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Characterization of the Opsonic and Protective Activity against *Staphylococcus aureus* of Fully Human Monoclonal Antibodies Specific for the Bacterial Surface Polysaccharide Poly-*N*-Acetylglucosamine

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Carbohydrate antigens are important targets of the immune system in clearing bacterial pathogens. Although the immune system almost exclusively uses antibodies in response to foreign carbohydrates, there is still much to learn about the role of different epitopes on the carbohydrate as targets of protective immunity. We examined the role of acetyl group-dependent and -independent epitopes on the staphylococcal surface of polysaccharide poly-*N*-acetylated glucosamine (PNAG) by use of human monoclonal antibodies (MAbs) specific for such epitopes. We utilized hybridoma technology to produce fully human immunoglobulin G2 (IgG2) MAbs from B cells of an individual post-*Staphylococcus aureus* infection and cloned the antibody variable regions to produce an IgG1 form of each original MAb. Specificity and functionality of the purified MAbs were tested in vitro using enzyme-linked immunosorbent assays, complement deposition, and opsonophagocytic assays. We found that a MAb (MAb F598) that bound the best to nonacetylated or backbone epitopes on PNAG had superior complement deposition and opsonophagocytic activity compared to two MAbs that bound optimally to PNAG that was expressed with a native level (>90%) of *N*-acetyl groups (MAbs F628 and F630). Protection of mice against lethality due to *S. aureus* strains Mn8 and Reynolds further showed that the backbone-specific MAb had optimal protective efficacy compared with the acetate-specific MAbs. These results provide evidence for the importance of epitope specificity in inducing the optimal protective antibody response to PNAG and indicate that MAbs to the deacetylated form of PNAG could be immunotherapeutic agents for preventing or treating staphylococcal infections.

Staphylococcus aureus continues to be a major pathogen for both hospital- and community-acquired disease (2, 4, 8, 12, 36). The rise in antibiotic resistance of *S. aureus* highlights the need for alternative treatments and preventative measures to combat this infectious agent (6, 15). There are several surface proteins and carbohydrates currently under investigation as targets for antibody-based immunotherapies (7, 9, 10, 32, 34). One such staphylococcal surface carbohydrate, poly *N*-acetylglucosamine (PNAG), also referred to as the polysaccharide intercellular adhesin, has been shown to elicit opsonic antibodies when used as a vaccine in goats and rabbits. In addition, these polyclonal antibodies passively protect mice against *S. aureus* bacteremia and renal infection as well as against lethality following a high-dose infection (17, 18, 20). Animal antibodies to PNAG also mediate killing of *S. epidermidis* strains that express this antigen (18), and these strains constitute a significant proportion of clinical isolates (36).

A key feature of the immune response to PNAG is the differing properties of antibodies with specificities for different epitopes on this molecule. Recent work showed that antibodies that bind well to PNAG with a native level (>90%) of acetate substituents on the glucosamine monomers, but poorly to the antigen when the majority of the acetates are chemically removed (~15% residual acetylation), are inferior in opsonic and protective properties compared to antibodies elicited against the deacetylated form of PNAG (dPNAG) (18). The latter antibodies bind comparably to the antigen regardless of the level of acetylation; these epitopes are referred to as backbone epitopes. Epitope specificity with respect to PNAG has also been studied using antibodies present in the sera of human cystic fibrosis patients who were colonized with *S. aureus* by comparing the opsonophagocytic activity of affinity-purified antibodies that bound to native PNAG with that of affinity-purified antibodies that bound to dPNAG (14). As with the animal-derived antibodies, the human backbone-specific antibodies were, in general, better able to mediate opsonophagocytic killing activity than antibodies that required the acetate groups to be present to bind well to PNAG.

To pursue further the role of epitope specificity as an important property distinguishing protective from nonprotective antibody to the PNAG antigen, we produced fully human monoclonal antibodies (MAbs) to this antigen that had different properties of binding to native PNAG and dPNAG and characterized their immunologic and protective characteristics. In addition, fully human MAbs are being developed as treatments for infections by bacterial, viral, and fungal pathogens (16, 19, 22, 38), and similar reagents are already in use for the

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treatment of numerous inflammatory diseases (21) and tumors (33). Fully human MAbs have been shown to have few side effects and low immunogenicity when given to patients (13). In light of these prior observations regarding immunity to staphylococcal PNAG, we hypothesized that MAbs specific to the backbone epitopes on PNAG would have superior *S. aureus* killing activity compared to MAbs that require the acetate substituents in order to bind well to PNAG. In this paper we describe the production of immunoglobulin G2 (IgG2)-secreting hybridomas as well as cell lines transfected with DNA to produce V region-identical recombinant IgG1 MAbs reactive with PNAG and dPNAG antigens. In addition, we compared the properties of the IgG1 and IgG2 MAbs by use of in vitro assays measuring complement deposition and opsonophagocytic killing and further studied the IgG1 MAbs by use of in vivo protection studies of mice. Overall, we found the IgG1 MAb with specificity to the dPNAG antigen had the greatest complement deposition and opsonic and protective activities against *S. aureus*.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains MN8 (capsular type 8 [CP8]), NCTC 10833 (ATCC 25904; CP untypable), Reynolds (CP5), and Newman (CP5) and *S. epidermidis* strain M187 were obtained and propagated as previously described (3). Methicillin-resistant *S. aureus* (MRSA) Panton-Valentine leukocidin (PVL)-producing strains NRS 123 (also known as MW2 and USA400), NRS 192, and NRS 193 were obtained from the repository of the Network on Antimicrobial Resistance in *Staphylococcus aureus*, as was methicillin-susceptible, PVL-producing strain NRS 157. Strains were grown in tryptic soy broth (TSB) supplemented with additional 1% glucose (TSBG) for opsonic killing assays, immunofluorescence studies, and protection studies.

Purification and chemical modification of PNAG. Purification of PNAG was performed as previously described (17), using the culture supernatant of *S. aureus* strain Mn8m grown in a chemically defined medium. To remove >80% of the *N*- and *O*-linked substituents from native PNAG, purified PNAG was dissolved at 0.5 mg/ml in 5 M NaOH and incubated for 18 h at 37°C with stirring. The solution was neutralized to a final pH between 6 and 8 and dialyzed against deionized water for 24 h and then freeze dried. The residual level of acetate groups was determined by nuclear magnetic resonance spectroscopy as described previously (18).

Hybridomas. Informed consent to take a blood sample from a patient 3 years after an episode of *S. aureus* bacteremia and then isolate B cells for processing for hybridoma production was obtained as stipulated by the Committee on Clinical Investigation at the Beth Israel Deaconess Medical Center. Hybridomas were created essentially as described previously (27, 28). In brief, B cells were transformed with Epstein-Barr virus and then dispersed into multiple culture wells and grown for several weeks. Supernatants were screened for the presence of antibody that could bind to highly acetylated, native PNAG (75% to 100% *N* acetylated) and/or to poorly acetylated PNAG (dPNAG; ~15% acetylated). Cells in positive wells were then fused with the HMMA 2.5 cell line (27) to generate stable, antibody-producing hybridomas, which were cloned for homogeneity by limiting dilution as described previously (26). Three MAb clones, designated F598, F628, and F630, were obtained for further study.

Cloning of antibody-variable regions. RNA was extracted from $\sim 6 \times 10^6$ cells of each hybridoma by use of an RNeasy kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. A 1-μg volume of total RNA was reverse transcribed using a QIAGEN Omniscript kit. A 1-μl volume of cDNA product was used as a template for PCRs. Each reaction consisted of 50 μl of PCR SuperMix High Fidelity (Invitrogen Corp., Carlsbad, CA), 100 pmol of each primer (see Table 1), and 1 μl of cDNA template. For PCR amplification ~30 cycles were used with the following protocol: 94°C for 30 s initially followed by cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products from at least three independent PCRs were sequenced at least three times until a consensus sequence for the variable regions could be determined. A consensus sequence was determined to be correct when the sequencing results from at least three different PCRs were found to be identical. Sequencing was done at the Harvard Medical School DNA core sequencing facility, and the resultant sequences were compared against known germ line sequences using Ig BLAST on the NCBI database. The final sequences have been deposited in GenBank.

TABLE 1. Primers used to clone hybridoma antibody variable regions

Primer	Sequence[a]
Light chains	
Hlambdasig1	5′AGATCTCTCACC**ATG**GCCRGCTTCCCTCTCCTC
Hlambdasig2	5′AGATCTCTCACC**ATG**ACCTGCTTCCCTCTCCTC
Hlambdasig3	5′AGATCTCTCACC**ATG**GCCTGGGCTCTGCT
Hlambdasig4	5′AGATCTCTCACC**ATG**ACTTGGATCCCTCTCTTC
Hlambdasig5	5′AGATCTCTCACC**ATG**GCATGGATCCCTCTCTTC
Hlambdasig6	5′AGATCTCTCACC**ATG**GCCTGGACCCCTCTCTGG
Hlambdasig7	5′AGATCTCTCACC**ATG**GCCTGGATGATGCTTCTC
Lambda constant	5′GACCGAGGGGGCAGCCTTGGGCTGACCTAGG
Heavy chains	
VH1LDRHU	5′GTCGAC**ATG**GACTGGACCTGGA
VH2LDRHU	5′GTCGAC**ATG**GACATACTTTGTTCCAC
VH3LDRHU	5′GTCGAC**ATG**GAGYYKGGGCTGAGC
VH4LDRHU	5′GTCGAC**ATG**AACAYCTGTGGTTCTT
VH5LDRHU	5′GTCGAC**ATG**GGGTCAACCGCCATCCT
VH6LDRHU	5′GTCGAC**ATG**TCTGTCTCCTTCCTCAT
VH7LDRHU	5′GTCGAC**ATG**AAACATCTGTGGTTCTTC
Heavy chain constant	5′TGGGCCCTTGGTGCTAGCTGAGGAGAC

[a] For the light chain primers, the Bgl II site is underlined and the starting ATG is shown in bold. For the lambda constant primer, the Avr II site is underlined. For the heavy chain primers, the SalI site is underlined and the starting ATG is shown in bold. For the heavy chain constant primer, the Nhe1 site is underlined.

Construction of IgG1 MAbs. The TCAE6 vector containing the human lambda and human IgG1 constant region was used as previously described (26, 30). Cloned heavy (H) chain V-region genes from the three hybridomas were digested with SalI and NheI restriction enzymes and ligated into the TCAE6 vector cut with the same enzymes. The ligation reaction mixture was then transformed into competent *Escherichia coli* TOP10F cells (Invitrogen). Plasmids within individual clones were purified using a QIAGEN plasmid Miniprep kit and V-region H chains digested and sequenced to ensure that the correct sequence had been inserted into the vector. Cloned light (L) chains were digested with BglII and AvrII restriction enzymes and ligated with the TCAE6 vector already containing the matching H chain variable region and cut with the same enzymes. Plasmids were transformed into *E. coli*, and then individual clones were isolated, plasmids were obtained, and inserted DNA was sequenced to insure that the correct L chain V-region sequence had been cloned. In the end, three constructs were created containing the H and L chains from F598, F628, and F630, the three initial MAbs. Each plasmid construct was transfected into CHO DHFR$^{-/-}$ cells by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stably transfected cells were selected using geneticin (Invitrogen), and clones were obtained by limiting dilution as described previously (26). Cloned cells were screened by enzyme-linked immunosorbent assay (ELISA) to identify those producing maximal levels of IgG1 for the native PNAG and dPNAG antigens.

Production of MAbs. Transfected cells were weaned to serum-free medium containing geneticin (CHO-SFMII medium; Invitrogen). To increase antibody production, methotrexate replaced geneticin in the medium and further clonal selection was undertaken as described previously (26). Bulk cultures were grown in flasks in 800-ml volumes, and after 2 to 3 liters of culture was obtained, the pH of the pooled supernatants was adjusted to 6.5 and then run over a protein G column (Invitrogen) according to the manufacturer's instructions except that the running buffer was adjusted to pH 6.5 and, after elution of bound MAb in 0.1 M glycine (pH 2.6), the eluate was neutralized using 1 M phosphate buffer (pH 6.4). Fractions containing protein were pooled and dialyzed against phosphate-buffered saline (PBS) (pH 6.5). MAb concentrations were determined by ELISA as described below.

ELISAs. Immulon 4 HBX microtiter plates (Thermo Lab Systems, Franklin, MA) were coated with 100 μl of a previously determined optimal binding concentration of each antigen (0.6 μg/ml for native PNAG or 3 μg/ml for dPNAG) dissolved in sensitizing buffer (40 mM phosphate buffer, 0.02% azide, pH 7.4) and incubated overnight at 4°C. Plates were washed three times with PBS (0.0144 g/liter KH_2PO_4, 0.9 g/liter NaCl, 0.0795 g/liter Na_2HPO_4) containing 0.05% Tween 20 (washing buffer) and blocked with 200 μl of a 5% solution of skim milk dissolved in PBS. Plates were again incubated overnight at 4°C. Plates were

washed, and purified MAbs diluted in 5% skim milk–0.05% Tween–PBS (dilution buffer) were added at various concentrations. Plates were then incubated for 1 h at 37°C and washed three times. A 100-μl volume of secondary antibody (anti-human IgG whole molecule conjugated to alkaline phosphatase) was added at a 1:1,000 dilution in the dilution buffer. Plates were incubated at 37° for 1 h and washed three times. A 100-μl volume of *p*-nitrophenyl phosphate at a concentration of 1 mg/ml in substrate buffer (800 mg $NaHCO_3$, 1.46 g Na_2CO_3, 10 mg MgCl, 20 mg Na_3N in 500 ml water) was added, plates were incubated at room temperature for 30 min, and then color development was read at 405 nm. To quantify the concentration of MAb preparations, rows on ELISA plates were first sensitized with anti-human IgG to which purified human IgG lambda (Sigma) was added. The optical density at 450 nm (OD_{450}) obtained was plotted versus the concentration of the IgG standard to derive a regression equation from which OD values of the test MAbs could be taken to calculate specific MAb concentrations.

Binding competition experiments. ELISA plates coated with native PNAG as described above were used to determine whether the MAbs competed for binding to related epitopes on this antigen. Constant amounts of the IgG2 form of one MAb (a concentration resulting in an OD_{405} reading of 1.0 under standard ELISA conditions) were mixed with various amounts of the IgG1 form of a competitor MAb, and the binding of the mixture to immobilized PNAG was performed as for the direct ELISA described above. Secondary monoclonal mouse anti-human IgG2 (Zymed) was then used to determine the ability of the IgG1 form of a different MAb to compete for binding to the PNAG antigen. Controls included uninhibited IgG2 MAb and PNAG-coated wells without primary MAb but probed with secondary antibody to determine only the background binding of the secondary antibody.

Immunofluorescence. Glass-bottom microwell plates (Mattek, MA) were coated with 4% Celltak (BD Biosciences, MA) in 0.1 M $NaHCO_3$ and 20 mM NaOH. Plates were coated for 30 min at room temperature and then washed twice with distilled water. A 1-ml volume of a bacterial suspension freshly grown in TSBG ($\sim 10^8$ CFU/ml) was added to the plates and allowed to adhere for 20 min at room temperature. After two washes with PBS, bacterial cells (*S. aureus* Mn8m and *S. aureus* Mn8Δ*ica*) were fixed with 1% formaldehyde in PBS for 30 min at 4°C. Cells were washed twice with PBS and then blocked with PBS–1% bovine serum albumin–2% normal rabbit serum for 30 min at 4°C with rocking. The blocking solution was replaced with 1 ml of the primary antibody solution, MAb F598 or F628 (2 μg/ml) in PBS with 1% bovine serum albumin and 2% normal rabbit serum, and allowed to incubate for 2 h at 4°C with rocking. After four washes with PBS, 1 ml of a secondary anti-human IgG, Alexa 488 (Molecular Probes, Corvallis, OR) (diluted 1:2,000), was added and the mixture was incubated at 4°C for 1 h. The plates were then washed four times with PBS. The plates were examined by phase-contrast and fluorescence microscopy. Images of the same field viewed by the two different microscopic methods were acquired by a camera, and images were processed by computer using the LSM 5 image analysis system.

Complement deposition assay. Determinations of the deposition of the opsonically active C3 component were based on assays described previously (29). Microtiter plates were coated, as described for the ELISA, with PNAG antigen. After incubation with dilutions of the MAbs and washing, normal human sera diluted 1:50 was used as a source of complement. Prior to use, this serum was absorbed with three different *S. aureus* strains to remove endogenous antibody to this microbe. Plates were incubated for 15 min at 37°C. After the plate was washed, goat anti-human C3 (MP Biomedicals, Irvine, CA) at a dilution of 1:2,000 was added and, after 1 h of incubation at 37°C followed by three washes, an anti-goat IgG-alkaline phosphate conjugate (Sigma) was added at a 1:2,000 dilution and the mixture was again incubated at 37°C for 1 h. Plates were developed as in the ELISA except that OD values were read after 15 min of substrate incubation.

Opsonophagocytic assays. The basic protocol has been previously described (17). In brief, the target *S. aureus* strain was grown to midlogarithmic phase (OD_{650} of 0.4; $\sim 1 \times 10^9$ CFU/ml) in TSBG and diluted 1:100 into RPMI medium with 15% fetal bovine serum (HyClone, Logan, UT). Complement (infant rabbit serum; Cedarlane Laboratories, Hornby, Ontario, Canada) (diluted 1:15) was absorbed for 1 h with *S. aureus* strain Mn8 (1 ml of diluted complement per 1 ml of packed cells obtained from suspensions of an OD_{650} of 1.0 prepared from cells grown on tryptic soy agar plates overnight at 37°C). Polymorphonuclear neutrophils (PMNs) were separated from freshly drawn human blood by use of heparin-dextran (1:1 mix) and adjusted to a concentration of 2×10^7 cells/ml before being added to the assay tubes. Components (MAb dilution, target bacteria, complement source, PMNs) (100 μl each) were added together and incubated for 1.5 h at 37°C on a rack rotating end over end. Bacterial enumeration was carried out by making serial 10-fold dilutions in TSB with 0.05% Tween, and dilutions were plated on tryptic soy agar. After plates were incubated overnight at 37°C, bacterial colonies were counted. Controls included tubes containing only PMNs, complement, and bacteria; PMNs and bacteria alone; and complement and bacteria alone. To rule out apparent decreases in bacterial CFU due to aggregation of the target bacteria by the MAbs, a control containing bacteria, complement, and monoclonal antibodies but lacking PMNs was also included. The percentage of reduction in CFU was calculated using as a denominator a control containing complement, PMNs, bacteria, and an irrelevant IgG myeloma protein (Sigma).

TABLE 2. Germline gene usage and changes from germ-line nucleotide sequences of PNAG-specific hybridomas

Hybridoma H or L chain	V gene usage	% Identity	No. of nucleotide mutations	No. of amino acid changes[a]		
				Total	FWK	CDR
F598L	IGLV4-69	95.00%	15	8	2	6
F598H	IGHV4-59	94.88%	15	9	4	5
F628L	IGLV4-69	94.30%	17	9	2	7
F628H	IGHV4-59	94.88%	15	8	5	3
F630L	IGLV4-69	93.62%	19	9	5	4
F630H	IGHV1-18	93.58%	19	13	6	7

[a] FWK, framework region; CDR, complementarity-determining region.

Animal models. For lethality studies, 4- to 6-week-old FVB female mice (Taconic Farms, Hudson, NY) (~20 g) were injected intraperitoneally with different amounts of the MAbs 4 h before bacterial challenge section. A purified myeloma protein, human IgG1 lambda, was used as an antibody control (Sigma). Bacteria were injected intraperitoneally at a challenge dose of 2.5×10^8 to 9.0×10^8 per mouse. Mouse survival was monitored for 5 days.

Statistics. Survival analysis and calculations of antibody binding and inhibiting properties used Prism software for a Macintosh computer. To determine half-maximal binding activities and epitope overlap of the MAbs, the log of the concentration of the MAbs was plotted against the activity being measured (defined as a percentage of maximal binding or inhibition); either linear or nonlinear regression was used to generate a formula to calculate the reported values. Microsoft Excel was used for regression analysis for antibody quantification.

Nucleotide sequence accession numbers. The final sequences determined as described above have been deposited in GenBank under accession numbers DQ231549 to DQ231554.

RESULTS

PNAG- and dPNAG-specific hybridomas and monoclonal antibodies. B cells from a patient who had recovered 3 years previously from *S. aureus* bacteremia were used to produce human hybridomas. Throughout the growth, cloning, and selection processes, the antibodies secreted by the hybridomas were screened for the ability to bind to the acetylated and/or deacetylated PNAG. We chose three hybridomas for further study and named them F598, F628, and F630 for their fusion numbers. All three hybridomas were of the IgG2 lambda subclass. This is consistent with observations of a predominantly IgG2 human immune response to polysaccharides. Due to the known superiority of IgG1 antibodies to IgG2 antibodies for both fixing complement and interacting with Fc receptors on neutrophils, two qualities important in antibody-mediated opsonophagocytosis and bacterial clearance, we chose to clone the heavy and light chains of the original hybridomas and create IgG1 equivalents. Table 1 describes the primers used to clone the heavy and light chain of each hybridoma into the TCAE6 vector, which contains both the human lambda and human IgG1 constant regions. Recombinant TCAE6 constructs containing the heavy and light chains of each hybridoma

TABLE 3. Results of CDR3 analysis

Hybridoma H or L chain	Size of CDR3 (no. of amino acids)	D region		J region	
		Gene usage	No. of mutations	Gene usage	No. of mutations
F598L	9			IGLJ3	1
F598H	18	IGHD3-22	3	IGHJ3	2
F628L	9			IGLJ3	1
F628H	18	IGHD3-22	4	IGHJ6	2
F630L	9			IGLJ3	1
F630H	17	IGHD3-22	2	IGHJ3	2

were transformed into CHO cells for MAb synthesis and purification.

The DNA V-region germ line genes used and mutational changes from consensus germ line genes that arose in the variable-region genetic elements to form each of the H and L chain V regions in the three MAbs are shown in Table 2 and Table 3. Sequences are deposited in GenBank under accession numbers DQ231549 to DQ231554. We found that common germ line genes were used to produce these three antibodies, and all three of the L chains used the same germ line IGLV4-69 and IGLJ-3 genes, but differences due to somatic mutation were found in the final sequences. All three heavy chains contained the same germ line IGHD gene (IGHD3-22). The F598 and F628 H-chain germ line gene sequences matched in every case except for the heavy chain IGHJ gene, and the F598 and F630 germ line gene sequences matched in every case except for the heavy chain IGHV gene sequence. This result agrees with previous observations indicating that a tightly restricted use of germ line genes occurs in the human immune response to carbohydrate antigens (37). Although the germ line genes used to make the different MAbs were highly related in their sequences, none of the three H or three L chains had identical amino acid sequences among the three hybridomas studied.

Comparative antigen binding and epitope specificities of the human MAbs. ELISAs were used to determine the comparative strength of antigen binding of each purified MAb by evaluating binding to both the acetylated and deacetylated forms of PNAG. As shown in Fig. 1A, all of the MAbs bound to native PNAG, with clear differences in the amounts of each MAb needed to achieve maximal binding to this antigen. Determinations of the amount of each MAb needed to achieve 50% maximal binding in the ELISA showed that MAb F598 was

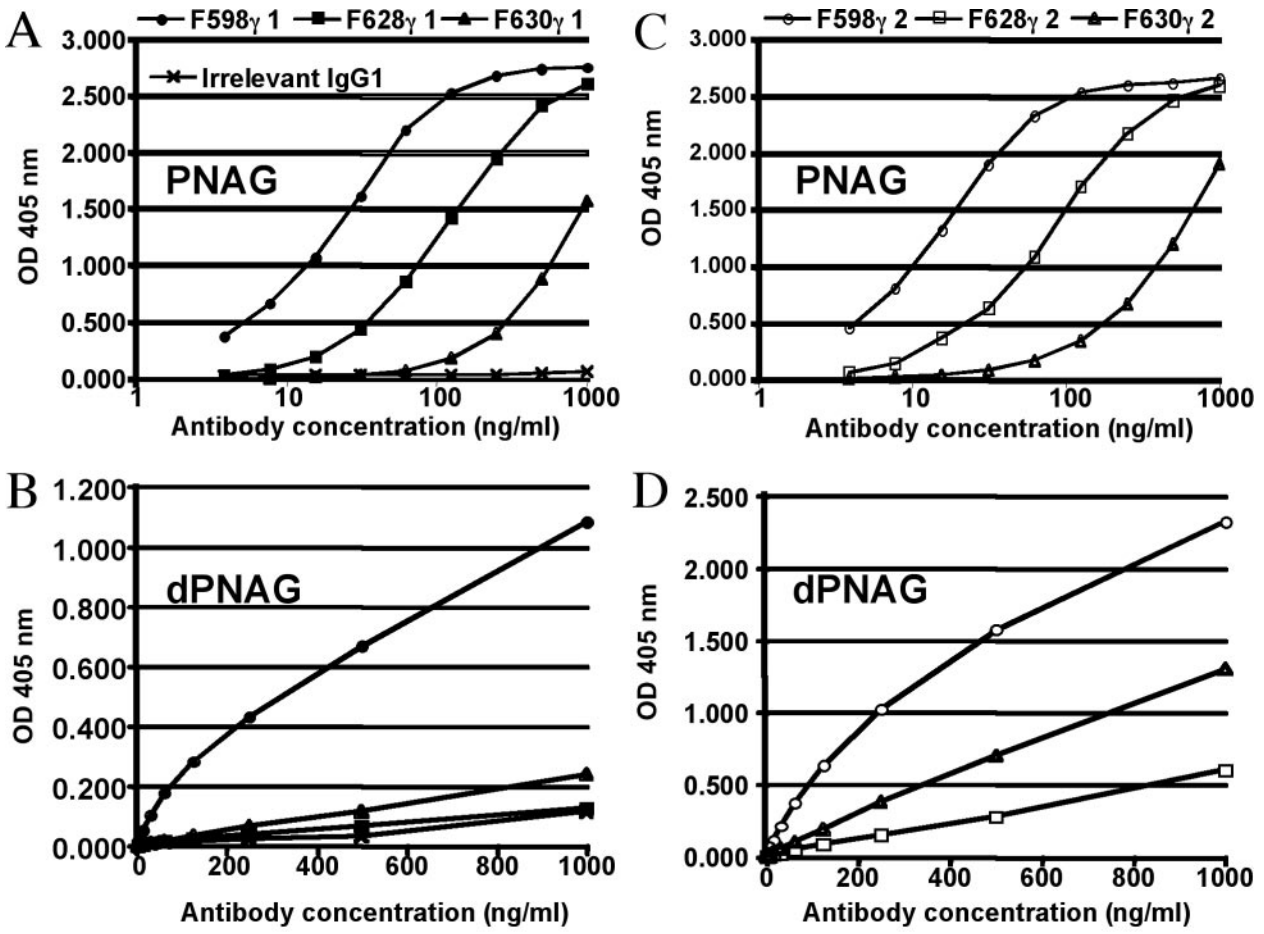

FIG. 1. ELISA reactivities of IgG1 (A and B) and IgG2 (C and D) MAbs to PNAG (A and C) and dPNAG (B and D) antigens. (A) Binding of isotype-switched IgG1 MAbs F598, F628, and F630 to native PNAG. (B) Binding of IgG1 MAbs to dPNAG antigen. (C) Binding of IgG2 MAbs to PNAG antigen. (D) Binding of IgG2 MAbs to dPNAG antigen. The irrelevant antibody is a human IgG1 antibody specific for *P. aeruginosa* alginate.

TABLE 4. Calculation of amount of each MAb needed to achieve 50% maximal binding to PNAG in an ELISA

MAb	50% Maximal binding (ng/ml)[a]	95% Confidence interval (ng/ml)	r^2 goodness of fit
F598	17.66	16.28–19.15	0.9997
F628	84.75	76.38–94.03	0.9994
F630	1051	913.4–1209	1.0

[a] Determined by nonlinear regression of data for IgG1 MAb binding to PNAG (as shown in figure 1A) by use of a sigmoidal curve-fitting function for a variable slope and plotting the $\log_{10}$ concentration of the MAb versus percent maximal binding.

significantly superior to MAb F628 in antigen binding, and both of these MAbs were greatly superior to MAb F630 in antigen binding (Table 4). Of note, there was little difference between the results for binding of the IgG1 and IgG2 forms of each MAb to PNAG (Fig. 1A and C). We also found that MAb F598 bound to dPNAG better than the other two MAbs (Fig. 1B and D), and the low level of binding of MAbs F628 and F630 to dPNAG precluded calculation of a half-maximal binding level with any biologic significance. Apparent differences in the final OD readings obtained for the IgG1 (Fig. 1B) and IgG2 (Fig. 1D) forms of the MAbs to dPNAG likely were due to reduced overall binding of the MAbs to this form of the PNAG antigen and use of a secondary antibody, directed to the whole IgG molecule, which likely had different reactivity to IgG1 and IgG2. MAbs F598 and F628 were used in immunofluorescence studies to compare binding to wild-type and *ica*-deleted *S. aureus* strain MN8. Results shown in Fig. 2 depict the strong binding of both MAbs to the wild-type strain but not to the *ica*-deleted strain. All three MAbs were also tested against a panel of antigens in an ELISA and found to be nonreactive against alginate from *Pseudomonas aeruginosa* strain PAO 581 (24), the *P. aeruginosa* LPS O antigens from serogroups 02 and 06 (25), and the PS/A antigen of *Bacteroides fragilis* (23) (data not shown).

Determination of epitope overlap by competitive binding assays using IgG1 forms of the MAbs to compete for binding to native PNAG of the IgG2 form of the MAbs (held at a concentration that would give rise to an OD_{405} of ~1.0 in a standard ELISA) revealed that MAbs F598 and F628 had an apparent partial overlap in epitope specificity. Inhibition of the binding of 72 ng/ml of the IgG2 form of MAb F598 by the IgG1

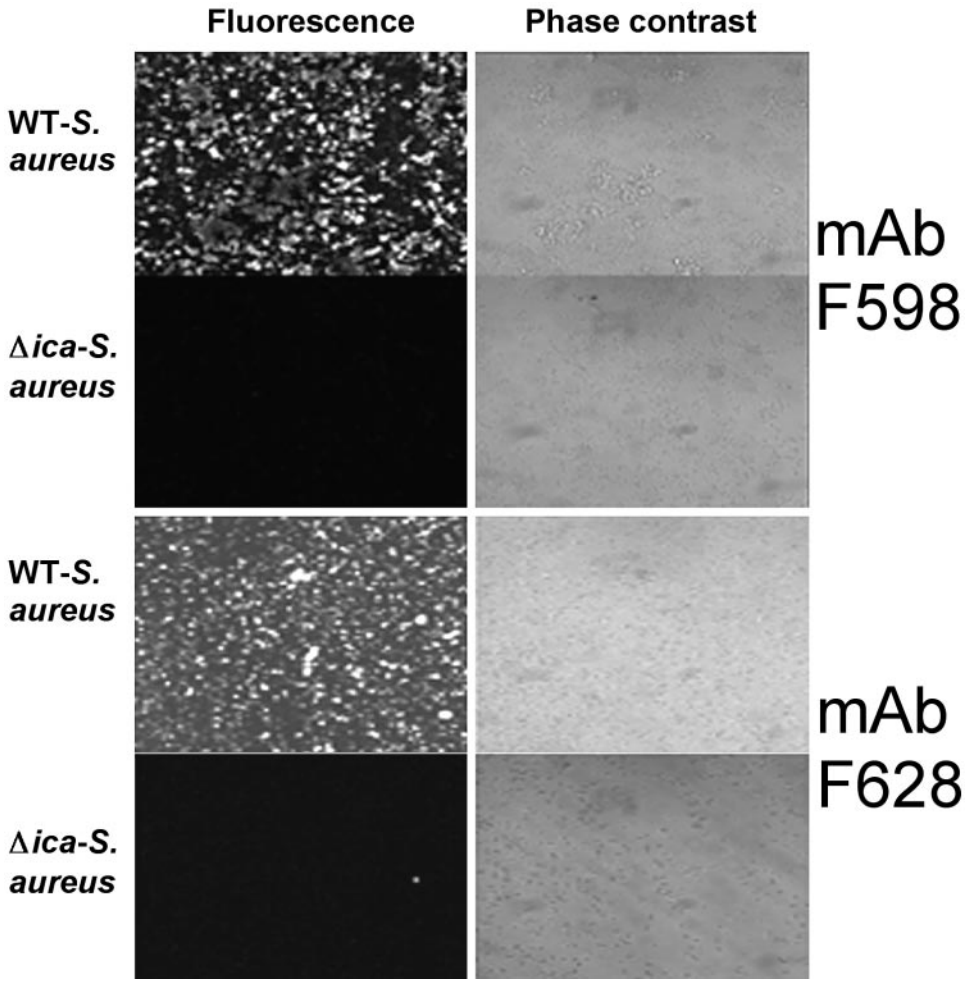

FIG. 2. Immunofluorescence study of binding of MAbs F598 and F628 to wild-type (WT) and Δ*ica S. aureus* strain Mn8, showing specificity of binding to the PNAG-producing parental strain.

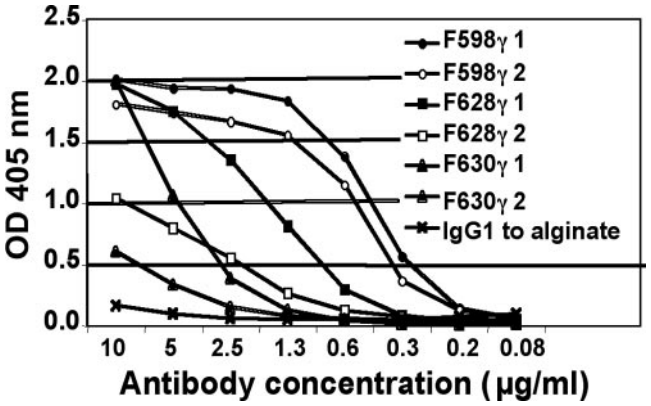

FIG. 3. Relative deposition onto purified PNAG of the third component of complement (C3) by IgG1 (filled symbols) and IgG2 (open symbols) MAbs. C3 deposition was measured by ELISA with a goat anti-human C3. The MAb to alginate is an irrelevant human IgG1 antibody specific for the *P. aeruginosa* alginate capsular antigen.

form of this MAb revealed that it took 1.8 μg/ml (95% confidence interval, 1.2 to 2.6 μg/ml) of the homologous MAb to reduce the binding by 50%. Similarly, it took 1.1 μg MAb F598/ml (95% confidence interval, 0.48 to 2.5 μg MAb F598/ml) to reduce binding by 50% of 600 ng MAb F628/ml to PNAG. We could not determine whether MAb F628 could reduce the binding of MAb F598 to PNAG, as the greater amount of binding of the latter MAb needed to achieve half-maximal binding (~4.8 times for half-maximal binding; Table 4) precluded adding sufficient amounts of MAb F628 as a competitor to achieve significant inhibition of binding of MAb F598. Neither MAb F598 nor F628 inhibited ≥50% of the binding of 1.2 μg MAb F630/ml when up to 10 μg/ml competitor MAb was used, suggesting there was minimal to no overlap in epitope specificity for the native PNAG antigen between MAb F630 and the other two MAbs.

Complement deposition activity of MAbs. As deposition of opsonically active fragments of the third component of complement (C3) often correlates with in vitro phagocytic killing and in vivo protection against gram-positive pathogens, we measured complement deposition onto immobilized PNAG antigen by both the IgG1 and IgG2 forms of each MAb. As seen in Fig. 3, the IgG1 form of the antibody was able to deposit the C3 component of the complement cascade to a greater extent than the IgG2 forms of the same antibody. Interestingly, there was a modest but clear difference for MAb F598, whereas a larger difference was seen with MAbs F628 and F630. Although changing MAbs F628 and F630 to the IgG1 form did increase complement binding ability over that achieved by the IgG2 form, this manipulation did not increase the overall complement deposition to the level achieved with either the IgG1 or IgG2 forms of MAb F598.

Opsonophagocytic activity of MAbs. The functional activity of the MAbs was also measured in vitro by utilizing an opsonophagocytosis assay. As seen in Fig. 4, MAb F598 in both the IgG1 and IgG2 isotypes had the most killing activity compared to the MAbs F628 and F630. In agreement with the complement deposition data, there was some difference in opsonophagocytic activity between the IgG1 and IgG2 forms of

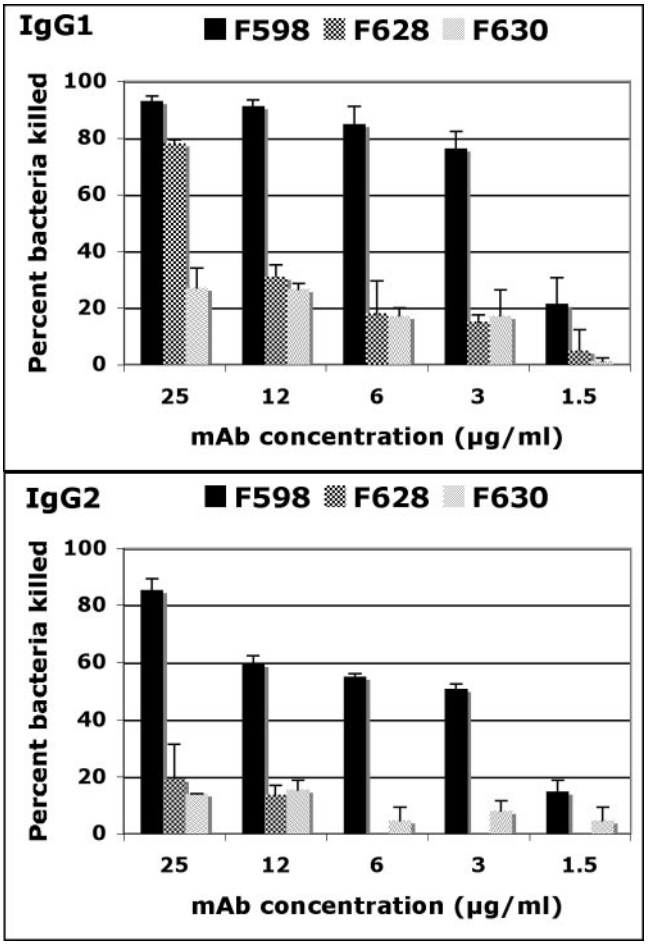

FIG. 4. Opsonophagocytic activity of IgG1 and IgG2 MAbs. Target strain is *S. aureus* Mn8. Bars represent means; error bars represent the standard errors of the means for three to eight replicates. Percent reduction in CFU was calculated compared to controls containing complement, PMNs, bacteria, and IgG control antibodies.

MAb F598: the IgG1 form killed 25% to 30% more bacterial CFU than did the IgG2 form at comparable MAb concentrations. More notably, changing MAb F628 from the IgG2 form to the IgG1 form resulted in a 60% increase in opsonophagocytic killing at the highest concentration tested (25 μg MAb/ml) whereas at the lower concentrations of MAb F628 the increased killing by the IgG1 isotype resulted in 15% to 20% more CFU killed. Changing MAb F630 to the IgG1 isotype had only a minimal effect, with only low levels of opsonic killing activity achieved with either the IgG1 or IgG2 isotype of MAb F630. Further opsonic assays found that MAb F598 mediated killing of all *S. aureus* strains tested, including four PVL-producing clinical isolates (three of which were also MRSA strains), as well as one *S. epidermidis* strain (Fig. 5).

Protective activity of MAbs. Because of the overall superiority of the IgG1 form of the antibodies in mediating complement deposition and opsonic killing, we chose to evaluate this subclass for the in vivo protection studies. A lethality model was used in which a single dose of an MAb was given 4 h prior to bacterial challenge. As seen in Fig. 6, 600 μg of either MAb F598 or MAb F628 could significantly protect mice from chal-

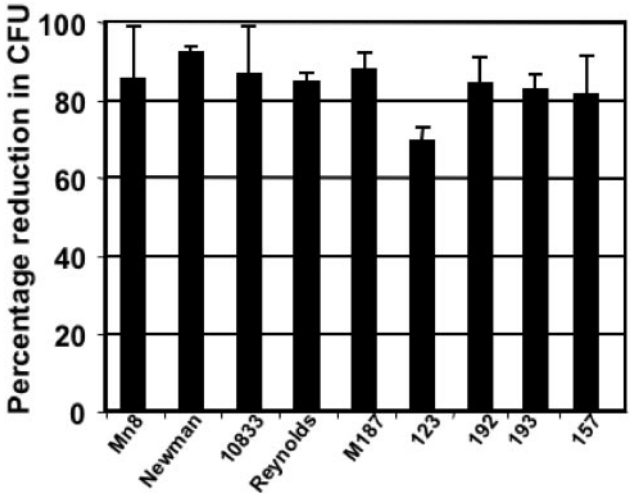

FIG. 5. Opsonophagocytic killing of several staphylococcal strains with MAb F598 at 12 μg/ml. Bars represent means; error bars represent the standard errors of the means. *S. aureus* strains Mn8, Newman, 10833, and Reynolds and *S. epidermidis* strain M187 represent methicillin-sensitive clinical and laboratory isolates. Strain 123 (also designated MW2 or USA400) is an MRSA strain carrying the PVL genes whose genome has been sequenced. Strains 192 and 193 represent MRSA, PVL-positive isolates from community-acquired infections. Strain 157 is a methicillin-sensitive, PVL-positive *S. aureus* isolate from a patient with lethal necrotizing pneumonia.

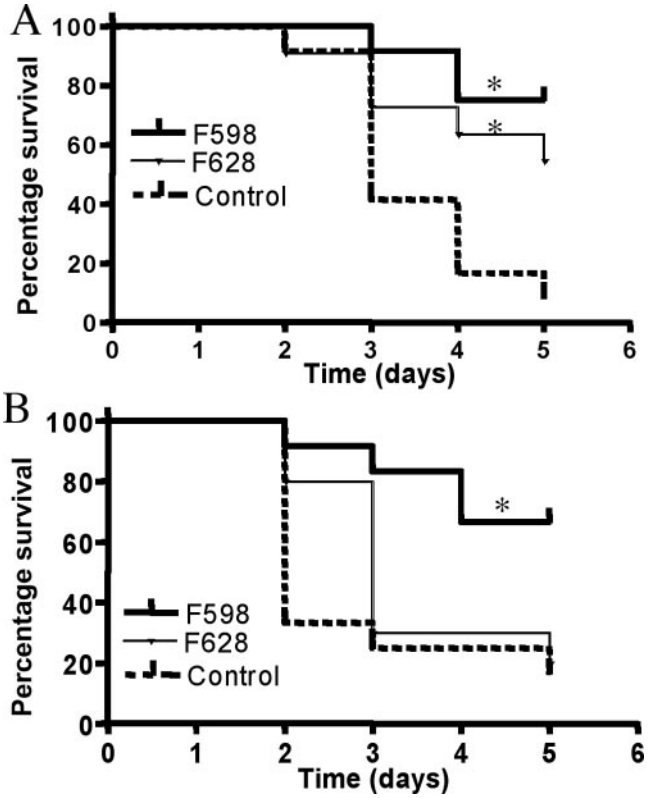

FIG. 6. Protection against *S. aureus* strain Reynolds with IgG1 MAbs (12 mice per group). (A) A 600-μg volume of each MAb was administered 4 h before bacterial challenge. (B) A 300-μg volume of each MAb was administered 4 h before bacterial challenge. Challenge dose, 2.5×10^8 CFU/mouse; *, *P* value less than 0.05 compared to IgG1 control MAb.

lenge with *S. aureus* strain Reynolds, a CP5 strain. When the amount of MAb passively administered was reduced to 300 μg per mouse, the F598 MAb continued to significantly protect the mice; however, MAb F628 was no longer providing significant protection ($P > 0.05$). We found with *S. aureus* strain MN8 (CP8) that somewhat lower doses of the MAbs could be used in passive protection studies. When we administered 400 μg of MAbs F598 and F628, we found that both MAbs mediated significant protection at this dose, but at a dose of 200 μg/mouse, only MAb F598 was protective (Fig. 7). A 300-μg volume of MAb F630 had no protective activity against challenge with *S. aureus* strain Mn8 (data not shown). Overall, MAb F598, which bound the best to dPNAG and had the optimal complement deposition and opsonic activity, was also the most protective MAb in these studies.

DISCUSSION

Previous findings have shown PNAG is an effective antigenic target for protecting animals against *S. aureus* and *S. epidermidis* infection via elicitation of polyclonal antibodies. Furthermore, there was a superiority in opsonic and protective properties of antibodies that bound well to the backbone epitopes on dPNAG compared with antibodies requiring acetate substituents to bind well to PNAG (18). In this study we confirmed and extended these findings by showing that a fully human MAb with good binding activity to the backbone epitopes on the dPNAG antigen had better in vitro opsonic killing activity and was more protective in vivo than two other MAbs that bound less well to both native PNAG and dPNAG. Importantly, we found that the better binding of MAb F598 to PNAG and dPNAG was correlated with superior C3 deposition, opsonic killing, and protection, indicating the interrelatedness of these properties for protective antibodies for the PNAG antigen. These in vitro correlates of protective immunity can be used in experiments to help identify improved antibody-based therapeutics and potentially find additive or synergistic combinations of antibodies to target PNAG-expressing pathogens. Of note, we did not find any additive or synergistic activities when we combined the three MAbs studied here in all possible combinations and evaluated them in opsonic killing assays (C. Kelly-Quintos, unpublished observation).

To improve the biologic properties of the initial IgG2 MAbs, we switched isotypes for the cloned V regions to produce human IgG1 MAbs. Interestingly, switching the F598 MAb from IgG2 to the IgG1 subclass gave a modest improvement in C3 deposition, with a concomitant 25% to 30% increase in opsonic killing activity. The F628 IgG2 MAb had a good improvement in both of these characteristics when switched to the IgG1 isotype, but significantly increased killing was achieved only with the highest concentration of MAb tested (25 μg/ml). Of note, the IgG2 isotype of MAb F598 showed two to three times more opsonic killing activity than the IgG1 form of MAb F628 at concentrations of 3 to 12.5 μg/ml, consistent with the increased level of C3 deposition achieved by the IgG2 MAb F598. This result suggests MAb F598 may recognize a densely packed epitope on the PNAG molecule, as it has been shown that IgG2 antibodies have increased com-

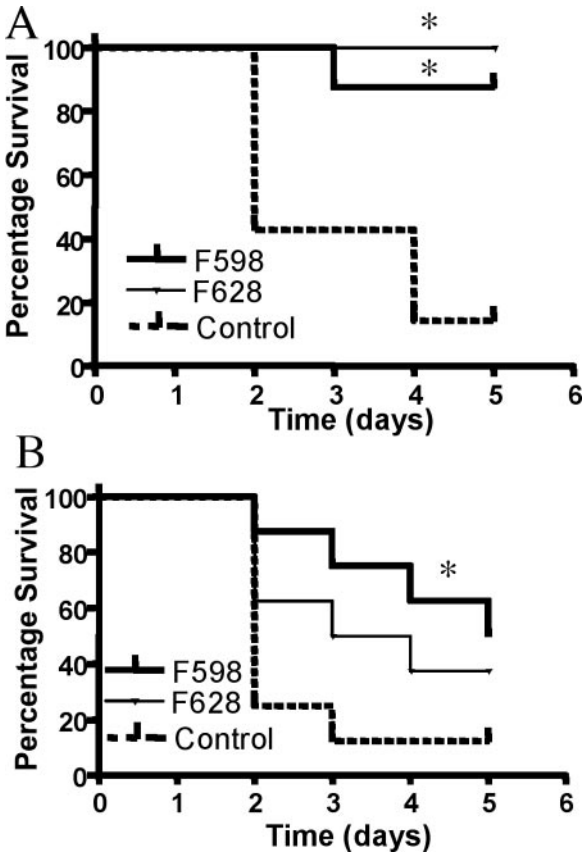

FIG. 7. Protection against *S. aureus* MN8 lethal infection with IgG1 MAbs to PNAG (eight mice per group). (A) A 400-μg volume of each MAb was administered 4 h before challenge. Strain Mn8 challenge dose, 5×10^8. (B) A 200-μg volume of each MAb was administered 4 h before challenge. Strain Mn8 challenge dose, 9×10^8; *, *P* value less than 0.05 compared to IgG1 control MAb.

plement deposition and opsonic activity when epitope density is high, as is commonly found on carbohydrate molecules (1). Although changing the F630 MAb from the IgG2 to the IgG1 form also increased complement deposition activity, this increase translated into only marginally better opsonophagocytic activity. Overall, converting the least opsonic F630 MAb into the IgG1 form did not improve the level of complement-fixing activity to that of MAbs F598 or F628, indicating a close relationship between the ability of these MAbs to deposit C3 onto the PNAG antigen and to mediate opsonic killing.

Even though it appeared that MAbs F598 and F628 had some partial epitope overlap, as revealed by competition binding experiments, the overall stronger binding of MAb F598 to the native PNAG and dPNAG antigens was likely a factor in its superior opsonic and protective properties. However, studies using human polyclonal antibodies produced in response to infection showed no effect of affinity on opsonic activity when comparing antibodies with specificity for dPNAG to those that bound optimally to native PNAG (14). This suggests that good binding to dPNAG, and not overall binding to native PNAG, is an important factor for the most opsonic and protective antibodies to *S. aureus* PNAG. Furthermore, when using diphtheria toxoid-conjugated PNAG or dPNAG antigens as active vaccines in animals, the native PNAG conjugate vaccine induced relatively low opsonic titers compared to the dPNAG conjugate vaccine, and all of the opsonic killing activity elicited by the native PNAG conjugate vaccine was inhibited by purified dPNAG antigen (18). Thus, a consistent picture emerges showing that even when there are antibodies present that bind better to native PNAG versus dPNAG, the opsonically active ones are those that bind to dPNAG. The findings reported here for the relative antigen-binding and immune-effector activities of MAbs F598 and F628 are consistent with this overall conclusion.

Also, consistent with previous findings using polyclonal antibodies (18), the dPNAG-binding MAb F598 had superior in vivo protective activity. The lowest dose of MAb F598 that we found was needed to achieve significant protection in the mice was ~10 mg/kg of body weight, which is quite comparable to the dose of the humanized MAb Palivizumab given clinically to infants for prophylaxis against respiratory syncytial virus infection. Palivizumab is administered to infants at a dose of 15 mg/kg (11). Similarly, a humanized MAb to *S. aureus* clumping factor A was used at 10 and 30 mg/kg to protect rabbits against consequences of *S. aureus* endocarditis (7) and this MAb has been evaluated in humans infused with doses of 2, 5, 10, or 20 mg/kg, with linear dose-response kinetics found for the final maximal serum concentration (31). These results further indicate that our MAbs are effective in mice at doses comparable to those likely to be used in humans. Thus, while it may be possible to improve the affinity or other properties of MAb F598 to make it more potent, based on the in vivo protection studies of mice, it is already in the potency range that is used for MAbs targeted to preventing or adjunctively treating infections in humans.

A possible explanation for the molecular basis of the superior activity of antibodies that bind well to the backbone epitopes on PNAG was recently described (35). Synthesis of PNAG is dependent on the protein products encoded by the genes in the intercellular adhesin (*ica*) locus (5). The protein encoded by the *icaB* gene is responsible for deacetylation of PNAG. It was found that in-frame deletion of the *icaB* gene in the *ica* locus of both *S. aureus* (N. Cerca, K. K. Jefferson, D. B. Pier, T. Maira-Litran, C. Kelly-Quintos, D. A. Goldmann, J. Azeredo, and G. B. Pier, unpublished data) and *S. epidermidis* (35) (where PNAG was referred to as PIA) resulted in production of only fully acetylated PNAG, and this form of PNAG was not retained on the cell surface. Instead, fully acetylated PNAG was all released into the culture supernatant. In contrast, when *icaB* was intact, most of the PNAG was retained on the cell surface and the overall level of acetylation was lower than that of the PNAG produced in the absence of functional IcaB (35). Thus, some amount of deacetylation is required for surface retention of PNAG. Further work showed that overexpression of the IcaB protein in a wild-type strain of *S. aureus* resulted in greater surface retention of PNAG and enhanced opsonophagocytic killing of dPNAG-specific antibodies without any effect on the opsonic killing activity of antibodies specific to native PNAG (N. Cerca, K. K. Jefferson, D. B. Pier, T. Maira-Litran, C. Kelly-Quintos, D. A. Goldmann, J. Azeredo, and G. B. Pier, unpublished data). These results indicate that dPNAG-specific antibodies may have an advantage over antibodies that require the presence of the acetate groups on PNAG to bind well to this antigen, based on the superior cell surface association of partially deacetylated PNAG. In

addition, the reduction in acetylation that accompanies cell surface binding of PNAG would reduce the density of acetate-dependent epitopes and concomitant binding of antibodies requiring acetate for maximal binding, resulting in a less effective antibody, as was shown here.

Overall, the findings in this study complement previous results regarding the importance of epitope specificity in the human immune response to *S. aureus* PNAG. The results further support the conclusion that antibodies that bind well to the backbone of PNAG are superior in their ability to kill and protect against *S. aureus* infection compared with antibodies that require the acetate groups for maximal binding to PNAG. In addition, the fully human MAb to staphylococcal dPNAG has excellent in vitro properties that translate into high levels of in vivo protective efficacy. These are key preclinical findings supporting further development of this reagent as an immunotherapeutic for prophylaxis and possibly for adjunctive treatment of *S. aureus* infection.

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