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Structure-Function Relationships for Human Antibodies to Pneumococcal Capsular Polysaccharide from Transgenic Mice with Human Immunoglobulin Loci

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To investigate the influence of antibody structure and specificity on antibody efficacy against *Streptococcus pneumoniae*, human monospecific antibodies (MAbs) to serotype 3 pneumococcal capsular polysaccharide (PPS-3) were generated from transgenic mice reconstituted with human immunoglobulin loci (XenoMouse mice) vaccinated with a PPS-3–tetanus toxoid conjugate and their molecular genetic structures, epitope specificities, and protective efficacies in normal and complement-deficient mice were determined. Nucleic acid sequence analysis of three MAbs (A7, 1A2, and 7C5) revealed that they use two different V_H3 genes (A7 and 1A2 both use V3-15) and three different V_κ gene segments. The MAbs were found to have similar affinities for PPS-3 but different epitope specificities and CDR3 regions. Both A7 and 7C5 had a lysine at the V_H-D junction, whereas 1A2 had a threonine. Challenge experiments with serotype 3 *S. pneumoniae* in BALB/c mice revealed that both 10- and 1-μg doses of A7 and 7C5 were protective, while only a 10-μg dose of 1A2 was protective. Both A7 and 7C5 were also protective in mice lacking either an intact alternative ($FB^{-/-}$) or classical ($C4^{-/-}$) complement pathway, but 1A2 was not protective in either strain. Our data suggest that PPS-3 consists of epitopes that can elicit both highly protective and less protective antibodies and that the superior efficacies of certain antibodies may be a function of their structures and/or specificities. Further investigation of relationships between structure, specificity, and efficacy for defined MAbs to PPS may identify antibody features that might be useful surrogates for antibody (and vaccine) efficacy.

The goal of vaccination with pneumococcal capsular polysaccharide (PPS)-based vaccines is to elicit type-specific antibodies to PPS that confer protection against *Streptococcus pneumoniae*. However, PPS is poorly immunogenic in many individuals who are at the highest risk for the development of invasive pneumococcal disease (3, 13, 24, 44). The poor immunogenicity of PPS in infants and young children has been overcome by the use of a PPS-protein conjugate vaccine (7). In contrast, the conjugated vaccine has not been found to be more immunogenic than unconjugated PPS vaccines in adults who are at increased risk for pneumococcal disease, including those with immunodeficiency and antibody defects (3, 13, 24, 44, 49, 50, 54). Moreover, the heptavalent vaccine in use in the United States (7) does not contain the serotype 3 PPS, which remains a major cause of invasive pneumococcal disease in adults (29, 35).

Serotype 3 *S. pneumoniae* is unique among pneumococcal serotypes because it causes disease predominantly in adults (29). Efforts to prevent invasive pneumococcal disease in individuals who are at the highest risk for disease have been plagued by poor PPS immunogenicity and reduced PPS-based vaccine efficacy (12). In addition, the lack of serologic surrogates that are predictive of antibody-mediated protection has hampered efforts to understand the determinants of vaccine efficacy or failure. Historically, the "gold standard" for serum protection against the pneumococcus was mouse protection (58). However, a therapeutic antiserum to serotype 3 *S. pneumoniae* could not be developed (14), and associations among parameters, such as serotype-specific antibody concentration, serum opsonic activity in vitro, and protection in experimental models, have been unpredictable (19, 39).

Efforts to understand PPS vaccine efficacy and failure have been limited by the use of polyclonal sera (15, 18), the lack of proven correlates of vaccine efficacy, and insufficient information regarding the characteristics of antibodies to PPS that mediate protection. Polyclonal sera often yield conflicting results with regard to antibody function in vitro and in vivo, because they are composed of antibodies of multiple specificities and isotypes that collectively, or individually, may be protective, nonprotective, or deleterious in vivo (14). In the hope of identifying surrogates of antibody efficacy against *S. pneumoniae* based on structure-function relationships, we have taken the approach of characterizing the molecular genetic structures and in vivo functional efficacies of defined, monospecific antibodies (MAbs) to PPS. In this study, we generated human MAbs to PPS-3 in a transgenic mouse strain that expresses human immunoglobulin genes, the XenoMouse mouse (37), and examined their molecular genetic structures and in vivo efficacies against serotype 3 *S. pneumoniae*.

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MATERIALS AND METHODS

Bacteria and PPS conjugate. *S. pneumoniae* serotype 3 strain 6303 (American Type Culture Collection [ATCC] Manassas, Va.) was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) to mid-log phase at 37°C in 5% CO_2 as

described previously (51). The PPS used for immunization was a conjugate that was produced using purified serotype 3 PPS (ATCC) and tetanus toxoid (TT) (PPS-3–TT) according to methods described for another TT conjugate produced by our group (21).

Generation of PPS-3-specific human MAbs from XenoMouse mice. XenoMouse mice obtained from Abgenix (Fremont, Calif.) were vaccinated subcutaneously at the base of the tail with a total dose of 2.5 μg of PPS-3–TT. Splenocytes were isolated on day 7 or day 14, and hybridomas were generated by fusion with the mouse myeloma cell line NSO as described previously (51) and propagated with a cell-cloning system, ClonaCell-HY (Stem Cell Technologies Inc., Vancouver, British Columbia, Canada) according to the manufacturer's instructions.

Isotypes and PPS specificities of MAbs. Hybridoma cell lines were tested for the secretion of antibodies that reacted with serotype 3 PPS as described previously (20). Briefly, hybridoma supernatants were adsorbed with purified pneumococcal cell wall polysaccharide (Statens Seruminstitut, Copenhagen, Denmark). Polystyrene enzyme-linked immunosorbent assay (ELISA) plates (Corning Glass Works, Corning, N.Y.) coated with 10 μg of PPS-3 (ATCC 6303)/ml were incubated with serial dilutions of the hybridoma supernatants, washed, and incubated at 37°C for 1 h with alkaline phosphatase-conjugated goat anti-human reagents to immunoglobulin G (IgG), IgM, IgA, and kappa light chains and a goat anti-mouse reagent to lambda light chains (Fisher Biotech, Fisher Scientific, Pittsburgh, Pa.). After the plates were washed, antibody binding was detected by developing the plates with *p*-nitrophenyl phosphate substrate (Sigma). Optical densities were recorded at 405 nm with an MRX Microplate Reader (Dynatech Laboratories, Chantilly, Va.). A human serum standard from a PPS vaccine (31) was used as a positive control, and an IgM myeloma protein (Calbiochem, San Francisco, Calif.) was used as a negative control. MAbs were also tested for binding to staphylococcal protein A (SPA) (Sigma) and double-stranded DNA as described previously (46, 51). The PPS-3 specificities of the MAbs were determined by two methods. First, the reactivities of the MAbs with the PPSs of *S. pneumoniae* serotypes 3, 4, 6B, 8, 9V, 14, 19F, and 23F (ATCC) were determined by a PPS capture ELISA as described above. Second, a modification of this ELISA was used in inhibition assays with soluble PPS-3. For these studies, plates were coated with 10 μg of PPS-3/ml and incubated with solutions consisting of 5-μg/ml constant amounts of the MAb and various concentrations of soluble PPS-3 ranging from 0.1 to 100 μg/ml in duplicate. After incubation at 37°C for 1 h, the plates were washed and incubated with alkaline phosphatase-conjugated goat anti-human IgM (Southern Biotechnology, Birmingham, Ala.) and developed as described above. The relative apparent affinity constant (aK_a) of each MAb for soluble PPS-3 was determined for the interaction of each MAb with soluble PPS-3 as described previously (40). The aK_a was defined as the inverse mole concentration of soluble PPS-3 needed to reduce maximal MAb binding to solid-phase PPS-3 by 50%. Although it is recognized that this method has limitations when applied to antibody-polysaccharide interactions, it is useful for comparing the relative affinities of antibodies binding to polysaccharide antigens for which defined epitopes are not available (38).

Epitope specificity. Competitive-binding assays were used to compare the epitope specificities of the different MAbs using a biotinylated MAb (A7 [see below]). MAb A7 was biotinylated as follows. First, the molar concentration of MAb A7 was determined, and a 0.1 M solution of EZ-link sulfo-NHS-LC biotin (Pierce, Rockford, Ill.) with *N*,*N*-dimethylformamide (Aldrich, Milwaukee, Wis.) was prepared. Next, 1 mg of biotin was slowly added while the solution was simultaneously vortexed. After a 1-h reaction at room temperature, the biotinylated MAb was dialyzed overnight in phosphate-buffered saline (PBS). The concentration of biotinylated MAb was determined by ELISA, and binding studies of PPS-3 showed that its binding was similar to that of unlabeled MAb (not shown). ELISA binding curves of each MAb on PPS-3-coated plates were used to determine the concentration of MAb resulting in 50% saturation, and this concentration was added in equal volume with dilutions of biotinylated MAb A7 and incubated with PPS-3-coated ELISA plates. In addition, the reverse assay was also performed: a fixed amount of the biotinylated MAb was added to serial dilutions of the unlabeled MAbs and incubated with PPS-3-coated plates. In both assays, the biotinylated antibody was also incubated with the plates without an inhibitor. The binding of the biotinylated MAb was detected with horseradish peroxidase-labeled streptavidin (Zymed, San Francisco, Calif.) and developed with peroxidase substrate (Kirkegaard & Perry Laboratories). Optical densities were recorded at 450 nm with an MRX microplate reader.

MAb-mediated complement activation. The abilities of the MAbs to promote pneumococcal activation of the classical and/or alternative complement pathway and mediate complement fixation to *S. pneumoniae* and/or PPS were assessed. These studies measure C3 deposition on either *S. pneumoniae* or PPS-3 by fluorescence-activated cell sorter (FACS) analysis (28) or ELISA (62) as described previously (63). For FACS analysis, heat-killed serotype 3 *S. pneumoniae* was incubated with 10 μg of the MAbs or the myeloma IgM/ml for 30 min at 37°C with 1% factor B-deficient human serum to evaluate classical complement pathway activation or with C2-deficient human serum (Calbiochem) to evaluate alternative complement pathway activation. The samples were then washed with Hanks balanced salt solution and incubated with fluorescein isothiocyanate-labeled goat anti-human C3 (Cappel, Durham, N.C.) for 1 h at room temperature, washed with Hanks balanced salt solution, and suspended in cold NaCl-EGTA. C3 deposition was analyzed by FACS at the FACS Facility of the Cancer Center at the Albert Einstein College of Medicine. Flow cytometric analysis was performed on a FACScan (Becton Dickinson Immunological Systems, San Jose, Calif.). For ELISAs, ELISA plates coated with 10 μg of PPS-3/ml were incubated with solutions consisting of a final concentration of 10 μg of either the MAbs or the IgM control/ml and factor B-deficient human serum or C2-deficient human serum at 37°C for 1 h. After incubation, the plates were washed again, incubated with goat anti-human C3 (Sigma) at 37°C for 1 h, washed, and then incubated with alkaline phosphatase-labeled rabbit anti-goat IgG (Sigma) at 37°C for 1 h. After being washed, the plates were developed with *p*-nitrophenylphosphate substrate (Sigma). The plates were then read as described above.

Nucleic acid sequence analysis. The nucleic acid sequences of the MAbs were determined by sequencing DNA amplified from RNA by PCR, as described previously (51). Briefly, V_H (heavy-chain) and V_L (light-chain) cDNAs were generated by reverse transcription of RNA with heavy- and light-chain constant-region primers. V_H and V_L were initially amplified with a set of sense primers complementary to human V_H and V_L sequences and the same antisense constant-region primers as for V_H and V_L. For the A7, 1A2, and 7C5 MAbs, the primers were as follows: V_H sense, 5′-GAGTTTGGGCTGAGCTGG-3′; V_H antisense, 5′-GGAATTCTCACAGGAGACGAG-3′; $V_{L(\kappa)}$ sense, 5′-GAA(CT)ATC(T)GAGCTCACC(GT)CAGTCTCCA-3′; and $V_{L(\kappa)}$ antisense, 5′-CCTG TTGAAGCTCTTTGTGAC-3′. The V_H and $V_{L(\kappa)}$ PCR products were gel purified and cloned into the PCR 1000 plasmid of the TA cloning system (Invitrogen, San Diego, Calif.) according to the manufacturer's instructions. The products of two independent PCRs were cloned. Inserts containing V_H and V_κ were identified by restriction endonuclease analysis. Plasmid DNA was isolated by the Maxi plasmid protocol (Qiagen, Inc., Chatsworth, Calif.). Oligonucleotide synthesis and DNA sequencing were performed by the DNA Synthesis Facility of the Cancer Center of the Albert Einstein College of Medicine. Variable-region sequences were compared to the database of human immunoglobulin sequences by using DNA PLOT (V Base Index; MRC Centre for Protein Engineering, Cambridge, United Kingdom) (22). The nucleic acid translation was performed by the BCM Search Launcher program (Human Genome Sequencing Center, Baylor College of Medicine, Houston, Tex.).

MZB-cell expression. Splenocytes from the spleen were prepared by passing the cells through a sterile nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, N.J.) in 10% fetal calf serum-RPMI 1640 medium (Sigma). Erythrocytes were lysed with an isotonic solution (0.155 M NH_4Cl, pH 7.4), and all cell suspensions were washed twice before being stained and subjected to flow cytometry analysis. Cell suspensions were stained by a combination of allophycocyanin anti-mouse B220 (Caltag Laboratories, Burlingame, Calif.), fluorescein–anti-mouse CD21, and phycoerythrin–anti-mouse CD23 (PharMingen, San Diego, Calif.) and analyzed by three-color flow cytometry using a FACSCalibur (Becton Dickinson Immunological Systems). Dead cells were excluded by using propidium iodide staining before gating B-220-positive cells. Marginal-zone B (MZB) cells $CD21^{high}$ $CD23^{low}$ were gated by using forward and side scatter profiles.

Mouse protection experiments. The protective efficacies of the MAbs were evaluated in models in which the antibody was administered via intraperitoneal (i.p.) injection 1 h prior to or (for complement reconstitution experiments) simultaneously with the injection of the organism. These models are based on the serum potency studies used to establish the efficacies of sera for antibody therapy (14, 39) and have been used by our group to establish the efficacies of human MAbs to PPS-8 (62) and *Cryptococcus neoformans* capsular polysaccharide (22). The following normal and complement-deficient mice were used for MAb protection experiments: female BALB/c mice (6 to 8 weeks old) obtained from the National Cancer Institute (Bethesda, Md.); factor B-deficient ($FB^{-/-}$) mice (129 Ola/Hsd) and their normal-background ($FB^{+/+}$) controls, which were used as a model of alternative complement pathway deficiency (41); C4-deficient ($C4^{-/-}$) and C3-deficient ($C3^{-/-}$) mice, which were used as models of classical and total complement pathway deficiency, respectively, generated as described previously(20, 56); and control F2 (C57BL/6 × 129) mice bred in the Animal Facility of Albert Einstein College of Medicine in sterile isolators. A myeloma IgM (Calbiochem) and PBS, which were used as controls, and either 10 or 1 μg of MAbs were given to the mice 1 h prior to the intraperitoneal (i.p.) administration of 50

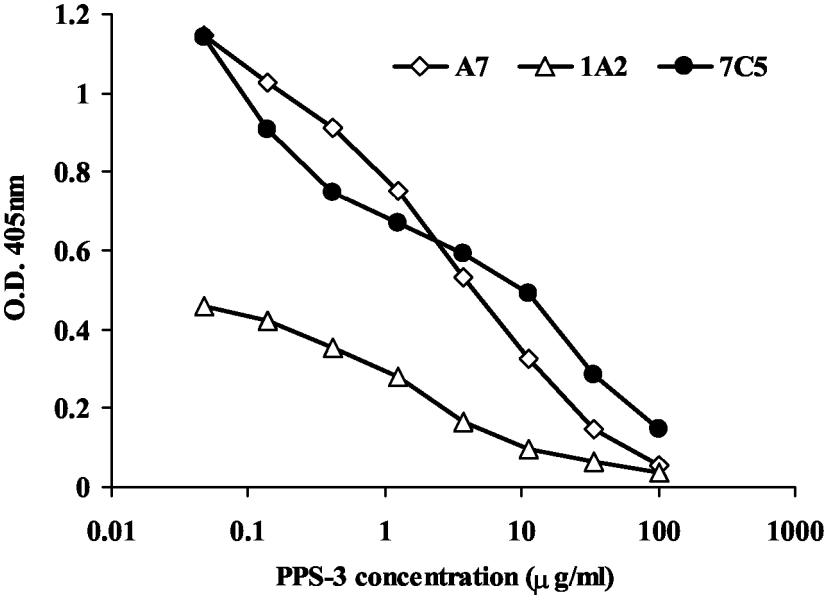


FIG. 1. Serotype 3 specificities of XenoMouse mouse-derived MAbs. O.D., optical density.

CFU (10 times the 50% lethal dose of ATCC strain 6303 in F2 and FB mice 48 h after infection) of serotype 3 *S. pneumoniae* (ATCC 6303). For reconstitution experiments in $C3^{-/-}$ mice, the inoculum, relevant MAb (or control), and 5% (vol/vol) either factor B-deficient or C2-deficient human serum (Calbiochem) were mixed immediately before administration and injected i.p. into groups of 10 mice each as described previously (22). The number of live bacteria used for the infections was confirmed by counting the CFU on blood agar plates (Difco) immediately before and after the mouse inoculations. All experimental groups had 10 mice, and the mice were monitored twice daily for survival.

Statistical analysis. Mouse survival data were analyzed statistically by using the Kaplan-Meier log rank survival test. A *P* value of 0.05 was taken to indicate significance.

Nucleotide sequence accession numbers. The GenBank accession numbers for V_H and V_L were as follows: 7C5 V_H, AF431049, and V_L, AF431050; A7 V_H, AF431055, and V_L, AF431056; and 1A2 V_H, F431053, and V_L, AF431054.

RESULTS

Specificities and isotypes of MAbs. Three MAbs are reported: A7, 1A2, and 7C5. Each MAb was an IgM(κ) and reacted with PPS-3 and SPA without significant binding to TT, bovine serum albumin, cell wall polysaccharide, double-

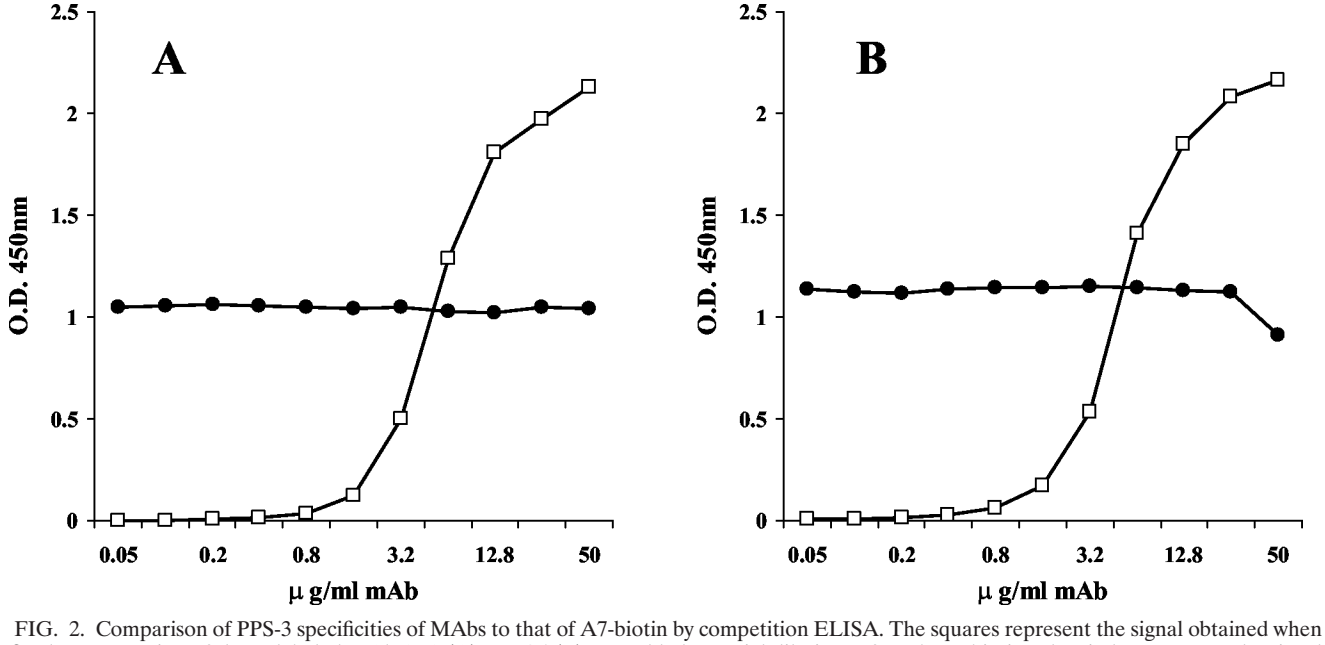


FIG. 2. Comparison of PPS-3 specificities of MAbs to that of A7-biotin by competition ELISA. The squares represent the signal obtained when a fixed concentration of the unlabeled MAb 1A2 (A) or 7C5 (B) was added to serial dilutions of MAb A7-biotin. The circles represent the signal obtained when a fixed concentration of MAb A7-biotin was added to serial dilutions of the unlabeled MAb 1A2 (A) or 7C5 (B). The signals are represented by the optical density (O.D.) as shown on the *y* axis for the concentrations of the indicated MAb on the *x* axis.

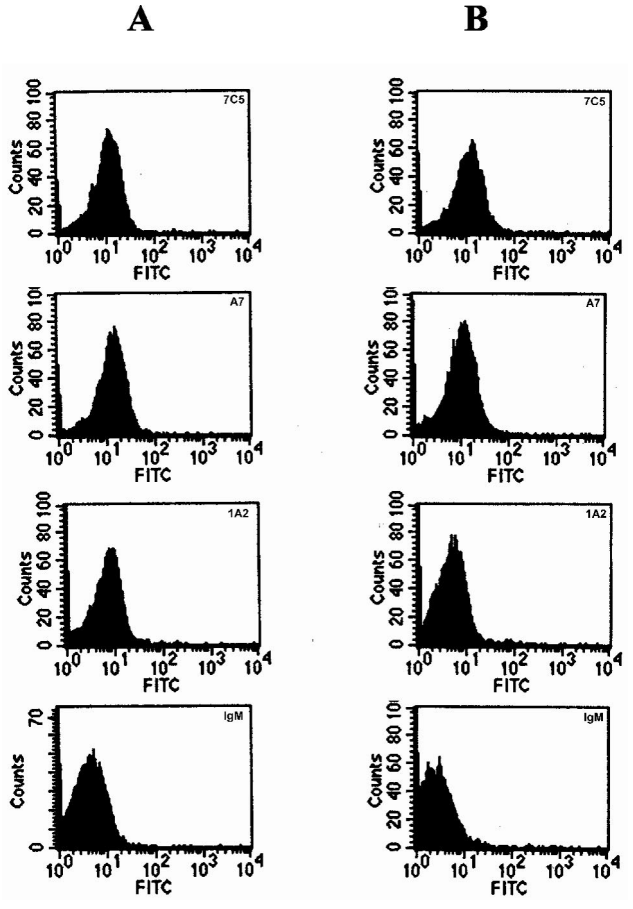


FIG. 3. FACS analysis of MAb-mediated C3 deposition on serotype 3 *S. pneumoniae*. C2 (A)- and factor B (B)-depleted human sera were used as complement sources as described in the text. The geometric mean median channel fluorescence intensity values in the histograms from top to bottom were 10.55 (7C5), 11.92 (A7), 6.6 (1A2), and 4.1 (IgM) (A) and 10.34 (7C5), 9.48 (A7), 4.46 (1A2), and 2.53 (IgM) (B).

stranded DNA, or other PPS serotypes (data not shown). None of the MAbs expressed the determinants recognized by the mouse anti-human MAbs D12, B6, and 16.84, which recognize determinants expressed by V_H3 genes (1). The specificities of the MAbs for PPS-3 were demonstrated by their exclusive binding to PPS-3 and inhibition of their PPS-3 binding by soluble PPS-3 (Fig. 1). The calculated aK_a values of the MAbs, defined as the inverse of the soluble PPS-3 antigen concentration at 50% maximal binding, were 1.7×10^7, 1.6×10^7, and 2.6×10^7 M^{-1} for A7, 7C5, and 1A2, respectively.

Epitope specificity. Competition ELISAs using biotinylated MAb A7 established that A7 has different epitope specificity than MAbs 1A2 and 7C5 (Fig. 2). Over most MAb concentrations, increasing concentrations of A7-biotin in the presence of a constant concentration of 1A2 or 7C5 resulted in an increase in PPS-3 binding, whereas incubation of constant concentrations of A7-biotin with increasing concentrations of 1A2 or 7C5 did not result in a change in absorbance. This result indicates that A7 does not compete with the other MAbs for PPS-3 binding. However, when the concentration of 7C5 was >25 μg/ml, A7-biotin binding to PPS-3 was inhibited by 19%, suggesting that these MAbs may recognize close or overlapping epitopes.

MAb-mediated complement activation. The MAbs promoted the deposition of C3 on serotype 3 *S. pneumoniae* and solid-phase PPS-3 when factor B- or C2-deficient human sera were used as complement sources (Fig. 3 and 4). The MAbs A7 and 7C5 mediated C3 deposition that was greater than that mediated by MAb 1A2 in the presence of both factor B- and C2-deficient sera by both FACS analysis and ELISA. The C3 deposition mediated by the control IgM was similar to that of 1A2.

Nucleic acid sequence analysis. The immunoglobulin gene segments used by the MAbs are shown in Table 1. All the MAbs express human heavy- (V_H) and light (V_L)-chain vari-

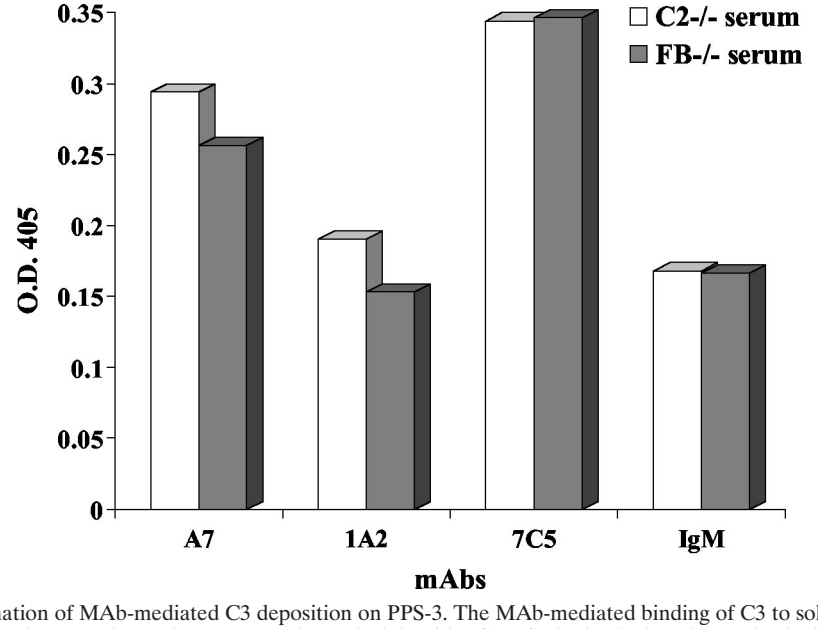


FIG. 4. ELISA determination of MAb-mediated C3 deposition on PPS-3. The MAb-mediated binding of C3 to solid-phase PPS-3 on the plate was evaluated as described in the text. The *y* axis represents the optical densities (O.D.) obtained when the MAbs depicted on the *x* axis were used.

TABLE 1. Human V_H and V_L gene segments used by IgM MAbs to PPS-3[a]

MAb	V_H (GenBank accession no.)	V_H3	D_H	J_H	CDR3 (length in amino acids)	V_L (GenBank accession no.)	$V_κ1$	$J_κ$	CDR3 (length in amino acids)
7C5	AF431049	DP-47/V3-23	D7-27	J_H4b	10	AF431050	DPK19/A1	Jκ1	9
A7	AF431055	DP-38/V3-15	D1-26	J_H1	11	AF431056	DPK15/A19	Jκ1	9
1A2	AF431053	DP-38/V3-15	D6-13	J_H4b	10	AF431054	DPK26/A26	Jκ1	9

[a] Variable-region gene usages were compared to the database of human immunoglobulin sequences by using DNA PLOT (V Base Index; MRC Center for Protein Engineering, Cambridge, United Kingdom).

able region gene transcripts. The MAbs all use V_H3 gene elements (A7 and 1A2 use the DP-38/V3-15 gene, and 7C5 uses the DP-47/V3-23 gene), but their D_H and J_H gene segment usages differ. The MAbs A7 and 1A2 have different CDR3 regions. The CDR3 regions of the MAbs all manifest 1- or 2-amino-acid differences compared with their closest germ line genes (Fig. 5). The MAbs 7C5 and A7 have a positively charged residue, K, at position 94, but 1A2 does not. For A7, there was a C-to-A base change that translated into a change from the germ line T to K. The K in 7C5 is in the germ line gene. All three MAbs use a $J_κ1$ light-chain gene element (A7 uses DPK15, 1A2 uses DPK26, and 7C5 uses DPK19) and have an arginine (R) at position 96 in the $J_κ1$ light chain compared to a tryptophan (W) in the closest germ line sequence (Fig. 5).

MZB cells. MZB cells in mice are phenotypically defined by IgM^{high}, Ig^{low}, $CD2^{high}$, and $CD2^{low}$ cell surface expression as determined by FACS analysis. Using flow cytometry, we measured this population in the spleens of XenoMouse mice. The proportions of all splenic B cells that were MZB cells in three XenoMouse mice were 24.23, 19.43, and 19.26%. The proportion of MZB cells in normal mice has been reported to be 5 to 10% (36).

Mouse protection experiments. Inoculation of all mouse strains with *S. pneumoniae* ATCC 6303 serotype 3 resulted in death within 48 h, with the mice appearing ill within 24 h of infection. For BALB/c mice, administration of A7, 1A2, and 7C5 significantly prolonged survival at a 10-μg dose compared to control IgM- and PBS-treated mice (Fig. 6A). Survival for 1A2- and A7-treated mice was prolonged for mice receiving the 10-μg dose compared to those receiving the 1-μg dose ($P = 0.0001$ and 0.05, respectively; Kaplan-Meier log rank survival test). The dose of MAb did not influence survival for 7C5 or the IgM control. MAbs A7 and 7C5, but not 1A2, significantly prolonged survival at a 1-μg dose compared to the IgM- and PBS-treated mice ($P < 0.01$; Kaplan-Meier log rank survival test). The studies of complement-deficient mice were all

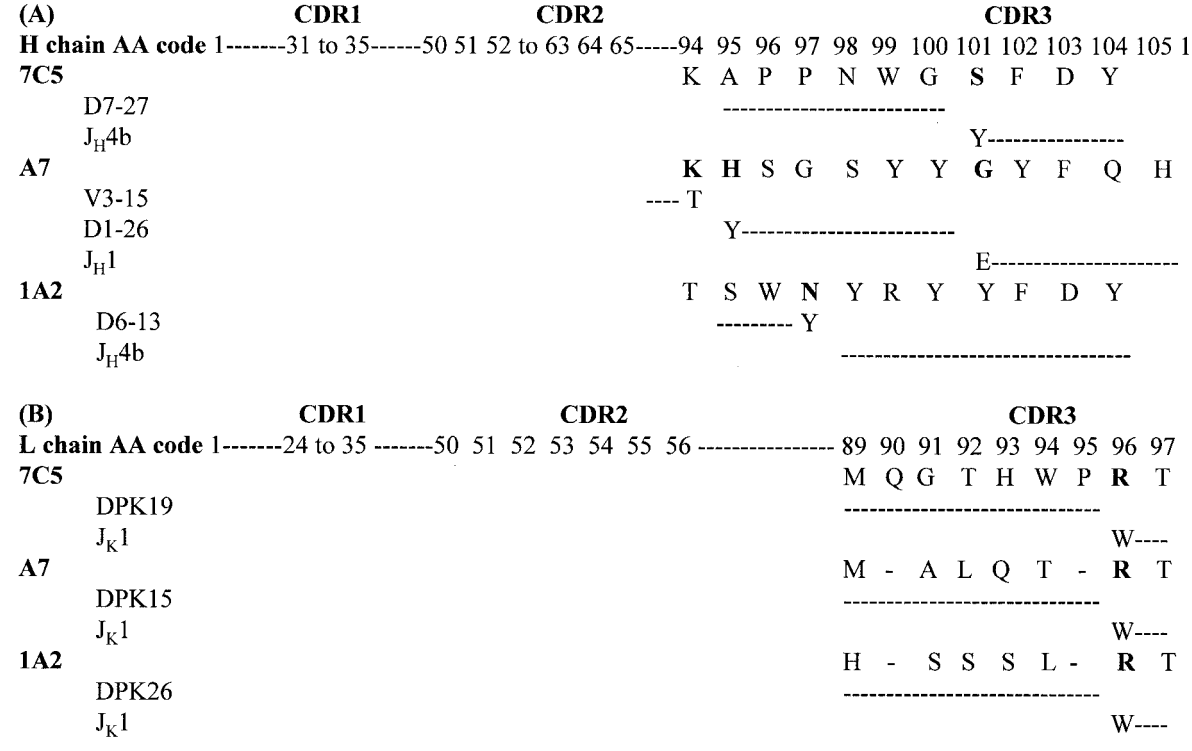
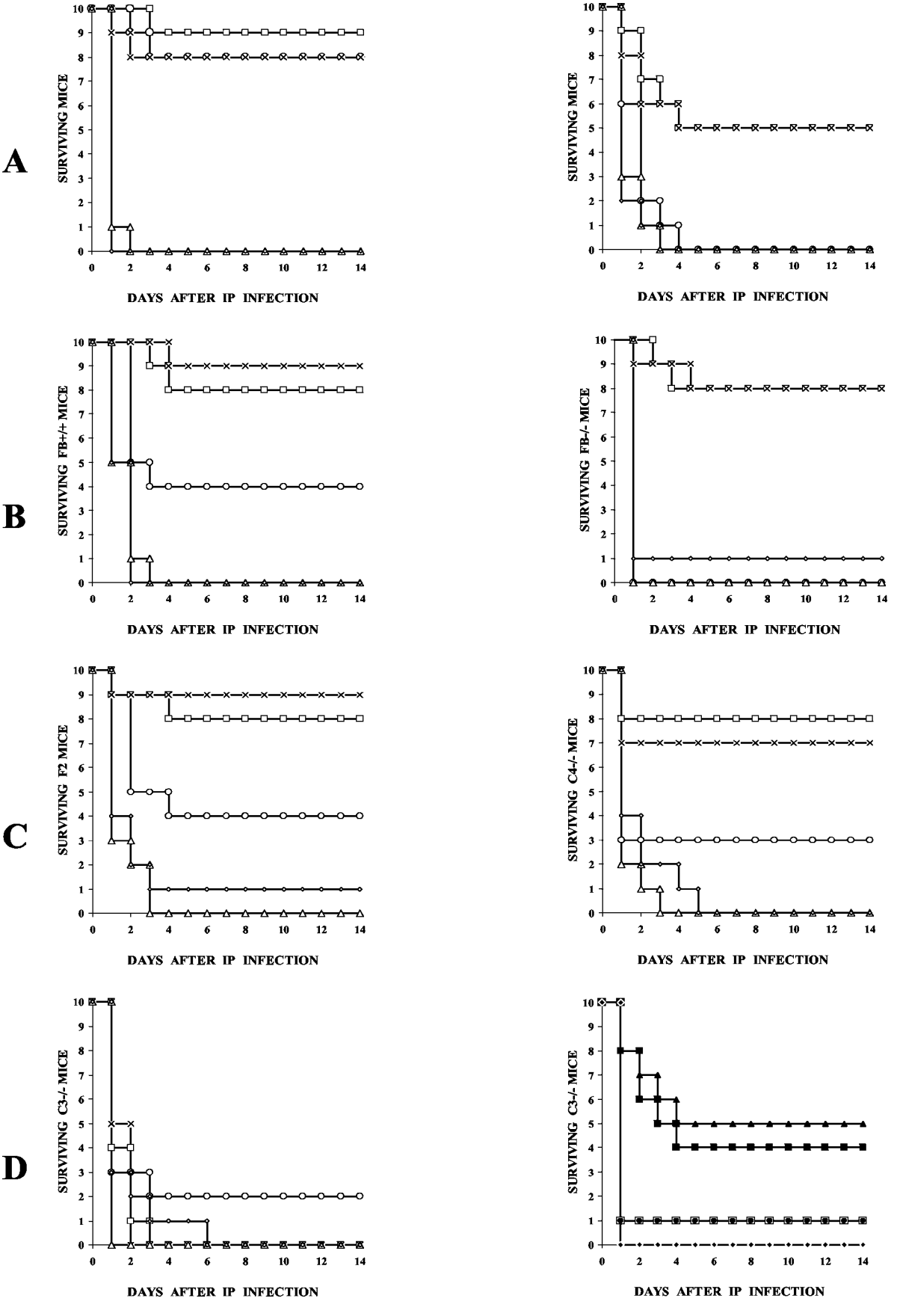


FIG. 5. CDR sequences of IgM MAbs to PPS-3. The amino acid (AA) sequences of the MAbs 7C5, A7, and 1A2 were compared to the closest germ line amino acid sequences. The dashes represent homology, and the boldface letters denote amino acid replacements. (A) MAb heavy (H)-chain amino acid codes in CDR3 (amino acids 95 to 108). (B) MAb κ light (L)-chain amino acid codes in CDR2 (amino acids 50 to 56) and CDR3 (amino acids 89 to 97). The numbering is according to V Base (MRC Centre for Protein Engineering). The nucleic acid translation was performed by the BCM Search Launcher program (Human Genome Sequencing Center, Baylor College of Medicine).


A
SURVIVING MICE
DAYS AFTER IP INFECTION
SURVIVING MICE
DAYS AFTER IP INFECTION
B
SURVIVING FB+/+ MICE
DAYS AFTER IP INFECTION
SURVIVING FB-/- MICE
DAYS AFTER IP INFECTION
C
SURVIVING F2 MICE
DAYS AFTER IP INFECTION
SURVIVING C4-/- MICE
DAYS AFTER IP INFECTION
D
SURVIVING C3-/- MICE
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performed with a 10-μg dose of MAb. In $FB^{+/+}$ mice, A7, 7C5, and 1A2 all prolonged survival compared to IgM- and PBS-treated mice ($P < 0.05$; Kaplan-Meier log rank survival test), and the survival of A7- and 7C5-treated mice was significantly greater than that of 1A2-treated mice (Fig. 6B). In $FB^{-/-}$ mice, only A7 and 7C5 significantly prolonged survival compared to A2-, IgM-, and PBS-treated mice (Fig. 6B). The survival of 1A2-treated $FB^{+/+}$ mice was greater than that of $FB^{-/-}$ mice ($P = 0$; Kaplan-Meier log rank survival test). For F2 mice, A7, 7C5, and 1A2 all significantly prolonged survival, but the survival of 1A2-treated mice was significantly less than that of A7- and 7C5-treated mice ($P < 0.05$; Kaplan-Meier log rank survival test). For $C4^{-/-}$ mice, A7 and 7C5 significantly prolonged survival compared to IgM- and PBS-treated mice ($P < 0.01$; Kaplan-Meier log rank survival test), and 1A2 did not significantly prolong survival compared to either IgM or PBS (Fig. 6C). There was no significant difference between the survival rates of 1A2-treated F2 and $C4^{-/-}$ mice ($P = 0.256$; Kaplan-Meier log rank survival test) or $FB^{+/+}$ and F2 mice ($P = 0.898$; Kaplan-Meier log rank survival test). There were no differences in survival for IgM- or PBS-treated BALB/c, 129, or F2 mice. None of the MAbs prolonged survival in $C3^{-/-}$ mice (Fig. 6D). The coadministration of either factor B-deficient or C2-deficient human serum with A7 and the inoculum to $C3^{-/-}$ mice resulted in significantly prolonged survival for each complement source compared to the complement sources alone ($P < 0.01$; Kaplan-Meier log rank survival test) (Fig. 6D). Taken together, the survival experiments show that (i) a 10-μg dose of A7, 1A2, and 7C5 prolongs survival in mice with both complement pathways intact (BALB/c, $FB^{+/+}$, and F2 mice); (ii) A7 and 7C5 mediate greater survival than 1A2 and prolong survival in mice deficient in either the classical ($C4^{-/-}$) or the alternative ($FB^{-/-}$) complement pathway, but 1A2 does not prolong survival in these strains; and (iii) the survival of 1A2-treated $FB^{-/-}$ mice is less than that of $FB^{+/+}$ mice.

DISCUSSION

MAbs can provide insights into antibody structure-function relationships that are not possible with polyclonal antibodies, because they are defined, monospecific reagents. In this study, we describe the molecular structures, specificities, and protective efficacies of three human MAbs to PPS-3—A7, 1A2, and 7C5—that were generated in a unique transgenic strain of mice, XenoMouse mice, that expresses human immunoglobulin genes (37). Our results extend the findings of our previous study (51) to XenoMouse mouse-derived MAbs generated by vaccination with PPS-3–TT, an immunogen that is representative of the conjugate vaccines that are used in humans. Although our panel was limited to three MAbs, a major finding of this study is that PPS-TT vaccination of XenoMouse mice can elicit PPS-3-specific antibodies that recognize different epitopes, including those that are highly protective and less protective (and nonprotective in certain models). These findings support the concept that the unpredictable efficacy of polyclonal sera may reflect the presence of antibodies that recognize epitopes that elicit protective as well as poorly protective and/or nonprotective antibodies, which can reduce the efficacy (14).

The human immunoglobulin repertoire of XenoMouse mice resembles that of mature human adults and displays no bias in gene expression in comparison to humans (23), except that the strain of mice used lacks human lambda light-chain genes. The V_H gene use of specific MAbs derived from XenoMouse mice, including the MAbs reported here, has been found to resemble that of human antibodies to antigens such as PPS (51), other bacterial polysaccharides (27), and mitochondrial proteins (52). The use of other immunoglobulin gene segments in XenoMouse mice also resembles that of human antibodies. For example, nearly all antigen-specific MAbs derived from XenoMouse mice use the most prevalent heavy-chain-joining (J_H) gene segments, J_H4 and J_H6 (23, 27, 51, 52). Interestingly, a protective MAb reported here, A7, uses J_H1, which is underrepresented in the repertoires of both humans and XenoMouse mice (23). This illustrates the power of these mice to probe the human antibody responses to specific antigens.

The seven MAbs to PPS-3 derived from XenoMouse mice reported in this and our previous studies (51) all use heavy-chain variable-region (V_H) genes from the V_H3 gene family, although there is no evidence for a predominant gene segment. This is consistent with other reports that human antibodies to PPS are restricted to the use of V_H3 genes (1, 16, 34, 53, 55, 62). In addition, the V_L use of the MAbs also resembles that of human antibodies to similar antigens (27). However, the degree to which the light-chain response resembles the normal human response to PPS cannot be determined from these mice, because they lack lambda light-chain genes. Although the mechanism for V_H restriction is unknown, it has been proposed that the structural characteristics of certain V_H genes, including the V_H3 genes in clan III, confer specificity for defined antigens (30, 31, 43, 57). Therefore, we have hypothesized that a "hole" in the V_H3 repertoire may translate into

FIG. 6. (A) Survival of BALB/c mice after infection with serotype 3 *S. pneumoniae.* On the left are the results obtained when a 10-μg dose of MAb was administered to each of 10 mice. On the right are the results obtained when a 1-μg dose of MAb was administered to each of 10 mice. The symbols for 7C5 and 1A2 (left) are superimposed after day 2. (B) Survival of factor B-deficient mice after infection with serotype 3 *S. pneumoniae.* On the left are the results obtained when a 10-μg dose of MAb was administered to each of 10 factor $B^{+/+}$ mice. On the right are the results obtained when a 10-μg dose of MAb was administered to each of 10 factor $B^{-/-}$ mice. The symbols for A7 and 7C5 (right) are superimposed after day 1. (C) Survival of C4-deficient mice after infection with serotype 3 *S. pneumoniae.* On the left are the results obtained when a 10-μg dose of MAb was administered to each of 10 F_2 mice. On the right are the results obtained when a 10-μg dose of MAb was administered to each of 10 $C4^{-/-}$ mice. (D) Survival of $C3^{-/-}$ mice after infection with serotype 3 *S. pneumoniae.* On the left are the results obtained when a 10-μg dose of MAb was administered to each of 10 $C3^{-/-}$ mice. On the right are the results obtained in a single-syringe injection with 10 μg of A7 in combination with C2- or factor B-deficient serum (see the text) as indicated. In each panel, the *y* axis represents the number of surviving mice, and the *x* axis depicts the number of days after i.p. infection. □, A7; ○, 1A2; ×, 7C5; △, IgM; ◇, PBS; ■, A7-$C2^{-/-}$; ▲, A7-$FB^{-/-}$; ●, $C4^{-/-}$; ◇, $FB^{-/-}$.

poor responses to PPS and other capsular polysaccharides (43). In support of this concept, human immunodeficiency virus (HIV) infection is associated with reduced antibody and B-cell V_H3 expression, increased susceptibility to pneumococcal infection, and poor PPS vaccine responses (4), and our group has shown that PPS vaccination induces an increase in antibodies and B cells expressing V_H3 determinants in non-HIV-infected, but not HIV-infected, individuals (1, 16). Hence, the finding of V_H3 use among protective antibodies to PPS-3 suggests that individuals with reduced V_H3 expression may be unable to mount a protective response to PPS-3.

To date, all six of the MAbs specific to PPS-3 that we have generated from XenoMouse mice are IgMs with limited diversity (reference 51 and this report). The benefit of IgM-based therapy has been shown for V_H3-positive IgMs to capsular polysaccharides of encapsulated pathogens (22, 51, 62) and the treatment of sepsis (8, 45). Along these lines, since IgM has superior complement-fixing properties and does not trigger potentially deleterious immune complex activation of Fc receptors (32), it may be useful in protection against pathogens that require opsonization to elicit effector cell antimicrobial activity. The XenoMouse mouse-derived MAbs to PPS-3 have features thought to be more characteristic of a T-cell-independent response in that they are IgMs and have little evidence of affinity maturation (see below), despite having been elicited by a PPS-protein conjugate. Interestingly, the adult response to polysaccharide-protein conjugate vaccines has also been noted to have T-cell-independent features (33, 47). The isolation of IgMs to PPS from XenoMouse mice compared to other antigens (27, 52) may reflect the anatomic location where polysaccharide responses are generated. The response to polysaccharides, including PPS, takes place in the MZ of the spleen (17, 26, 36, 42, 61). Based on their anatomical location and activated phenotype, MZB cells predominantly express germ line-encoded V_H genes and are thought to respond more rapidly than other B cells (17). Our data show that unimmunized XenoMouse mice have an increased proportion of MZB cells compared to that reported for other mouse strains (36). Hence, the response of XenoMouse mice to PPS-3 may also arise in the MZ, which may limit the diversity of the response.

The MAbs A7 and 1A2 use the same V_H gene. However, these MAbs have different CDR3s and PPS-3 specificities, and A7 is more protective in that 10 times less A7 protects BALB/c mice and it protects complement-deficient mice, whereas 1A2 does not. In contrast, A7 and 7C5 are equally protective in mice, use different V_H genes, and have distinct PPS-3 specificities. Since our data indicate that the three MAbs recognize at least two different PPS-3s and have similar affinities, their efficacies are most likely a function of their structures and/or epitope specificities. Each of the MAbs has nucleotide changes from its putative germ line gene at the V_H-D and D-J_H junctions that result in amino acid changes and a $J_\kappa1$ R at the V_κ-J_κ junction that is not present in the germ line $J_\kappa1$ sequence. Somatic mutation cannot be ruled out in the generation of these changes. However, their junctional location suggests they are most likely due to terminal deoxynucleotidyltransferase-mediated N nucleotide addition (48). Irrespective of the mechanism for their diversity, our discovery of antibodies with non-germ line sequences suggests they were probably selected in the immunized animals. In this regard, both A7 and 7C5 have a positively charged residue, K, at position 94 of the V_H, whereas the less protective 1A2 has a (germ line) T in this position. The A7 K resulted from a C-to-A base change from this germ line T, but the 7C5 K is germ line encoded. Therefore, the K at position 94 (the J_H-D_H junction) may confer specificity for a PPS-3 epitope(s) that elicits more-protective antibodies. Our finding of two additional MAbs to PPS-3 with a positively charged residue at position 94 of the V_H confirms and extends our previous finding of a positively charged residue (R or K) in this position in two protective XenoMouse mouse-derived MAbs to PPS-3 (51) and the finding by another group of an R in this position in a protective IgM to PPS-3 derived from a lymphoblastoid cell line (53). However, the importance of this residue for PPS-3 binding and protection can only be determined by mutational analysis. Notably, each of the MAbs also has a non-germ line V_κ-J_κ junctional R. V_κ specificity differences can confer specificity differences among antibodies to *Haemophilus influenzae* type b polysaccharide (2). However, the role that V_κ may play in conferring PPS-3 specificity remains to be determined, and available evidence suggests that V_H gene use and CDR3 structure confer specificity (60).

The MAbs reported in this study promoted in vitro complement activation via both the classical and the alternative complement pathways. The MAbs that produced the greatest C3 deposition in vitro, 7C5 and A7, were also the most protective in vivo. Given that the MAbs did not protect $C3^{-/-}$ mice, their efficacies were dependent on the availability of C3. This is underscored by the fact that A7 was protective in $C3^{-/-}$ mice when given with serum containing an intact alternative or classical complement pathway. These findings are consistent with other reports of an association between IgM-mediated complement deposition in vitro and efficacy in mice (25, 62). In addition, they confirm and extend to IgM longstanding evidence that type-specific antibody-mediated C3 deposition on the pneumococcal surface is required for host defense against the pneumococcus (10, 11, 59). However, 1A2 also led to C3 deposition in vitro, albeit to a lesser extent than A7 and 7C5, but was not protective in complement-deficient mice. This finding parallels studies in which in vitro measures of antibody biological activity were insufficient to predict antibody efficacy in experimental pneumococcal infection (9, 19) and suggests that in vivo studies may be more predictive of antibody efficacy than available in vitro assays. Despite being nonprotective in complement-deficient mice, 1A2 protects BALB/c mice at a 10-μg dose, albeit to a lesser degree than A7 and 7C5. However, its capacity to deposit C3 on whole organisms or PPS-3 is similar to that of a nonprotective IgM, suggesting that its efficacy cannot be fully reconciled by in vitro C3 deposition.

We found no difference in survival between 1A2-treated $C4^{-/-}$ and F2 mice, whereas the survival of 1A2-treated $FB^{+/+}$ mice was greater than that of $FB^{-/-}$ mice. This suggests that an intact alternative complement pathway is required for 1A2 to mediate protection. However, the survival difference between 1A2-treated $C4^{-/-}$ and $FB^{-/-}$ mice did not reach significance ($P = 0.067$), and the survival of 1A2-treated $C4^{-/-}$ mice was the same as that of control mice, suggesting that 1A2 cannot depend upon the alternative complement pathway alone to mediate protection. These results illustrate the complexity of antibody efficacy and comparing data across mouse

strains and raise the possibility that factors unrelated to complement may influence 1A2 efficacy. Nonetheless, our data demonstrate that 1A2, which protects 40% of F2, 30% of $C4^{-/-}$, and 40% of $FB^{+/+}$ mice but no $FB^{-/-}$ mice, is not as protective as either 7C5 or A7. C3 deposition on the pneumococcal surface via the alternative pathway is a function of antibody specificity in that it requires the $F(ab')_2$ but not the Fc region of PPS-specific antibodies (5, 6). Our data support the concept that the most protective antibodies might be distinguished from less protective and/or nonprotective antibodies by reactivity with defined PPS-3 epitopes. If identified, such epitopes would represent rationally based antigens for the development of therapeutic antibodies and/or more effective vaccines (43).

In summary, the MAbs reported here further document the fact that the human antibody response to PPS is restricted to the use of V_H3 gene segments. This provides indirect support for the concern that PPS-based vaccines may fail to be effective in individuals with B-cell repertoire defects and calls for efforts to identify the characteristics of antibodies to PPS that are protective. A fuller characterization of the structures and specificities of antibodies to PPS-3 that protect against serotype 3 *S. pneumoniae* may be useful in predicting vaccine efficacy and in the identification of novel vaccine antigens and/or the development of antibody therapy for those who are unable to respond to existing vaccines.

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