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Antibody-Independent, Interleukin-17A-Mediated, Cross-Serotype Immunity to Pneumococci in Mice Immunized Intranasally with the Cell Wall Polysaccharide

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Serotype-specific immunity to *Streptococcus pneumoniae* is conferred by antibodies to the capsular polysaccharides, which define the 90 known serotypes. Whether antibody to the species-common cell wall polysaccharide (C-Ps) is protective has been a matter of controversy. Here we show that C-Ps given intranasally with mucosal adjuvant increased the resistance of mice to experimental nasopharyngeal colonization by capsulated *S. pneumoniae* of serotype 6B. This immunity could be induced in mice congenitally lacking immunoglobulin but was dependent upon $CD4^+$ T cells. Elimination of the charged amino group on the polymer backbone by N acetylation of C-Ps reduced the immunity, as did treatment of the mice with antibody to the cytokine interleukin-17A at the time of challenge, both consistent with the hypothesis of T-cell activation due to the zwitterionic motif of the polymer. C-Ps also protected in a model of fatal aspiration pneumonia by heavily capsulated serotype 3. These findings suggest a novel immunization strategy against *S. pneumoniae*.

Streptococcus pneumoniae (pneumococcus) can asymptomatically colonize the human nasopharynx, but it also causes pneumonia and, particularly in infancy, other diseases ranging from otitis media to fatal systemic infections. Pneumococcal otitis is a major cause of morbidity and expense in industrialized nations, and in the developing world almost one million children yearly die of pneumococcal diseases (57). The capsular polysaccharides (PS), which define the 90 known serotypes, impede the phagocytosis of pneumococci. Antibodies to the PS are opsonic, confer serotype-specific protection, and have been called the only significant mechanism of acquired immunity (20). Current vaccines are based on injected mixtures of PS chosen for prevalent serotypes: plain PS vaccine includes 23 serotypes and immunizes mature humans but generally is not efficacious in infancy. Protein-conjugated PS vaccine protects infants against seven serotypes prevalent in systemic infections (4) but is costly to make and administer and subject to evasion by the increasing prevalence of nonvaccine serotypes (24). Simpler approaches with broader coverage are being sought.

Certain pneumococcal "species" antigens (common to all serotypes) have been shown to have immunoprotective potential despite the PS encapsulation, e.g., the surface proteins PspA, PspC, and PsaA and the cytolysin pneumolysin (6); the recent use of genomics has identified several dozen additional species proteins (56). Immunity has been induced by such antigens in animal models, but no vaccine based on species antigens has been licensed. Evidence was recently presented that natural immunity to pneumococci increases with age in early childhood without detectable antibodies to the PS, implying the possibility that other antigens are involved (31). Pneumococcal cell wall polysaccharide (C-Ps), a ribitol teichoic acid linked to the muramic residues of the cell wall peptidoglycan (9), and the membrane-bound lipoteichoic acid (LTA), consisting of the identical teichoic acid with a glycolipid end group (13), are much-studied species antigens. Natural antibody to the phosphorylcholine (PCho) determinant of pneumococcal teichoic acid was reported in 1981 to be protective in mice (8), and accordingly, the elicitation of antibodies with protein-coupled C-Ps or PCho as a species vaccine has been explored (29, 47, 48, 53). Protection was found in some model systems; in others, however, C-Ps or PCho antibodies were reported to be nonprotective (38, 39, 47), a result attributed to exclusion by the capsular PS (45). Similarly, antibodies to the "F antigen" expressed in LTA were once thought possibly to confer species protection, but follow-up studies discounted this view (1).

Most research on induction of pneumococcal immunity has used mice challenged by the intraperitoneal or intravenous route. Although the pathogenesis of pneumococcal systemic infection has been analyzed in detail, the mechanism of nasopharyngeal (NP) carriage, which precedes much of natural pneumococcal disease is less well understood (49, 50). Several workers recently have investigated the role of virulence factors in mucosal colonization (2, 3, 41, 42). Immunity to colonization can be induced: PS conjugate vaccine reduces carriage in children and induces herd immunity in adults (12, 30). Certain of the species protein antigens have also been shown to induce resistance to colonization in animal models (2, 7) and perhaps also in humans (35). Investigation of "phase variation" by Weiser and colleagues has revealed a mechanism whereby the

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subcapsular antigens of pneumococci, particularly PCho, may be more accessible in colonization than in bacteremia (26, 54). Pursuing an economical method to immunize with multiple species antigens, we found that intranasal (i.n.) vaccination with killed noncapsulated pneumococci (whole-cell vaccine [WCV]) plus mucosal adjuvant protected rats against serotype 3 pneumonia and protected mice against NP colonization by several other serotypes (32, 33). Unexpectedly, protection by various WCV lots correlated with their C-Ps antigenic expression (unpublished), so in the present study we tried i.n. immunization with purified C-Ps (given without coupling) plus mucosal adjuvants. The responses of wild-type and mutant mouse strains showed an antibody-independent $CD4^+$ T-cell-dependent protective activity similar to that of the WCV (34) and revealed an unexpected role of the cytokine interleukin-17A (IL-17A). The results suggest both a mechanism of immunity and an approach to immunization against pneumococci not heretofore considered.

MATERIALS AND METHODS

Biologics. Purified pneumococcal cell wall polysaccharide was bought from the Pneumococcal Reference Laboratory, Statens Seruminstitut (SSI), Copenhagen, Denmark. The purification from strain CSR SCS2 and properties have been described previously (25): the teichoic acid component has the repeating unit -6)-β-D-Glcp-(1–3)-α-D-AATGalp-(1–4)-α-D-[6-PCho]GalNAc-(1–3)-β-D*GalNAc-(1–1)-D-ribitol-5-P(O-. The asterisk indicates the position of a second phosphorylcholine side group in some strains (13, 25). The linkage of teichoic acid to the cell wall muramic acid, which has not been defined, is difficult to break without degrading the former (13), so C-Ps preparations, including that from SSI (25), typically include fragments of peptidoglycan. The SSI preparations are free of F antigen and contain about 1% "protein," consisting mainly of oligopeptides of the residual peptidoglycan rather than proteins per se (I. Skovsted, SSI, personal communication); here, no F antigen was detectable by enzyme-linked immunosorbent assays (ELISAs) (see below) in samples assayed at up to 1,000 μg/ml, and the 1% protein value was confirmed by using the Bradford assay kit (Bio-Rad, Hercules, CA) and A_{280}. Unless otherwise mentioned, the SSI preparations were used as furnished and referred to as C-Ps. For one study, C-Ps was further purified here by a 24-hour exposure at 4°C to 10% (wt/vol) trichloroacetic acid followed by centrifugation and reisolation from the supernatant by ethanol-acetone fractionation as previously described (9); recovery of C-Ps antigenicity as assayed by ELISA inhibition with TEPC-15 antibody (see below) was 63%, while the density of silver-stained bands in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGs) (presumably peptides) was reduced by ca. 90% (see Fig. 2A). C-Ps was periodate oxidized by 16 h of incubation at 25°C in a 0.28-ml aqueous solution containing 5 mg of C-Ps and 3 mg of $NaIO_3$. Five microliters of glycerol was added to reduce any excess periodate. Glycerol, sodium iodate, and oxidized C-Ps fragments of <3 kDa were removed by ultrafiltration with a Centricon YM-3 device (Millipore Corp., Bedford, MA). Recovery of C-Ps antigenicity was 6% that of a control preparation made by first incubating the $NaIO_3$ with glycerol, then mixing with C-Ps, and ultrafiltration. C-Ps was N acetylated with acetic anhydride in 5% (wt/vol) $NaHCO_3$ and dialyzed as previously described (52); in this procedure, which should not disrupt the polymer, C-Ps antigenicity was 84% conserved.

Cholera toxin (CT) and cholera toxin B subunit (CTB) were from List Biological Laboratories (Campbell, CA), muramyl dipeptide (MDP), and the TEPC-15 monoclonal antibody to the PCho determinant of C-Ps were from Sigma (St. Louis, MO).

ELISA. Assays for murine antibodies to C-Ps were done in NUNC-Immuno 96-microwell plates (Nalge Nunc International, Rochester, NY) coated overnight with C-Ps (5 μg/ml). After antigen adsorption, the plates were washed with phosphate-buffered saline containing 0.05% Tween (PBS-T) and blocked with 5% fetal bovine serum (FBS) in PBS-T. To distinguish PCho-specific antibodies from C-Ps "backbone"-specific antibodies, the diluted antibody samples were first coincubated with PBS-T alone or PBS-T with phosphorylcholine (100 μg/ml; Sigma) for 30 min at room temperature, after which samples were added to the ELISA plates and incubated at room temperature for 2 h. Plates were washed with PBS-T, and secondary antibody to mouse immunoglobulin G (IgG) or IgM (Sigma) was added and incubated at room temperature for 1 hour. The plates were washed and developed with SureBlue 3,3′,5,5′-tetramethylbenzidine microwell peroxidase substrate (KPL, Gaithersburg, MD). Anti-C-Ps antibody titers were determined against a standard serum sample from a C-Ps-immunized mouse whose value was arbitrarily set at 1,000 units/ml. Percent reduction by phosphorylcholine was calculated to ascertain to what extent the measured antibodies were directed against PCho. C-Ps antigenicity of various teichoic acid preparations was assayed by inhibition of the ELISA in which mouse monoclonal antibody to PCho (TEPC-15; Sigma) was premixed with serial dilutions of C-Ps (as the standard) or the modified antigens before application to the C-Ps-coated plates. Antibodies to whole pneumococci were assayed by coating the plates with 10^8 killed cells/ml before the blocking step as described previously (34). F-antigen activity was assayed by ELISAs in which the wells were coated with TEPC-15 (1 μg/ml in PBS) to capture antigens expressing PCho. Blocking and dilution of samples and secondary reagents were with PBS containing 5% FBS (no Tween), and washing was with PBS alone. Samples were added and incubated at room temperature for 2 h; after the wells were washed, they were incubated with a 1:2,000 dilution of rabbit antiserum to group C *Streptococcus* (SSI, Denmark), known to bind F antigen (46), then washed, and incubated with horseradish peroxidase-conjugated antibody to rabbit IgG (Southern Biotech, Birmingham, AL). Results were expressed as the concentration of sample generating a 0.2 increase in A_{450} compared to wells reacted without sample.

Immunization of mice. Mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, unless otherwise noted. The age of the mice at the time of the first immunization was between 4 and 6 weeks. C57BL/6J mice were used as wild-type animals. C57BL/6J muMT$^{-/-}$ mice (B6.129S2-*Igh-6tm1Cgn*/J, in which B-cell development is blocked at the pro-B stage [27]) are referred to as antibody deficient. With age, some of these mice tend to develop low levels of IgM and IgA antibodies; however, ELISAs of serum or salivary samples obtained following immunization showed no detectable antibodies to noncapsulated pneumococci. T-cell-deficient mice, *nu*$^{-/-}$, were compared to their respective *nu*$^{+/-}$ littermates. Major histocompatibility complex (MHC) class II-deficient mice (B6.129-H2-*Ab1tm1Gru* N12, with a disruption of the H2-Ab1 gene [16]) were from Taconic Inc. (Germantown, NY); these mice lack $CD4^+$ T cells. Immunization i.n. with C-Ps or its derivatives was done by instilling 10 μl of adjuvant mixed with antigen; secondary immunizations were given after 1 week. Separate study with methylene blue showed that, in unanesthetized mice, this volume of immunizing solution does not reach the lung (data not shown).

Colonization model. To determine susceptibility to NP colonization, i.n. challenge with live encapsulated pneumococci was done as described previously (32) 4 to 5 weeks after the second immunization, with the one noted exception in which challenge was done 5 months postimmunization. Briefly, serotype 6B strain 0603 (32), containing predominantly the transparent phenotype (55) was grown to mid-log phase in Todd-Hewitt broth with 0.5% yeast extract, harvested by centrifugation, resuspended in saline at a concentration of 10^8 CFU/ml, stored at −80°C, and thawed just prior to challenge. In unimmunized animals this challenge results in CFU in the nasopharynx increasing through day 4 and remaining about constant thereafter through at least day 14 postchallenge (33); all the studies here determined colonization at day 6 or 7 postchallenge. The mice were euthanized by CO_2 inhalation. To determine NP colonization, an upper respiratory culture was done by instilling sterile saline retrograde through the transected trachea, collecting the first 6 drops (about 0.1 ml) from the nostrils, and plating neat or diluted samples on blood agar plates containing 2.5 μg gentamicin/ml; thus, 1 CFU/100 μl of wash fluid or 10 CFU/ml was detectable. For calculations of geometric means, a sterile sample was assigned half the lower limit of detection, or 5 CFU/ml.

Model of postcolonization inhalation pneumonia. The heavily capsulated serotype 3 strain WU2, known to be highly virulent in mice (8), was used. The mice were given 10^6 CFU in 10 μl of saline i.n. to establish NP colonization; after 2 days, to mimic the aspiration of pneumococci, the mice were anesthetized with isoflurane and given an additional dose of 10^6 CFU of WU2 in 100 μl by the i.n. route. In control animals, this challenge produced death within 4 or 5 days in most and bacteremia in all.

Measurement of cytokine secretion by splenocytes. Cellular suspensions of splenocytes were obtained by passing spleens from immunized mice through a 70-μm cell strainer (BD Biosciences, Bedford, MA). The cells were washed, and red blood cells were removed by hemolysis. The cells were plated into 24-well tissue culture plates at a concentration of 10^5 cells/well in 500 μl of Dulbecco modified Eagle medium (BioWhittaker, Walkersville, MD) containing 10% low-endotoxin defined FBS (HyClone, Logan, UT) and ciprofloxacin (10 μg/ml, gift from Miles Pharmaceuticals). Following 72 hours of stimulation with concanavalin A (5 μg/ml; Sigma), C-Ps (at 10 and 100 μg/ml), or heat-killed pneumococcal strain 0603 (equivalent to 10^6 CFU/ml), supernatants were collected following centrifugation and stored at −80°C until analyzed by ELISA for gamma interferon,

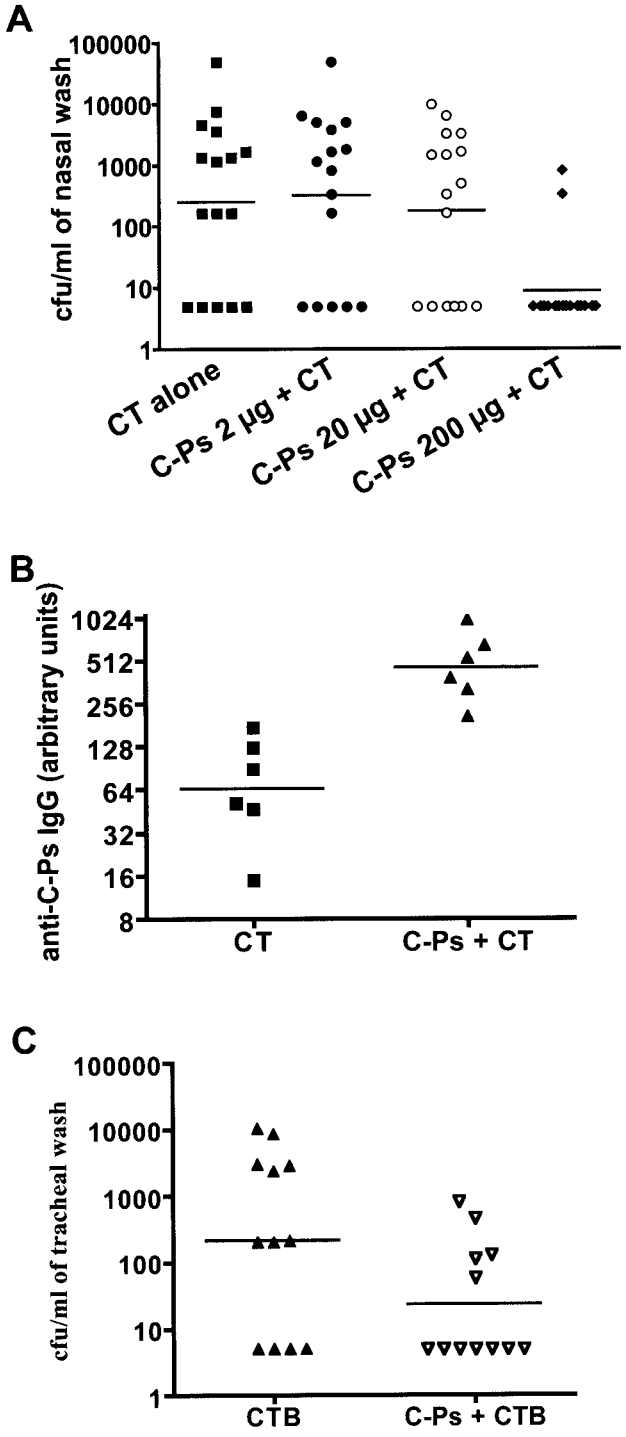

FIG. 1. Effect of intranasal immunization with C-Ps upon pneumococcal colonization in C57BL/6 mice. For panels A and C, each data point represents the density of nasopharyngeal colonization in CFU/ml of tracheal wash for each mouse, with sterile samples assigned a value of 5 (half the lower limit of detection of 10). The horizontal bar shows the geometric mean CFU/ml for each group. A. Immunization was with C-Ps in doses indicated and with 1 μg of cholera toxin where indicated. Three weeks after secondary immunization, the mice ($n = 16$ for each group) were challenged intranasally with a strain of serotype 6B. Reduction of CFU recovered from tracheal wash was significant for mice immunized with 200 μg C-Ps plus CT versus CT alone ($P = 0.004$ by the Kruskal-Wallis test with Dunn's correction). B. Serum antibody determination in mice ($n = 6$ for each group) that received CT or CPS (200 μg) plus CT. IgG serum antibodies to C-Ps were measured by ELISA; mice that received CPS (200 μg) plus CT had significantly more anti-C-Ps IgG 4 weeks after the second intranasal immunization ($P = 0.002$ by the Mann-Whitney U test). C. Immunization was with 200 μg of C-Ps plus 4 μg of cholera toxin B subunit or CTB alone. Mice ($n = 12$ for each group) were challenged with a strain of serotype 6B (comparison of density of colonization between mice that received C-Ps plus CTB versus CTB alone, $P = 0.049$ by Student's t test of log-transformed data).

IL-4, or IL-17A concentration (R&D Systems, Minneapolis, MN). Supernatants were analyzed in duplicate and read against a standard, following directions provided by the manufacturer.

Neutralization of IL-17A in vivo. Rabbit antibody to mouse IL-17A was obtained as described previously (10). The IgG fraction was purified using the Melon gel IgG purification kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Mice that had been previously immunized with C-Ps plus CT were randomized to receive either an intraperitoneal injection of 1 ml of normal rabbit serum (Sigma) or the same serum containing about 200 μg of anti-mouse IL-17A antibody. Serum was administered just prior to challenge with pneumococci and 3 days later. Mice were sacrificed on day 6 after challenge for determination of NP colonization.

Statistical analysis. Serum antibody and NP colonization density was compared by Student's t test for normally distributed data, the Mann-Whitney U test for nonnormally distributed data, or by the Kruskal-Wallis test with Dunn's correction for multiple comparisons using PRISM (version 4.0a; GraphPad Software, Inc.). Survival analysis by Kaplan-Meier analysis was performed using PRISM as well.

RESULTS

Intranasal C-Ps with adjuvant protects mice against pneumococcal nasopharyngeal colonization. C-Ps (200 μg per immunization) given i.n. twice at 1-week intervals as a droplet without adjuvant was nonprotective against pneumococcal colonization (data not shown); therefore, mucosal adjuvants were used. Because enterotoxin-type adjuvants like cholera toxin can induce transient nonspecific protection in the NP (32), the experiments below always compared antigen plus adjuvant versus adjuvant alone. Mice were immunized i.n. (sequentially, twice) with 2, 20, or 200 μg of C-Ps mixed with 1 μg of CT as adjuvant or with CT alone and challenged i.n. 4 weeks later with serotype 6B (Fig. 1A). The mice receiving 200 μg of C-Ps plus CT had significantly lower colonization density than mice that received the adjuvant alone (comparison of CFU recovered from tracheal washes, $P = 0.004$ by the Mann-Whitney test). The immunity was tested here 4 weeks after the second immunization; a separate study showed that immunity persisted at least 5 months postimmunization (comparison of CFU between mice immunized with C-Ps plus CT versus CT alone, $P = 0.04$ by the Mann-Whitney U test [data not shown]).

Four lots of the SSI C-Ps (D2, E2, E6, and G4) were tested, and all induced immunity (data not shown). In general, the CFU of adjuvant-only controls varied among experiments; however, particularly when control CFU was high, immunity was apparent in a significant reduction of CFU in the C-Ps-vaccinated animals, although there may not have been a difference in the incidence of culture negativity (e.g., see Fig. 3C). CTB, the nontoxic binding subunit of CT, also was an effective adjuvant: with 4 μg of CTB, C-Ps reduced NP colonization by serotype 6B ($P = 0.049$) (Fig. 1C).

Serum antibody responses. Mice receiving the immunizing dose of 200 μg C-Ps with CT had significantly higher serum IgG anti-C-Ps antibody titers than the CT-only controls: the

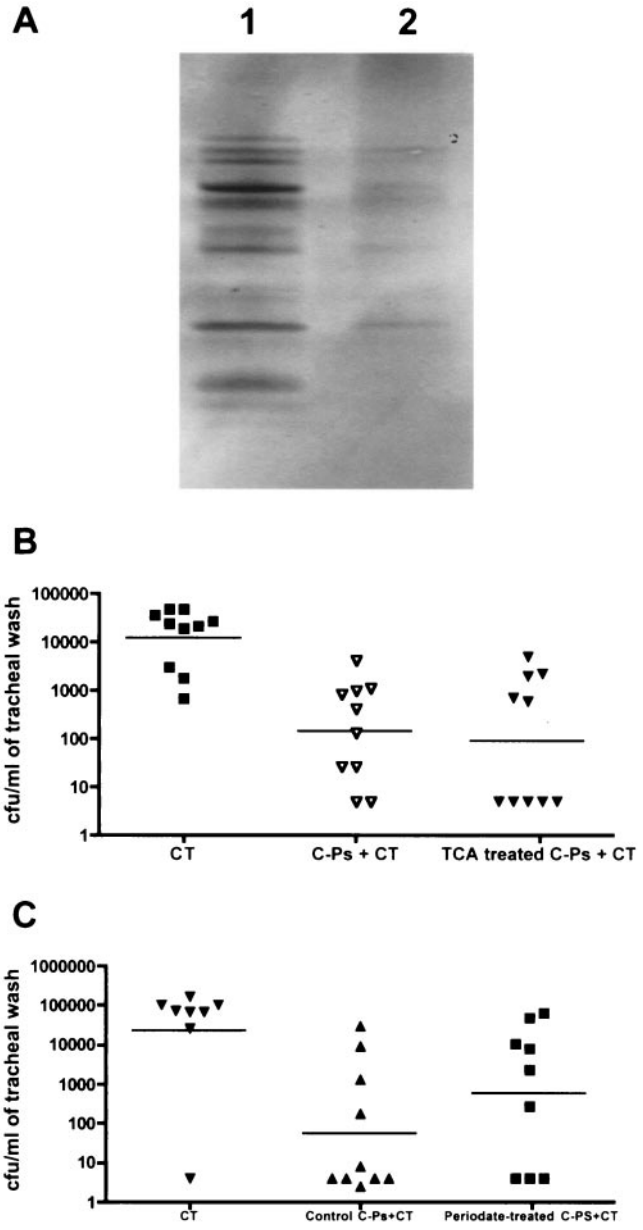

FIG. 2. Effect of further treatment of C-Ps with trichloroacetic acid or periodate. A. Silver-stained SDS-PAG of 100-μg samples of C-Ps (Statens Seruminstitut) before (lane 1) and after (lane 2) extraction with TCA for 1 day at 4°C as described in Materials and Methods. B and C. Effect of immunization with TCA-treated C-Ps plus CT (B) or periodate-oxidized C-Ps plus CT (C) upon nasopharyngeal colonization of C57BL/6 mice by serotype 6B. Mice were immunized intranasally with 1 μg of CT and 200 μg of C-Ps with and without prior treatment as indicated. The horizontal bar shows the geometric mean CFU/ml for each group. Protection by immunization with TCA-treated C-Ps plus CT was significant versus that achieved by CT alone ($P = 0.0007$ by the Mann-Whitney U test) and similar to that with C-Ps plus CT ($P = 0.74$). Oxidation of C-Ps with periodate reduced protection ($P = 0.21$ versus immunization with CT alone by the Mann-Whitney U test), whereas the control preparation of C-Ps incubated with glycerol-pretreated periodate was significantly effective ($P = 0.01$ versus CT alone by the Mann-Whitney U test) ($n = 8$ to 10 mice per group).

geometric mean was about sixfold greater in C-Ps-immunized mice compared to controls ($P = 0.002$ by the Mann-Whitney U test; Fig. 1B). Anti-C-Ps IgG antibodies in both control and C-Ps-immunized mice were predominantly directed against the PCho component (median reduction by phosphorylcholine, >90%).

Effect of further purification or modification of C-Ps. To investigate possible contribution of coisolated proteins or peptidoglycan, the SSI C-Ps was further purified with 10% (wt/vol) trichloroacetic acid (TCA) at 4°C, which can precipitate proteins and also slowly detach the peptidoglycan from the teichoic acid (9, 13). In SDS-PAGs with silver staining, C-Ps displayed ca.12 bands; the TCA-purified derivative exhibited a great reduction in the intensity of these bands (Fig. 2A), but TCA-treated C-Ps reduced colonization from a serotype 6B challenge as well as the untreated C-Ps did (Fig. 2B). To further explore whether residual peptidoglycan fragments in C-Ps could account for its activity in our model, MDP, a peptidoglycan derivative known to be immunologically potent (58) was tested directly; however, 20-μg doses of MDP (with CT) were nonprotective against pneumococcal colonization (data not shown). The vicinal hydroxyls of the glucose and ribitol residues make the teichoic acid subject to fragmentation by periodate oxidation, while peptidoglycan is not. C-Ps was oxidized with periodate and cleared of fragments by ultrafiltration: the oxidized C-Ps did not reduce pneumococcal colonization in a test in which the control C-Ps was protective (Fig. 2C).

Protection against pneumococcal colonization by C-Ps is antibody independent and $CD4^+$-T-cell dependent. C-Ps was tested to determine whether, like a whole-cell vaccine given i.n., its protective effect against colonization could be antibody independent and $CD4^+$-T-cell dependent (34). In $muMT^{-/-}$ (immunoglobulin-deficient) mice, C-Ps was as effective as in wild-type mice (Fig. 3A). In $nu^{-/-}$ (T-cell-deficient) mice, C-Ps was ineffective ($P > 0.05$ versus CT alone), but it was effective in $nu^{-/+}$ controls (Fig. 3B). In MHC class II-deficient mice, C-Ps did not confer any protection (Fig. 3C). Thus, like WCV, C-Ps given i.n. with adjuvant elicited an antibody-independent, $CD4^+$ T-cell-dependent protection against colonization by capsulated pneumococci.

Role of the zwitterionic motif of C-Ps. The observation of T-cell dependence suggested that the protection by C-Ps might include a mechanism previously described in a different context, whereby injection of C-Ps or other zwitterionic polysaccharides (containing positive and negative charges within the polymer backbone) into rats can induce abscess formation by stimulating $CD4^+$ T cells (11, 52). The primary amino group on each AATGal residue of C-Ps is positively charged, and N acetylation to convert the amino to N acetyl groups (uncharged) inhibited abscess formation (52). Accordingly, C-Ps was N acetylated by the identical method. Figure 4 shows that acetylated C-Ps, although retaining 84% of original TEPC-15 ELISA antigenicity, did not significantly reduce colonization ($P > 0.05$) in an experiment where untreated C-Ps was effective as expected ($P < 0.01$ versus CT alone).

IL-17A mediates C-Ps-induced immunity to colonization. An additional feature of the zwitterionic induction of abscess formation is the participation of IL-17A (10). The pertinence to the present work was evaluated in two ways. First, the ability

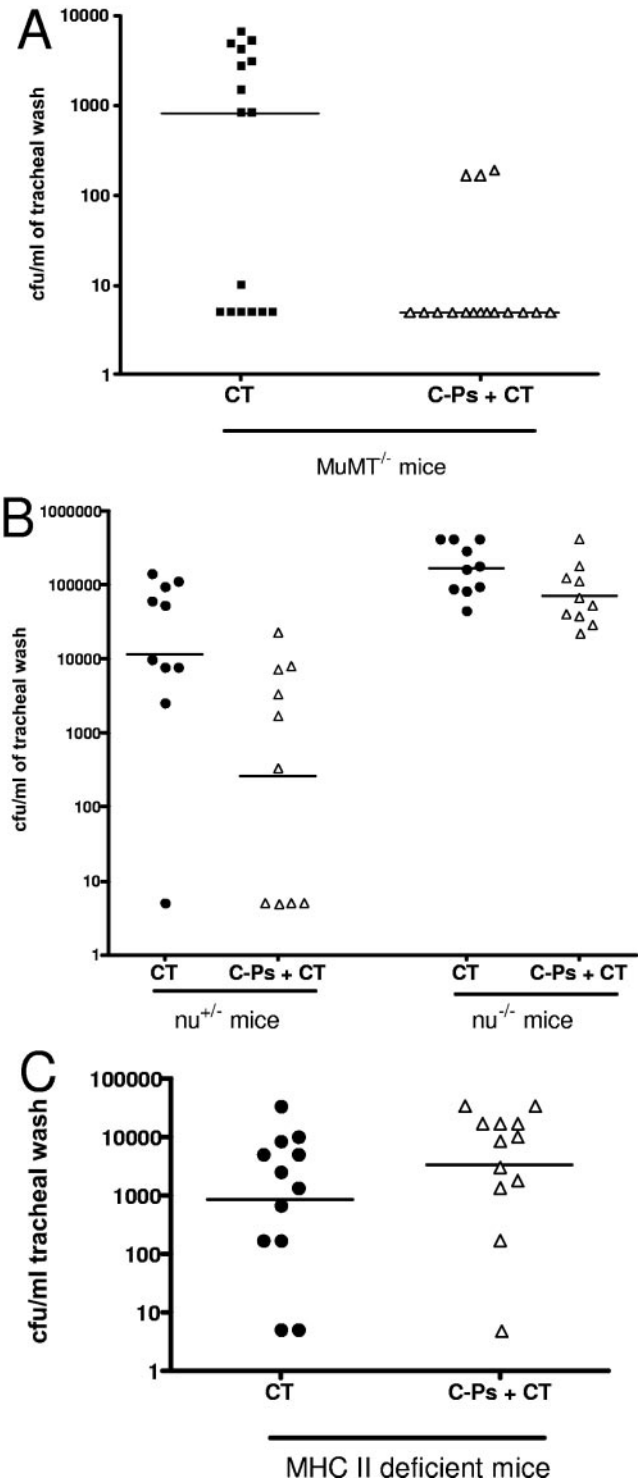

FIG. 3. Effect of intranasal immunization with C-Ps upon pneumococcal serotype 6B nasopharyngeal colonization in immunodeficient mice. Mice were immunized with 1 μg of CT and 200 μg of C-Ps or CT alone as indicated. The *P* values are calculated by the Mann-Whitney U test for protection versus CT alone. A. muMT$^{-/-}$ mice, deficient in immunoglobulin were significantly protected compared with CT controls ($P = 0.01$; 16 mice per group). B. Nude mice ($nu^{-/-}$) were not protected ($P > 0.05$) in contrast to their heterozygote controls ($nu^{+/-}$) ($P = 0.01$; 10 mice per group). C. Mice with a disruption of the MHC class II H2-Ab1 gene were not protected ($P > 0.1$; 12 mice per group). The horizontal bar shows the geometric mean CFU/ml for each group.

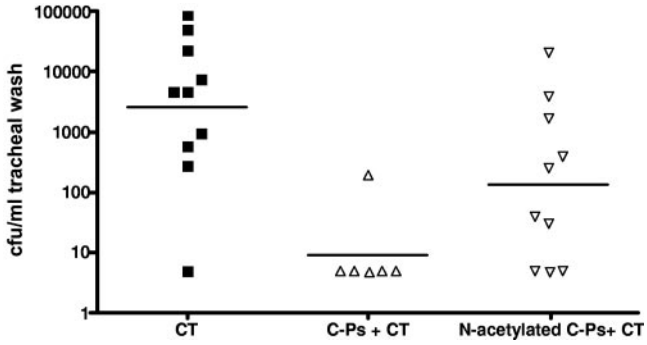

FIG. 4. Effect of N acetylation of C-Ps upon immunity to NP colonization. C-Ps was acetylated to convert the free amino to N acetyl groups (52). C57B/6 mice were immunized with 1 μg of CT and 200 μg of C-Ps or N-acetylated C-Ps as indicated. The density of colonization in the tracheal wash of individual mice is shown. The horizontal bar shows the geometric mean CFU/ml for each group. N-acetylated C-Ps plus CT was not effective in reducing colonization ($P > 0.05$ versus CT alone by the Kruskal-Wallis test with Dunn's correction), whereas untreated C-Ps plus CT was effective ($P < 0.01$) ($n = 6$ to 10 mice per group).

of splenocytes from mice immunized with C-Ps plus CT versus CT to secrete IL-17A in response to stimulation with C-Ps or killed pneumococci was compared. As shown in Fig. 5A, IL-17A secretion in response to stimulation with these pneumococcal antigens was observed in splenocytes only from mice that had been immunized with C-Ps plus CT, whereas the response to a nonspecific stimulus, such as concanavalin A, was similar in both groups. There was no detectable production of gamma interferon or IL-4 in response to stimulation with C-Ps or killed pneumococci (data not shown). Second, to test whether IL-17A is a critical effector cytokine for immunity in this model, mice immunized with C-Ps plus CT were treated with antiserum to IL-17A just before challenge, and immunity to pneumococcal colonization was reduced in comparison to treatment with a control serum (Fig. 5B).

Intranasal immunization with C-Ps protects against fatal pneumonia. Mice were inoculated i.n. with 10^6 CFU of serotype 3 strain WU2 in saline and 2 days later were given an additional 10^6 CFU under isoflurane anesthesia. In pilot studies this inoculum resulted in 80% mortality within 7 days, with the remainder of mice all showing signs of illness and/or bacteremia (data not shown). Mice were immunized with C-Ps plus CT or CT alone, then challenged as described above, and observed for 7 days; blood cultures were obtained on all survivors on day 7. As shown in Fig. 6, C-Ps plus CT significantly protected against death (survival in mice that received C-Ps plus CT versus CT alone, 60% versus 22%, respectively, $P = 0.046$ by Kaplan-Meier analysis). In addition, all the surviving mice in the CT control group had bacteremia, whereas survivors in the group treated with C-Ps plus CT did not.

DISCUSSION

After the 1981 report that PCho antibody in mice could be protective against pneumococcal challenge (8), a number of workers investigated this lead to develop a species immunogen. Since C-Ps by itself is a poor systemic immunogen (29), protein conjugates of PCho or C-Ps were used (29, 47, 53). Evidence of

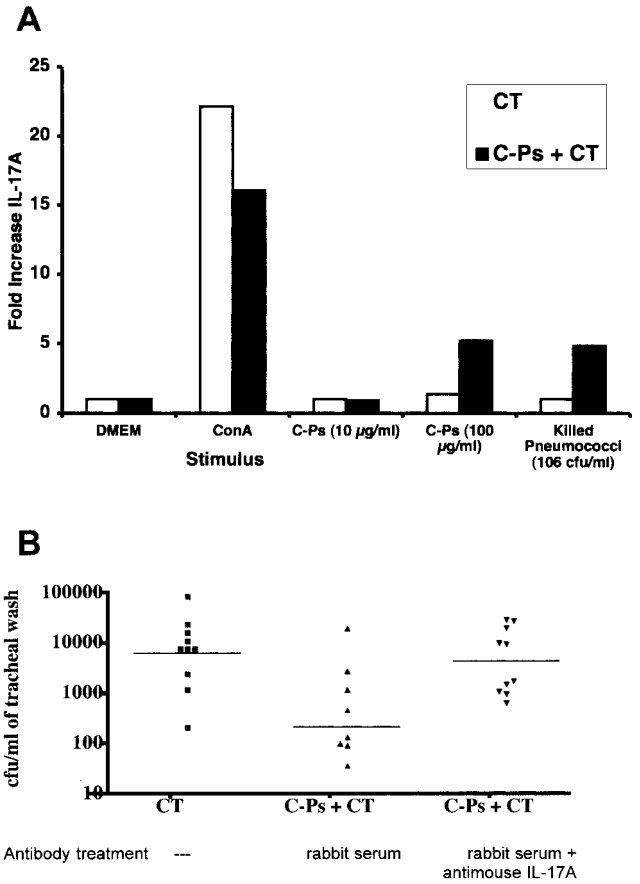

FIG. 5. Secretion of IL-17A following immunization with C-Ps and role in immunity to NP colonization. A) Cultured splenocytes from mice immunized with C-Ps plus CT or CT alone were stimulated with the agents indicated for 72 h, after which IL-17A production was measured by ELISA. Increased IL-17A production due to C-Ps or killed pneumococci was noted in splenocytes from mice immunized with C-Ps plus CT. Shown is a representative experiment of at least three experiments, which gave similar results. dmem, Dulbecco modified Eagle medium; conA, concanavalin A. B) Mice immunized with C-Ps plus CT 4 weeks earlier received intraperitoneal injections of rabbit serum with or without anti-mouse rabbit IL-17A antibody at the time of pneumococcal i.n. challenge and 3 days later. Mice were sacrificed on day 6, and nasal washes were collected for quantification of pneumococcal colonization. The horizontal bar shows the geometric mean CFU/ml for each group. Immunized mice that received IL-17A antibody had no reduction in the density of NP colonization (nonsignificant versus CT alone), whereas immunized mice that received only rabbit serum had significantly fewer recovered pneumococci from tracheal washes ($P = 0.01$ versus mice that received CT alone) ($n = 8$ to 10 mice per group).

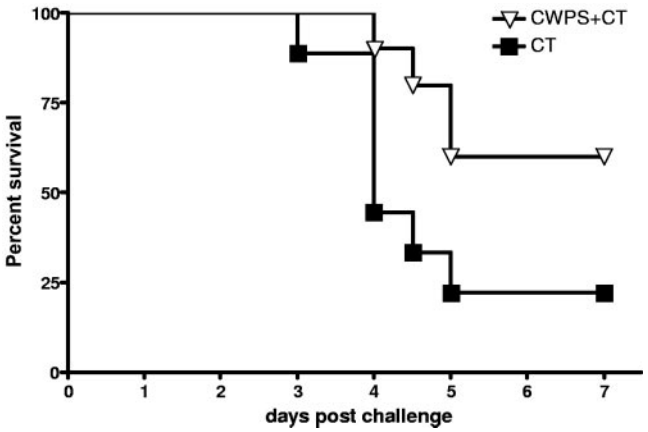

FIG. 6. Effect of intranasal immunization with C-Ps upon invasive disease in C57BL/6 mice. Mice immunized with C-Ps plus CT or CT alone were challenged by intranasal aspiration of type 3 strain WU2, and survival was assessed twice daily. Mice immunized with C-Ps plus CT survived significantly longer than mice that received CT alone ($P = 0.046$ by Kaplan-Meier analysis) ($n = 9$ or 10 mice per group).

protection was found in some studies (for example, references 48 and 53). In others, however, antisera raised by such conjugates (47) or monoclonal antibodies to PCho or backbone determinants (39) appeared nonprotective, and an ultrastructural study concluded that pneumococci with "intact capsules" would exclude C-Ps antibody (45). In humans, naturally acquired C-Ps serum IgG antibody appeared uncorrelated with immunity (38). However, interest in C-Ps as a protective antigen, particularly with respect to colonization, was increased by the description of pneumococcal phase variation (26, 55). Compared to the "opaque" phenotype (predominant in the bloodstream), the "transparent" phenotype (predominant in the nasopharynx) expresses less capsular PS and more C-Ps; thus, the PCho determinant may facilitate colonization by binding to the platelet-activating factor receptor on human mucosa. The transparent phenotype is susceptible to passively administered human PCho antibody (14) and to the acute-phase reactant C-reactive protein (15), which could have protective roles against pneumococci in the nasopharynx.

The present study has revealed what appears to be an additional C-Ps-induced host defense mechanism, one that can be elicited by i.n. vaccination with uncoupled C-Ps. In the wild-type mice, PCho-specific serum IgG antibody titers were increased, which may have contributed to the protection observed; however, these were unnecessary for protection in the NP colonization model, as indicated by the protection in the muMT$^{-/-}$ animals. The immunity resembles that which we described for a killed noncapsulated cell vaccine, WCV, in the requirement for adjuvant, antibody independence, and CD4$^+$ T-cell dependence (32–34). The antibody independence also resembles recent observations that clearance of pneumococci from the nasopharynx of naïve mice can occur without antibody (36) and that systemic clearance likely involves CD4$^+$ T cells (21), but whether the underlying mechanisms, including the critical role of IL-17A, are identical is presently uncertain.

To explore the protective potential of C-Ps in case pneumococci evaded NP clearance and invaded the lung, we sought a model to reproduce the natural pathogenesis in which, sometime after carriage is established, some insult, such as viral infection or aspiration, overcomes nonspecific barriers to lower-tract access. Thus, two days after an initial inoculation, an additional bolus of bacteria was given with anesthesia, which gave a consistently high fatality rate against which the effect of vaccination could be determined. C-Ps protected significantly in the model, indicating a potential for preventing invasion of the lung. The result superficially resembles the study of Trolle et al. in which a PCho-protein conjugate administered i.n. induced both mucosal and serum antibodies to PCho and protected against lung infection of anesthetized mice challenged

i.n. with serotype 3 (48); however, the extent of mechanistic overlap is unknown. Although the PCho dosages in the two studies were similar (15 μg coupled to 500 μg of carrier protein compared to 200 μg of C-Ps containing a maximum of 26 μg of PCho, assuming 100% purity), the conjugate was given in 50-μl volumes to anesthetized mice and reached the lung (known to increase the systemic antibody response [18]), while we gave (the nonconjugated) polysaccharide to nonanesthetized mice in small volumes shown not to reach the lung. It remains to be determined whether C-Ps will induce lung protection in muMT$^{-/-}$ mice, i.e., could be antibody independent, as is the immunity to NP colonization.

The immunizing dosage of C-Ps is high in relation to immunizing dosages of purified polysaccharides given systemically. The i.n. administration by droplet, however, does not maximize exposure of the immunogen to the nasal mucosa; unknown proportions are quickly swallowed by the animals. Also, as noted above, the mouse-immunizing dose of PCho-protein conjugate used i.n. by Trolle et al. was similarly high (48). In humans, the i.n. immunizing dosage of a meningococcal outer membrane antigen was about 10-fold the systemic dosage (40). Nonetheless, we have attempted to rule out components other than the teichoic acid of the SSI C-Ps preparations that could be responsible for the immunity observed. Residual capsular PS is unlikely to contribute, because strain CSR SCS 2 was derived from a heterologous capsular type (serotype 2) (5). The SSI purification of C-Ps includes deproteinization with chloroform-butanol and trypsin treatment (25); this and our finding that activity remained after TCA repurification reduces the likelihood that protection is attributable to coisolated pneumococcal proteins. TCA also has the potential to detach peptidoglycan from the teichoic acid (9, 13), and it seems likely that the silver-staining PAG bands diminished by the TCA procedure (Fig. 2A) represent residual peptidoglycan oligopeptides known to be present in SSI C-Ps preparations. Another approach to the peptidoglycan question was testing muramyl dipeptide directly, which was nonprotective against pneumococcal colonization. Finally, peptidoglycan, lacking vicinal hydroxyls, is periodate resistant. Thus, the observed reduction of immunity by periodate (Fig. 2C) indicates that residual peptidoglycan is not solely responsible and that the teichoic acid component of the C-Ps preparations is involved. Although the SSI C-Ps lacks detectable F antigen, not all molecules in pneumococcal lipoteichoic acid preparations equally express F activity (13). Thus, it is possible that the C-Ps contained some coisolated LTA, a known Toll-like receptor 2 agonist (17, 44), that contributed to the protection we observed.

The immunologic response to polysaccharides in general has been considered T-cell independent and characterized by a primary humoral response of IgM production, rare IgG class switching, and no immunologic memory (20). In contrast, adaptive major histocompatibility complex class II-restricted CD4$^+$ T-cell activation had been considered to be limited to proteins or protein-polysaccharide-conjugated antigens. These antigens are presented to T cells by MHC class II, leading to an immune response characterized by the production of Th1 or Th2 cytokines as well as IgG and the induction of immunologic memory. However, beginning with capsules of *Bacteroides fragilis*, Kasper and colleagues showed that polysaccharides with a zwitterionic charge motif within the repeating unit could activate CD4$^+$ αβ T cells to form abscesses in a process restricted by MHC class II. They found that such polymers are oxidized by nitric oxide and presented by MHC class II by B cells to T cells via interactions with αβ T-cell receptors (11, 51) and that IL-17A is an effector of abscess formation (10). Among the examples of polymers with such abscess induction activity is pneumococcal C-Ps, and N acetylation of the polysaccharide abrogated the activity (52). Our present observations that immunity to pneumococcal colonization is inducible by C-Ps in uncoupled form, that immunity is antibody independent and MHC class II T cell dependent, and that immunity is IL17A mediated all suggested that the activity (like abscess induction) could be due to the zwitterionic motif of the polymer. Consistent with this hypothesis, N acetylation of the polysaccharide, which removes a positive charge in the polymer backbone, resulted in an ineffective immunogen (Fig. 4). The precise mechanism whereby IL-17A mediates protection remains to be determined. There is abundant evidence from mouse models that the IL-17 cytokine family plays a critical role in neutrophil recruitment and abscess formation (10, 37, 59, 60) as well as induction of antimicrobial peptides (22, 23). Both of these mechanisms may be responsible for the protection observed and are currently under investigation.

This hypothesis aside, it is noteworthy that the SSI C-Ps, if presented mucosally with suitable adjuvant, induced immunity to NP colonization against a serotype 6 strain and protection against sepsis by serotype 3. The immunity to colonization required CD4$^+$ T cells and the activity of IL-17A. This cytokine, although much studied in the context of inflammation (19, 28, 43), to our knowledge has not been viewed as an effector of vaccine-induced bacterial immunity. Conceivably, once primed for enhanced production in the nasopharynx, its release could be triggered by a variety of potential pathogens presenting the same signal as pneumococci. Studies are under way to define the structural basis of activity of the C-Ps preparations, to further characterize the effector mechanisms, and to extend the observations to additional pneumococcal isolates and related respiratory pathogens. In general, our findings and recent reports from other laboratories (21, 36) commend attention to antibody-independent, T-cell-mediated mechanisms of immunity to pneumococci.

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We declare that we have no conflicting interests.

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