duplex encoding 27 amino acids of *P. aeruginosa* PAK strain pilin (ntPEpilinPAK) that includes a C-terminal disulfide-bonded loop (DSL) producing a protein having 619 amino acids. Coinciding cysteine residues of ntPE and ntPEpilinPAK in the Ib region of ntPE are underlined. This insertion strategy also resulted in replacement of a portion of domain II of ntPE with an amino acid sequence of pilin. Three amino acids irrelevant to both ntPE and *P. aeruginosa* that were introduced by this cleavage and ligation strategy are italicized.

Cell detachment assay. Cell-substrate detachment was measured using a noninvasive electric cell-substrate impedance sensing (ECIS) method (67). A549 cells, prepared in antibiotic-free media, were seeded onto 8-well 1-electrode culture arrays (8W1E) (Applied BioPhysics, Troy, NY), with a working electrode area of 5×10^{-4} cm^2 and a counter electrode area of 0.15 cm^2, and grown in a humidified incubator at 37°C in 5% CO_2. Cell attachment was monitored for 22 h to ensure confluent lawns of ~1×10^5 cells/well with a resistance reading of 2 to 3 kOhms. Cells were further stabilized by replenishing with fresh media for 2 to 3 h prior to introduction of bacteria. Cell detachment was monitored as a measure of electrical impedance at 0.5-min time points, 40 kHz, for 24 h.

Assessment of exotoxin A neutralization. A549 cells were grown in Dulbecco's modified Eagle's medium F12 (DMEM F12) supplemented with 10% HI-FBS, 2.5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Cell toxicity assays using A549 cells were performed essentially as previously performed using L929 cells (48). Apoptosis was assessed using the ApoAlert Caspase-3/7 Colorimetric Assay kit (Becton Dickinson, Franklin Lakes, NJ) according to the manufacturer's instructions. Collection of antibody fractions from serum samples was achieved using ImmunoPure (L) Immunoglobulin Purification kit from Pierce Chemical Company (catalog no. 20550) following the manufacturer's instructions.

RESULTS

Protein chimera has the anticipated characteristics. The ntPEpilinPAK protein used in these studies was prepared by genetically grafting the terminal 24 amino acids of the *P. aeruginosa* PAK strain pilin protein in place of 20 amino acids normally present in ntPE (Fig. 1). This modification resulted in exchange of the 6-amino-acid Ib loop cysteine disulfide bridge of ntPE with the 12-amino-acid C-terminal disulfide-bonded loop (DSL) of pilin as well as the 8 amino acids preceding the

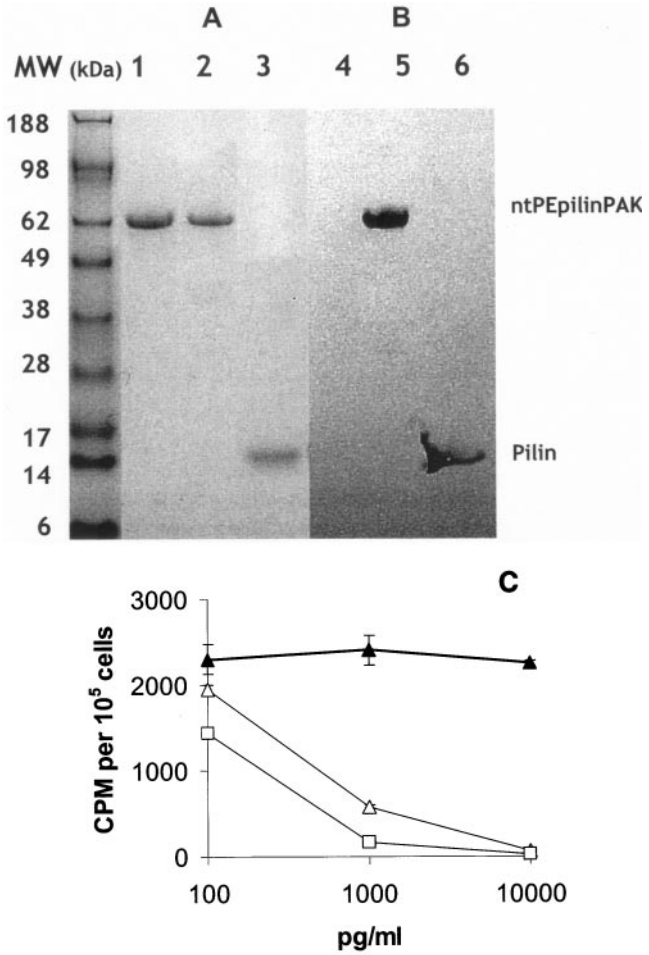

FIG. 2. Characterization of ntPEpilinPAK. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (A) and Western blot (B) analysis of ntPE (lanes 1 and 4), ntPEpilinPAK (lanes 2 and 5), and pilin protein purified from the PAK strain of *P. aeruginosa* (lanes 3 and 6) with 1 μg of protein loaded per lane. (C) A toxic form of the protein construct (PEpilinPAK) was prepared and compared to native PE and ntPEpilinPAK for its capacity to inhibit protein synthesis (monitored by radioactive leucine incorporation in trichloroacetic acid-precipitated material) in L929 cells in vitro. ▲, ntPEpilinPAK; △, PEpilinPAK; □, PE. MW, molecular size; CPM, counts per million.

DSL (63). Following expression in *E. coli*, ntPEpilinPAK was isolated from inclusion bodies and renatured in a redox shuffling buffer as described previously (4, 25). The product had the anticipated mass of ~68 kDa, similar to that observed for similarly purified and refolded ntPE (Fig. 2A). Other physical characteristics of ntPEpilinPAK are described in Materials and Methods. A monoclonal antibody, 1D10, that recognized the DSL of the PAK pilin protein also recognized ntPEpilinPAK (Fig. 2B). Crystal structure projections of PE (66) and pilin (24) suggest β-sheet conformations immediately prior to their respective disulfide-constrained loops. Therefore, we hypothesized that the 8 amino acids exchanged preceding the Ib loop with those of the pilin protein sequence have minimal structural impact on ntPE. To test this we compared the ability of a toxic form of the protein chimera (PEpilinPAK) to kill toxin-sensitive cells in vitro (Fig. 2C). Indeed, PEpilinPAK was as toxic as PE, while ntPEpilinPAK showed no toxicity.

We next examined whether the inserted pilin loop of ntPEpilinPAK could interact with the epithelial cell surface receptor previously identified to bind this structure: the ganglioside asialo-G_{M1}. An ELISA-based binding protocol showed that ntPEpilinPAK selectively interacted with asialo-G_{M1} relative to monosialo-G_{M1} (Fig. 3A). Control studies showed that ntPE (lacking the pilin loop insert) did not interact with asialo-G_{M1} (Fig. 3A). The human respiratory epithelial cell line A549 has been shown to bind *P. aeruginosa* through a pilin DSL-specific mechanism in vitro (25, 68). Introduction of an antibody directed against the *P. aeruginosa* PAK strain DSL, 1D10 monoclonal (at 1 μg/ml), reduced binding of PAK strain *P. aeruginosa* to A549 lawns in vitro by ~50% (Fig. 2B). This same 1D10 antibody bound to ntPEpilinPAK, but not ntPE, in a Western blot format (Fig. 2A). A standard curve (Fig. 3B) correlating real-time PCR product specific for the mRNA of *P. aeruginosa* PAK strain pilin protein to the number of bacteria is shown. This correlation was used to monitor the concentration of bacteria unable to adhere to A549 cells in vitro. The presence of 10 μg/ml ntPEpilinPAK was found to also reduce bacteria binding to A549 cells in vitro (Fig. 3C). Similarly, the average number of bacteria adhering to A549 cells in vitro determined by a direct microscopic counting method was reduced by the addition of ntPEpilinPAK relative to ntPE (Fig. 3D).

ntPEpilinPAK uses a novel mucosal vaccination strategy to deliver intact, conformation antigens across epithelial barriers in a system that results in their targeting to APCs (46). Thus, it was important to verify that incorporation of the pilin DSL structure and adjacent amino acids into ntPE to generate ntPEpilinPAK did not impede the inherent capacity of ntPE to transport across intact (in this case, nasal) epithelium. Previous studies have implicated CD91 as a receptor involved in *trans*-epithelial ntPE (and PE) transport (46). Distribution of CD91 in isolated naïve nasal mouse tissue demonstrated extensive labeling in epithelial cells and specific cells in the submucosal region consistent with distribution of APCs (Fig. 4A). Distribution of biotin-labeled ntPEpilinPAK 30 min following i.n. application was similar to that of CD91 in this tissue (Fig. 4B). These results suggest that ntPEpilinPAK can migrate across mouse nasal epithelia in a manner consistent with CD91 cellular distribution without apparent disruption of gross modification of the epithelium and deliver antigenic components in a manner consistent with systemic and mucosal immunization outcomes.

Systemic and mucosal humoral immune responses. Four i.n. administrations of ntPEpilinPAK, even at doses as low as 1 μg, resulted in systemic anti-ntPEpilinPAK IgG responses detected in serum diluted 1:20 with PBS (Fig. 5A). Serum IgG responses achieved with 100 μg applied by i.n. administration were approximately half of that obtained by s.c. injection of 10 μg ntPEpilinPAK with a cocktail of Freund's complete and incomplete adjuvant. Although in this particular study the 10 μg i.n. group was not consistent with a dose-dependent immune response, a dose-dependent response to i.n. administration of ntPEpilinPAK was typically observed in other studies (data not shown). Measurement of anti-ntPEpilinPAK IgA antibodies in these same 1:20-diluted serum samples demon-

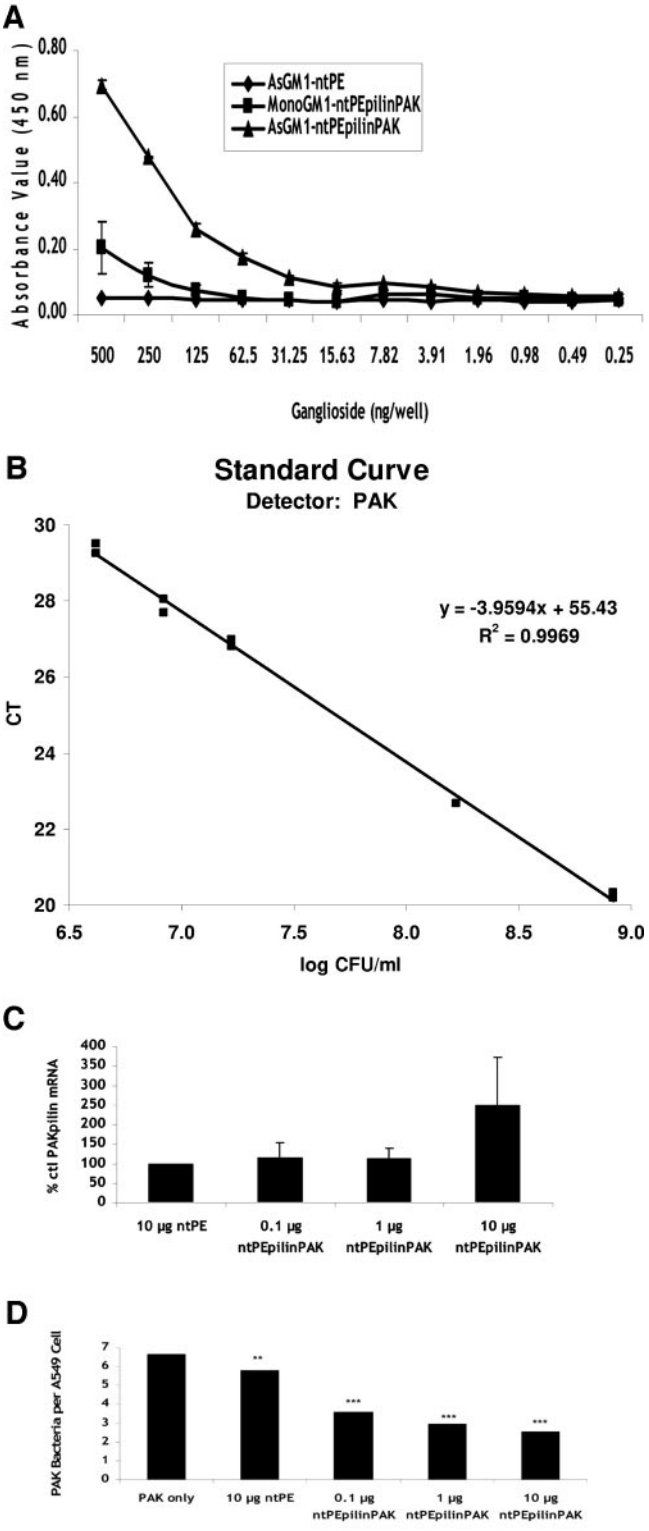

FIG. 3. Pilin loop insert binds to native epithelial cell receptor. (A) An ELISA-based method was used to determine binding of ntPE or ntPEpilinPAK to the ganglioside asialo-G_{M1} (AsGM1) or monosialoG_{M1} (MonoGM1) after a 3-h incubation at 25°C. Values refer to means ± standard errors of the means (SEM) for triplicate samples. (B) Typical standard curve comparing PAK *P. aeruginosa* concentration with values obtained for real-time PCR measurements using SYBR green. Plot shows values for input amount (log CFU/ml) versus threshold cycle (C_T) for target amplification. (C) *P. aeruginosa* PAK strain bacteria were allowed to bind to near-confluent cultures of A549 cells for 2 h at 37°C in the presence of increasing concentrations of ntPEpilinPAK or constructs lacking the pilin loop insert (ntPE). Nonadherent bacteria were then quantitated by real-time PCR. Data represent four separate experiments performed in duplicate each time, with values obtained in the ntPE group used as the control (ctl). (D) In vitro adherence of *P. aeruginosa* PAK strain bacteria to A549 cell lawns was reduced by the presence of ntPEpilinPAK in a dose-dependent manner (0.1 to 10 µg) relative to binding observed in the presence of PBS (PAK only) alone ($n = 4$). Introduction of 10 µg ntPE, lacking the pilin loop insert, was less effective at reducing this interaction. Statistical assessment was performed using one-way ANOVA, and data are expressed as means ± SEM; $P < 0.001$ (***) and $P < 0.01$ (**) compared to PBS (PAK only) values.

strated that only mice receiving 100 µg i.n. produced a response detectable in this assay (Fig. 5B). Saliva samples obtained from mice and diluted 1:10 with PBS were found to have increased anti-ntPEpilinPAK IgG immune responses in the 100-µg i.n. and 10-µg–Freund's s.c. groups (Fig. 5C), while only saliva obtained from mice receiving 100 µg i.n. could be shown to have significant anti-ntPEpilinPAK IgA antibodies (Fig. 5D). No anti-ntPEpilinPAK IgE responses were detected (data not shown).

We also examined the importance of multiple i.n. exposures in the BALB/c model by administering ntPEpilinPAK either on the schedule of days 0, 14, and 28 (three total exposures) or days 0, 7, 14, and 28 (four total exposures; data are shown in Fig. 5) and comparing salivary IgA responses to ntPEpilinPAK and serum IgG responses to the pilin loop insert antigen 7 days following the day-28 dosing. No statistical differences could be detected between these animal groups (data not shown), suggesting that the ELISA data shown here (Fig. 5) represent complete or near-complete immune responses produced by i.n. application of ntPEpilinPAK. Overall, these results suggest that i.n. administration of ntPEpilinPAK can generate a systemic anti-ntPEpilinPAK immune response that compares closely to that observed using an s.c. injection protocol involving a regimen of complete and incomplete Freund's adjuvant. Further, i.n. administration of ntPEpilinPAK stimulated mucosal immune outcomes that were not observed in animals receiving s.c. injections of ntPEpilinPAK in a Freund's adjuvant cocktail.

Efforts to measure anti-ntPEpilinPAK IgA antibodies in saliva were compromised by lack of a high-affinity antibody that selectively recognized mouse secretory IgA (sIgA) and the requirement to dilute these saliva samples to eliminate viscosity-related high-assay background. Using a commercially available secondary antibody determined to have the greatest capacity to cross-react with sIgA (these reagents are typically generated using serum IgA), statistically significant levels of salivary IgA antibodies specific for ntPEpilinPAK could be observed only in the 100-µg i.n.-dosed mouse group (Fig. 5D). No similar responses could be detected in either the 1- or 10-µg i.n. dose groups or in the 10-µg s.c. group administered with Freund's adjuvant. Assessment of anti-ntPEpilinPAK IgA antibodies in serum similarly showed that only the 100-µg i.n. dose group generated detectable antibodies with these characteristics. A detailed study of immune responses to *Chlamydia pneumoniae* demonstrated that active infection resulted in ap-

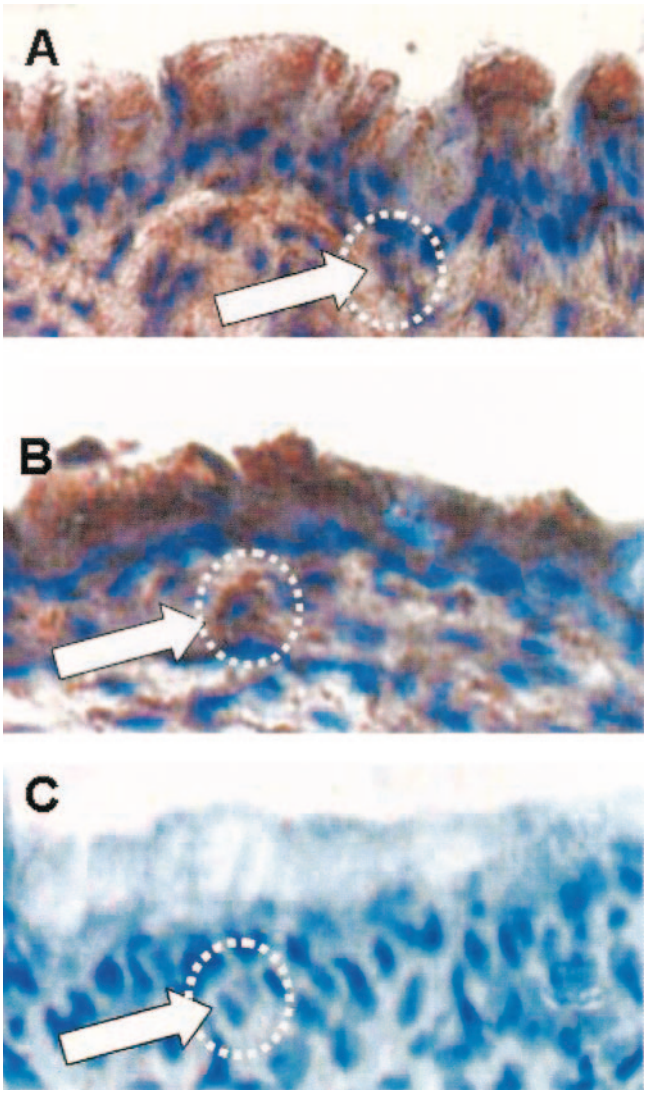

FIG. 4. Cellular distribution of CD91 and uptake of biotin-labeled ntPEpilinPAK in mouse nasal mucosa. A) Distribution of an anti-CD91 polyclonal antibody in mouse nasal tissue was detected using a streptavidin-HRP conjugate. Note extensive labeling in epithelial cells and specific cells in submucosal regions consistent with phagocytic cell characteristics (dashed circle highlighted by an arrow). B) Streptavidin-HRP distribution in nasal mucosa 30 min after exposure with biotin-ntPEpilinPAK. Note extensive labeling in epithelial cells and specific cells in submucosal regions consistent with phagocytic cell characteristics (dashed circle highlighted by an arrow). C) Labeling control showing mouse nasal tissue exposed to streptavidin-HRP conjugate (the dashed circle outlines a cell having phagocytic cell characteristics).

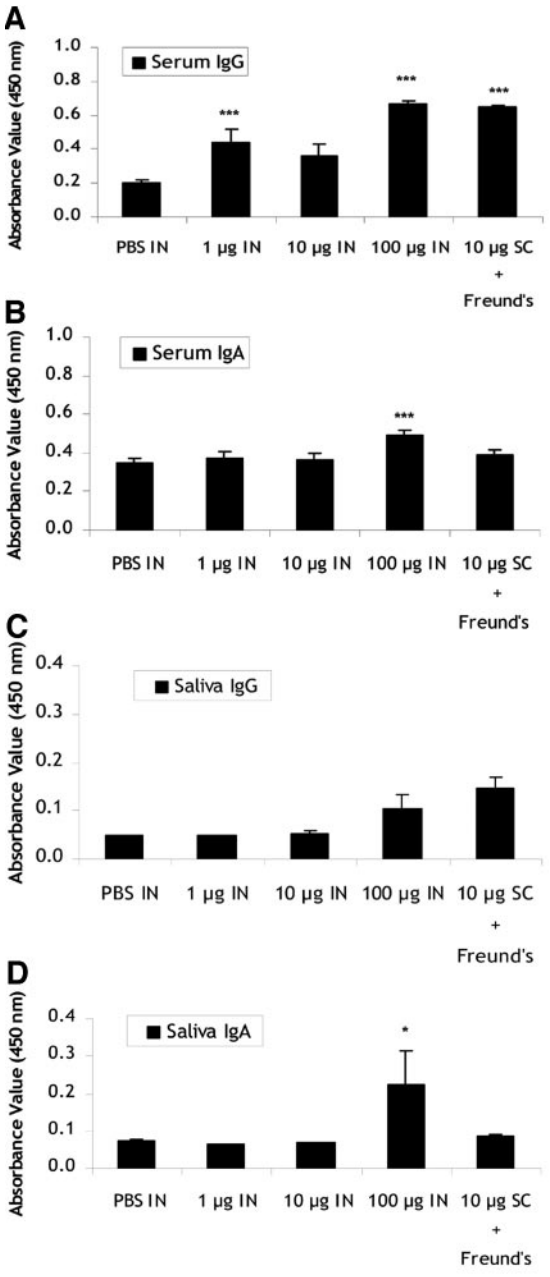

FIG. 5. Anti-ntPEpilinPAK antibody responses in serum and saliva. Standard format ELISA protocols were used to detect anti-ntPEpilinPAK serum IgG (A), serum IgA (B), salivary IgG (C), and salivary IgA (D) antibodies induced in mice following i.n. immunization with 1, 10, or 100 μg ntPEpilinPAK ($n = 8$ per group). Negative control animals received an equal volume of carrier buffer by i.n. instillation (PBS IN), and positive controls (SC + Freund's) received 10 μg ntPEpilinPAK injected s.c. in a regimen of complete and incomplete Freund's adjuvant. Prior to analysis, serum and saliva samples were diluted 1:20 and 1:10 with PBS, respectively. Statistical assessment was performed using one-way ANOVA, and data are expressed as means ± standard errors of the means; $P < 0.001$ (***) and $P < 0.05$ (*) compared to PBS i.n. values.

proximately 40-fold less pathogen-specific serum IgA antibodies than serum IgG antibodies (65). Although the ELISA results obtained in our studies cannot be directly compared, mucosal immunization with 100 μg ntPEpilinPAK produced a similar ratio of observed serum IgG and serum IgA responses.

Mucosal immunization with ntPEpilinPAK is designed to provide immunity against both PE and the DSL domain of *P. aeruginosa*. Immune responses to ntPEpilinPAK chimera should be dominated by antigenic epitopes present on ntPE relative to the engrafted pilin DSL of 12 amino acids. The latter was identified as most relevant to blocking pilin-mediated bacteria-host cell interactions that could occur at epithelial surfaces of the oral-pharyngeal cavity and trachea. While

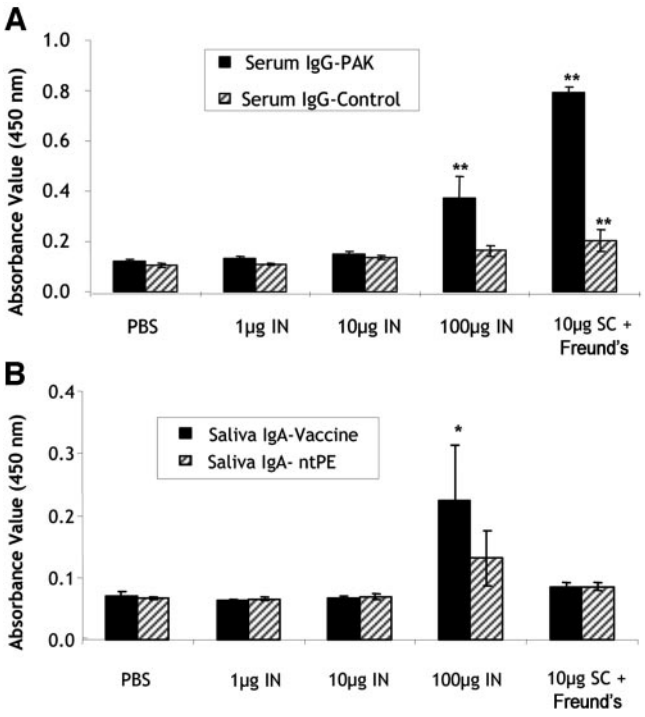

FIG. 6. Induction of anti-pilin antibody responses. A) Serum IgG antibodies specific for the PAK pilin loop sequence, relative to a scrambled peptide (Control) sequence, was determined using a standard ELISA format for mice dosed i.n. with 1, 10, or 100 μg ntPEpilinPAK. B) Salivary IgA antibodies recognizing ntPE and ntPEpilinPAK were detected by ELISA. Negative control animals received an equal volume of carrier buffer (PBS) by i.n. instillation, and positive controls received 10 μg ntPEpilinPAK injected s.c. (SC + Freund's) in a regimen of complete and incomplete Freund's adjuvant ($n = 8$ per group). Statistical assessment was performed using one-way ANOVA, and data are expressed as means ± standard errors of the means; $P < 0.01$ (**) and $P < 0.05$ (*) compared to PBS i.n. values.

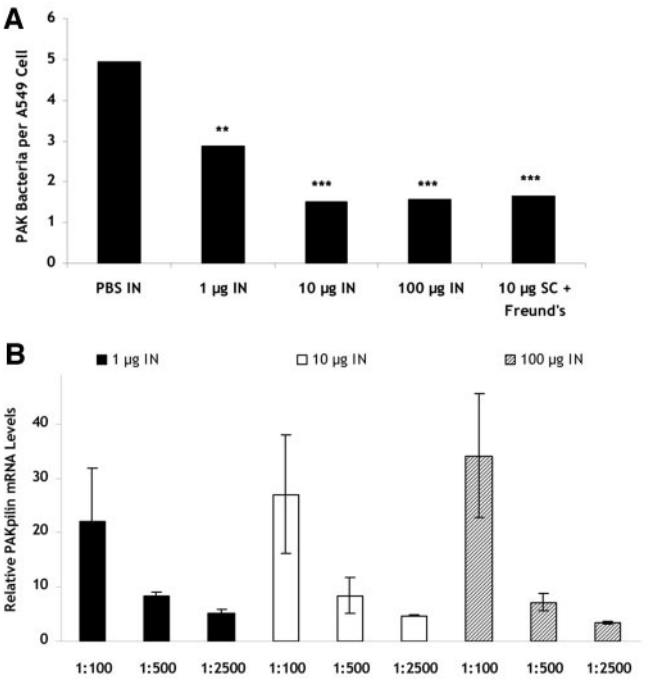

FIG. 7. Effect of immune saliva on pilin-mediated bacteria binding to A549 cells. A) Saliva obtained from mice immunized with ntPEpilinPAK by intranasal (IN) instillation or by s.c. injection with a cocktail of compete and incomplete Freund's adjuvant (SC + Freund's) and diluted 1:100 with PBS reduced bacteria adherence relative to saliva obtained from mice dosed i.n. with PBS ($n = 4$) after 2 h of incubation at 37°C. B) A549 bacteria media were collected following 2-h incubations at 37°C with PAK strain of *P. aeruginosa* with saliva samples obtained from mice immunized i.n. with 1, 10, or 100 μg ntPEpilinPAK and diluted to various extents prior to introduction into the binding assay. Unbound bacteria levels were determined by real-time PCR performed in duplicate. Statistical assessment was performed using one-way ANOVA, and data are expressed as means ± standard errors of the means; $P < 0.001$ (***) and $P < 0.01$ (**) compared to PBS i.n. values.

the dominant IgA isotype in saliva was assumed to represent sIgA resulting from active transport of dimeric IgA following interaction with the poly Ig receptor (61), IgG present in the saliva was presumed to reach that site as an exudate from serum (22). Possibly due to the poor sensitivity of the sIgA ELISA, we could not demonstrate DSL-specific sIgA responses in saliva of animals dosed with ntPEpilinPAK (data not shown). DSL-specific serum IgG responses were detectable in both 100-μg i.n. and 10-μg s.c.–Freund's adjuvant groups, although the immune response generated by injection also demonstrated a nonspecific immune response as demonstrated by increased recognition of a control (scrambled) peptide used for this assay (Fig. 6A). The level of insert-specific systemic immunity demonstrated in these studies was comparable to that previously observed using an ntPE-based mucosal vaccination that incorporated the V3 loop of human immunodeficiency virus gp120 protein (45). Although PBS-diluted samples of saliva collected from mice that received four i.n. administrations of 100 μg ntPEpilinPAK failed to demonstrate significant anti-pilin loop responses in the ELISA protocol, these same saliva samples did show greater reactivity with ntPEpilinPAK relative to ntPE in an ELISA format (Fig. 6B). Importantly, saliva from animals receiving ntPEpilinPAK by injection with Freund's adjuvant failed to show any specificity of anti-ntPEpilinPAK IgA responses.

Immune saliva blocks host-pathogen interactions. An in vitro model of A549-*P. aeruginosa* interactions was established and validated to reflect pilin-mediated interactions using the pilin-specific antibody 1D10 (data not shown) and comparing relative binding of ntPE (lacking the DSL insert) with ntPEpilinPAK (Fig. 3). Saliva samples collected from mice immunized by four i.n. administrations of either 1, 10, or 100 μg ntPEpilinPAK significantly decreased the mean number of PAK strain *P. aeruginosa* attached to A549 cell lawns in vitro (Fig. 7A). Importantly, saliva obtained from mice that received 10 μg ntPEpilinPAK by s.c. injection with Freund's complete and incomplete adjuvant also blocked A549-*P. aeruginosa* interactions (Fig. 7A). These studies were preformed using saliva samples diluted 1:100 in PBS and counted the average number of PAK strain *P. aeruginosa* bound to an A549 cell. Greater reductions in A549-*P. aeruginosa* interactions, similar to those obtained with the 1D10 antibody, could be achieved with undiluted or 1:10-diluted saliva, but such studies were impossible to perform on a regular basis due to limited quantities of saliva that could be collected. Studies were also performed using

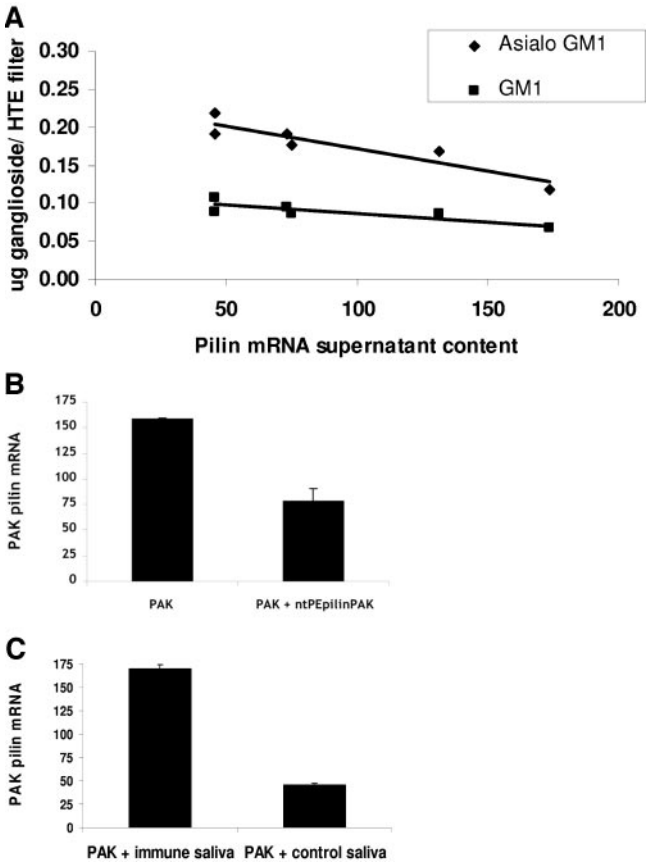

FIG. 8. Specificity of pilin-mediated bacteria binding to polarized human tracheal epithelial (HTE) cells in vitro. A) Differences in cellular levels of the ganglioside asialo-G_{M1} (Asialo GM1) but not monosialoG_{M1} (GM1) correlated with increased *P. aeruginosa* binding to polarized HTE cells. The amount of pilin-specific mRNA present in the supernatant (determined by real-time PCR) was used as a measure of piliated *P. aeruginosa* not bound to the apical surface of these same polarized HTE cell sheets. B) Binding of piliated *P. aeruginosa* (PAK) to polarized HTE cell sheets was reduced by the presence of 1 μg/ml ntPEpilinPAK (PAK + ntPEpilinPAK) in vitro. C) Binding of piliated *P. aeruginosa* PAK strain bacteria to polarized HTE cell sheets was reduced by the presence of saliva (diluted 1:20 with PBS) obtained from mice immunized by i.n. administration of 100 μg/ml ntPEpilinPAK relative to saliva collected from mice that received PBS (ntPEpilinPAK carrier buffer) by i.n. administration. Data represent four separate experiments performed in duplicate each time.

increased dilutions of saliva samples where binding of PAK strain *P. aeruginosa* to A549 cells was determined by real-time PCR. Greater dilutions of saliva samples obtained from mice immunized by i.n. administrations of either 1, 10, or 100 μg ntPEpilinPAK showed a concomitant decrease in the capacity to block PAK strain *P. aeruginosa* binding to A549 cells in vitro (Fig. 7B).

Confluent sheets of human tracheal epithelial (HTE) cells were examined for their capacity and characteristics of piliated *P. aeruginosa* binding in vitro. Initial studies demonstrated that HTE cell sheets enriched in asialo-G_{M1}, but not monosialoG_{M1}, showed increased binding of piliated PAK *P. aeruginosa* (Fig. 8A). Further, the presence of 10 μg ntPEpilinPAK significantly reduced the binding of PAK strain *P. aeruginosa* bacteria to HTE cell sheets compared to those exposed to 10 μg ntPE (Fig. 8B). These results suggest that this in vitro binding assay reflects pilin loop-asialo-G_{M1} binding interactions. Introduction of PBS-diluted (1:100) saliva obtained from mice immunized i.n. with 100 μg ntPEpilinPAK resulted in a significant reduction of piliated *P. aeruginosa* PAK strain binding to HTE cell sheets in vitro when measured as an assessment of nonadherent bacteria using real-time PCR relative to saliva obtained from mice that received i.n. administrations of PBS (Fig. 8C). Overall, these studies suggest that some factor(s) in mouse saliva, following i.n. administrations of ntPEpilinPAK, can reduce pilin-mediated *P. aeruginosa* binding to human airway epithelial cells in vitro.

Immune saliva protects epithelial cells from toxic actions of *P. aeruginosa*. Pilin-mediated interactions have been shown to enhance mRNA levels for a spectrum of proinflammatory-related genes in the human respiratory cell line A549 (26) and induce cytotoxicity in epithelial cells in vitro (9). We monitored an in vitro phenomenon where A549 cells round up and lift from their substrate following several hours of contact with piliated PAK strain *P. aeruginosa* using electric cell-substrate impedance sensing (ECIS). This technique uses an electrode array to continuously monitor cell-substrate interactions (67). Increasing amounts of *P. aeruginosa* PAK strain, from 20 to 200 bacteria per A549 cell, demonstrated accelerated rates of cell rounding and lifting as demonstrated by ECIS and corroborated by microscopic assessment (data not shown). Four hours following inoculation with ~50 bacteria per A549 cell, there was extensive rounding of A549 cells and loss of epithelial cell-substrate association characterized by reduction of resistive properties of the system (Fig. 9A). Saliva samples (diluted 1:100 with PBS) obtained from mice immunized either i.n. with 100 μg ntPEpilinPAK or 10 μg ntPEpilinPAK injected s.c. with Freund's adjuvant cocktail blocked *P. aeruginosa*-induced A549 cell rounding and lifting events (Fig. 9A). Saliva obtained from mice inoculated i.n. with PBS had no protective effect.

Additional studies verified a role for pilin in the *P. aeruginosa*-induced A549 cell rounding and lifting event-mediated induction of the lifting response. The DSL of *P. aeruginosa* binds selectively to a disaccharide, 4-*O*-(2-acetomido-2-deoxy-β-D-galatopyranosyl)-D-galactose, also known as GalNAcβ1-4Gal, that represents a water-soluble portion of asialo-G_{M1} (6). Addition of 10 μg GalNAcβ1-4Gal to *P. aeruginosa* PAK strain bacteria rescued A549 cells from rounding and lifting events induced by addition of these bacteria (data not shown). Similarly, addition of 1 μg 1D10 monoclonal antibody, which recognizes the PAK pilin DSL region, blocked *P. aeruginosa* PAK strain-induced rounding and lifting of A549 cell in vitro (data not shown). Similar addition of M40-1, an antibody that recognizes PE, did not affect PAK strain-induced A549 cell rounding and lifting events (data not shown). Interestingly, introduction of pilin protein purified from *P. aeruginosa* PAK strain accelerated and enhanced A549 rounding and lifting induced by *P. aeruginosa* PAK strain bacteria (data not shown). Although the exact mechanism(s) involved in the pilin-mediated lifting response observed in A549 cells is unclear, such a morphological outcome is generally associated with cytotoxic events such as those observed with the type III secretion system of *P. aeruginosa* (9). This might be similar to induction of virulence factors following pili-mediated adherence as observed with uropathogenic *E. coli* (70). Measurement of hu-

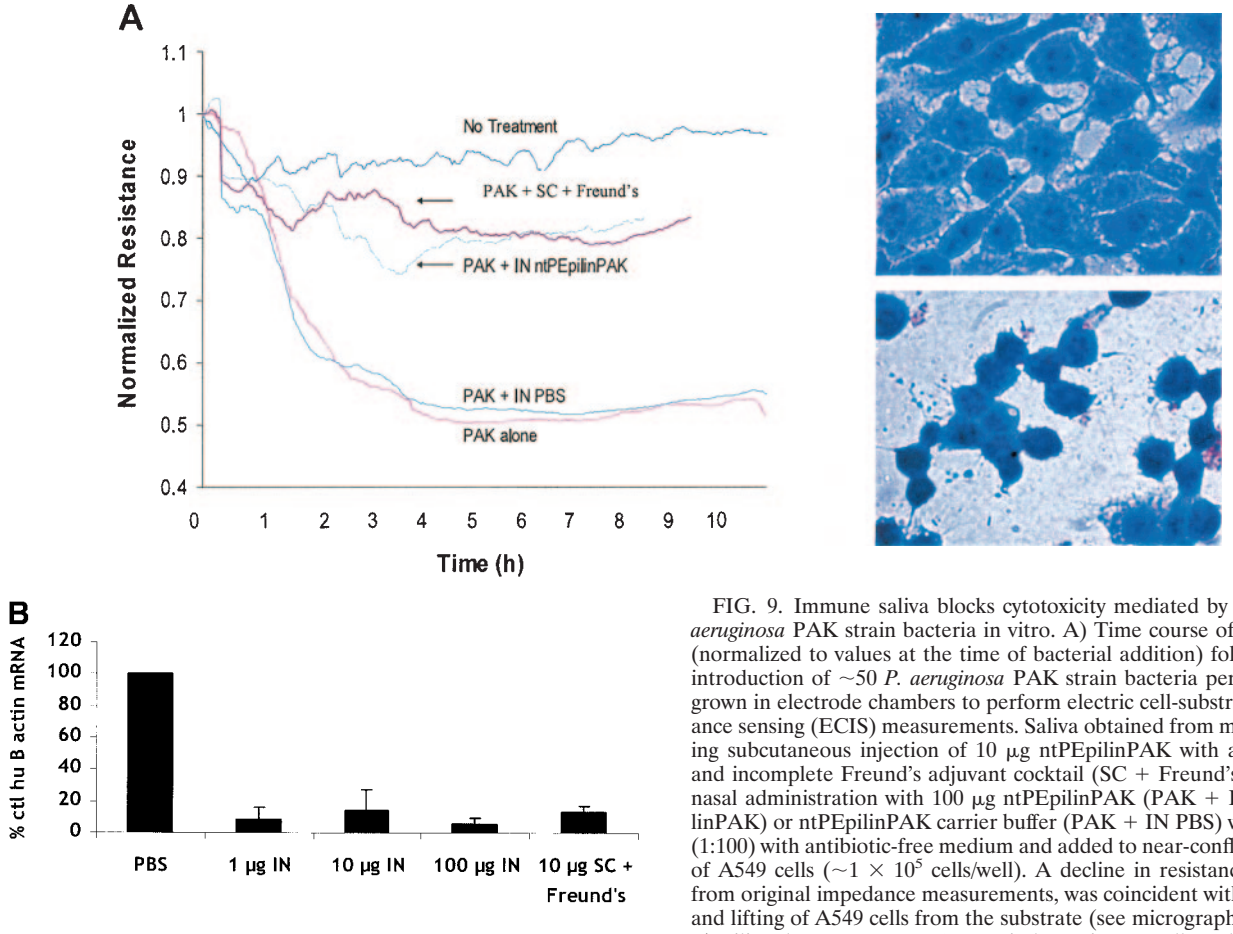

FIG. 9. Immune saliva blocks cytotoxicity mediated by piliated *P. aeruginosa* PAK strain bacteria in vitro. A) Time course of resistance (normalized to values at the time of bacterial addition) following the introduction of ~50 *P. aeruginosa* PAK strain bacteria per A549 cell grown in electrode chambers to perform electric cell-substrate impedance sensing (ECIS) measurements. Saliva obtained from mice following subcutaneous injection of 10 μg ntPEpilinPAK with a complete and incomplete Freund's adjuvant cocktail (SC + Freund's) or intranasal administration with 100 μg ntPEpilinPAK (PAK + IN ntPEpilinPAK) or ntPEpilinPAK carrier buffer (PAK + IN PBS) was diluted (1:100) with antibiotic-free medium and added to near-confluent lawns of A549 cells (~1 × 10^5 cells/well). A decline in resistance, derived from original impedance measurements, was coincident with rounding and lifting of A549 cells from the substrate (see micrographs at right). B) Piliated *P. aeruginosa* PAK strain bacteria were allowed to bind to near-confluent cultures of A549 (~50 bacteria/cell) for 2 h at 37°C in the presence of saliva (diluted 1:20 with PBS) obtained from mice that had received PBS or ntPEpilinPAK (at 1, 10, or 100 μg) or had been immunized by s.c. injection of ntPEpilinPAK along with a cocktail of complete and incomplete Freund's adjuvant (SC + Freund's). The extent of A549 cell lifting was monitored by measuring the amount of human β-actin mRNA present in supernatants collected from these cultures relative to control (ctl) samples. Data represent four separate experiments performed in duplicate each time and are expressed as means ± standard errors of the means.

man β-actin mRNA in the supernatant of these lifting assays was also performed as a correlating parameter of A549 cell lifting. Saliva obtained from mice immunized i.n. with 1, 10, or 100 μg ntPEpilinPAK substantially reduced, relative to saliva from mice that received i.n. PBS, the amount of A549 β-actin mRNA in the media after addition of piliated *P. aeruginosa* PAK strain (Fig. 9B). Also consistent with ECIS data (Fig. 9A), supernatant A549 β-actin mRNA were reduced in samples treated with diluted saliva obtained from mice immunized by s.c. ntPEpilinPAK injection with Freund's adjuvant cocktail (Fig. 9B).

PAK strain-induced A549 cell lifting did not appear to involve actions of PE, since none of this toxin was ever detected in any incubation (ELISA studies; data not shown), consistent with an observation that PE is secreted by *P. aeruginosa* under times of iron-deficient stress (60), and culture media used in A549 lifting assays was not iron deficient. PE, however, still represents a potent cytotoxic virulence factor for *P. aeruginosa* infection (21). Previous studies where a chimera protein similar to ntPEpilinPAK (containing an abbreviated pilin protein sequence) was injected into rabbits demonstrated serum immune responses capable of neutralizing PE toxicity in vitro (25). A549 cells challenged with PE showed increased caspase-3/7 activity after 24 h in vitro (Fig. 10), indicating induction of the apoptosis mechanism used by PE to kill cells (43). Saliva obtained from mice following i.n. immunization with ntPEpilinPAK neutralized PE toxicity in this assay in a dilution-dependent fashion (Fig. 10A). Interestingly, PE-stimulated caspase-3/7 activity in A549 cells in vitro was not affected by serum from mice immunized with ntPEpilinPAK (Fig. 10B). However, the potential for serum factors to activate caspase-3/7 appears to have confounded this assay, since serum antibody fractions captured by protein L showed a marked reduction in PE in this in vitro cytotoxicity assay using relevant control conditions (Fig. 10C). Protection from PE cytotoxicity was greater in the antibody fraction obtained from i.n.-immunized mice compared to those that received ntPEpilinPAK by injection with a cocktail mixture of complete and incomplete Freund's adjuvant or a control i.n. administration of PBS (Fig. 10C).

DISCUSSION

Prophylactic vaccine approaches to protect CF patients against pulmonary *P. aeruginosa* infection frequently focus on

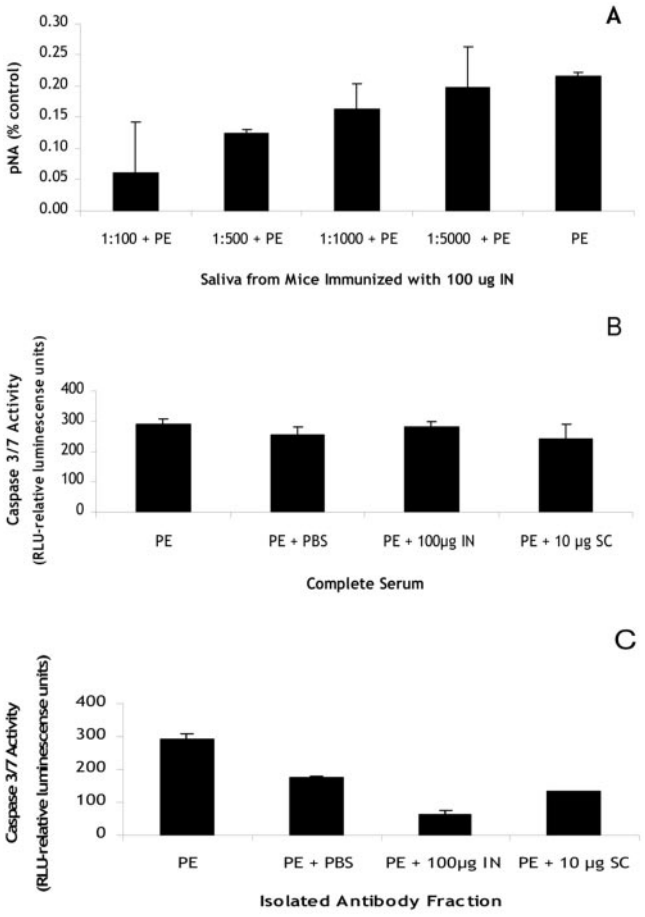

FIG. 10. Immunized saliva attenuates PE-induced caspase-3/7 activation. A) Saliva obtained from mice immunized i.n. with 100 μg ntPEpilinPAK added to confluent A549 cells after dilution with PBS (1:100, 1:500, 1:1,000, and 1:5,000) in the presence of 10 μg/ml PE for 24 h showed a concentration-dependent reduction of caspase-3/7 activation. B) Serum obtained from mice receiving intranasal ntPEpilinPAK carrier buffer (PBS), ntPEpilinPAK (100 μg IN), or ntPEpilinPAK by s.c. injection with 10 μg ntPEpilinPAK with a cocktail of complete and incomplete Freund's adjuvant (10 μg SC) failed to affect caspase-3/7 activity stimulated by the presence of 10 μg/ml PE. C) Antibody pools enriched from serum samples described for panel B using protein L column chromatography of serum samples demonstrated reductions in caspase-3/7 activity induced by PE in A549 cells. Caspase-3/7 activity was assayed by measuring the enzymatic release of *p*-nitroaniline (pNA) from A549 cells. Data are presented as the percentage of control and are shown as the means ± standard errors of the means for four separate experiments performed in duplicate each time.

preventing critical early host-pathogen interactions. Oligosaccharide- and flagella-based interactions involving *P. aeruginosa* and human airway epithelia have been suggested to incite early host-pathogen interactions (34, 40). Exposure of integrins and fibronectin on the surface of dedifferentiated epithelial cells may also incite some early interactions of *P. aeruginosa* adherence as a prelude to infection (55). Several studies have suggested a role for pilin structures in early binding events of *P. aeruginosa* to epithelial cells, and the potent virulence factor PE may act to further facilitate *P. aeruginosa* infections (outlined in the introduction). We have evaluated the potential for an i.n. immunization strategy with a novel immunogen designed to induce both a mucosal and systemic immune response against a domain of pilin delivered across intact epithelial barriers in a near-native conformation. Our studies support the hypothesis that active mucosal immunity to a conformational pilin structure can lead to diminished host cell interactions involving *P. aeruginosa* and that i.n. immunization with ntPEpilinPAK can also generate immune outcomes that reduce cytotoxic actions associated with both piliated bacteria and secreted PE.

E. coli-expressed ntPEpilinPAK exhibited the anticipated physical and chemical properties of the designed chimera as well as the ability for absorption across intact nasal mucosa through its interaction of epithelia-expressed CD91 (also known as low-density lipoprotein receptor-like protein, LRP1). This uptake and potential targeting to CD91-expressing APCs (62) in draining lymph nodes and spleen (13) was considered a plausible explanation for the observed simultaneous stimulation of mucosal and systemic immune responses demonstrated by ntPEpilinPAK-specific serum IgG and salivary sIgA. Based upon the fact that initial *P. aeruginosa*-epithelial cell interactions are likely to occur in the oropharyngeal cavity of CF patients, we examined the efficacy of saliva from immunized animals to affect in vitro assays that describe bacterial-epithelial cell interactions and PE toxicity. Our results demonstrate that saliva from immunized mice can impede host-pathogen binding and reduce pathogen-mediated cytotoxicity. This combination of immune-related outcomes, although only demonstrated using in vitro models, presents a promising outlook for this vaccination approach and point out that the apparent low levels of measurable antibody responses do not provide an adequate picture of the potential for this vaccination approach.

The salivary immune responses observed in our studies may be due to a combination of both sIgA and IgG. Although salivary antigen-specific IgG likely results from an exudate from serum (22), antigen-specific salivary sIgA should have reached this location through selective translocation of dimeric IgA across epithelial cells (61). The relative amounts of antigen-specific sIgA observed in serum and saliva levels likely represents antigen-specific precursors of IgA plasma cells that correlate with and precede the appearance of sIgA antibodies in external secretions (12). i.n. immunization with ntPEpilinPAK was considered crucial to generation of an optimal protective immune response against oropharyngeal *P. aeruginosa* challenge, because nasally induced IgA antibody secreting cells, based upon their complement of surface adhesion molecule, should traffic back to tissues that include salivary glands and the respiratory tract while systemically injected antigen would not induce this outcome (30). Since PE has been shown to act as a potent virulence factor for *P. aeruginosa* infection, efforts to induce a neutralizing immunization against this toxin have been extensive (8, 17, 18, 21). It is important to note that immunization with ntPEpilinPAK resulted in neutralizing salivary immune responses, although it is unclear whether sIgA or exudates of IgG are more critical.

Numerous studies have examined the potential of bacterial toxins as potent mucosal adjuvants (46). ntPEpilinPAK takes advantage of several unique aspects of the protein PE. First, a completely nontoxic form of PE (ntPE) can be generated by the genetic deletion of one critical amino acid (29). Second,

ntPE, like PE, has been shown to efficiently transport across intact epithelial barriers and selectively target APCs (13). Third, bacterial toxins such as PE frequently provide a mechanism for potent antigen presentation, possibly through their ability to deliver exogenous antigens and activate both major histocompatibility complex class I and class II processing pathways (36). Indeed, injection of ntPE chemically conjugated to capsular polysaccharide from *Salmonella enterica* serovar Typhi has demonstrated this material to generate a safe and effective vaccine to protect children from infection (35). Fourth, a simple i.n. application was sufficient to stimulate both mucosal and systemic immunity. Finally, from a pharmaceutical perspective, ntPE-based chimeras are relatively easy to prepare in a sufficiently pure and properly folded form.

P. aeruginosa can generate a spectrum of cytotoxic agents, such as PE, and can incite cell toxicity by pilin-mediated interactions (9). Additionally, pili are required for cell intoxication induced by the type III secretion system of *P. aeruginosa* (9), and the type III system can disrupt cellular architecture (51). We modeled pili-mediated cell toxicity using an assay that monitored rounding and lifting of a human pulmonary epithelial cell line from its substrate and found that saliva obtained from mice immunized i.n. with ntPEpilinPAK could block these events in a manner consistent with impeding pilin-mediated cell toxicity. Previous studies have shown selective interactions between the DSL and asialo-G_{M1} as an early critical interaction event (23). Our data suggest that by blocking this interaction through generation of a mucosal immune response it may be possible to reduce pathogen binding and subsequent cellular toxicity events involving increased access asialo-G_{M1} on poorly differentiated cells (16). Even though antibody-independent mechanisms have been suggested to be useful in the clearance of chronic *P. aeruginosa* lung infections (44), prophylaxis from initial infection may require stimulation of an antibody response against a surface structure on *P. aeruginosa* involved in epithelial cell binding.

Although our results demonstrate that i.n. administration of ntPEpilinPAK can generate salivary immune responses capable of blocking binding of a piliated strain of *P. aeruginosa* to human airway epithelial cells, no clinical data are yet available to determine the importance of this blockade in CF patients. We have used poorly polarized A549 epithelial cells in some of our studies, since others have suggested that differences in *P. aeruginosa* binding to airway epithelial cells may be enhanced with reduced differentiation (16, 32), but it is unclear if this is essential to infection of CF airway epithelia. We have also shown that saliva from i.n.-immunized mice also reduced bacterial binding to confluent polarized sheets of primary human tracheal cell cultures. Since CF patients harbor unique isolates of *P. aeruginosa* (5, 15), an argument could be made that a cocktail of pilin DSL sequence chimeras may be needed for a broadly effective vaccine. Previous studies have demonstrated, however, that despite amino acid sequence variability within the DSL of *P. aeruginosa* pilin (7), there exists a common type I β-turn followed by a type II β-turn structure within this domain (6). Due to increased side chain structure flexibility within the rigid backbone of this region (63), the DSL can interact through several backbone atoms with the βGalNAc1-4βGal sugar domain present in asialo-G_{M1} and asialo-G_{M2} (28), suggesting an induced fit mechanism (63). Thus, an antibody response generated to a correct conformation rather than a specific amino acid sequence may be required for a broadly protective vaccine. Indeed, serum obtained from rabbits injected with an ntPE chimera containing a shorter PAK pilin amino acid sequence blocked the binding of a wide range of *P. aeruginosa* strains to A549 cells in vitro (25). Overall, ntPEpilinPAK looks to be a promising approach to generate simultaneous mucosal immune responses against a β-turn structure common within this domain (1). The usefulness of such an approach, however, must await clinical assessment, since no viable animal model reflecting early *P. aeruginosa* pulmonary infection events currently exists.

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