Oral Immunization with PspA Elicits Protective Humoral Immunity against *Streptococcus pneumoniae* Infection

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Streptococcus pneumoniae is a major respiratory mucosal pathogen affecting infants and children. Although a polysaccharide-based vaccine has been useful in adult populations, it does not elicit protective immunity in infants and young children. Pneumococcal surface protein A (PspA) is a highly immunogenic surface protein produced by all strains of *Streptococcus pneumoniae*. Previous studies have shown that systemic immunization of mice with PspA can elicit protective immunity against fatal pneumococcal infection. In this study, we demonstrated that oral immunization with PspA could elicit protective immune responses against pneumococcal infection. When mice were orally immunized with PspA alone, low levels of PspA-specific immunoglobulin G (IgG) responses were induced in serum; none was induced in secretion. On the other hand, when PspA was given orally with the mucosal adjuvant cholera toxin (CT), significant levels of IgG and IgA anti-PspA responses were induced in serum. The major IgG subclass was IgG1, followed by IgG2b, a profile of antibody response supported by Th2-type cells. In addition, all mice orally immunized with PspA and CT were protected from the lethal challenge with capsular serotype 3 *S. pneumoniae* A66. These results suggested that an oral PspA vaccine may be a useful means of preventing pneumococcal disease.

Streptococcus pneumoniae is a major respiratory mucosal pathogen which causes meningitis, otitis media, bacteremia, and pneumonia (7, 14, 30, 34). It has been well established that specific antibodies to the capsular polysaccharides can elicit a protective immune response against pneumococcal infection in adults (22). In the case of children, however, the polysaccharide vaccines do not effectively elicit pneumococcus-specific protective antibody responses (1, 8, 12, 19), and children can have repeated infections involving strains of the same capsular serotype (13). Protein polysaccharide conjugates and pneumococcal proteins alone have been considered as alternative means to induce protective immunity in infants and children from pneumococcal infection.

Pneumococcal surface protein A (PspA) is a surface protein of *S. pneumoniae* and is a virulence factor (3, 4, 27). PspA is highly immunogenic and is found on all isolates of *S. pneumoniae* (9, 25–27). Although PspA exhibits variability among different strains, it appears to elicit cross-protection against different capsular type strains (29). Previous studies have shown that monoclonal antibodies to PspA are able to protect mice from fatal pneumococcal infection (24, 25). Systemic immunization with full-length PspA and the isolated N-terminal half of PspA have been shown to elicit protective immunity (5, 32). It has also been demonstrated that immunization of X-linked immunodeficient (XID) mice, which are not able to respond to polysaccharide antigens, with purified PspA or non-encapsulated pneumococci expressing PspA are protected from subsequent fatal infection with pneumococci; isogenic

pneumococci lacking PspA do not elicit protective immune responses in these mice (5, 28). It has been shown that recombinant *Mycobacterium bovis* bacillus Calmette-Guerin expressing the N-terminal half of PspA as a secreted protein or as a chimeric exported membrane-associated lipoprotein can elicit cross-protective humoral immunity against different pneumococcal strains (20).

In this study, we have used oral administration of PspA with the mucosal adjuvant cholera toxin (CT) to assess whether protective immunity could be achieved by this route of immunization.

MATERIALS AND METHODS

Mice. C57BL/6 mice of 8 to 12 weeks of age were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, Md.). They were kept in horizontal laminar-flow cabinets and provided with sterile food and water ad libitum.

Antigens and immunization. Full-length native PspA was prepared from R36A pneumococci (rough variant of the type 2 strain D39) which were grown in a defined medium in which choline had been replaced with ethanolamine. Under these conditions, PspA is secreted rather than bound to the cell surface (5, 37). Supernatant from pneumococcal cells secreting PspA was passed over a choline-Sepharose column. Following washing with Tris-acetate (TA) buffer (pH 6.9), the PspA was eluted with a 2% choline-TA buffer. For parenteral immunization, 0.5 µg of PspA emulsified in complete Freund's adjuvant was given subcutaneously on day 0 and followed by a boost in incomplete Freund's adjuvant on day 14. For oral immunization, mice were deprived of food for 2 h and then given a solution of sodium bicarbonate to neutralize stomach acidity prior to the immunization (16). Thirty minutes later, mice were orally immunized by gastric intubation with 7.5 µg of PspA either in the presence or absence of 10 µg of the mucosal adjuvant CT in 200 µl of phosphate-buffered saline (PBS) per mouse. Oral immunization were carried out on days 0, 7, 14, and 35.

ELISA. Serum and fecal extracts were prepared as described previously (16, 23). Serum and fecal extracts were collected at weekly intervals and assayed for PspA-specific immunoglobulin M (IgM), IgG, and IgA antibodies by enzyme-linked immunosorbent assay (ELISA). Ninety-six-well plates (Immuno Plate; Nunc, Roskilde, Denmark) were coated with PspA in PBS overnight at 4°C. After washing three times with PBS, the wells were blocked for 1 h with PBS containing 10% goat serum. Serial twofold dilutions of samples starting at a

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FIG. 1. Induction of PspA-specific systemic immune responses by subcutaneous immunization with PspA. (A) Mice were subcutaneously immunized with 0.5 μ g of PspA emulsified in complete Freund's adjuvant on day 0; a boost in incomplete Freund's adjuvant on day 14 followed. Serum samples were examined to determine the levels of PspA-specific IgM (■), IgG (□), and IgA (▒) antibody titers. Nonimmunized mice always gave titers of less than $\log_2$ of 3. (B) To confirm production of PspA-specific AFC, splenic mononuclear cells were isolated from these parenterally immunized mice at 4 days postimmunization and then examined by ELISPOT assays. The results are expressed as means $\pm$ standard deviations obtained for six mice per group.

dilution of 1:2 (fecal extract) and 1:32 (serum) were tested. The plates were incubated for 4 h at room temperature, and antibodies were detected with biotinylated anti-mouse μ , γ , and α heavy-chain-specific antibodies (Southern Biotechnology Associates, Birmingham, Ala.) and horseradish peroxidase-streptavidin (GIBCO, Gaithersburg, Md.). ABTS [2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), diammonium salt; Sigma Chemical Co., St. Louis, Mo.] was used for color development. Titers were recorded as the last dilution which gave an A_{414} of 0.1 U greater than background. In no case did nonimmunized sera give titers greater than $\log_2$ of 3.

Luminometry. To assess PspA-specific fecal IgA responses, we applied the method of luminometry as described previously (17). Briefly, 96-well plates (Microtiter; Dynatech, Chantilly, Va.) were prepared exactly as described for ELISA. Briefly, following the incubation of the secondary biotinylated antibody, the plates were washed with PBS-Tween containing 2 mM EGTA and Aqualite streptavidin (Sealite Science Inc., Bogard, Ga.) and incubated for 1 h at room temperature. The plates were washed with PBS-Tween-EGTA, dried, and placed in the luminometer. The wells containing the acquirin derivatives were developed in a Dynatech ML-3000 luminometer by injection of 100 μ l of a buffer containing 20 mM Ca^{2+} acetate in 50 mM Tris (pH 7.5). The luminometer was programmed to integrate counts over 3-s intervals. Results were expressed as relative light units following subtraction of blank values. To determine endpoint titers for the luminometric assay, the straight line generated following background subtraction was extrapolated (E titer) to a baseline 1,000 relative light units above background.

ELISPOT assay. To assess numbers of PspA-specific antibody-forming cells (AFC), an ELISPOT assay was performed (16, 23). Single-cell suspensions were prepared from the spleen by mechanical dissociation. Ninety-six-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, Mass.) were used for the assay. The plates were coated with 0.5 μ g of PspA, and after incubation overnight at 4°C, they were washed and blocked with 10% fetal calf serum (FCS)-RPMI 1640. The blocking solution was discarded, and 100 μ l of cells in 10% FCS-RPMI 1640 at various dilutions was added. Cell suspensions were incubated for 4 h at 37°C in 5% CO_2 . The plates were washed with PBS and PBS-0.05% Tween 20. The detection antibody consisted of 100 μ l of a 1:1,000 dilution of goat anti-mouse μ , γ , and α heavy-chain-specific horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates) in PBS-0.05% Tween 20 containing 1% bovine serum albumin (BSA). Following overnight incubation, the plates were washed with PBS and developed by the addition of 200 μ l of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 per well. The plates were incubated at room temperature for 15 to 20 min and washed with water, and AFC were counted with the aid of a stereomicroscope. In nonimmunized mice, the number of AFC was always less than 1 per 10^6 cells.

Mouse protection assay. The pneumococcal in vivo protection analysis was performed by a previously described protocol (29). Briefly, 14 days after the fourth immunization, mice were challenged intravenously with 3.6×10^3 CFU of capsular serotype 3 *S. pneumoniae* A66. The survival of mice was monitored for 21 days. The number of CFU of *S. pneumoniae* per milliliter of peripheral blood

was determined by plating serial dilutions of peripheral blood obtained 24 h postchallenge (2).

Statistics. The results were analyzed statistically by the two-tailed Wilcoxon two-sample rank test.

RESULTS

Analysis of PspA-specific immune responses following systemic immunization. The immunogenicity of the PspA used in these studies was first evaluated by subcutaneous immunization to obtain serum antibody levels for comparison with oral immunization. PspA-specific IgG responses were detected by 14 days after the primary immunization, and levels of PspA-specific titers were increased by a second immunization. These PspA-specific IgG responses persisted for more than 70 days (Fig. 1A). To examine these responses at the level of PspA-specific IgG-secreting cells, mice were sacrificed 4 days after secondary immunization. Splenic mononuclear cells were isolated and assayed for PspA-specific AFC by the ELISPOT assay. Significant numbers of PspA-specific IgG AFC were detected in splenocytes isolated from mice subcutaneously immunized with PspA when compared with that of the control group of unimmunized mice. On the other hand, numbers of IgM and IgA AFC were either low or undetectable (Fig. 1B). These results are consistent with previous findings that systemic immunization with PspA induces high levels of antigen-specific serum responses (29).

Induction of PspA-specific serum antibody responses by oral immunization with PspA without CT. To evaluate the ability of oral immunization with PspA to induce serum antibody responses, a group of mice were orally immunized with PspA. When the antigen-specific immune response was analyzed by ELISA, PspA-specific serum IgG responses were noted after primary immunization; however, the antibody titers were low (1:32 to 1:128) when compared with the titers of 1:512 to 1:16,384 obtained by systemic immunization (Fig. 1A and 2A). Furthermore, serum antibodies induced by oral immunization were not enhanced by an oral booster. Very small serum IgA responses (1:32) were detected 3 weeks after a

FIG. 2. Kinetics of antigen-specific serum antibody responses in mice orally immunized with PspA. Mice were orally immunized with 7.5 μ g of PspA (A) or 7.5 μ g of PspA together with 10 μ g of CT (B). Mice were orally immunized on days 0, 7, 14, and 35. Serum samples were examined to determine the levels of PspA-specific IgM (■), IgG (□), and IgA (▒) antibodies. The results are expressed as means $\pm$ standard deviations of $\log_2$ ELISA antibody titers obtained for six mice per group. Nonimmunized mice always gave titers of less than $\log_2$ of 3.

fourth oral immunization (day 35). No antigen-specific IgM responses were detected during the analysis period (Fig. 2A). In the case of fecal extracts, no detectable level of antigen-specific immune responses was observed (data not shown).

Oral administration of PspA mixed with CT induced high levels of PspA-specific serum IgG1 and IgG2b as well as mucosal IgA responses. It has been shown that oral administration of mucosal adjuvant CT with soluble protein antigens not only induces specific serum antibody responses to CT, but it also proved to be an effective regimen for the generation of antigen-specific antibody responses in mucosal sites (16, 23, 36). When PspA was given orally with CT on days 0, 7, 14, and 35, antigen-specific IgM, IgG, and IgA responses were induced in the serum. After the second immunization, PspA-specific IgM antibodies were observed (1:512). After the third immunization, a secondary type of response was obtained with high levels of IgG (1:16,000) on day 21 (Fig. 2B). These PspA-specific IgG responses were further elevated (1:524,000) after a fourth oral immunization with PspA plus CT. PspA-specific serum IgA antibody responses were not detected until days 42 and 49 (Fig. 2B). In addition, antigen-specific IgA antibodies were detected in fecal extracts obtained 2 weeks after the third oral immunization, and their levels increased following the fourth immunization (Fig. 3). During the immunization period, all of the mice maintained their body weight and none showed symptoms of diarrhea. When the subclasses of PspA-specific IgG were evaluated, the major subclass was IgG1, with IgG2b being second highest (Fig. 4). Taken together, these results indicate that oral immunization of PspA, with CT as the mucosal adjuvant, was capable of inducing PspA-specific serum as well as mucosal antibody responses.

FIG. 3. Antigen-specific fecal IgA responses in mice orally immunized with PspA plus CT. These are the same mice whose serum responses are illustrated in Fig. 2. Antibody titers were examined in pooled fecal extracts collected from six mice by ELISA using luminometry. Nonimmunized mice or mice immunized with PspA alone gave titers of less than $\log_2$ of 2 in this assay.

Oral immunization with PspA plus CT elicits protective immune responses against lethal challenge with *S. pneumoniae*. To determine whether orally administered PspA elicited protective immune responses, mice orally immunized with PspA or PspA plus CT were challenged intravenously with 3.6×10^3 CFU of *S. pneumoniae* A66 2 weeks following the fourth oral immunization. The mice orally immunized with PspA plus CT showed antigen-specific serum IgG titers of $\log_2$ of 19 (1:524,000) prior to challenge (Fig. 2 and Table 1). In the case of mice orally immunized with PspA alone, the levels of antigen-specific IgG responses were only $\log_2$ of 6 (1:64). When challenged intravenously with a lethal dose of A66, oral immunization with PspA and CT protected all the mice from the infection. Oral immunization with PspA alone failed to provide complete protection; however, three of eight mice did survive the challenge (Table 1).

FIG. 4. PspA-specific IgG subclass responses in mice orally immunized with PspA and CT as adjuvant. The data shown are from mice 49 days following primary immunization; results are expressed as means $\pm$ standard deviations of $\log_2$ ELISA antibody titers obtained for six mice per group. A similar pattern but of lower magnitude for antigen-specific responses was noted when samples were obtained 42 days following primary immunization. Nonimmunized mice always gave titers of less than $\log_2$ of 3 in this assay.

TABLE 1. Oral immunization with PspA plus CT induces protective immunity to systemic challenge with capsular type 3 strain A66 of *S. pneumoniae*

Vaccine	Serum IgG titer (reciprocal log ₂)	Day of death after challenge ^a	Median day of death	P value ^b
PspA + CT	19	>21, >21, >21, >21, >21, >21, >21	>21	0.003
PspA	6	1, 1, 2, 3, 4, >21, >21, >21	3.5	NS ^c
None	<4	1, 1, 2, 2, 3	2	NA ^d

^a Groups of mice were challenged intravenously with 3.6×10^3 CFU of strain A66. The day of death is shown for each mouse in each group.

^b Compared with group receiving no vaccine.

^c NS, not statistically different.

^d NA, not applicable.

The protective effects of oral immunization with PspA were further demonstrated when we examined the number of CFU in the peripheral blood of the mice 24 h postchallenge. Whereas all nonimmunized mice had very high numbers of *S. pneumoniae* bacteria in their peripheral blood, no bacteria were detected in the peripheral blood of mice orally immunized with PspA and CT (Table 2). In comparison with non-immunized mice, the number of *S. pneumoniae* bacteria in the peripheral blood was reduced 1,000-fold or more in mice that had been orally immunized with PspA (Table 2).

DISCUSSION

The demonstrations that PspA can elicit protective immunity against *S. pneumoniae* in mice (20, 25, 29) has caused PspA to be considered as a candidate antigen for the development of a human vaccine. PspA-specific monoclonal antibodies have been shown to be protective against fatal infection with *S. pneumoniae* (24, 25), and immunization can provide cross-protection against heterologous serotypes of *S. pneumoniae* (29, 33). A significant milestone would be the development of an oral PspA vaccine for the pediatric population.

In the present study, we thus used PspA as a model system to study the nature and kinetics of antigen-specific antibody responses induced by oral immunization and their protective capabilities against pneumococcal infection. We have demonstrated that oral immunization with PspA vaccine can induce protective humoral immune responses against *S. pneumoniae* infection. In addition, the levels of protection induced by oral immunization with PspA was enhanced by use of the mucosal adjuvant CT. Titers of PspA-specific serum IgG antibodies reached log₂ of 19 to 20 (1:500,000 to 1:1,000,000) and provided complete protection against systemic challenge with pathogenic *S. pneumoniae* (Tables 1 and 2). These findings suggest that oral immunization with PspA can be considered to be a practical and effective route of immunization for the induction of specific immunity against pneumococcal infections.

When 7.5 µg of PspA was administered without CT via the oral route, small but significant serum IgG anti-PspA levels were induced (1:32 to 1:128) (Fig. 2A), and three of eight mice were protected against the lethal dose of *S. pneumoniae* challenge (Table 1). Soluble proteins are generally not strong immunogens when given by the oral route. This result, however, suggested that small amounts of PspA could be sufficient to induce serum responses by the oral route which are able to protect against pneumococcal infection.

It is well established that oral immunization with protein

antigens mixed with CT effectively induces antigen-specific serum antibody responses, mainly of IgG and IgA isotypes as well as a mucosal IgA response. Previous studies have shown that 10 µg of CT is an effective oral dose for the induction of anti-CT-B antibody responses (36). This dose of CT also effectively promotes antibody responses to the coadministered protein (11, 16, 21, 23, 35). Thus, 10 µg of CT was coadministered with PspA in this study. PspA given orally with CT induced high levels of PspA-specific IgG responses (Fig. 2B). Antigen-specific IgA antibodies appeared in the serum a week after the fourth immunization (Fig. 2B). When orally immunized mice were intravenously challenged with a lethal dose of *S. pneumoniae*, all mice survived the infection (Table 1). It has been shown that a single PspA can elicit significant cross-protection against pneumococcal sepsis of isolates of the major mouse virulent capsular types, 3, 4, 5, 6A, and 6B (6, 29, 33). Furthermore, rabbit antiserum raised against a single PspA showed cross-reactivity with all PspAs (6). These findings indicate that despite the variability of PspA, there are cross-reactive epitopes shared by all PspAs. Thus, oral immunization with PspA plus CT could induce protective levels of serum antibody responses against strains of the major capsular serotypes.

Examination of IgG subclasses showed that oral PspA together with CT induced mainly IgG1 and IgG2b antibody responses (Fig. 4). IgA antibody to PspA appeared in the fecal extracts following the third immunization, and the response increased after the fourth immunization. In this regard, CT is known to selectively induce antigen-specific Th2 type responses which promote IgG1 in serum as well as mucosal IgA responses following oral immunization with protein antigens (23, 35). Thus, these results suggest that oral immunization with PspA plus the adjuvant CT leads to the generation of antigen-specific Th2-type responses accounting for the induction of PspA-specific IgG1 as well as secretory IgA (S-IgA) responses in systemic and mucosal sites, respectively. In general, oral immunizations have been shown to result in the induction of antigen-specific S-IgA in various remote mucosal effector sites such as the lamina propria of the gastrointestinal tract, the upper respiratory tract, and the genitourinary tract (18). In this regard, several studies have shown that oral immunization with antigen induces protective immune responses in the respiratory tract of humans (31), pigs (15), and mice (10). Thus, it is likely that oral immunization with PspA plus adjuvant CT is also capable of inducing S-IgA responses in the respiratory tract. Since the main route of *S. pneumoniae* invasion is the respiratory tract, it is important to induce immune responses not only in the systemic compartment but also in the mucosal sites (e.g., respiratory tract). Thus, oral immunization of PspA would be a powerful vaccine regimen for the induction

TABLE 2. Oral immunization with PspA and CT reduces the number of *S. pneumoniae* recovered from peripheral blood of mice after systemic challenge with capsular type 3 strain A66 of *S. pneumoniae*

Vaccine	No. of mice	CFU ^a (log ₁₀)	P value ^b
PspA + CT	7	<2	0.005
PspA			
Mice survived	3	<2	0.02
Mice died	5	5.1 ± 2.1	0.04
None	5	8.0 ± 0.6	NA ^c

^a CFU of *S. pneumoniae* were determined by plating serial dilutions of peripheral blood.

^b Compared with group receiving no vaccine.

^c NA, not applicable.

of a pneumococcus-specific immune response in both systemic and mucosal sites. Whether oral immunization with PspA will protect against a mucosal challenge with pneumococci remains to be determined.

In summary, our present study provided evidence that oral immunization with a vaccine containing PspA and the mucosal adjuvant CT induced PspA-specific humoral immune responses. Furthermore, PspA-specific responses generated by the oral immunization have provided protective immunity against pneumococcal infection. In addition, our study demonstrated that effective mucosal antipneumococcal IgA responses could be induced with this immunization regimen.

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