Protective Humoral Response Against Pneumococcal Infection in Mice Elicited by Recombinant Bacille Calmette-Guérin Vaccines Expressing Pneumococcal Surface Protein A

By Solomon Langermann,* Susan R. Palaszynski,* Jeanne E. Burlein,* Scott Koenig,* Mark S. Hanson,* David E. Briles,† and C. Kendall Stover$ 

From *MedImmune, Inc, Gaithersburg, Maryland 20878; the †Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294; and $PathoGenesis Corp, Seattle, Washington 98119

Summary

Pneumococcal surface protein A (PspA), a cell-surface protein present on all strains of pneumococci, has been shown to elicit protective antibody responses in mice in the absence of capsular polysaccharide. Whereas PspA is polymorphic, considerable cross-reactivity and cross-protection have been demonstrated among PspA proteins of pneumococci exhibiting different capsular and PspA serotypes. A gene segment encoding the nonrepetitive variable NH₂-terminal portion of PspA has been cloned into three distinct recombinant Bacille Calmette-Guérin (rBCG) vectors, allowing for expression of PspA as a cytoplasmic or secreted protein, or a chimeric exported membrane-associated lipoprotein. All rBCG-PspA strains elicited comparable anti-PspA ELISA titers, ranging from 10⁴ to 10⁵ (reciprocal titers) in both BALB/c and C3H/HeJ mice. However, protective responses were observed only in animals immunized with the rBCG-PspA vaccines expressing PspA as a secreted protein or chimeric exported lipoprotein. In addition, anti-PspA immune sera elicited by the rBCG vaccines passively protected X-linked immunodeficient mice from lethal challenge with the highly virulent, encapsulated WU2 strain of Streptococcus pneumoniae and two additional virulent strains exhibiting heterologous PspA and capsular serotypes. These studies confirm previous PspA immunization studies showing cross-protection against heterologous serotypes of S. pneumoniae and demonstrate a potential for rBCG-based PspA vaccines to elicit protective humoral responses against pneumococcal disease in humans.

Streptococcus pneumoniae is the most frequent causative agent of bacterial pneumonia and otitis media, one of the major causes of meningitis, and a primary source of death worldwide (1–3). Among infants and young children, acute otitis media (AOM) is the most common disease caused by this pathogen (4–6). It has been estimated that approximately three fourths of all children experience at least one episode of AOM and that >50% of these cases are caused by S. pneumoniae. S. pneumoniae, therefore, accounts for >10⁶ cases of middle ear infection in the United States alone in children under 2 yr of age (1). In addition, S. pneumoniae continues to be a major respiratory tract pathogen of adults, especially the elderly, and is responsible for ~40,000 deaths per year among adults in the United States (7). The increasing frequency world-wide of antibiotic-resistant S. pneumoniae, as well as the morbidity and mortality associated with pneumococcal disease, argue for prophylactic vaccination as a means to protect against infection.

The pneumococcal vaccine currently available is a polyvalent vaccine composed of unconjugated, purified, pneumococcal capsular polysaccharides. Whereas this vaccine has been shown to be protective in adults (8, 9), it is of limited use in infants and children since the latter populations are poor responders to polysaccharide antigens and generally fail to generate protective antibodies to isolated polysaccharides (10–13). Protein antigens, in contrast to polysaccharides, have the advantage that they are immunogenic in infants as well as adults. The need for a pneumococcal vaccine that is effective in young children and infants has prompted studies to identify protein antigens of the pneumococcus that are able to elicit protective immune responses. The pneumococcal surface protein A (PspA)¹ is a protein antigen of S. pneumoniae which has been shown to elicit protective immunity in the mouse model for pneumococcal disease (14–16).

PspA is present on all pneumococcal isolates (17). Although a great number of antigenic variants of PspA exist, PspA also

1Abbreviations used in this paper: BCG, Bacille Calmette-Guérin; OspA, outer-surface protein A; PspA, pneumococcal surface protein A.
exhibits considerable cross-reactivity and is capable of eliciting cross-protection against strains of different PspA and capsular types (18, 19, and Briles, D. E., and L. S. McDaniels, manuscript in preparation). Furthermore, mAbs to PspA provide passive protection in mice against fatal pneumococcal infection (16, 18, 20). In addition, it has been shown that X-linked immunodeficient (Xid) mice, which like young children are unable to mount immune responses to polysaccharide vaccines (21), produce antibodies to PspA (22). The anti-PspA antibodies are able to protect against death even in the absence of other antipneumococcal antibodies. Taken together, these studies suggest that the PspA protein may be a good candidate antigen for incorporation into a pneumococcal vaccine.

Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, offers a number of advantages as a vaccine vehicle for delivering foreign antigens such as PspA. BCG is already the most widely administered live vaccine, having been given as an antituberculosis vaccine to >2.5 billion people worldwide (23–24). Because of its safety record, it is suitable for immunization of young children and infants. BCG is also relatively inexpensive to produce and has strong immunostimulatory properties that produce long-lasting sensitization to mycobacterial antigens.

Recently developed genetic systems for the expression of foreign antigens in mycobacteria (25–28) have enabled the evaluation of rBCG as a vaccine delivery vehicle. Subsequent studies have demonstrated that rBCG can elicit antigen-specific systemic antibody responses after parenteral or mucosal (intrasinal) immunization and that these antibodies are protective (29, 30, 30a). For example, humoral antibodies to the outer-surface protein A (Ospa) of *Borrelia burgdorferi* elicited by a rBCG vaccine have been shown to be protective against challenge with *B. burgdorferi* in a mouse model for Lyme borreliosis (29, 30). Also, BCG expressing gp63 from *Leishmania major* have been shown to induce strong protective responses in a mouse model for *Leishmania* (30) and antibodies to tetanus toxin fragment C expressed in rBCG have been shown to protect against challenge with tetanus toxin (31). Thus, rBCG vaccines expressing PspA (rBCG-PspA) were constructed to test their efficacy as candidate vaccines against pneumococcal disease. In this paper we report on protective humoral responses elicited by such rBCG-PspA vaccines in the mouse model for pneumococcal disease.

### Materials and Methods

**Construction of BCG/Escherichia coli Shuttle Expression Vectors for Expression of pspA.** Plasmid vectors pMV206 and pMV261 (25) are the parental plasmids for all the plasmids constructed described below. Plasmid pMV206 is composed of DNA cassettes encoding Kanamycin resistance (Kan), an *E. coli* origin of replication (Erep), a mycobacterial plasmid replicon derived from plasmid pHAL5000 (Mrep), and a multiple cloning site (MCS) all assembled into a mycobacterial-*E. coli* shuttle vector. pMV206 is similar to pMV261 but also includes the promoter, ribosomal binding site (RBS), and initial six codons of the BCG heat shock protein 60 (hsp60) gene inserted between XbaI and BamHI sites of the multiple cloning site of plasmid pMV206. Plasmid pRB26 differs from pMV261 in that it contains only the BCG hsp60 promoter but not the hsp60 RBS nor any of the hsp60 coding sequence (29). Plasmid expression/secretion vector p2619s was constructed by inserting a PCR-derived DNA segment encoding the *Mycobacterium tuberculosis* hsp60-kD (Mtb19) RBS and 5' lipoprotein signal peptide downstream from the hsp60 promoter of plasmid pRB26. For two of the plasmids encoding PspA, pMV261-pspA33 and p2619-pspA33, a pspA gene segment encoding the NH2-terminal region of the PspA protein, spanning amino acids +6 to +299, was amplified by PCR from DNA derived from *S. pneumoniae* strain Rx1 (32) and cloned between the BamHI and SalI sites in the MCS of plasmids pMV261 and p2619 to yield plasmids pMV261-pspA33 and p2619-pspA33, respectively, (see Fig. 1); 5' BamHI and 3' SalI restriction sites were incorporated into the primers for subcloning. The predicted size of the PspA-derived peptide encoded by these plasmids, 33 kD, is larger than that encoded by the original ppsA truncation mutation as reported by Talkington et al. (pJY2008) (33). For plasmid plgPspA, the cloned pspA fragment encodes a protein that extends from the first residue of the PspA leader sequence (31) through residue +299 (see Fig. 1). A PCR-amplified BamHI-Sall pspA gene segment (pspA69) encoding the full-length 69-kD PspA protein without signal peptide was cloned into another vector, the hsp60- lacZ fusion vector pSL26 which is a derivative of plasmid pMV261. pSL26 incorporates the 5' region of *E. coli* lacZ (encoding the first 71 codons) upstream of the first six hsp60 codons of pMV261. The resulting vector was termed pSL26-pspA69.

**Growth of BCG and Transformation of Shuttle Plasmids into BCG.** All liquid cultures of BCG strain Pasteur 1173P2 were grown at 37°C in stationary tissue culture flasks (25 cm² with 5–10 ml or 75 cm² with 15–25 ml) or roller bottles (490 cm² with 100–200 ml or 1,700 cm² with 200–500 ml) using Dubos (Difco Laboratories, Detroit, MI) media supplemented with 1/10th volume of AD enrichment consisting of 5% BSA fraction V (Sigma Chemical Co., St. Louis, MO), 2% dextrose, and 0.85% sodium chloride. Liquid culture media included 0.02% Tween 80 (T80) to prevent clumping of BCG cells. BCG colonies were grown at 37°C on Middlebrook 7H10 agar media (Difco Laboratories) supplemented with 1/10th volume of AD enrichment. For transformation, BCG cultures were grown to densities of ~10⁸ CFU/ml, sedimented at 4,000 g, and washed twice by resuspension and centrifugation (4,000 g) in 10% glycerol at 4°C, and finally resuspended in 1/20th of the original culture volume of cold 10% glycerol. Then 200 μl of the cold BCG suspension was mixed with plasmid DNA (50–500 ng) in a prechilled 0.2-cm electroporation cuvette and transformed using the Biorad Gene Pulser electroporator (both from Bio-Rad Laboratories, Richmond, CA) at 2.5 kV, 25 μF, and 1,000 W. After electroporation, 50 μl 5 X Dubos media was added to the BCG-DNA suspension, and the mixture was incubated at 37°C for 1 h before plating on Middlebrook 7H10 plates supplemented with AD enrichment and kanamycin (15 μg/ml).

**Analysis of pspA Expression in rBCG,** BCG transformants were grown to mid-logarithmic phase in Dubos liquid media containing kanamycin (15 μg/ml). After washing the cells in PBS plus 0.05% T80, the cell suspension was concentrated 20-fold in RIPA buffer (1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) to lyse the cells. Culture lysates were analyzed by SDS-PAGE and Western blot with the PspA-specific mAb Xi126 (15). Expression of PspA by rBCG was compared with an *S. pneumoniae* lysate derived from strain Rx1 and to purified recombinant PspA protein kindly provided by Dr. P. H. McVerry (Connaught Laboratories Inc., Swiftwater, PA). Protein bands reacting with Xi126 were visualized after incubation with a secondary antibody (goat...
anti-mouse conjugated to horseradish peroxidase) using the (Amer-

sham, Arlington Heights, IL) enhanced chemiluminescence (ECL)
system.

Immunizations with rBCG Strains. rBCG cultures were grown
in roller bottles to optical densities of ~1.2 A_600 U (=2 × 10^6
CFU/ml), concentrated 20-fold by centrifugation at 4,000 g, and
resuspended in 1 ml of 15% glycerol. The rBCG glycerol suspen-
sions were gradually frozen at a rate of −1°C per minute, from
4°C to −40°C and then stored at −70°C until use. CFU were
assayed by growing thawed material on Middlebrook 7H10 plates
plus kanamycin at various dilutions.

4–6-wk-old female BALB/c and C3H/HeJ mice (The Jackson
Laboratory, Bar Harbor, ME) were immunized by the intraperitoneal
route with 10^4 rBCG CFU in 100 μl of PBS-T80.

Anti-PspA Response of Immunized Mice. Sera were collected from
the tail veins of immunized mice at 4-wk intervals and pooled for
each group to monitor antibody responses by ELISAs with purified
PspA protein (provided by Dr. P. H. McVerry. ELISA plates (Im-
mulon 4; Dynatech Laboratories, Inc., Chantilly, VA) were coated
with 50 μl of PspA protein (0.1 μg/ml) in 0.1M carbonate buffer,
ph 9.6, and incubated for 2–4 h at room temperature or overnight
at 4°C. The antigen solution was removed and plates were incubated
with blocking solution (0.5% BSA and 0.5% nonfat dry milk in
H_2O) for 1 h at room temperature. Twofold serial dilutions of
serum starting at 1:200 were made in blocking solution and 50
μl of each dilution was added to duplicate wells of the antigen-
coated plate. After an incubation at room temperature for 1 h, the
coated plates were washed with PBS plus 0.1% Tween-20 (T20) and
incubated with 50 μl of a 1:4,000 PBS-T20 dilution of peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry
Laboratories, Gaithersburg, MD) secondary antibody for 1 h at room
temperature. Color was developed with ABTS substrate reagent
(Kirkegaard & Perry Laboratories) and measured by absorbance at
405 nm on a Dynatech ELISA reader. Endpoint titers were defined
as the highest dilution at which the A_600 values were twice the
values for preimmune sera diluted 1:200 in blocking solution.

Mouse Protection Experiments in rBCG-PspA Immunized Mice.
BALB/c and C3H/HeJ mice immunized with rBCG-PspA vaccine
strains were challenged intraperitoneally with 10^4 CFU (100 ×
LD_50) of the highly lethal S. pneumoniae strain WU2 (34). Im-
mEDIATELY after challenge, samples of the S. pneumoniae challenge
inoculum were plated on trypticase soy agar plates to deter-
mine the CFU and verify the challenge dose. Deaths were recorded
at 24-h intervals. Most deaths occurred between days 2 and 4 after
challenge, however the mice were observed for a total of 10–12 d
as indicated.

Passive Protection Experiments with Anti sera from rBCG-PspA Im-
imunized Mice. Anti-PspA antisera collected from mice vaccinated
with rBCG containing p2619-pspA was administered passively
in 0.1 ml volumes into CBA/N mice by the intraperitoneal route
at a 1:40 dilution on days 0, 2, and 3. Mice were challenged by
the intravenous route on day 0 with 500 CFU of each of five highly
virulent strains of S. pneumoniae (35), in a total volume of 0.2 ml,
1 h after passive administration of the antibody. The virulent
S. pneumoniae challenge strains varied either in their capsular or
PspA serotype. The mice were observed for survival for a period of
12 d after challenge with the virulent S. pneumoniae.

Results

Expression and Localization of PspA in rBCG. Previously
we have shown that rBCG can elicit both humoral and cel-
lular immune responses to foreign antigens expressed in the
cytoplasm of rBCG (25, 31). More recently, we reported on
the development of rBCG expression vectors that allow for
the export or secretion of foreign antigens from rBCG by
fusing the recombinant proteins to the Mtb19 lipoprotein
signal peptide (29). In the case of at least one antigen, the
Ospa of B. burgdorferi, secretion and surface expression resulted
in much stronger antigen-specific and protective humoral and
cellular responses than was possible with cytoplasmic expres-
sion in rBCG.

A PspA gene segment (pspA33) encoding the NH_2-
terminal variable protective domain of the PspA protein from
S. pneumoniae strain Rxl (type 25 PspA [36]) spanning amino
acids +4 to +299 of the protein, was cloned into rBCG ex-
pression vectors pMV261 and p2619s to result in vectors
pMV261-pspA33 and p2619-pspA33. In both plasmids, the
DNA fragment encoding the NH_2-terminal portion of PspA
did not contain the PspA signal peptide. For pMV261-pspA33,
the pspA33 gene segment was fused to the first six codons
of the cytoplasmically expressed BCG hsp60 gene product.
For p2619-pspA33, the pspA33 fragment was fused to the 5’
region of the Mtb19 structural gene encoding the NH_2-
terminal 28 amino acids including the 21-residue signal pep-
tide for the M. tuberculosis Mtb19 surface-expressed lipoprotein.
In addition to constructing these two plasmids, a slightly
larger pspA33 gene segment (extending from the −31 resi-
due of PspA through residue +299, thus including the nat-
ural PspA signal peptide, as well as the PspA ribosomal binding
site), was cloned into vector pRB26, resulting in a nonfused
pspA33 gene segment expressed in BCG (pRB26-pspA33).
In all three vectors, expression of the pspA33 gene segment
was driven by the BCG hsp60 promoter on a multi-copy extra
chromosomal plasmid vector (Fig. 1). In addition, a gene seg-
ment encoding the full-length 69-kD PspA protein, with or
without the 5’ region encoding the PspA signal peptide, was
cloned into a variety of BCG expression vectors that all util-
ized the BCG hsp60 promoter (Fig. 2 legend).

Expression of PspA gene segments from all of the rBCG
expression vectors was confirmed by SDS-PAGE and Western
blot analysis of whole-cell BCG lysates. The highest level
of expression was seen in rBCG transformed with the cyto-
plasmic expression vector pMV261-pspA33 encoding the
NH_2-terminal protective PspA33 domain without signal
peptide. Cytoplasmically expressed PspA33 (C-PspA) in rBCG-
pMV261-pspA33 accounted for ~15% of the total BCG pro-
tein, and the amount produced was two- to fivefold greater
than that seen with rBCG carrying plasmids pRB26-pspA33
or p2619-pspA33 encoding either PspA33 with the natural
pneumococcal signal peptide (S-PspA) or the Mtb19 lipopro-
tein signal peptide (L-PspA), respectively (Fig. 2).

Expression of the full-length 69-kD PspA protein was seen
only in lysates of BCG strains transformed with the
pSL26-pspA69 plasmid (Fig. 2). Whereas rBCG-pSL26-pspA69
expressed low levels of a full-length Hsp60-LacZ-PspA fusion
protein without signal peptide, this rBCG strain was
difficult to maintain in culture and was therefore not pur-
sued further. The inability to obtain stable rBCG strains ex-
Expression vector derivative pMV261, also includes the promoter, ribosomal binding site (RBS), and initial six codons of the BCG hsp60 gene cloned between the XbaI and BamHI site of pMV206. Plasmid pRB26 differs from pMV261 in that it only includes the BCG hsp60 promoter. Plasmid p2619s is a derivative of pRB26 and includes a DNA segment encoding the Mtb19 RBS and 5' lipoprotein signal peptide downstream from the hsp60 promoter. A pspA gene segment encoding the NH2-terminal 33-kD region of the pspA gene from S. pneumoniae strain Rx1 (34) was PCR amplified with or without the 5' region encoding the PspA signal peptide and cloned into either pMV261, pRB26, or p2619s.

Immunization with rBCG expressing the unrelated OspA antigen as a lipoprotein did not protect against pneumococcal infection in either C3H or BALB/c mice. In a subsequent experiment, C3H and BALB/c mice were immunized intraperitoneally with 10^6 CFU of the rBCG-PspA vaccines described above and evaluated for PspA-specific IgG responses. Both strains of mice mounted strong antibody responses to PspA regardless of whether PspA was expressed in the cytoplasm, exported to the surface of BCG, or secreted (Fig. 3). In all cases, reciprocal ELISA titers to PspA ranged from 10^3 to 10^4 after a single immunization and increased 10-fold (10^4 to 10^5 reciprocal titers) after a booster immunization at ~17 wk postprimary immunization. However, only the rBCG vaccines expressing PspA as a secreted protein (pRB26-pspA) or as a chimeric lipoprotein (pMV261-pspA) elicited protective immunity against pneumococcal challenge with 100 LD_{50} of virulent pneumococci (strain WU2) in both C3H and BALB/c mice (Fig. 3). The level of protection against pneumococcal challenge ranged from 50% for C3H mice to as high as 90% for BALB/c. In this particular experiment, the secreted form of PspA afforded a slightly higher level of protection than the lipidated PspA. It is surprising that whereas comparable ELISA end-point titers to PspA were observed in animals immunized with the nonsecreted, cytoplasmic rBCG containing the pMV261-pspA clone, only modest protection against pneumococcal challenge was seen, and only in the CH3 strain of mice. Immunization with rBCG expressing the unrelated OspA antigen as a lipoprotein did not protect against pneumococcal challenge in either C3H or BALB/c mice.

| Promoter | RBS | Secretion Signal | pspA Gene |
|----------|-----|-----------------|-----------|
| pMV261   | None|                 |           |
| pRB26-pspA |     |                 |           |
| p2619-pspA |     |                 |           |

Figure 1. BCG/E. coli shuttle expression vectors used to express recombinant antigens. Plasmid vector pMV206, the parent vector of all vectors used in this study, is composed of DNA cassettes encoding Kanamycin resistance (Kan), an E. coli origin of replication (ErEp), a mycobacterial plasmid replicon derived from plasmid pALS000 (Mrep) and a multiple cloning site all assembled into a BCG/E. coli shuttle vector as described by Stover et al. (25). Expression vector derivative pMV261, also includes the promoter, ribosomal binding site (RBS), and initial six codons of the BCG hsp60 gene cloned between the XbaI and BamHI site of pMV206. Plasmid pRB26 differs from pMV261 in that it only includes the BCG hsp60 promoter. Plasmid p2619s is a derivative of pRB26 and includes a DNA segment encoding the Mtb19 RBS and 5' lipoprotein signal peptide downstream from the hsp60 promoter. A pspA gene segment encoding the NH2-terminal 33-kD region of the pspA gene from S. pneumoniae strain Rx1 (34) was PCR amplified with or without the 5' region encoding the PspA signal peptide and cloned into either pMV261, pRB26, or p2619s.

In a subsequent experiment, C3H and BALB/c mice were
To verify that the observed protection against pneumococcal challenge was antibody mediated and not dependent in part on simultaneous inflammatory responses induced by live BCG, serum was collected from rBCG-PspA-immunized mice and administered passively to CBA/N mice before challenge with a lethal dose of *S. pneumoniae*. CBA/N, which carry the Xid trait, are highly sensitive to pneumococcal infection (21, 22). Three doses of anti-PspA antibody diluted 1:40 and administered to CBA/N mice by intraperitoneal injection, protected against challenge with 100 times the lethal dose of three highly virulent strains of pneumococci that differed in either their PspA or capsular serotypes. Whereas the anti-PspA antisera directed against the type 25 PspA serotype (the type cloned into BCG) protected against challenge with *S. pneumoniae* strains expressing heterologous PspA serotypes (types 1, 13, and 24), it did not protect against passive challenge with a *S. pneumoniae* strain expressing PspA type 18, as shown (Table 1). The anti-PspA antibody prevented death or delayed the time of death by at least 2 d. CBA/N mice that did not receive any passive serum died within 2 d of challenge. Furthermore, virtually all CBA/N mice given passive intraperitoneal injections of anti-OspA antiserum also died within 2 d of challenge (Table 1).

Discussion

Previous studies have shown that antibody raised against purified PspA protein, a surface antigen present on all *S. pneumoniae* clinical isolates (17), protects against fatal *S. pneumoniae* infection in mice (16, 18, 20, 22). In this report, we demonstrate that a single inoculation with a live BCG bacterial vaccine expressing PspA as a secreted mature protein or a chimeric exported lipoprotein (rBCG-PspA), followed by a booster at 17 wk, results in a protective humoral antibody response against virulent *S. pneumoniae*. These results lend further support to a role for anti-PspA antibodies in protecting against pneumococcal infection and demonstrate that BCG is a powerful vaccine vehicle for eliciting such a protective response.

BCG, an attenuated bovine tubercle bacillus used as a vaccine for tuberculosis, offers considerable advantages as a safe, live vaccine vehicle for the expression and delivery of protective recombinant antigens. It is the most widely used vaccine in the world with a low incidence of serious complications, it can be given at birth, and is unaffected by maternal antibodies (23, 24, 37). Furthermore, given that BCG can accommodate large pieces of foreign DNA, it is also possible to develop rBCG which allow for the simultaneous expression of multiple protective antigens derived from different pathogens (25, 27, 28, 38, 39). All of these factors are particularly important to consider in the development of a pneumococcal vaccine that is targeted predominantly to infants.

In addition, it is important to consider the potential side effects associated with a live bacterial vaccine vector such as BCG. The most frequent adverse reaction to BCG is suppurative lymphadenitis (40). However, it has clearly been shown in studies conducted with BCG strains used for im-

Figure 2. Expression of PspA gene segments in rBCG. rBCG lysates were subjected to SDS-PAGE (top) and Western blot analysis with PspA-specific mAb Xi126 (bottom). Expression of the PspA33 gene product from vector pMV261 (261::pspA33) was estimated to be ~100 ng per 10^6 CFU of rBCG whereas expression from pRB26 (RB26::pspA33) and p2619s (2619::pspA33) was five- and twofold less, respectively. A whole cell lysate derived from *S. pneumoniae* strain Rx1 and purified preparations of recombinant PspA protein were run as positive controls. The 261::lac which expresses Lac rather than PspA in the same BCG background strain was run as a negative control. Expression of a full-length Hsp60-LacZ-PspA fusion protein without a signal peptide was observed with rBCG containing vector pSL26::pspA69.

In a subsequent experiment, C3H and BALB/c mice were immunized intraperitoneally with 10^6 CFU of either rBCG expressing the secreted (S-PspA) or the lipidated (L-PspA) form of PspA, and were challenged with a lethal dose of virulent pneumococci to confirm the protective efficacy of these two rBCG vaccine strains. Once again, reciprocal titers to PspA ranged from 10^3 to 10^4 after a single immunization and increased 10-fold (10^4 to 10^5 reciprocal titers) after a booster immunization at 17 wk. When the mice were challenged with 100x LD_{50} virulent pneumococci, 80% of the C3H and 100% of the BALB/c mice receiving either of the rBCG-PspA vaccine strains survived (Fig. 4). A small percentage of the control mice survived presumably due to nonspecific immunity to *S. pneumoniae*.

Passive Transfer of Antibody from rBCG-PspA–immunized Mice Protects CBA/N Mice from Lethal Challenge with Diverse Strains of Pneumococci. To verify that the observed protection against pneumococcal challenge was antibody mediated and not dependent in part on simultaneous inflammatory responses induced by live BCG, serum was collected from rBCG-PspA-immunized mice and administered passively to CBA/N mice before challenge with a lethal dose of *S. pneumoniae*. CBA/N, which carry the Xid trait, are highly sensitive to pneumococcal infection (21, 22). Three doses of anti-PspA antibody diluted 1:40 and administered to CBA/N mice by intraperitoneal injection, protected against challenge with 100 times the lethal dose of three highly virulent strains of pneumococci that differed in either their PspA or capsular serotypes. Whereas the anti-PspA antisera directed against the type 25 PspA serotype (the type cloned into BCG) protected against challenge with *S. pneumoniae* strains expressing heterologous PspA serotypes (types 1, 13, and 24), it did not protect against passive challenge with a *S. pneumoniae* strain expressing PspA type 18, as shown (Table 1). The anti-PspA antibody prevented death or delayed the time of death by at least 2 d. CBA/N mice that did not receive any passive serum died within 2 d of challenge. Furthermore, virtually all CBA/N mice given passive intraperitoneal injections of anti-OspA antiserum also died within 2 d of challenge (Table 1).
Figure 3. Mice immunized with rBCG-PspA and challenged with S. pneumoniae are protected against death: challenge experiment No. 1. C3H/HeJ and BALB/c mice were immunized and boosted by the intraperitoneal route with 10^6 CFU of rBCG expressing the PspA antigen from S. pneumoniae. The recombinant PspA protein contained either an exported, lipid-acylated mycobacterial lipoprotein sequence (p2619::pspA), the endogenous PspA signal sequence (pRB26::pspA), or no signal sequence (pMV261::pspA). Control mice were inoculated with 10^6 rBCG expressing a lipid-acylated form of the OspA protein from B. burgdorferi (2619::ospA). (A) Sera were collected every 4 wk as well as 2 wk postbooster immunization (week 17), pooled for each group, and evaluated for anti-PspA antibody by ELISA. (B) 2 wk postboost mice from each group were challenged intraperitoneally with 10^4 CFU S. pneumoniae (100 x LD_{50}). Survival was determined in all five groups over the course of 12 d after challenge. Data are presented as the percent survival for a total of 10 mice per experimental group. For the C3H mice, p <0.001 for pRB26::pspA and P2619::pspA immunized vs. naive mice; p <0.05 for pRB26::pspA and P2619::pspA immunized mice vs. the p2619::ospA immunized control. For the BALB/c mice, p <0.02 for pRB26::pspA immunized vs. naive mice; p <0.05 for pRB26::pspA and P2619::pspA immunized mice vs. the p2619::ospA immunized control. For both pRB26::pspA and P2619::pspA vs. p2619::ospA immunized control (two-sample rank test [53]).
Antibody responses against PspA were observed in BALB/c and C3H/HeJ mice with all rBCG vaccines. Whereas peak antibody ELISA titers elicited by rBCG expressing PspA with or without secretion signal peptides did not differ markedly, protective responses were observed only in mice immunized with rBCG expressing PspA with its native signal peptide or as a fusion with the Mtb19 lipoprotein signal peptide. In contrast, prior studies had shown that export of a different foreign antigen, B. burgdorferi OspA, from the rBCG cytosol, seemed to be required for maximum levels of both total antibody and protective immunity against challenge (29). One possible explanation for this difference is that, unlike OspA, some chimeric PspA is transported across the membrane even in the absence of a signal peptide, by some as yet unexplained mechanism. This possibility is suggested by limited fraction-
with its natural signal peptide or as a chimeric lipoprotein. Its "transported" form. Conversely, PspA protein expressed in the appropriate conformation can induce a protective response even in vivo. The cytoplasmic form of PspA might not be conformationally correct to induce a protective response, however (18, 19). The PspA vaccine elicits protection against pneumococci of more than one capsular serotype. Whereas the rBCG-PspA vaccines were highly protective against S. pneumoniae challenge, absolute protection was not always observed. The lack of protection in some mice was not a function of their failure to respond to the rBCG-PspA vaccine; all mice given the rBCG-PspA vaccine mounted strong antigen-specific responses (data not shown).

It should be noted that CBA/N mice were used in the passive protection studies rather than BALB/c or C3H mice, because they have become a standard model for passive protection with antibody to pneumococcal antigens (34, 35, 44-46). CBA/N (Xid) mice are more susceptible to most pneumococcal infections than BALB/c and C3H mice as well as most other strains of mice (47). Thus, protection of Xid mice against pneumococcal challenge is a particularly stringent test of protective efficacy of antipneumococcal antibodies. Furthermore, as another measure of stringency in the passive protection assay, Xid mice were challenged intravenously rather than intraperitoneally. Whereas in general strains that are virulent intravenously are also virulent when inoculated intraperitoneally (35, 48), death occurs a day or two earlier in mice challenged by the intravenous route. Another reason for choosing to do passive protection studies in Xid mice was that whereas most strains of mice including BALB/c and C3H/HeJ have antiphosphocholine antibodies in their serum, which is protective against challenge with low inocula of many pneumococcal strains (35), antibodies against phosphocholine and most carbohydrates are absent in Xid mice (35). Therefore, Xid mice provide the best opportunity to assay for the protective effects of specific, passively administered antibodies in the absence of potentially protective, endogenous antibodies. Nonetheless, recent studies in one of the investigators' laboratory have demonstrated that the anti-PspA antibodies protect against pneumococcal infection regardless of the route.
of challenge and independent of the Xid status of the mouse (Benton, K., and D. Briles, manuscript in preparation).

Overall, the mouse model for pneumococcal disease is useful in terms of identifying and characterizing protective, systemic antibody responses that clear disseminated infections. However, initial colonization by *S. pneumoniae* occurs along mucosal surfaces in the upper respiratory tract, a site where secretory antibodies of the IgA subclass may play an important role in prevention of mucosal surface colonization by pathogenic organisms (49–52). Thus, it may be important to stimulate immune responses at mucosal surfaces to block colonization and to evaluate the protective efficacy of such responses in an appropriate challenge model. Recently, we demonstrated that mucosal immunization with a live rBCG vaccine expressing the OspA antigen results in a prolonged secretory IgA response that is disseminated throughout the mucosal immune system, including the upper respiratory tract, as well as a prolonged and protective systemic immune response against a target pathogen (30). A similar approach may now be taken with rBCG expressing PspA to determine if mucosal delivery of such a vaccine results in protection against pneumococcal infection and disease.

We thank Nita Patel for her technical assistance and Dr. Larry McDaniel for his helpful suggestions.

Address correspondence to Dr. S. Langermann, MedImmune, Inc., 35 West Watkins Mill Road, Gaithersburg, MD 20878.

Received for publication 31 May 1994 and in revised form 5 August 1994.

References

1. Gray, B.M., and H.C. Dillon, Jr. 1986. Clinical and epidemiological studies of pneumococcal infections in children. *Pediatr. Infect. Dis. J.* 5:201.
2. Klein, J.O. 1981. The epidemiology of pneumococcal disease in infants and children. *Rev. Infect. Dis.* 3:246.
3. Austrian, R. 1984. Pneumococcal infection. In *Bacterial Vaccines.* R. Germanier, editor. Academic Press, Inc., New York. 257.
4. Austrian, R., V.W. Howie, and J.H. Ploussard. 1977. The bacteriology of pneumococcal otitis media. *J. Infect. Dis.* 141:104.
5. Luotonen, J., E. Herva, P. Karma, M. Timonen, M. Leionen, and P. Mäkelä. 1981. The bacteriology of acute otitis media in children with special reference to *Streptococcus pneumoniae* as studied by bacteriological and antigen detection methods. *Scand. J. Infect. Dis.* 13:177.
6. Giebenk, G.S. 1989. The microbiology of otitis media. *Pediatr. Infect. Dis. J.* 8:518.
7. Gardner, P., and W. Schaffner. 1993. Immunization of adults. *N. Engl. J. Med.* 328:1252.
8. Shapiro, E.D., A.T. Berg, and R. Austrian. 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N. Engl. J. Med.* 325:1453.
9. Bolan, G., C.V. Broome, R.R. Facklam, M.S. Plikaytis, D.W. Fraser, and W.F. Schlech III. 1986. Pneumococcal vaccine efficacy in selected populations in the United States. *Ann. Intern. Med.* 104:1.
10. Douglas, R.M., and H.B. Miles. 1984. Vaccination against *Streptococcus pneumoniae* in childhood: lack of demonstrable benefit in young Australian children. *J. Infect. Dis.* 149:861.
11. Douglas, R.M., J.C. Paton, S.J. Duncan, and D.J. Hansman. 1983. Antibody response to pneumococcal vaccination in children younger than five years of age. *J. Infect. Dis.* 148:131.
12. Cowan, M.J., A.J. Ammann, D.W. Wara, V.M. Howie, L. Schultz, N. Doyle, and M. Kaplan. 1978. Pneumococcal polysaccharide immunization in infants and children. *Pediatrics.* 62:721.
13. Gotschlich, E.G., I. Goldschneider, and M. Lepow. 1977. The immune response to bacterial polysaccharides in man. In Antibodies in Human Diagnosis and Therapy. E. Haber and R. Krause, editors. Raven Press, Ltd. New York. 391.
14. Briles, D.E., J. Yother, and L.S. McDaniel. 1988. Role of pneumococcal surface protein A in the virulence of *Streptococcus pneumoniae*. *Rev. Infect. Dis.* 10:S372.
15. McDaniel, L.S., G. Scott, K. Widenhofer, J. Carroll, and D.E. Briles. 1986. Analysis of a surface protein of *Streptococcus pneumoniae* recognized by protective monoclonal antibodies. *Microb. Pathog.* 1:519.
16. McDaniel, L.S., G. Scott, J.F. Kearney, and D.E. Briles. 1984. Monoclonal antibodies against protease-sensitive pneumococcal antigens can protect mice from fatal infection with *Streptococcus pneumoniae*. *J. Exp. Med.* 160:386.
17. Crain, M.J., W.D. Walmian, J.S. Turner, J. Yother, D.F. Talkington, L.S. McDaniel, B.M. Gray, and D.E. Briles. 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. *Infect. Immun.* 58:2393.
18. McDaniel, L.S., J.S. Sheffield, P. Delucchi, and D.E. Briles. 1991. PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one capsular serotype. *Infect. Immun.* 59:222.
19. McDaniel, L.S., C. Forman, and, D.O. McDaniel. 1993. Pneumococcal surface protein A (PspA) type 12 elicits protection against pneumococci of different capsular and PspA types. *Abstracts of 1993. ASM Mtg.* 61.
20. McDaniel, L.S., and D.E. Briles. 1986. Monoclonal antibodies against surface components of *Streptococcus pneumoniae*. In *Monoclonal Antibodies Against Bacteria.* A.J.L. Macario and E.C. Macario, editors. Academic Press, Inc., Orlando, FL. 143.
21. Wicker, L.S., and I. Scher. 1986. X-linked immune deficiency
22. McDaniel, L.S., J. Yother, M. Vijayakumar, L. McGarry, W.R. Guild, and D.E. Briles. 1987. Use of insertional inactivation to facilitate studies of biological properties of mycobacterial surface protein A (PspA). J. Exp. Med. 165:381.

23. Bloom, B.R. 1989. Vaccines for the third world. Nature (Lond.). 342:115.

24. Fine, E.M. 1989. The BCG story: lessons from the past and implications for the future. Rev. Infect. Dis. 11:5353.

25. Stover, C.K., V.F. de la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, I.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull, et al. 1991. New use of BCG for recombinant vaccines. Nature (Lond.). 351:456.

26. Aldovini, A., and R.A. Young. 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature (Lond.). 351:479.

27. Jacobs, W.R., Jr., S.B. Snapper, L. Lugosi, and B.R. Bloom. 1990. Development of BCG as a recombinant vaccine delivery vehicle. Curr. Top. Microbiol. Immunol. 155:153.

28. Jacobs, W.R., Jr., M. Tuckman, and B.R. Bloom. 1987. Introduction of foreign DNA into mycobacteria using a shuttle plasmid. Nature (Lond.). 327:532.

29. Stover, C.K., G.P. Bansal, M.S. Hansen, J.E. Burlein, S.R. Palaszynski, J.F. Young, S. Koenig, D.B. Young, A. Sadziene, and A.G. Barbour. 1993. Protective immunity elicited by recombinant BCG expressing OspA lipoprotein: a candidate lysyme disease vaccine. J. Exp. Med. 178:197.

30. Connell, N.D., E. Medina-Acosta, W.R. McMaster, B.R. Bloom, and D.G. Russell. 1993. Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the Leishmania surface proteinase gp63. Proc. Natl. Acad. Sci. USA. 90:11473.

30a. Langermann, S., S. Palaszynski, A. Sadziene, C.K. Stover, and S. Koenig. Induction of sustained systemic and mucosal immunity by a single intranasal immunization with recombinant BCG expressing the OspA antigen of Bordetella burgdorferi. Nature (Lond.). In press.

31. Cassatt, D.R., V.F. de la Cruz, J.F. Burlein, J. Young, S. Koenig, and C.K. Stover. 1993. Protection of mice against tetanus challenge using an experimental tetanus vaccine based on recombinant BCG. In Modern Approaches to New Vaccines Including Prevention of AIDS. H.S. Ginsberg, F. Brown, R.M. Chanoek, and R.A. Lerner, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 385–390.

32. Shoemaker, N., and W.R. Guild. 1974. Destruction of low efficiency markers is a slow process occurring at a heteroduplex stage of transformation. Mol. & Gen. Genet. 128:283.

33. Talkington, D.F., D.L. Criminns, D.C. Voellinger, J. Yother, and D.E. Briles. 1991. A 43-kilodalton pneumococcal surface protein, PspA: isolation, protective abilities, and structural analysis of the amino-terminal sequence. Infect. Immun. 59:1285.

34. Briles, D.E., C. Forman, and M. Crain. 1992. Mouse antibodies to phosphocholine can protect mice from infection with mouse-virulent human isolates of Streptococcus pneumoniae. Infect. Immun. 60:1957.

35. Briles, D.E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 Streptococcus pneumoniae. J. Exp. Med. 153:694.

36. McDaniel, L.S., J.S. Sheffield, E. Swazio, J. Yother, M.J. Crain, and D.E. Briles. 1992. Molecular localization of variable and conserved regions of pspA, and identification of additional pspA homologous sequences in Streptococcus pneumoniae. Microb Pathog 13:261.

37. Lotte, A., O. Wätz-Hockert, N. Poisson, N. Dumitrescu, M. Verron, and E. Couvet. 1984. BCG complications. Estimates of the risks among vaccinated subjects and statistical analysis of their main characteristics. Adv. Tuberc. Res. 21:107.

38. Snapper, S.B., L. Lugosi, A. Jekkel, R.E. Melton, T. Kieser, B.R. Bloom, and W.R. Jacobs, Jr. 1988. Lysogeny and transformation in Mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. USA. 85:687.

39. Burlein, J.E., C.K. Stover, S. Offutt, and M.S. Hanson. 1994. Expression of foreign genes in mycobacteria. In Tuberculosis: Pathogenesis, Protection and Control. B.R. Bloom, editor. American Society for Microbiology, Washington, DC. 239-252.

40. Lugosi, L. 1992. Theoretical and methodological aspects of BCG vaccine from the discovery of Calmette and Guerin to molecular biology. A review. Tuber. Lung Dis. 73:252.

41. Gheorghiu, M., P.H. Lagrange, and C. Fillastre. 1988. The stability and immunogenicity of a dispensed-grown freeze-dried Pasteur BCG vaccine. J. Biol. Stand. 16:15.

42. Munoz, R.J., J.M. Musser, M. Crain, D.E. Briles, A. Marton, A.J. Parkinson, U. Sorenson, and A. Tomasz. 1992. Geographic distribution of penicillin-resistant clones of Streptococcus pneumoniae: characterization by penicillin-binding protein profile, surface protein A typing, and multilocus enzyme analysis. Clin. Infect. Dis. 15:112.

43. Wältman, W.D. II, L.S. McDaniel, B.M. Gray, and D.E. Briles. 1990. Variation in the molecular weight of PspA (pneumococcal surface protein A) among Streptococcus pneumoniae. Microb Pathog 8:61.

44. Briles, D.E., C. Forman, S. Hudak, and J.L. Clafflin. 1982. Anti-PC antibodies of the T15 idiotype are optimally protective against Streptococcus pneumoniae. J. Exp. Med. 156:1177.

45. McDaniel, L.S., W.H. Benjamin, Jr., C. Forman, and D.E. Briles. 1984. Blood clearance by anti-phosphocholine antibodies as a mechanism of protection in experimental pneumococcal bacteremia. J. Immunol. 133:3308.

46. Briles, D.E., C. Forman, J.C. Horowitz, J.E. Volanakis, W.H. Benjamin, Jr., L.S. McDaniel, J. Eldridge, and J. Brooks. 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. Infect. Immun. 57:1457.

47. Briles, D.E., J. Horowitz, L.S. McDaniel, W.H. Benjamin, Jr., J.L. Clafflin, C.L. Booker, G. Scott, and C. Forman. 1986. Genetic control of susceptibility to pneumococcal infection. Curt. Top. Microbiol. Immunol. 124:103.

48. Briles, D.E., M.J. Crain, B.M. Gray, C. Forman, and J. Yother. 1992. A strong association between capsular type and mouse virulence among human isolates of Streptococcus pneumoniae. Infect. Immun. 60:111.

49. Mestecky, J. 1987. The common mucosal immune system and current strategies for the induction of immune responses in external secretions. J. Clin. Immunol. 7:265.

50. McGhee, J.R., J. Mestecky, M.T. Detzbaugh, J.H. Eldridge, M. Hirsubara, and H. Kiyono. 1992. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine. 10:75.

51. Brandtzaeg, P. 1992. Humoral immune response patterns of human mucosae: induction and relation to bacterial respiratory tract infections. J. Infect. Dis. 165 (Suppl. 1):S167.

52. McDermott, M.K., and J. Bienenstock. 1979. Evidence for the migration of B immunoblasts into intestinal, respiratory, and genital tissues. J. Immunol. 122:1892.

53. Zar, J.H. 1984. Biostatistical Analysis. 2nd ed. Prentice Hall, Inc., Englewood Cliffs, NJ. 718 pp.