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A Nontoxic Adjuvant for Mucosal Immunity to Pneumococcal Surface Protein A[1]

Masafumi Yamamoto,*[†] David E. Briles,* Shingo Yamamoto,* Mari Ohmura,* Hiroshi Kiyono,*[†] and Jerry R. McGhee[2]*

In this study, we demonstrated that pneumococcal surface protein A (PspA) nasally administered with a nontoxic A subunit mutant of cholera toxin (mCT) S61F elicited a protective immune response. Immunization with PspA and mCT elicited higher levels of PspA-specific IgG and IgA Abs in serum and of IgG and IgA anti-PspA Ab-forming cells in spleens, cervical lymph nodes (CLN), and lung tissue when compared to nonimmunized mice. Furthermore, significant PspA-specific IgA Abs were induced in saliva and nasal secretions. These responses were dependent on the use of mCT as a mucosal adjuvant. The PspA-specific Ab responses induced by mCT S61F were comparable with those induced by native CT (nCT). Analysis of cytokine responses showed that nasal PspA plus mCT S61F enhanced the induction of PspA-specific CD4$^+$ T cells producing IL-4 but not IFN-γ in CLN at both the protein and mRNA levels. Importantly, significant numbers of mice intranasally immunized with PspA plus mCT S61F were protected from lethal challenge with capsular serotype 3 *Streptococcus pneumoniae* A66. These results show that intranasal administration of PspA together with mCT S61F is an effective mucosal vaccine against pneumococcal infection and induces CD4$^+$ Th2-type cells, which provide help for both mucosal and systemic Ab responses. *The Journal of Immunology,* 1998, 161: 4115–4121.

*S*treptococcus pneumoniae is known to be a major cause of pneumonia, meningitis, otitis media, ocular infection, and bacteremia. Abs specific for capsular polysaccharides can elicit a protective immune response against pneumococcal infection in adults (1) but not in children (2, 3). Vaccines composed of purified pneumococcal polysaccharides show little promise of protecting against pneumococcal infections in children less than 2 years of age (4).

Protein vaccines have been considered as alternative approaches to induce protective immune response in infants and children to protect from pneumococcal infection. In particular, pneumococcal surface protein A (PspA),[3] which is a surface protein of *S. pneumoniae* and is highly immunogenic, is considered to be a promising vaccine candidate for combating pneumococcal infection (5–7). Not only is it a major virulence factor common to all isolates of *S. pneumoniae* (5–10), but mAbs to it have been shown to protect mice from fatal pneumococcal challenge (11). Immunization studies using PspA have also shown that full-length PspA and the isolated N-terminal half of PspA both induce protective immunity (10, 12). Furthermore, recombinant bacillus Calmette-Guérin expressing PspA as a secreted protein or as a chimeric exported membrane-associated lipoprotein can protect against systemic challenge with different strains of *S. pneumoniae* (13). Thus, PspA appears to be the most attractive candidate Ag for future development of a protein-based vaccine to protect from pneumococcal infections.

Recently, much effort has been focused on inducing immune responses in mucosal tissues since pneumococcal infection is acquired through the mucus membranes of the upper respiratory tract. It has been shown that intranasal immunization with PspA plus cholera toxin B subunit (CT-B) as an adjuvant induces mucosal IgA Ab responses and provides protection against carriage of *S. pneumoniae* (14). In a previous study, we also demonstrated that oral administration of PspA with cholera toxin (CT) as adjuvant induced PspA-specific mucosal IgA Abs as well as protective immunity (15). Although the combination of PspA with the adjuvant CT or CT-B was then shown to be effective against pneumococcal infection, CT, an enterotoxin that causes clinical manifestations of cholera, is unsuitable for use in humans.

In order to eliminate the diarrheagenicity but retain the adjuvanticity of CT, we generated two mutants of CT (mCT) (S61F and E112K), which harbor single amino acid substitutions in the ADP-ribosyltransferase active center that render them enzymatically inactive and thus nontoxic (16). Interestingly, these mutants support Ag-specific immune responses when administered parenterally (16). Furthermore, we have found that mCT S61F acts as a mucosal adjuvant by inducing CD4$^+$ Th2 cells secreting IL-4, IL-5, IL-6, and IL-10, which provide effective help for Ag-specific secretory IgA as well as serum IgG1, IgE, and IgA Ab responses (17).

In this study, we have evaluated the efficacy of using a mucosal vaccine to prevent pneumococcal infection. The results obtained in

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this study suggest that nasal PspA plus mCT S61F would be a practical and effective vaccine candidate for induction of Ag-specific Ab responses in both mucosal and systemic compartments.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory Animal Resources Center (Bar Harbor, ME) and were maintained and bred in this facility under pathogen-free conditions in microisolator cages. All mice were provided sterile food and water ad libitum and were used in this study at 8 to 12 wk of age.

PspA and adjuvants

Full length native PspA was prepared from R36A pneumococci (10). Briefly, supernatants were prepared from pneumococci grown in defined medium in which choline had been replaced with ethanolamine. Pneumococci grown under these conditions release PspA into the medium. This PspA was isolated by passing the PspA-containing supernatants over a choline-Sepharose column. After washing the column with tris-acetate (TA) buffer, the PspA was eluted with a 2% choline in TA buffer.

Escherichia coli containing the plasmid for the mCT S61F gene were grown in LB medium (NaCl, 10 mg/ml; yeast extract, 5 mg/ml; tryptone, 10 mg/ml) with 100 μg/ml of ampicillin. The mCT S61F was purified using a D-galactose-immobilized column (Piece, Rockford, IL) from a cell suspension prepared by sonication of the bacteria, as described previously (16). The purity of mCT S61F was assessed by SDS/PAGE and no contaminating protein bands were noted (17). Native CT (nCT) was purchased from List Biologic Laboratories (Campbell, CA).

Immunization and sample collection

Mice were intranasally immunized on days 0, 7, and 14 with a 10-μl aliquot (5 μl per nostril) containing 100 ng of PspA alone or combined with either 5 μg of mCT S61F or 0.5 μg of nCT (17). Saliva was obtained from mice following i.p. injection with 100 μl of 1 mg/ml pilocarpine (Sigma, St. Louis, MO). Nasal washes were collected by gently flushing the nasal passage with 100 μl of sterile PBS.

Detection of Ag-specific Ab isotype and IgG subclass responses

Ab titers in serum, saliva, and nasal wash were determined by ELISA as described previously (18). Briefly, plates were coated with PspA (100 ng/ml) and blocked with 10% goat serum in PBS. After washing, serial dilutions of serum or fecal extracts were added in duplicate. Following incubation, the plates were washed and peroxidase-labeled goat anti-mouse μ, γ, or α heavy chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to appropriate wells. Finally, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) with H_2O_2 (Moss, Pasadena, CA) was added for color development. For IgG subclass analysis, biotinylated rat monoclonal anti-mouse γl (G1-7.3), γ2a (R19-15), γ2b (R12-3), or γ3 (R40-82) heavy chain-specific Abs (PharMingen) and streptavidin-conjugated peroxidase were employed. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD at 414 nm of 0.1 greater than background after 15 min of incubation. In no case did non-immunized mice give titers greater than $\log_2$ of 5 in sera and $\log_2$ of 2 in external secretions.

Ab-forming cell (AFC) assay

Cervical lymph nodes (CLN) and spleens were aseptically removed and single cell suspensions were obtained as described (19). The nasal passage, submandibular gland (SMG), and lung tissues were carefully excised, teased apart, and dissociated using collagenase type IV (Sigma) (19, 20). The mononuclear cells were obtained at the interface of the 40 and 75% layers of a discontinuous Percoll gradient (Pharmacia). To assess number of PspA-specific AFCs, an enzyme-linked immunospot (ELISPOT) assay was performed as previously described (15). Briefly, 96-well nitrocellulose plates (Milliliter HA; Millipore, Bedford, MA) were coated with PspA (100 ng/ml), incubated, and then plates were washed and blocked with 10% goat serum. The blocking solution was discarded, cells at various dilutions were added, and the cell suspensions were incubated for 4 h at 37°C in 5% CO_2 in moist air. The detection Ab consisted of goat anti-mouse α or γ heavy chain-specific, horseradish peroxidase-conjugated Abs (Southern Biotechnology Associates). Following overnight incubation, plates were washed with PBS and developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 (Moss) to each

well. Plates were incubated at room temperature for 15 to 20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope.

PspA-specific CD4+ T cell responses

CD4+ T cells from CLN cell suspensions were purified by use of the magnetic-activated cell sorter system (Miltenyi Biotec, Sunnyvale, CA). Cells were added to a nylon wool column (Polysciences, Warrington, PA) and incubated at 37°C for 1 h to remove adherent cells. The enriched T cell populations were then incubated with biotinylated anti-CD8 (53-6.7), anti-Mac-1 (M1/70), and anti-B220 (RA3-6A2) mAbs followed by streptavidin-conjugated microbeads before being passed through the magnetic column. Two cycles of the above procedures yielded CD4+ T cell preparations that were >98% pure. Purified CLN CD4+ T cells (2×10^6 cells/ml) were cultured with T cell-depleted, irradiated splenic feeder cells (2.5×10^6 cells/ml) from naive mice in complete medium (RPMI 1640, Cellgro Mediatech, Washington, DC) containing 10% FCS, 1% L-glutamine, 50 μM 2-ME, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamicin. The cultures were supplemented with 10 U/ml of mouse rIL-2 (Genzyme, Cambridge, MA). PspA (1 μg/ml) was added for restimulation of CD4+ T cells. The CD4+ T cell cultures were incubated for 4 days at 37°C in 5% CO_2 in air. To measure cell proliferation, 0.5 μCi of [^{3}H]thymidine (DuPont/New England Nuclear Products, Boston, MA) was added to individual culture wells 15 h before termination, and the uptake of counts per minute by dividing cells was determined by scintillation counting.

Quantitative analysis of cytokine-specific mRNA

For evaluation of cytokine-specific mRNA levels, a quantitative RT-PCR was employed. Total RNA was isolated by the acid guanidinium thiocyanate phenol chloroform extraction procedure. Cytokine-specific rDNA for IFN-γ and IL-4 were used as the internal standards as described in detail previously (16, 21). Briefly, aliquots of total RNA were subjected to standard RT. RT products with a series of diluted rDNA internal standards were amplified by PCR. For quantification, capillary electrophoresis with the laser-induced fluorescence detection system (CE-LIF) (LIF-P/ACE, Beckman Instruments, Fullerton, CA) was applied as described previously (22, 23). An analysis of PCR products was conducted by using a coated capillary tube in Tris-borate EDTA containing replaceable linear polyacrylamide and fluorescent intercalator. The fluorescence content of each cytokine-specific RT-PCR product was expressed as the peak area of relative fluorescence light units.

Analysis of secreted cytokines

Cytokine levels in culture supernatants were determined by a cytokine-specific ELISA as described previously (24, 25). Nunc MaxiSorp Immunoplates (Nunc, Naperville, IL) were coated with monoclonal anti-IFN-γ (R4-6A2) or anti-IL-4 (BVD4-1D11) Abs (PharMingen). After blocking, samples and serial twofold dilutions of standards were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated with biotinylated monoclonal anti-IFN-γ (XMG 1.2) or anti-IL-4 (BVD6-24G2). After incubation, peroxidase-labeled anti-biotin Ab (Vector Laboratories, Burlingame, CA) was added and developed with ABTS containing H_2O_2 (Moss). Standard curves were generated using mouse rIFN-γ (Genzyme) and rIL-4 (Endogen, Boston, MA).

Mouse protection assay

The pneumococcal in vivo protection assay was carried out as described previously using log-phase pneumococci diluted in Ringer's lactate (26). Four weeks after the last immunization, mice were challenged i.v. with 2.5×10^6 CFU of capsular serotype 3 S. pneumoniae A66. The survival of the mice was monitored for 12 days.

Statistics

The data are expressed as the mean ± SEM and compared using one-tailed Wilcoxon two-sample rank test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for Macintosh computers.

Results

Analysis of PspA-specific immune responses

In the initial study, we sought to determine if intranasal administration of PspA with mCT S61F as mucosal adjuvant could induce

FIGURE 1. PspA-specific serum Ab isotype responses (*A*) and numbers of IgG and IgA AFC in spleen, CLN, and lung tissues (*B*). Groups of C57BL/6 mice were intranasally immunized with 100 ng of PspA plus 5 μg of mCT S61F (■), 100 ng of Psp A plus 0.5 μg of nCT (□), or PspA alone (▨) on days 0, 7, and 14 and serum Ab titers were assessed on day 21. Mononuclear cells were isolated from spleen, CLN, and lung tissues and then examined by ELISPOT. The results are expressed as the mean ± SEM obtained for five mice per group. The data are representative of three separate experiments.
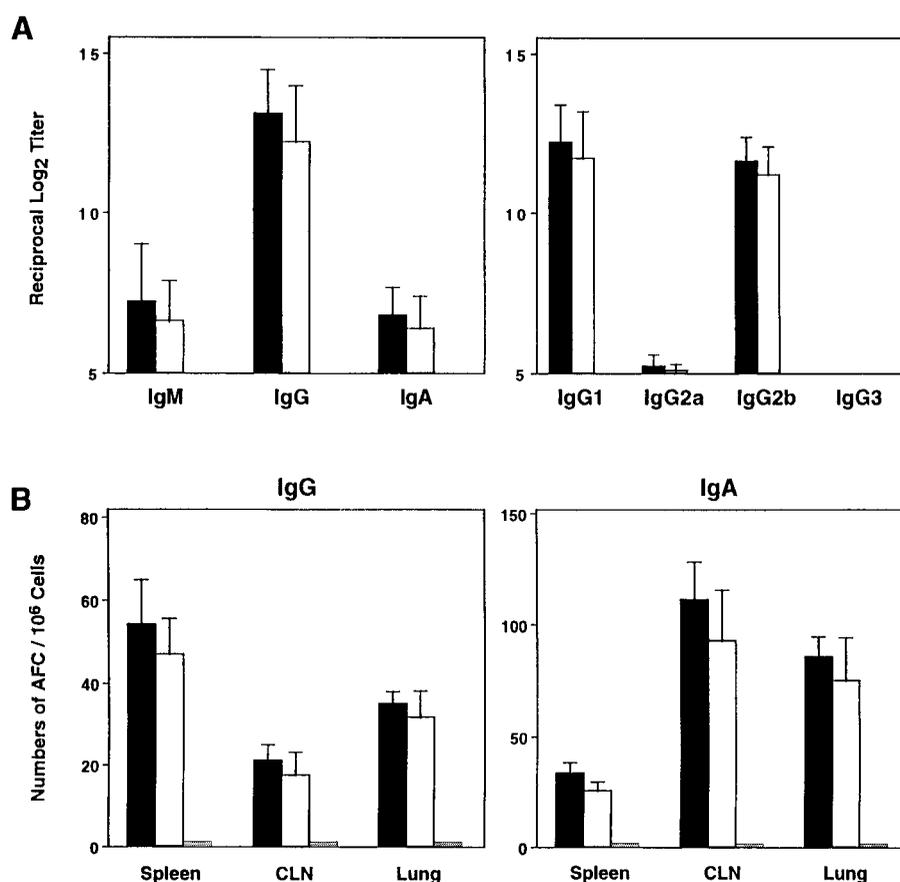
PspA-specific Ab responses. Mice intranasally immunized with PspA plus mCT S61F showed significant PspA-specific serum IgM, IgG, and IgA Ab responses, which were comparable to those induced by PspA with nCT as adjuvant. In contrast, Ab responses were not detected after immunization with PspA alone (Fig. 1*A*). As expected, administration of mCT S61F or nCT alone did not induce PspA-specific Ab responses that were above the dilution cutoff ($\log_2$ of 5) used in our experiments. Analysis of IgG subclass responses in mice given PspA plus mCT S61F or nCT revealed that the major subclasses were IgG1 and IgG2b (Fig. 1*A*). AFC responses supported the serum Ab titers and showed that significant numbers of PspA-specific IgG- and IgA-producing cells were found in the spleens of mice given PspA plus mCT S61F or nCT as adjuvants. Furthermore, high numbers of IgG and IgA AFCs were detected in cells isolated from CLN and lung tissues of mice given PspA with mCT S61F or nCT as mucosal adjuvants (Fig. 1*B*).

Since mCT S61F given intranasally has been shown to be an effective adjuvant for the induction of Ag-specific mucosal IgA Abs to coadministered proteins (17), it was important to determine whether PspA-specific IgA Ab responses were induced in mucosal secretions when mCT S61F was given with PspA. Our results showed that intranasal coadministration of PspA and mCT S61F induced PspA-specific mucosal IgA Ab responses in saliva and nasal wash samples that were comparable to those induced by nCT as adjuvant. In contrast, essentially no Abs were detected in either saliva or nasal washes of mice that received PspA alone (Fig. 2). As expected, nasal delivery of mCT S61F or nCT alone failed to elicit PspA-specific Ab titers greater than $\log_2$ of 2. AFC analysis confirmed the above results by revealing significant numbers of PspA-specific IgA AFC in SMG and nasal passages following in-

tranasal administration of PspA plus mCT S61F or nCT and by showing low AFC responses in SMG and nasal passages of mice given PspA alone (Fig. 2).

PspA-specific CD4⁺ T cell responses

Since intranasal immunization with PspA plus mCT S61F induced PspA-specific Ab responses in both mucosal and systemic compartments, it was important to establish the nature of the CD4⁺ T cell help supporting PspA-specific Ab responses. When CD4⁺ T cells from spleen or CLN of mice immunized with PspA plus mCT S61F were restimulated with PspA in vitro, levels of proliferative responses induced were identical to those seen with CD4⁺ T cells from mice immunized with PspA plus nCT. Essentially no increased proliferation occurred in spleen or CLN taken from mice given PspA alone (Fig. 3). These results indicate that mCT S61F, like nCT, is an effective adjuvant for the induction of PspA-specific CD4⁺ T cells in both mucosal and systemic tissues.

Analysis of cytokine-specific mRNA by quantitative RT-PCR revealed that PspA-specific CD4⁺ T cells from CLN of mice given PspA plus mCT S61F or nCT produced high levels of IL-4 (Th2-type)- but only low levels of IFN-γ (Th1-type)-specific mRNA. In contrast, no IL-4-specific mRNA was detected in CD4⁺ T cells from mice given PspA alone. Furthermore, IFN-γ-specific mRNA was detectable in mice given PspA alone but in much lesser quantity than that observed in mice given mCT S61F or nCT (Fig. 4*A*).

IL-4 and IFN-γ production were further analyzed at the protein level. Increased levels of IL-4 production with low IFN-γ were seen in PspA-specific cultures from mice nasally immunized with PspA plus mCT S61F or nCT, a finding in complete agreement with results of cytokine analysis at the transcriptional level (Fig. 4*B*). As expected, CLN CD4⁺ T cells from mice given PspA alone
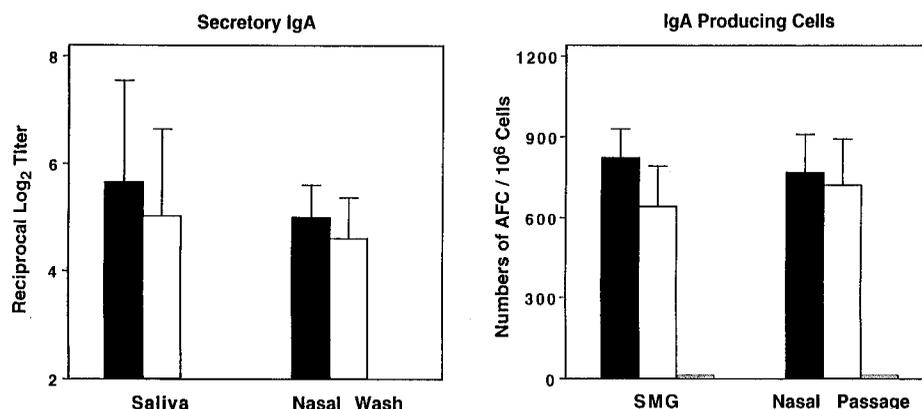
FIGURE 2. Detection of PspA-specific IgA Ab responses in saliva and nasal washes and numbers of IgA AFC in SMG and nasal passages. Groups of C57BL/6 mice were immunized with PspA plus mCT S61F (■), PspA plus nCT (□), or PspA alone (▨) as described in the legend of Figure 1. Tissue samples and external secretions were assessed for IgA AFC or IgA Ab titers at day 21. The results are expressed as the mean ± SEM obtained for five mice per group. The data are representative of three separate experiments.

produced low or undetectable levels of IFN-γ or IL-4. Taken together, these studies show that intranasal administration of PspA plus mCT S61F as adjuvant induces Th2-type cytokine responses to support PspA-specific mucosal IgA as well as serum IgG1 and IgG2b Ab responses.

Intranasal immunization with PspA plus mCT S61F elicits protective immunity

In the present study, we sought to determine whether intranasal PspA plus nontoxic mCT S61F, which elicited immune responses to PspA, were also protective. Mice given PspA plus mCT S61F, PspA plus nCT, or PspA alone were challenged i.v. with virulent A66 pneumococci. As expected, intranasal immunization with PspA plus nCT provided significant protection ($p = 0.0011$) against a lethal dose of A66 (Fig. 5). It is important to note that PspA given intranasally with nontoxic mCT S61F possessed protective ability ($p = 0.0009$) comparable to or even better than that induced by nCT. In contrast, all mice given PspA alone, mCT S61F alone, or nCT alone were not protected and died within 3 days (Fig. 5). These findings indicate that Ab responses elicited by PspA plus mCT S61F were protective against pneumococcal infection.

Discussion

In the present study, we have assessed the potential of a combined intranasal vaccine, PspA with nontoxic mutant of CT S61F, to induce an immune response that would protect the host from challenge. PspA has been shown to induce protective humoral immune responses when given parenterally (10, 13, 26, 27). Furthermore, oral or intranasal delivery of PspA with CT or with CT-B as adjuvant is known to elicit mucosal IgA Ab responses in addition to serum Abs (14, 15). In this study, we have shown that intranansal administration of PspA with mCT S61F as nontoxic adjuvant induced significant PspA-specific IgM, IgG, and IgA Ab responses in serum and IgA Abs in mucosal secretions of the upper respiratory tract. Furthermore, these Ab responses provided significant protection against systemic challenge with pneumococci. Thus, PspA is an effective Ag for induction of protective immune responses against pneumococcal infection; however, mucosal immunization with PspA alone failed to enhance anti-PspA Abs in both serum and mucosal secretions. Indeed, previous studies have also reported that PspA without adjuvant is a weak immunogen when given via the mucosal route (14, 15), although parenteral immunization did elicit protective immunity to *S. pneumoniae* infection
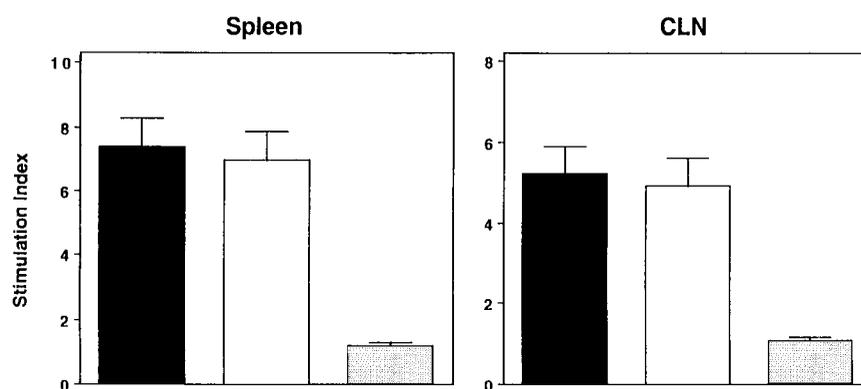

FIGURE 3. PspA-specific CD4^{+} T cell proliferative responses. Groups of C57BL/6 mice were immunized with PspA plus mCT S61F (■), PspA plus nCT (□), or PspA alone (▨) as described in the legend of Figure 1. CD4^{+} T cells were isolated from spleen or CLN on day 21 and were cultured with 1 μg/ml of PspA in the presence of irradiated, T cell-depleted spleen cells and rIL-2. The results were similar and are representative of three separate experiments. Counts for control cultures containing rIL-2 alone in medium were 1994 ± 254 cpm (spleen cells from mice given PspA with mCT S61F), 1956 ± 129 cpm (spleen cells from mice given PspA with nCT), 1986 ± 213 cpm (spleen cells from mice given PspA only), 2087 ± 146 cpm (CLN from mice given PspA with mCT S61F), 2531 ± 29 cpm (CLN from mice given PspA with nCT), and 2112 ± 213 cpm (CLN from mice given PspA only).
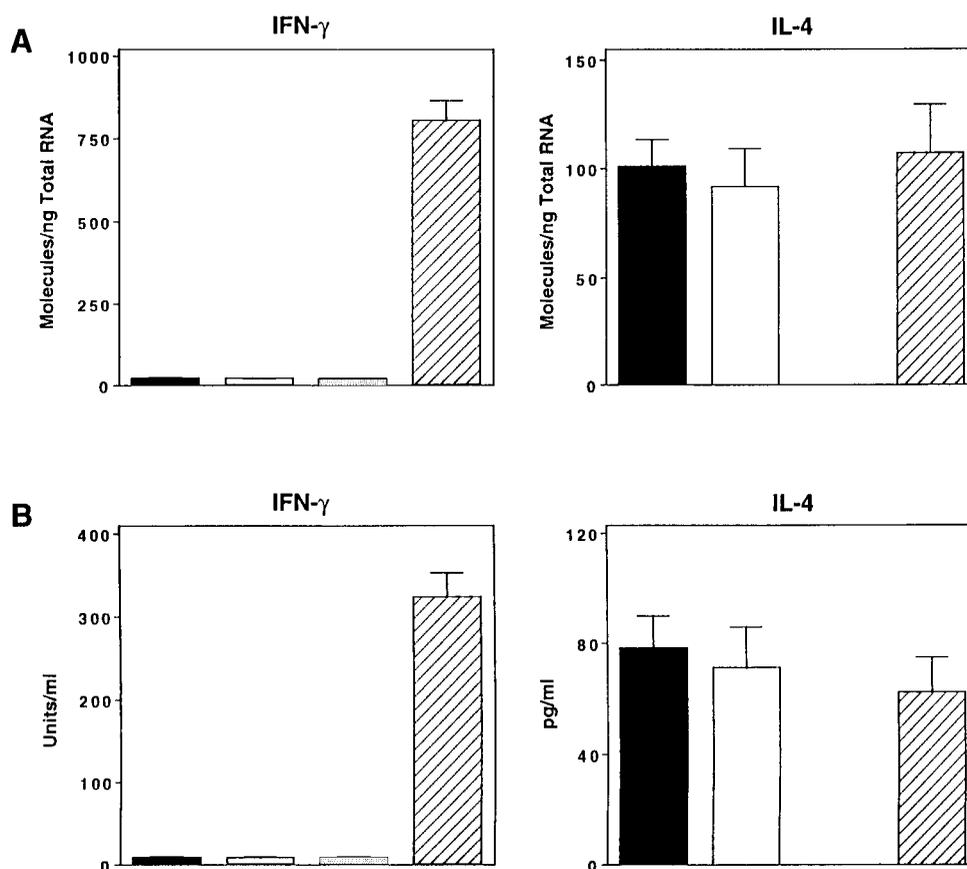
FIGURE 4. Quantitative analysis of Th1 (IFN-γ) and Th2 (IL-4) cytokine-specific mRNA expression (*A*) and protein synthesis (*B*) by CD4$^+$ T cells isolated from CLN of mice intranasally immunized with PspA plus mCT S61F (■), PspA plus nCT (□), or PspA alone (▨). Cytokine molecules per nanogram of total RNA were determined by a quantitative RT-PCR using competitive rDNA and CE-LIF analysis. Culture supernatants were harvested and then analyzed for the elucidation of secreted cytokines using the appropriate cytokine-specific ELISA. mRNA molecules and protein levels produced by nonimmunized CD4$^+$ T cells stimulated with anti-CD3 mAb were used as controls (▨). The results are expressed as the mean ± SEM from five mice per group and were taken from a total of three separate experiments. IL-4 was not detected in cells incubated with rIL-2 alone at both mRNA and protein levels. The mRNA levels of IFN-γ for control cultures containing rIL-2 alone were 19.2 ± 2.1 (PspA plus mCT S61F), 22.3 ± 3.1 (PspA plus nCT), and 21.6 ± 2.4 (PspA alone). Protein levels of IFN-γ for control cultures were 8.2 ± 1.4 (PspA plus mCT S61F), 10.3 ± 0.6 (PspA plus nCT), and 8.7 ± 0.9 (PspA alone).

in the absence of adjuvant (10). These earlier studies together with our findings indicate that a mucosal adjuvant such as CT is required for induction of PspA-specific Ab responses in both mucosal and systemic compartments when PspA is given by mucosal routes such as the intranasal one.

CT and purified CT-B have been used widely as adjuvants for mucosal immunization (14, 15, 18, 28–34), and our results indicate that intranasal administration of nCT possesses adjuvant effects to PspA and induces PspA-specific mucosal IgA, as well as serum IgM, IgG, and IgA Ab responses. However, despite these beneficial attributes, CT is unsuitable for humans since it causes severe diarrhea (35). CT consists of one A subunit (CT-A) and five B subunits (35). CT-B selectively binds GM1 cell surface receptors and promotes the entry of CT-A. Following entry into cells, CT-A catalyzes ADP-ribosylation of Gsα, resulting in elevated intracellular cyclic AMP levels, which in epithelial cells cause secretion of water and chloride ions into the small intestine with a characteristic watery diarrhea (36, 37). Thus, in order to develop an adjuvant suitable for use with the pneumococcal vaccine for humans, ADP-ribosytransferase activity of CT-A must be eliminated. The studies presented here again demonstrate that mutant CT is an effective mucosal adjuvant for intranasal immunization, and that when given with PspA, it facilitates the development of

protective levels of Ab to PspA. The results presented here are the first to show that a nontoxic mutant of CT, designated S61F and carrying a single amino acid substitution in the A subunit, induces Ab responses in both serum and mucosal secretions to coadministered PspA. These findings suggest that mCT S61F may be a more promising mucosal adjuvant for PspA than nCT.

Intranasally coadministered mCT S61F, which was shown to lack ADP-ribosyltransferase activity and diarrheagenicity (16), possessed adjuvant effects for PspA. Earlier studies had suggested that the adjuvanticity of CT and that of the related *E. coli* heat-labile toxin (LT) were closely associated with ADP-ribosyltransferase activity (38). However, our previous studies have shown that two mutants of CT (S61F and E112K), which lack ADP-ribosyltransferase activity, still supported Ag-specific immune responses (16, 17). Furthermore, several groups have reported that single amino acid substitution mutants of LT (R7K, S63K, and R192G), which lack toxicity, retain their adjuvant properties (39–41). These studies suggest that ADP-ribosyltransferase activity is not required for the adjuvant effects of CT and LT. However, it should be noted that although mCT S61F possesses adjuvanticity in the absence of ADP-ribosyltransferase activity, 10-fold higher doses of mCT S61F were required to elicit PspA-specific Ab responses comparable with nCT, implying that adjuvanticity of CT
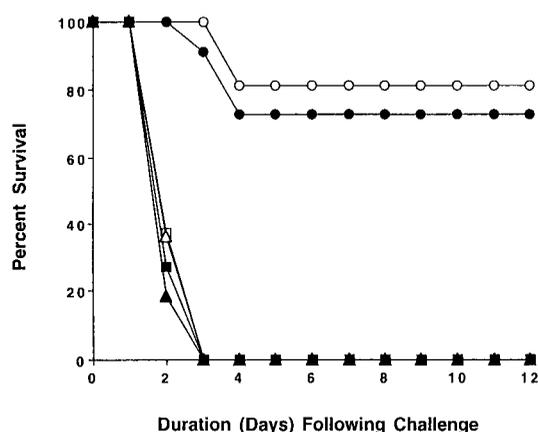
FIGURE 5. Protection against fatal challenge with pneumococci. Groups of C57BL/6 mice were intranasally immunized with 100 ng of PspA plus 5 μg of mCT S61F ($\bigcirc$), 100 ng of PspA plus 0.5 μg of nCT ($\bullet$), 100 ng of PspA alone ($\square$), 5 μg of mCT S61F alone ($\blacksquare$), or 0.5 μg of nCT alone ($\triangle$) on days 0, 7, and 14. Four weeks after the last immunization, mice were challenged i.v. with 2.5×10^6 CFU of capsular serotype 3 *S. pneumoniae* A66. PspA + mCT vs nonimmunized group ($\blacktriangle$) at *p* = 0.0009; PspA + nCT vs nonimmunized group at *p* = 0.0011.

may be derived from two or more mechanisms and that ADP-ribosyltransferase activity may also contribute to the adjuvanticity of CT and derivatives. Studies are underway to elucidate these additional steps.

Although past studies have demonstrated that CT and purified CT-B possess adjuvant effects for PspA and induce PspA-specific Ab responses in serum and mucosal secretions (14, 15), the nature of helper CD4$^+$ T cells for these Ab responses has not been established. In this study, we have shown that PspA plus mCT S61F induced PspA-specific CD4$^+$ T cells in CLN and spleen when given intranasally. Furthermore, analysis of cytokine patterns produced by CD4$^+$ T cells revealed significant IL-4 synthesis with minimal IFN-γ synthesis, indicating that PspA with mCT S61F elicits PspA-specific CD4$^+$ Th2-type cells in both mucosal and systemic tissues. Previous studies by our group support this finding by showing that mCT, like nCT, induces significant Th2-type cytokine responses with subsequent serum IgG1, IgG2b, IgE, and IgA, and mucosal IgA Abs to coadministered Ag when given intranasally (17) or orally (unpublished observation). Taken together, these findings indicate that PspA plus mCT S61F as mucosal adjuvant generates PspA-specific Th2-type responses that account for the induction of IgG1, IgG2b, and IgA Ab responses in serum, and IgA Abs in external secretions of the respiratory tract.

The cellular and molecular basis for induction of Ab responses by the mucosal adjuvant CT has not yet been elucidated. A previous study has shown that B7-1 (CD80) and B7-2 (CD86) expression on B cells is enhanced by the administration of CT or a fusion protein retaining an intact A1 subunit and so exhibiting ADP-ribosyltransferase enzyme activity (42). Other studies have shown that CT enhances B7-2 but not B7-1 on macrophages and that macrophages treated with CT enhance proliferative responses of T cells activated by anti-CD3 or alloantigens (43). Both studies suggested that CT adjuvanticity was associated with B7 cosignaling and that ADP-ribosyltransferase enzyme activity was required for the induction of B7 expression. However, our separate study has indicated that mCT, which completely lacks ADP-ribosyltransferase activity, induced both B7-1 and B7-2 expression on Peyer's patch B cells and macrophages (M.Y., unpublished observation). Furthermore, our present and past studies demonstrate that mCT

induces Ag-specific serum IgG and mucosal IgA Ab responses to coadministered proteins (17). Based upon our studies, we conclude that ADP-ribosyltransferase activity can be separated from the adjuvant properties of CT.

The immunization protocol used in this study was designed to induce significant PspA-specific Ab responses in mucosal secretions and serum. We chose intranasal administration as the delivery route because it offered several advantages over oral immunization. First, intranasal administered Ag interacts directly with IgA-inductive sites termed nasal-associated lymphoreticular tissue. Second, lower doses of vaccine Ag are required because this mode of delivery does not subject the Ag to the enzyme and acids of the gastrointestinal tract and so minimizes Ag loss through the process of digestion. Only 100 ng of PspA were required to generate an Ab response equivalent to that elicited by 7.5 μg of orally administered PspA (15). Third, it has been suggested that since intranasal immunization induces lower IgE Ab responses than those induced by oral immunization, this immunization regimen may have less risk for anaphylactic reactions (17).

In summary, the present study has provided evidence that intranasal immunization with a vaccine containing PspA plus mCT S61F elicited PspA-specific CD4$^+$ Th2 cells in both mucosal and systemic tissues with subsequent IgA Ab responses at the mucosal surfaces of the respiratory tract, and IgG and IgA Abs in serum. Furthermore, PspA-specific responses induced by PspA plus mCT S61F provided protective immunity against pneumococcal infection. The combination of PspA and mCT provides a very effective means of eliciting protective levels of Ab to PspA, and therefore should be considered as a candidate adjuvant for vaccinating humans against pneumococci.

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