A Peptide Mimotope of Type 8 Pneumococcal Capsular Polysaccharide Induces a Protective Immune Response in Mice

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Increasing antibiotic resistance and a rising patient population at risk for infection due to impaired immunity underscore the importance of vaccination against pneumococci. However, available capsular polysaccharide vaccines are often poorly immunogenic in patients at risk for pneumococcal disease. The goal of this study was to explore the potential of peptide mimotopes to function as alternative vaccine antigens to elicit a type-specific antibody response to pneumococci. We used a human monoclonal immunoglobulin A (IgA) antibody (NAD) to type 8 *Streptococcus pneumoniae* capsular polysaccharide (type 8 PS) to screen a phage display library, and the phage PUB1 displaying the peptide FHLPYNHNWFAL was selected after three rounds of biopanning. Inhibition studies with phage-displayed peptide or the peptide PUB1 and type 8 PS showed that PUB1 is a mimetic of type 8 PS. PUB1 conjugated to tetanus toxoid (PUB1-TT) induced a type 8 PS-specific antibody response in BALB/c mice, further defining it as a mimotope of type 8 PS. The administration of immune sera obtained from PUB1-TT-immunized mice earlier (days 14 and 21) and later (days 87 and 100) after primary and reimmunization resulted in a highly significant prolongation of the survival of naive mice after pneumococcal challenge compared to controls. The survival of PUB1-TT-immunized mice was also prolonged after pneumococcal challenge nearly 4 months after primary immunization. The efficacy of PUB1-TT-induced immune sera provides proof of principle that a mimotope-induced antibody response can protect against pneumococci and suggests that peptide mimotopes selected by type-specific human antibodies could hold promise as immunogens for pneumococci.

Type-specific antibodies to the polysaccharide capsule are essential for protection against pneumococci, and the rationale for polysaccharide-based vaccines is that they induce such antibodies. Unconjugated pneumococcal capsular polysaccharide vaccines elicit antibodies that are largely restricted in their immunoglobulin G (IgG) subclass distribution, with a preponderance of IgG2 in humans (IgG3 in mice), even after absorption of nonspecific antibodies (3, 6, 25, 31, 34, 42, 45, 55). Type-specific antibodies to pneumococcal polysaccharide are also restricted in their V-region gene use, being predominantly derived from members of the V_H3 immunoglobulin gene family (1, 13, 39). Although the importance of antibody restriction for pneumococcal resistance is unknown, it may translate into a poor response to polysaccharide-based vaccines in patients with IgG subclass deficiencies and/or dysregulation of V_H3 gene expression, such as human immunodeficiency virus-infected individuals (1, 11, 12, 47).

Despite the generation of protective human monoclonal antibodies (MAbs) to several pneumococcal capsular polysaccharides (9, 13, 43, 46), the type-specific determinants that elicit protective antibodies are unknown. Since polysaccharides can elicit antibodies that are nonprotective (13, 28), in addition to those that are protective, surrogates for certain polysaccharide antigens might be useful in focusing the immune response on “protective” epitopes, e.g., those that elicit only a protective

response and/or overcome the restricted nature of the polysaccharide response (5, 39). Since they have been shown to induce antibody responses to native carbohydrate and/or microbial capsular polysaccharides (39), peptide mimetics or mimotopes have been proposed as potential surrogate antigens for polysaccharide epitopes. As such, these antigens may hold promise as alternative and/or adjunctive immunogens to induce immunity against pneumococci.

It has been proposed that molecular mimicry between peptides and polysaccharides enables peptides to serve as surrogates for polysaccharide antigens (39). However, the mechanisms by which mimicry translates into an immune response to the nominal polysaccharide antigen remain incompletely understood. Only a few peptides characterized as polysaccharide mimetics have been found to induce mimotope responses to the native polysaccharide, and fewer have been investigated for efficacy in animal models (16, 21, 24, 40). The difficulties of identifying immunologically active mimetics (mimotopes) have called into question the utility of commonly used parameters for selecting peptides as immunogens, such as high affinity to the selecting MAb, high antigenicity (defined as induction of a peptide-specific response), and cross-reactivity with immune sera to the nominal antigen (39).

Herein, we describe the identification of a mimotope of the capsular polysaccharide of serotype 8 *Streptococcus pneumoniae* (type 8 PS) by screening a phage display library with a human MAb IgA against type 8 PS. When conjugated to a protein carrier, the mimotope induced a long-lasting protective immune response to type 8 pneumococci in BALB/c mice,

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which mediated protection against pneumococcal challenge upon transfer to naive mice.

MATERIALS AND METHODS

Antibody reagents. NAD is a human monoclonal IgA1 antibody that binds specifically to type 8 PS (46). The efficacy and nucleotide sequence of NAD have been described previously (9, 46). NAD was purified as described elsewhere (9). A human myeloma IgA1 (Calbiochem Novabiochem Corp., San Diego, Calif.) and polyclonal human plasma IgA1, IgA, IgM and IgG (all from Calbiochem) were used as controls in binding and inhibition studies.

Bacteria and capsular polysaccharides. *S. pneumoniae* type 8 (ATCC 6308), type 3 (ATCC 6306), and purified type 8 PS (from strain 6308; molecular mass, 140 kDa [9]), and type 3 PS (from strain 6306; molecular mass, 220 kDa) were obtained from the American Type Culture Collection (ATCC; Manassas, Va.). Type 8 pneumococci were grown, frozen, and prepared for challenge experiments as described previously (54). Purified capsular polysaccharide of *Cryptococcus neoformans* (GXM) was prepared as described elsewhere (17).

Biopanning of a phage display peptide library. A linear 12-mer phage display peptide library (New England Biolabs Inc., Beverly, Mass.) was screened with NAD in a solid-phase support system. Three subsequent biopanning rounds were carried out according to the manufacturer's instructions, using 100 µg of NAD/ml in the first panning round and decreasing NAD concentrations in the second (10 µg/ml) and third (10 and 1 µg/ml) rounds, respectively. Between panning rounds 1 and 2, a subtractive panning was performed on a blank, blocked polystyrene plate to remove nonspecific plastic binders.

Sequencing of phage-displayed peptides. DNA of amplified phage clones was isolated with the QIAprep Spin M13 kit according to the manufacturer's instructions (QIAGEN, Valencia, Calif.). Sequence analysis of the peptide inserts was done by the DNA Sequencing Facility of the Albert Einstein College of Medicine. The sequencing primer used was the -96 gIII primer, 5'-GTA TGG GAT TTT GCT AAA CAA A-3' (New England Biolabs Inc.).

Peptide and peptide-conjugate synthesis. The peptide insert of phage PUB1, FHLPYNHNWFAL (molecular mass, 1,559 Da), was synthesized by the Laboratory for Macromolecular Analysis and Proteomics of the Albert Einstein College of Medicine. PUB1 with an N-terminal cysteine residue was conjugated to dextran and tetanus toxoid (TT) as described previously (16). Two 12-mer peptides were used as controls in inhibition studies: peptide 315, CKVMVHDPHSLA (5) (molecular mass, 1,337 Da; kindly provided by Matthew Scharff, Albert Einstein College of Medicine) and peptide 1496, QRKPIMIRQMK (molecular mass, 1,542 Da; synthesized as control).

Inhibition ELISAs with phage PUB1 and peptide PUB1. To determine the specificity of phage PUB1 for NAD, inhibition enzyme-linked immunosorbent assays (ELISAs) were performed as described elsewhere (53). Serial dilutions of the inhibitor were incubated with a fixed sample concentration previously shown to bind close to the middle of the linear portion of the binding curve. For each inhibition study, wells were included which did not contain any inhibitor. The percent inhibition was calculated as follows: $[(A_{405} \text{ without inhibitor} - A_{405} \text{ with inhibitor}) / (A_{405} \text{ without inhibitor})] \times 100$.

For inhibition of phage PUB1 binding to NAD, serial dilutions of NAD or the control monoclonal IgA1 were mixed with 2^{10} UV-inactivated phage-transforming units (PFU)/ml, preincubated at 4°C overnight, transferred to NAD-coated plates, and incubated for 2 h at 37°C. After washing, a mouse anti-phage M13 MAb (Amersham Biosciences Corp., Piscataway, N.J.) was added at 1 µg/ml for 1 h at 37°C, followed by an alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Southern Biotechnology Associates). To determine whether phage PUB1 was a mimetic of type 8 PS, 1.25 µg of NAD/ml and serial dilutions of phage PUB1 or the control phages (range, 5^{12} to 2^{10} PFU/ml) were added to type 8 PS-coated plates and incubated for 1 h at 37°C, and NAD binding was detected with an AP-labeled goat anti-human IgA (Southern Biotechnology Associates). Phage 4B4, a melanin binding phage with the peptide insert YERKFWHGRH (36) and phage 33, which does not contain a peptide insert (48), were used as negative controls. Inhibition of NAD binding to phage PUB1 was determined by incubating anti-phage M13 antibody-coated plates with 2.5^{10} PFU/well for 2 h at 37°C. After washing, 1.25 µg of NAD/ml together with serial dilutions of type 8 PS or the control polysaccharide (range, 500 to 1 µg/ml) were added for 2 h at 37°C. NAD binding was detected as described above.

The reactivities of polyclonal plasma IgA, IgA1, IgM, and IgG with type 8 PS-reactive antibodies and whether or not such antibodies recognized PUB1 were determined by ELISA. Type 8 PS-coated plates were incubated with serial dilutions of the plasma antibodies (range, 1.5 to 50 µg/ml) mixed with 50 µg of peptide PUB1 or peptide 315 (control)/ml as inhibitor and preincubated on blank, blocked plates overnight at 4°C. The mixture was then transferred to type

8 PS-coated plates, and the assay was developed as described above with AP-labeled goat anti-human IgA, IgA1, IgM, and IgG (all from Southern Biotechnology Associates).

Animals and immunization protocol. BALB/c and C57BL/6 mice, purchased from the National Cancer Institute (Bethesda, Md.), were housed at the Animal Facility of the Albert Einstein College of Medicine. All animal studies complied with the relevant federal guidelines and the policies of the Institute for Animal Studies of the Albert Einstein College of Medicine. Groups of five 6- to 8-week-old female BALB/c mice were immunized subcutaneously at the base of the tail on day zero and boosted on days 14 and 35. Mice were given 10 µg of PUB1-TT conjugate diluted in phosphate-buffered saline (PBS) without azide (BioWhittaker, Walkersville, Md.) to a final volume of 200 µl with or without the addition of an adjuvant mixture consisting of 10 µl of CpG oligodeoxynucleotide preparation (ImmuneEasy mouse adjuvant; QIAGEN) and 50 µl of Alhydrogel 2.0% (aluminum hydroxide gel adjuvant; HCL Biosector, Frederikssund, Denmark) per animal. Control groups received 10 µg of TT with or without CpG-Al or PBS alone. Mice were bled from the retroorbital plexus before vaccination and weekly thereafter.

Serological studies. Sera were separated by centrifugation of whole blood. Following absorption with 2.5 µg of serum cell wall polysaccharide (Statens Serum Institut, Copenhagen, Denmark)/ml, type 8 PS-specific antibodies were detected in pooled sera. PUB1-specific antibodies were detected in a modification of a previously described protocol on Immuno plates with a Maxisorp surface (Nalge Nunc Int. Corp., Rochester, N.Y.) coated with 2 µg of PUB1-dextran/ml in 0.1 M NaHCO₃ (pH 8.6) (16). ELISA data were plotted on a semi-log scale; the titer was defined as the serum dilution extrapolating to an absorbance of 0.1.

To determine the specificity and mutual cross-reactivity of type 8 PS- and PUB1-specific antibodies, inhibition studies with soluble type 8 PS and PUB1 were performed (16). PUB1-TT and PUB1-TT/CpG-Al immune sera from days 21 and 87 were mixed with serial dilutions of type 8 PS (range, 200 to 2 µg/ml) or PUB1 (range, 250 to 7 µg/ml), preincubated overnight at 4°C, transferred to type 8 PS- and PUB1-coated plates, incubated for 1 h at 37°C, and processed as described above. Type 3 PS and P1496, respectively, were used as negative controls. In another experiment, immune sera from day 28 to 87, used at a dilution in the linear portion of the binding curve to type 8 PS or PUB1, respectively, were absorbed with 2.5×10^6 CFU of heat-killed type 8 and type 3 pneumococci and incubated for 4 h at 4°C with continuous rotation. The bacteria were then centrifuged at 8,000 rpm (Savant µSpeedfuge model SFR1300) for 10 min, and the sera were transferred to a new tube containing the same number of bacteria. After four absorption rounds, sera were tested in serial dilutions for type 8 PS and PUB1 binding by ELISA as described above.

Active and passive protection experiments. On day 110 after primary immunization, PUB1-TT- and PUB1-TT/CpG-Al-immunized mice or control animals were challenged by intraperitoneal (i.p.) injection with 900 CFU of type 8 pneumococci. Challenge was performed at this time to follow the stability of type 8 PS-specific titers as long as possible. At the time of challenge, titers had been stable for 2 months. The 50% lethal dose (LD₅₀) of type 8 pneumococci in mice has been reported to be <10 CFU (54). A preliminary study for this experiment revealed that the inoculum used represented 1 LD₅₀ for type 8 pneumococci in BALB/c mice of the same age. Infected mice were monitored daily for survival. Bacteremia was assessed 6, 12, 24, 48, and 72 h after infection as described previously (54). CFU numbers were log transformed, and the mean of each dilution series was calculated.

The efficacy of sera from immunized mice was evaluated with several experiments in a passive protection model similar to that used to establish the efficacy of immune sera from mice immunized with a cryptococcal polysaccharide mimotope (29). In one experiment, the efficacy of immune sera was evaluated for each PUB1-TT and control immunization group with pooled sera obtained on days 28 to 57 after primary immunization. In another experiment, the efficacy of PUB1-TT-induced immune sera obtained at an early time after primary immunization was compared to that of sera from a later time. Sera from days 14 and 21 after primary immunization were used for the early time, sera from days 87 and 100 were used for the later time, and controls received PBS. Sera were diluted 1:5, 1:10, or 1:40 in PBS and heat inactivated for 30 min at 56°C. Groups of 10 6- to 8-week-old female C57BL/6 mice were given 100 µl of the diluted serum i.p. 1 h prior to the i.p. injection of 900 CFU of type 8 pneumococci (the LD₅₀ for C57BL/6 mice 72 h after infection) in 100 µl of tryptic soy broth/mouse. Survival was recorded daily.

Statistical analysis. Statistical comparisons were performed with Prism version 3.0.2. (GraphPad Software Inc., San Diego, Calif.). Mouse survival data were compared statistically by using the Kaplan-Meier log-rank test. For bacteremia studies, CFU counts between different treatment groups at the same time point postinfection

FIG. 1. Immunization with PUB1-TT induces type 8 PS- and PUB1-specific IgM and IgG. The y axis shows the inverse titer of antibodies to either type 8 PS or PUB1 on the days post-primary immunization, indicated on the x axis. The bar on day zero represents the titer of each specific antibody type in pooled preimmune sera. (A) IgM to type 8 PS; (B) IgG to type 8 PS; (C) IgM to PUB1; (D) IgG to PUB1.

were compared using the unpaired *t* test. A *P* value of 0.05 was taken to indicate statistical significance. Since all serological studies were performed on pooled sera, antigen-specific serum titers were not statistically compared.

RESULTS

Phage PUB1 binds to NAD and is a mimetic of type 8 PS.

After the third panning round, 12 clones of the phage library screened with 10 μ g of NAD/ml and 7 clones of the library screened with 1 μ g of NAD/ml were picked and amplified. Upon sequencing, all clones had the same amino acid sequence, FHLPYNHNWFAL, designated phage PUB1. ELISAs to determine the specificity of phage PUB1 revealed that soluble NAD inhibited $\geq 90\%$ of phage PUB1 binding to solid-phase bound NAD in a concentration-dependent manner, while a human myeloma IgA did not block phage PUB1-NAD binding (data not shown). Studies to determine whether PUB1 is a mimetic of a type 8 PS determinant showed that phage PUB1 inhibited 62% of NAD binding to solid bound type 8 PS, whereas the control phage 4B4 inhibited only 9%. In the reverse experiment, type 8 PS inhibited 67% of NAD binding to phage UB1, and the control polysaccharide (glucuronoxylomannan of *C. neoformans* [GXM]) did not inhibit any NAD binding to phage PUB1.

Since NAD uses gene elements that are used in naturally

occurring antibodies (9), we tested whether polyclonal human IgA, IgM, and IgG preparations bind type 8 PS and, if so, whether they recognize the epitope mimicked by PUB1. All polyclonal preparations showed reactivity with type 8 PS in ELISA-based assays (data not shown). Cross-inhibition studies showed that soluble PUB1 inhibited 39 and 68% of the binding of IgA and IgG to type 8 PS, respectively, but the binding of IgM and IgG was only inhibited by 3 and 9%, respectively. The inhibition studies were repeated with higher and lower antibody and peptide concentrations with the same results.

PUB1 is a mimotope of type 8 PS. Preimmune serum from PUB1 conjugate-immunized mice reacted with type 8 PS at titers of 1:540 for IgM and 1:210 for IgG. On day 7, type 8 PS-specific IgM in the PUB1-TT and PUB1-TT/CpG-AI groups increased 5.5- to 6.5-fold compared to preimmune serum values (Fig. 1A). Type 8 PS-specific IgM peaked on day 14, with titers of 1:7,000 in the PUB1-TT group and 1:8,000 in the PUB1-TT/CpG-AI group (13- and 15-fold increases compared to the preimmune serum, respectively) and decreased thereafter. A slight boost response was observed on day 49, 14 days after the second boost. Type 8 PS-specific IgG appeared between days 7 and 14 (Fig. 1B). The response of the group that received the adjuvants was more rapid than that of the one

FIG. 2. IgG subclass distribution of PUB1-TT-induced type 8 PS- and PUB1-specific antibodies. The y axis shows the sum of the IgG1, IgG2a, IgG2b, and IgG3 titers and their proportional distribution. The sum of the titers here may differ from the total IgG titer shown in Fig. 1 because polyclonal and monoclonal detection reagents were used, respectively. (A) IgG to type 8 PS of the PUB1-TT group; (B) IgG to type 8 PS of the PUB1-TT/CpG-Al group; (C) IgG to PUB1 of the PUB1-TT group; (D) IgG to PUB1 of the PUB1-TT/CpG-Al group.

that did not. The PUB1-TT/CpG-Al group showed a titer of 1:2,600 on day 14 and a peak titer of 1:3,000 at day 28 (14-fold increase compared to preimmune serum values). The PUB1-TT group not receiving adjuvants had a slower increase of type 8 PS-specific IgG, with a peak titer of 1/2,200 occurring on day 49 (10-fold increase). Following day 49, the titers decreased in both groups but remained 6- to 11-fold above baseline values. Compared to preimmune values, the control groups manifested increases in type 8 PS-specific IgM and IgG that were substantially less than those of the PUB1 conjugate-immunized mice (2.5- to 6-fold and 1.3- to 3-fold, respectively) (Fig. 1A and B). Control increases in type 8 PS-specific IgG were not sustained (Fig. 1B). There was no measurable induction of type 8 PS- or PUB1-specific IgA upon PUB1-TT (with or without CpG-Al) immunization.

There was an induction of PUB1-specific IgM upon PUB1-TT immunization, with peak titers of 1:1,300 in the PUB1-TT group and 1:3,300 in the PUB1-TT/CpG-Al group on day 28 (Fig. 1C). There was a strong induction of PUB1-specific IgG, with a peak titer of 1:50,000 in the PUB1-TT group on day 49 and a peak titer of 1:700,000 in the group

receiving CpG-Al on day 28 (Fig. 1D). The preimmune serum of BALB/c mice had IgM to PUB1 (Fig. 1C) but no PUB1 binding IgG.

The IgG subclass distributions of PUB1-TT-induced type 8 PS- and PUB1-specific antibodies are shown in Fig. 2. IgG1 and IgG2a were the predominant subclasses of PUB1-specific antibodies at all times. In contrast, IgG3 dominated the early type 8 PS-specific response, but IgG1 titers increased over time. In the PUB1-TT group, type 8 PS-specific IgG2a titers were below the lowest tested serum dilution (1:150) at most times. CpG-Al was associated with higher proportions of type 8 PS-specific IgG2a and IgG3 and PUB1-specific IgG1 and IgG2a.

PUB1-induced antibodies to type 8 PS and PUB1 are not cross-reactive with the nonhomologous antigen. The binding of serum IgG from PUB1-TT- and PUB1-TT/CpG-Al-immunized mice to solid-phase type 8 PS was almost completely inhibited by 200 μ g of soluble type 8 PS/ml, and 90% of the binding of serum IgG to solid-bound PUB1 was inhibited by 250 μ g of soluble PUB1/ml (data not shown). Inhibition ELISAs performed to evaluate cross-reactivity of type 8 PS- and PUB1-reactive antibodies did not reveal specific inhibition

FIG. 3. Cross-inhibition of binding to type 8 PS and PUB1 by PUB1 and type 8 PS, respectively. PUB1-TT-induced immune serum from day 87 was preincubated with soluble PUB1, type 8 PS, or the respective controls (P1496 and type 3 PS) and then tested in an ELISA against the nonhomologous antigen. The optical density (OD) at 405 nm is shown on the y axis, and the inhibitor concentration is shown on the x axis. (A) IgG binding to type 8 PS in the presence of peptides PUB1 or P1496. (B) IgG binding to PUB1 in the presence of type 8 and type 3 PS. Similar results were obtained with PUB1-TT immune serum from day 21 and with PUB1-TT/CpG-Al-induced immune serum from days 21 and 87. The point designated 1 on the logarithmic x axis represents the relevant uninhibited value.

of IgG binding to type 8 PS by soluble PUB1 or of IgG binding to PUB1 by type 8 PS (Fig. 3). The increased binding to PUB1 in the presence of high polysaccharide concentrations (Fig. 3B) has been previously observed for a mimetic of group B meningococcal polysaccharide (33). Absorption of PUB1-TT-induced immune serum with heat-killed type 8 PS pneumococci decreased IgG binding to type 8 PS by 60% (PUB1-TT/CpG-Al) and 76% (PUB1-TT) at the highest serum dilution tested by ELISA, but IgG binding to PUB1 was inhibited by <10% (data not shown). Therefore, within the limitations of our assays, we did not find evidence for significant cross-reactivity of type 8 PS- and PUB1-specific antibodies. Inhibition studies with soluble type 8 PS and PUB1 to evaluate affinity maturation of the type 8 PS and PUB1 response revealed a 5-fold decrease in the 50% inhibitory concentration of the non-CpG group, a 12-fold decrease for the CPG-group for the type 8 PS response, and a minor decrease for the PUB1 response (data not shown).

PUB1-TT-immunized mice manifest prolonged survival after a systemic type 8 pneumococcal challenge. All PUB1-TT- and PUB1-TT/CpG-Al-immunized animals survived an i.p. challenge with type 8 pneumococci compared to 60% of TT- or PBS-immunized mice and 20% of TT/CpG-Al-immunized mice (Fig. 4). The survival of PUB1-TT- and PUB1-TT/CpG-Al-immunized animals was significantly longer than that of TT/CpG-Al-vaccinated mice ($P = 0.01$). However, the survival difference between the PUB1-TT-immunized, TT- and PBS-treated mice did not reach statistical significance ($P = 0.13$), and that of the TT- and PBS-treated mice was not different from that of TT/CpG-Al-immunized mice. Bacteremia was detected in a total of four PUB1-TT/PUB1-TT/CpG-Al-immunized mice 12 or 24 h postinfection (Fig. 5A and B). All PUB1-TT-immunized mice and one control mouse that developed bacteremia were no longer bacteremic at 48 h postinfection (Fig. 5C). Only one control mouse that became bacter-

mic survived, and the other control mice that survived were never bacteremic 6, 12, 24, 48, or 72 h after challenge. All other control mice that became bacteremic had increasing bacteraemia until they succumbed (Fig. 5D).

PUB1-induced immune serum protects naive mice against systemic type 8 pneumococcal infection. Passive transfer of pooled sera from days 28 to 57 in a 1:10 dilution protected 100% of mice receiving PUB1-TT/CpG-Al immune sera and 90% receiving PUB1-TT immune sera against an i.p. challenge

FIG. 4. Survival of PUB1-TT-immunized BALB/c mice after i.p. challenge with type 8 pneumococci. The lengths of survival of PUB1-TT- and PUB1-TT/CpG-Al-immunized animals were significantly greater than that of TT/CpG-Al-immunized animals. *, $P = 0.01$ for PUB1-TT versus TT/CpG-Al and $P < 0.01$ for PUB1-TT/CpG-Al versus TT/CpG-Al by the Kaplan-Meier log-rank test. $n = 5$ mice per group.

FIG. 5. Bacterial burden in blood of PUB1-TT and control immunization groups of BALB/c mice after i.p. challenge with type 8 pneumococci. The y axis shows the log-transformed CFU of individual mice in the groups designated on the x axis at 12 (A), 24 (B), 48 (C), and 72 (D) h after infection. The mean CFU count of each group is designated by a solid line. A log CFU of 2.3 was assigned to mice in which no bacteremia was detected, consistent with the lower detection limit of the assay. *, $P \leq 0.05$ comparing PUB1-TT to control groups; #, $P < 0.05$ comparing PUB1-TT/CpG-Al to control groups (unpaired t test). $n = 5$ mice per group.

with type 8 pneumococci. The survival of mice receiving sera from PUB1-TT- or PUB1-TT/CpG-Al-immunized mice was significantly longer than that of all mice that received sera from the control groups (Fig. 6A). Passive protection experiments were also performed with a 1:5 (PUB1-TT and PUB1-TT/CpG-Al) and a 1:40 (PUB1-TT/CpG-Al) serum dilution. In both experiments, survival of mice receiving PUB1-TT-induced immune sera was 80 to 100% and significantly longer than that of all mice receiving control sera (data not shown). In another experiment, passive transfer of a 1:10 dilution of PUB1-TT-induced immune sera obtained on days 14 and 21 after primary immunization (before a second boost was administered) protected 90%, and sera obtained on days 87 and 100

after primary immunization protected 100% of animals (Fig. 6B).

DISCUSSION

Herein we report the isolation and immunogenicity of PUB1, a human IgA-selected type 8 PS mimetic which, like other PS mimetics, is rich in hydrophobic and aromatic amino acids (22). Our data show that a conjugate of PUB1 and TT induced a long-lasting type 8 PS-specific response in BALB/c mice which was protective in naive mice. Rational vaccine design strategies have predicted that the most desirable peptide mimotopes would be those with high affinity to their selecting antibody (39). However, this criterion has not proven to

FIG. 6. Survival of C57BL/6 mice receiving immune sera from PUB1-TT- and control-immunized mice after i.p. challenge with type 8 pneumococci. (A) Survival of mice receiving PUB1-TT- or PUB1-TT/CpG-Al-induced immune sera was significantly longer than that of control animals. *, $P \leq 0.05$ comparing PUB1-TT to control groups; #, $P \leq 0.05$ comparing PUB1-TT/CpG-Al to control groups (Kaplan-Meier log-rank test). $n = 10$ mice per group. (B) Survival of mice receiving PUB1-TT-induced immune sera obtained early, days 14 to 21, or later, days 87 to 100, was significantly longer than that of mice receiving PBS. *, $P \leq 0.05$ comparing early serum to PBS; #, $P \leq 0.05$ comparing late serum to PBS (Kaplan-Meier log-rank test).

be sufficient to predict the ability of certain mimetics to be successful immunogens against the antigen of interest (5, 48). Similar to a cryptococcal capsular polysaccharide epitope mimotope selected by a human IgM (53) that induced a protective antibody response (16, 29, 30), PUB1 was selected by a human IgA, an antibody isotype that could be more representative of an innate or primary antibody response. The IgM-selected GXM mimotope was recognized by naturally occurring antibodies (16, 53). IgMs have also been used to select mimotopes of other capsular polysaccharides (24, 38), and an IgA successfully selected mimotopes of *Shigella flexneri* (37). However, the efficacy of the foregoing peptides was not evaluated in vivo. IgA is the predominant isotype in mucosal tissues (50). Interestingly, the IgA used to select PUB1, NAD, shares molecular genetic characteristics with naturally occur-

ring antibodies, some of which are reactive with pneumococcal polysaccharides (9, 27). Our data showing that PUB1 inhibited the type 8 PS binding of human IgAs, but not other isotypes, suggest that PUB1 could be a surrogate for an epitope that elicits naturally occurring type 8 PS-reactive IgA. In view of evidence that isotype influences polysaccharide and peptide specificity (30, 32, 35), our data suggest that the specificity of type 8 PS-specific IgA could differ from that of IgM or IgG. Alternatively, our findings could reflect differences in affinity or avidity or unique characteristics of the primary or secondary reagents that were used.

We were unable to demonstrate inhibition of the binding of PUB1-induced antibodies to either type 8 PS or PUB1 with the nonhomologous antigen. This could in part be a limitation of our ELISA-based assays. However, similar results were also reported for polysaccharide- and peptide-reactive antibodies induced by a GXM mimotope in human transgenic immunoglobulin (30) and BALB/c mice for certain conjugates (16). In light of these observations and studies showing that binding to carbohydrate antigens and peptide mimetics is conferred by different paratopes (5, 19, 35, 44), our findings suggest that type 8 PS- and PUB1-specific antibodies could be derived from different precursors. Validation of this hypothesis requires analysis of the molecular derivation of PUB1- and PS-reactive antibodies, which was beyond the scope of the present study. Nonetheless, the concept is supported by evidence that antibodies to carbohydrates and proteins use different variable region genes (49) and the proposal that discrete antigen specificities are conferred by separate immunoglobulin clans (23).

The possibility that type 8 PS- and PUB1-specific antibodies have different precursors is also supported by their IgG subclass profiles. Although the subclass profiles could reflect the sensitivity of our reagents or other methodological factors, it is notable that the predominant subclass of type 8 PS-reactive IgG was IgG3, which is a minor constituent of mouse IgG but the predominant subclass of murine antibodies to polysaccharide antigens and highly protective against murine pneumococcal infection (6, 31). In contrast, the PUB1-specific response was dominated by IgG1 and IgG2a, subclasses often associated with T-cell-dependent responses to protein antigens. Variable region identical antibodies with different IgG subclasses can manifest different polysaccharide specificities (15, 32). Mice expressing human IgG4 were only able to produce peptide-specific antibodies, whereas mice expressing IgG2 were able to produce both peptide- and polysaccharide-specific antibodies after immunization with a capsular polysaccharide mimotope (30). In light of these observations, our findings support the proposal that subclass restriction among certain PS-specific antibodies could have a biological basis in avidity (52) and/or specificity. Although this hypothesis requires validation, it implies that subclass restriction of the polysaccharide response may not be overcome by changing the antigen from a polysaccharide to a peptide. In fact, other groups have shown that when mimotopes did not elicit the restricted isotype or idiotype of the native antigen, they were often unable to confer protection (5, 20, 48).

The shift towards IgG1 during the type 8 PS response to PUB1-TT suggests that PUB1 induced a T-cell-dependent response (31). Although comparison of the mimotope and a type 8-protein conjugate-induced response was beyond the scope of

the present study, the IgG subclass response resembled that observed for some PS-protein conjugates (18, 31, 42). Similar to the response to a GXM mimotope (16) and polysaccharide on the surface of whole pneumococci (51), the PUB1-TT-induced type 8 PS response was also characterized by the appearance of IgG early in the response and a lack of significant boosting. Regarding the appearance of IgG, the preimmune antibody levels in the mice used in this study suggest that the mimotope-induced type 8 PS response could have been a memory response. In support of this concept, the induction of specific PS responses has been proposed to depend upon the presence of a specific preimmunization repertoire (2, 10, 41), and vaccine-induced pneumococcal polysaccharide-specific antibodies have been found to originate from memory B cells (4, 26, 55). Analysis of the molecular derivation of type 8 PS- and PUB1-specific B cells is required to establish whether or not they originate from naive or memory cells.

Our finding that PUB1-TT-induced immune sera conferred almost complete protection from pneumococcal challenge in naive mice provides clear evidence that PUB1-TT immunization induced protective antibodies in BALB/c mice. Some control animals in the active challenge study survived, but 10 of 10 PUB1-TT and PUB1-TT/CpG-immunized mice survived, and the survival of these groups was significantly longer than that of TT/CpG-immunized controls. The survival of TT- and PBS-immunized controls could reflect several factors, including mouse strain, age, or experiment-to-experiment variation, since pneumococci may not be consistently lethal in mice (7). Regarding the latter, our finding that seven control mice (three of five TT, one of five TT/CpG-Al, and three PBS) never became bacteremic raises the possibility that they may not have been infected. This question could not be evaluated with the study design we used. However, since our data revealed the presence of a preimmune type 8 PS-reactive repertoire, the survival of control mice could be attributable to naturally occurring antibody as reported in another model (8). For both passive and active pneumococcal challenge studies, we used an inoculum that was equivalent to, or larger than, that used to study the efficacy of human MAbs to type 8 PS (9, 54). Interestingly, even though NAD selected PUB1 and PUB1 elicited protective antibodies, NAD itself was not protective against a similar systemic challenge (9). While more work is needed to understand this finding and to determine the robustness of PUB1-induced protection and how it compares to conventional polysaccharide vaccines, our results establish the efficacy of a mimotope-induced response against *S. pneumoniae*.

CpG has been found to be beneficial for some (30), but not other (20), mimotope vaccines. In our model, PUB1-TT immunization with CpG appeared to produce a faster induction of type 8 PS-specific IgG than immunization without CpG and increased the type 8 PS responses as reported for a polysaccharide vaccine. However, CpG use resulted in only a minimal effect on affinity maturation, which did not translate into a difference in efficacy. Hence, there was no functional benefit of CpG in our model, although the use of higher inocula or different conditions might have revealed a benefit. In light of a report that CpG inhibited the reactivity to one mimotope and altered the idotype of antibodies induced by another phosphorylcholine mimotope (20), our data underscore that the utility of CpG must be established empirically. Towards this end,

studies of the efficacy, specificity, idotype, or other qualitative characteristics of PUB1-TT-induced antibodies elicited with or without CpG could provide a rational approach for the use of CpG and/or other adjuvants.

In summary, our findings that PUB1-TT-induced immune sera were protective against pneumococcal challenge in naive mice provide proof of principle that a pneumococcal polysaccharide mimotope can induce a protective response, with the caveat that more studies are needed to establish mimotope efficacy against active challenge and efficacy relative to conventional polysaccharide vaccines. Future studies will address these questions and evaluate PUB1-TT efficacy in the setting of IgG subclass or T-cell deficiency; the data presented herein suggest that further studies of the use of mimotope-based reagents for antibody-based therapy and/or as alternative or adjuvative vaccines are warranted.

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