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# Efficacy of a Conjugate Vaccine Containing Polymannuronic Acid and Flagellin against Experimental *Pseudomonas aeruginosa* Lung Infection in Mice▽

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**Vaccines that could effectively prevent *Pseudomonas aeruginosa* pulmonary infections in the settings of cystic fibrosis (CF) and nosocomial pneumonia could be exceedingly useful, but to date no effective immunotherapy targeting this pathogen has been successfully developed for routine use in humans. Evaluations using animals and limited human trials of vaccines and their associated immune effectors against different *P. aeruginosa* antigens have suggested that antibody to the conserved surface polysaccharide alginate, as well as the flagellar proteins, often give high levels of protection. However, alginate itself does not elicit protective antibody in humans, and flagellar vaccines containing the two predominant serotypes of this antigen may not provide sufficient coverage against variant flagellar types. To evaluate if combining these antigens in a conjugate vaccine would be potentially efficacious, we conjugated polymannuronic acid (PMA), containing the blocks of mannuronic acid conserved in all *P. aeruginosa* alginates, to type a flagellin (FLA) and evaluated immunogenicity, opsonic killing activity, and passive protective efficacy in mice. The PMA-FLA conjugate was highly immunogenic in mice and rabbits and elicited opsonic antibodies against mucoid but not nonmucoid *P. aeruginosa*, but nonetheless rabbit antibody to PMA-FLA showed evidence of protective efficacy against both types of this organism in a mouse lung infection model. Importantly, the PMA-FLA conjugate vaccine did not elicit antibodies that neutralized the Toll-like receptor 5 (TLR5)-activating activity of flagellin, an important part of innate immunity to flagellated microbial pathogens. Conjugation of PMA to FLA appears to be a promising path for developing a broadly protective vaccine against *P. aeruginosa*.**

Vaccination to prevent infection by *Pseudomonas aeruginosa* would be a welcome advance in many regards, but particularly for preventing the chronic infection that is the major cause of morbidity and mortality in cystic fibrosis (CF) patients (20, 24). Colonization of the CF lung by *P. aeruginosa* occurs early in life (23, 42) predominantly by low-alginate-producing, nonmucoid, motile strains expressing a single polar flagellum. Components of the *P. aeruginosa* flagella as well as the constituent flagellin proteins are also involved in adhesion to host tissues, binding to mucins and also possibly to the glycolipid asialo-GM1 and to Toll-like receptor 5 (TLR5) (1, 39, 40), where host innate immune effectors are activated. The onset of increased yearly declines in CF lung function is associated with the conversion of *P. aeruginosa* from a nonmucoid and motile to a mucoid and nonmotile phenotype in the lung (42). Mucoid strains overproduce alginate, a random polymer of partially O-acetylated D-mannuronic acid and L-guluronic acid residues linked β1→4 (22, 46). Alginate plays many roles in the pathogenesis of the respiratory tract infection in CF. It serves as a mechanism for the formation of microcolonies or biofilms, confers antiphagocytic properties to mucoid strains, and protects *P. aeruginosa* from the consequences of inflammation, such as lethal oxygen radicals (17).

While early antibiotic treatment of the initial colonizing population of *P. aeruginosa* might prevent or at least delay chronic pulmonary infection (14, 42), vaccination that could prevent the establishment of chronic infection in the CF lung would have many advantages. Epidemiologic studies have linked opsonic antibodies specific to alginate in the sera of CF patients with a lack of chronic mucoid *P. aeruginosa* colonization and better overall lung function (13, 23, 31, 35, 37), but these antibodies are rarely induced by infection in most CF patients (31, 37). Purified *P. aeruginosa* alginate is safe when administered to humans (35) but alginate overall is poorly immunogenic in most humans, failing to elicit high titers of opsonic, protective antibodies. In an attempt to increase the immunogenicity of alginate, the molecule has been conjugated to carrier proteins (10, 13, 20, 47) by using detoxified exotoxin A, keyhole limpet hemocyanin (KLH), and tetanus toxoid (TT) as carrier proteins. However, a major challenge to this approach is that alginate from different mucoid strains has variable ratios of mannuronic to guluronic acids (~10:1 to close to 1:1) (35), making it difficult to find a preparation of alginate that gives rise to antibodies reactive with multiple strains of mucoid *P. aeruginosa.* Nonetheless, within most *P. aeruginosa* alginates are blocks of O-acetylated polymannuronic acid (PMA) (44), suggesting that a preparation of alginate rich in PMA residues could induce broadly reactive antibodies. O-acetyl groups on *P. aeruginosa* alginate affect the physical

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properties including the ability of *P. aeruginosa* to form biofilms *in vivo* (30). Acetate residues confer bacterial resistance to phagocytosis and complement by preventing activation of the alternative pathway of complement (34). It has been previously demonstrated that the O-acetyl groups of alginate are the epitopes recognized by antibody raised to purified alginate, alginate conjugates, or murine monoclonal antibodies (MAbs) that were opsonic and promoted protection against mucoid strains of *P. aeruginosa* in mice (15, 34, 47). However, a human MAb to alginate that protected against infection by both mucoid and nonmucoid strains recognized a different epitope on the PMA molecule (33), encompassing the uronic acid on C-6 of nonacetylated PMA blocks (33). Additional human MAbs generated for this prior study that bound to acetylated PMA blocks mediated opsonic killing of mucoid but not nonmucoid strains, leading to the conclusion that the optimal epitope for antibodies to *P. aeruginosa* alginate able to protect against both phenotypes of this organism were directed to the nonacetylated blocks of PMA contained with the alginate.

As for being a good carrier protein for a conjugate vaccine, *P. aeruginosa* flagellin and whole flagellar protein have potential desirable properties, as these molecules have been shown to be protective antigens (5, 11, 18, 19, 25, 27, 29, 38, 49), and the interaction of flagellin with TLR5 can also provide an adjuvant effect for vaccines containing TLR5-active flagellins (6, 26, 45). Additionally, a bivalent *P. aeruginosa* flagellar vaccine administered to CF patients in a phase III clinical trial was well tolerated, elicited high IgG titers, and achieved a 34% reduction in the number of patients with a first positive culture for *P. aeruginosa* (12). In another study, a fusion protein vaccine containing OprF epitope 8, OprI, and type a and b flagellins administered to young African green monkeys generated high-affinity antibodies that, after passive transfer to mice, protected against nonmucoid *P. aeruginosa* lung infection (49). We have found in animal studies that whole flagella are a superior vaccine compared with flagellin (8), but the large, polymeric, and unstable nature of whole flagella makes them difficult to use as a carrier protein in conjugate vaccines. Therefore, we used the flagellin protein monomer as a carrier for conjugation as it was considerably more suitable for making this type of vaccine. However, use of flagellin in a vaccine has potential negative effects, wherein antibodies that inhibit the binding of both flagellin from *P. aeruginosa* and other organisms to TLR5 might be generated, thus interfering with an interaction that contributes to innate immunity to *P. aeruginosa* (41, 45) and other pathogens.

In this study, we describe the synthesis and characterization of a polymannuronic acid-flagellin (PMA-FLA) conjugate, using purified PMA and type a flagellin to evaluate the immunogenic response in rabbits and mice and the protective efficacy of passive immunization with antibody to PMA-FLA against mucoid as well as nonmucoid, low-alginate-producing *P. aeruginosa* strains (3, 36) in a murine model of lung infection. We also investigate whether this conjugate avoids potentially deleterious induction of antibodies that could inhibit the TLR5 activation that occurs with *P. aeruginosa* infection.

## MATERIALS AND METHODS

**Bacterial strains.** The *P. aeruginosa* strains used for these studies were as follows: PAK, a serogroup O6, type a flagellated strain; PAO1, a serogroup O2/O5, type b flagellated strain; and PAO1 ExoU$^+$, a PAO1 strain expressing the ExoU cytotoxin (2). Clinical isolates obtained from CF patients included nonmucoid strains N6 and N13, which are type a flagellated strains, and nonflagellated mucoid strains 2192, FRD1, and 8050. Also, FRD1 Δ*algD* is an FRD1 strain defective for alginate production (9).

**Animals.** C57BL/6 and C3H/HeN mice were obtained from Charles River Laboratories. New Zealand White rabbits were from Millbrook Breeding Labs, Amherst, MA. All animal studies were conducted in accordance with protocols approved by the Harvard Medical Area Institutional Animal Care and Use Committee.

**Reagents.** Nonacetylated polymannuronic acid derived from *Pseudomonas fluorescens* was provided by Gudmund Skajk-Braek (Trondheim, Norway). Protanal LF 120 M sodium alginate was obtained from FMC Biopolymers, Philadelphia, PA. These polysaccharides had <0.01% endotoxin as determined by *Limulus* amebocyte assay (Cape Cod Associates, Hyannis, MA). 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC), dithiothreitol (DTT), dithionitrobenzoic acid (Ellman's reagent), cystamine, and cysteine were from Sigma-Aldrich, St. Louis, MO. The *Limulus* amebocyte lysate assay was from Cape Cod Associates, Woods Hole, MA. Superose 6 prep-grade and PD-10 columns (Sephadex G-25 M) were from GE Healthcare. GMBS [*N*-(γ-maleimidobutyryloxy)succinimide ester] was from Thermo Scientific Pierce Protein Research Products. Alkaline phosphatase conjugates of goat anti-rabbit IgG (whole molecule) were from Sigma. Bio-Rad protein assay dye reagent concentrate was from Bio-Rad Laboratories, Inc., Hercules, CA.

**Purification of flagellin.** Recombinant type a flagellin was purified from *Escherichia coli* BL21(DE3) carrying the pET15BVP vector with His-tagged type a *fliC* gene as previously described (48).

**Ultrasonic depolymerization of PMA.** Polymannuronic acid was depolymerized by using a sonicator (60 sonic dismembrator; Fisher Scientific) as previously described (32). Briefly, PMA was dissolved in deionized water at a concentration of 1 mg/ml and placed on ice. The sonication cycle consisted of 10 min of exposure at 3 W followed by 10 min of rest. PMA was sonicated until a cumulative time of 200 min was achieved.

**PMA-flagellin conjugation.** PMA was conjugated to flagellin as previously described with some modifications (47). Sonicated PMA (2 mg/ml) and cystamine (35 mM) were dissolved in 0.05 M MES [2-(*N*-morpholino) ethanesulfonic acid] buffer, and the pH was adjusted to 5.0. EDC was added (0.4 M final concentration), and the reaction mixture was stirred at room temperature for 2 h while the pH was maintained between 4.9 and 5.1. The polysaccharide was then passed through a PD-10 column equilibrated with the conjugation buffer (50 mM phosphate-buffered saline [PBS], 0.9 M NaCl, 5 mM EDTA; pH 7.4) for removal of low-molecular-weight material. For conjugation to flagellin, PMA-cystamine was reduced with 0.05 M DTT for 2 h. The mixture was then desalted with a PD-10 column equilibrated with conjugation buffer, and volume fractions that assayed positive for free sulfhydryl groups and uronic acid were pooled and concentrated to a final concentration of 2.5 mg/ml.

To derivatize the type a flagellin protein, the protein (2 mg/ml) was dissolved in conjugation buffer, 2 mg of GMBS [*N*-(γ-maleimidobutyryloxy)succinimide ester] in 100 μl of dimethyl sulfoxide (DMSO) were added, and the reaction mixture was stirred at room temperature for 1 h. The GMBS-derivatized flagellin was then passed through a PD-10 column equilibrated with the conjugation buffer.

For conjugation, GMBS-derivatized flagellin was added to the reduced cystamine derivative of PMA, and the mixture was stirred at room temperature for 1 h. After incubation, the conjugate was passed through a 1.6 by 95-cm Superose 6 column with phosphate-buffered saline (PBS) used as running buffer. Void-volume fractions that assayed positive for both protein and uronic acid were designated polysaccharide-protein conjugate and were pooled, dialyzed against deionized water, and lyophilized in aliquots. The amounts of protein and PMA present in the conjugate were quantified by assaying for protein by the Bio-Rad Protein assay using bovine serum albumin (BSA) as the standard and for uronic acid by the carbazole assay with sodium alginate as the standard (Protanal LF 120 M; FMC Biopolymers).

**Immunization of animals.** C3H/HeN mice (8/group), 6 to 8 weeks old, were immunized subcutaneously (s.c.) three times at weekly intervals with 3 μg of sugar in the PMA-FLA conjugate (determined in preliminary experiments to be the optimal dose) or a noncovalent mixture of PMA/FLA suspended in the adjuvant Specol. Serum samples were obtained on days 0, 28, 35, 42, and 56 for analysis.

A female New Zealand White rabbit was immunized with 120 μg of PMA on days 1 (in complete Freund's adjuvant) and 8 (in incomplete Freund's adjuvant [Sigma]). The rabbit was boosted intravenously with three 120-μg doses the following week on alternate days. A second rabbit was immunized with 100 μg of flagellin on days 1 and 8 in incomplete Freund's adjuvant. The rabbit was boosted intravenously with three 100-μg doses the following week. A third rabbit was immunized with 10 μg of conjugated PMA-FLA in incomplete Freund's adjuvant on days 1 and 8. The rabbit was boosted intravenously with three 10-μg doses the following week. Further booster doses were given to each rabbit intravenously at intervals of 2 to 4 months.

**ELISA.** Enzyme-linked immunosorbent assays (ELISAs) were performed by standard methods as described previously (8). Briefly, microtiter plates were coated with 1 μg flagellin/ml of 0.2 M carbonate buffer (pH 9.6) or 10 μg PMA/ml of 0.04 M phosphate buffer (pH 7.4) and kept overnight at 4°C. Between incubation steps, plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Blocking was performed with 1% BSA in PBS overnight at 4°C. Serum samples diluted 2-fold in 1% BSA in PBST were incubated for 1 h at 37°C. An alkaline phosphatase conjugate diluted 1:1,000 was used as secondary antibody, and *p*-nitrophenyl phosphate was used as a substrate (Sigma; 1 mg/ml in diethanolamine buffer, 0.5 mM $MgCl_2$ [pH 9.8]). After 60 min of incubation at 37°C, the absorbance was measured at 405 nm. ELISA titers were calculated by linear regression analysis of the average of duplicate measurements; the titer was the serum dilution giving a final optical density value at 405 nm ($OD_{405}$) of 0, as calculated from the linear regression curve.

An ELISA for inhibition, based on the competitive inhibition by PMA or PMA-FLA antigens of the binding of the antibodies to PMA to PMA antigen, was used to evaluate the modification of epitopes in the conjugated polysaccharide. Briefly, an ELISA was performed as described above, and serum samples were added at the same time with PMA as the inhibitor.

**Opsonophagocytic assay.** An opsonophagocytic assay was used as previously described (8), with only one modification. To prepare mucoid bacteria for use in the assay, 5 ml of tryptic soy broth (TSB) was inoculated with bacteria from a tryptic soy agar (TSA) plate grown overnight at 37°C, with the inoculum placed into the TSB tubes and grown at 37°C by tumbling the tubes end over end on a rotator until an $OD_{650}$ of 0.4 was obtained.

**Motility inhibition assays.** Motility assays were performed as previously described (8). Briefly, bacteria were grown statically in TSB at 37°C. Approximately $10^5$ log-phase organisms were inoculated onto plates made with lysogeny broth (LB) and 0.3% agar in the presence of antisera raised to flagellin or PMA-FLA at a dilution of 1:10 and incubated at 30°C for 18 h. Normal rabbit serum (NRS) was used as a negative control. Results were photographically recorded after 18 h.

**Flagellar typing of CF strains.** Flagellar typing of CF strains was accomplished by PCR amplification of the central region of the flagellin gene, as previously described (8).

**Mouse model of clearance of lung infection.** Antibody-promoted clearance of mucoid *P. aeruginosa* 4 h postinfection was used to measure vaccine efficacy against this phenotype of *P. aeruginosa* strains, as previously described (33). This clearance model was used due to the fact that the lipopolysaccharide (LPS) rough, mucoid *P. aeruginosa* strains are readily cleared by nonimmune mice unless very high challenge doses are used (33). After anesthetizing mice by intraperitoneal (i.p.) injection of 0.2 ml of xylazine (1.3 mg/ml) and ketamine (6.7 mg/ml), 6- to 8-week-old female C3H/HeN mice were given 50 μl of anti-PMA or anti-PMA-FLA or NRS intranasally (i.n.) 48 h and 24 h before infection.

*P. aeruginosa* mucoid strains were grown on TSA plates overnight, and bacteria from these plates were inoculated into TSB at an $OD_{650}$ of 0.1 and grown to an $OD_{650}$ of 0.4 at 37°C. Bacteria were recovered by centrifugation, resuspended in PBS to the desired dose for infection, and washed 3 times in this buffer. Prior to administration to animals, bacterial cells were plated on MacConkey agar plates to determine the inoculum. For infection, anesthetized mice were given 25 μl of *P. aeruginosa* i.n., prepared as described above. Infected mice were sacrificed 4 h postinfection, both lungs were removed, weighed, and homogenized, and the homogenate was diluted and plated for determinations of CFU per gram of lung tissue.

**Protection against infection in a setting of acute pneumonia.** An acute pneumonia model of infection was used to measure efficacy against nonmucoid strains of *P. aeruginosa*. After anesthesia, 6- to 8-week-old female C57BL/6 mice were given 50 μl of antibody i.n. raised to either PMA, PMA-FLA, flagellin type a, or NRS 48, 24, and 4 h prior to infection. For infection, bacteria were prepared and inoculated i.n. as described above, and animals were observed for survival twice a day for up to 5 days. Animals found moribund were sacrificed and counted as deceased for the purposes of these experiments.

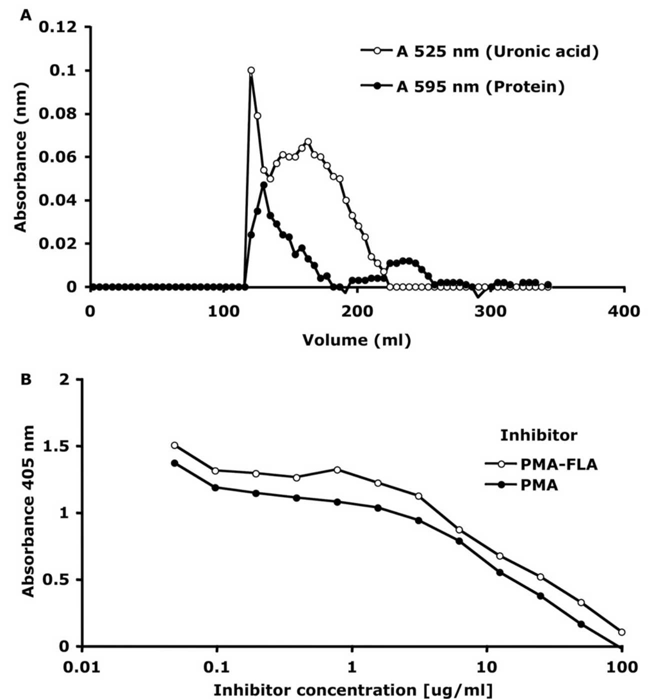


FIG. 1. Superose 6 prep-grade gel filtration profile of sonicated PMA conjugated to type a flagellin. (A) Fractions were assayed for protein by the Bradford assay (595 nm) and for uronic acid by the carbazole assay (525 nm). (B) ELISA for inhibition based on the competitive inhibition by PMA or PMA-FLA of the binding of the anti-PMA antibodies to PMA.

**Cell culture and Toll-like receptor 5 inhibition assay.** A TLR5-expressing A549 lung epithelial cell line stably transfected with a nuclear factor-kappa B (NF-κB) luciferase reporter plasmid (NFκB-luc, Panomics, Freemont, CA) was used to detect cellular activation by *P. aeruginosa* flagellin or PMA-FLA conjugate as previously described (8). Briefly, the cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and selected by 100 μg hygromycin B/ml in T75 flasks. After ~90% of confluence was reached, A549/NFkB-luc cells were transferred to 96-well solid white plates (Costar) at a concentration of $5 \times 10^4$ cells/well. Antisera were diluted 1:50 and then added along with flagellin or PMA-FLA to the plates, which were then incubated for 5 h. After 5 h, a Steady-Glo luciferase reagent (Promega) was added and the resultant luminescence read in a luminometer after 10 min of incubation at room temperature.

## RESULTS

**Composition of conjugates.** To synthesize the PMA-FLA conjugate vaccine, we used nonacetylated PMA from *P. fluorescens*, as it is a polymeric version of the epitope found to bind highly protective human MAb F429 (33), and *P. aeruginosa* type a flagellin, as it is the most prevalent type of flagellin expressed by *P. aeruginosa* isolates (4, 50). Initial attempts to conjugate native, high-molecular-weight (>250,000) PMA to type a flagellin were unsuccessful; we therefore reduced the molecular size of the polysaccharide in order to facilitate the conjugation process. Repeated sonication of PMA for short periods of time significantly reduced its molecular weight (~60,000). Gel filtration of the PMA-FLA conjugate on a Superose 6 prep-grade column yielded a peak at void volume containing both protein and polysaccharide, a second minor

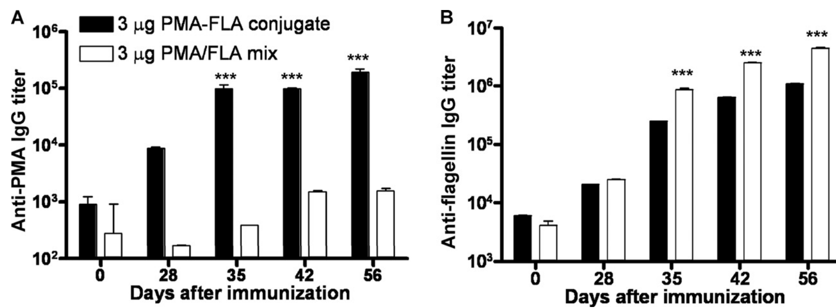


FIG. 2. Titers of IgG antibodies to PMA and flagellin in the sera of mice immunized s.c. with 3 μg of PMA-FLA vaccine three times at weekly intervals. (A) Titer to the homologous immunizing antigen PMA. (B) Titer to the homologous immunizing type a flagellin protein. Values represent means of triplicate determinations in sera pooled from 4 animals, and error bars represent standard errors of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. ***, $P < 0.001$.

protein peak at higher elution volumes, and a broad major peak containing material reacting positively for the presence of uronic acid (Fig. 1A). Since sonicated PMA elutes from this resin over a broad range of molecular sizes, only fractions eluting at the void volume were presumed to be free of non-conjugated polysaccharide and used as the PMA-FLA conjugate. The conjugate failed to enter a 4%-to-12% polyacrylamide gel. In an ELISA for inhibition, it was demonstrated that the conjugation process did not modify critical epitopes of the polysaccharide, as both purified PMA and the PMA-FLA conjugate equally inhibited the binding of antibodies to PMA to the PMA antigen (Fig. 1B). The PMA-FLA conjugate contained 75% (wt/wt) polysaccharide and 25% (wt/wt) protein.

**ELISA determination of immune responses.** We evaluated the ability of the PMA-FLA conjugate to elicit a humoral response in mice. Mice were immunized s.c. three times at weekly intervals with 3 μg of PMA conjugated to type a flagellin (PMA-FLA) or a mixture of purified PMA and flagellin. Mice were bled weekly starting 1 week after the last immunization, and sera were evaluated by ELISA for IgG titers to PMA and type a flagellin antigens. As expected, vaccination with the PMA flagellin mixture failed to induce a significant rise in IgG titers to the PMA antigen, whereas immunization with the PMA-FLA conjugate elicited high levels of PMA-specific IgG (Fig. 2A), suggesting that the enhanced IgG response to PMA is a result of the covalent conjugation between flagellin and PMA. Immunization of mice with the PMA-FLA conjugate also induced high antibody titers to alginate purified from *P. aeruginosa* strains 2192 and FRD1 (data not shown).

We noted that the immune response to type a flagellin elicited by both the PMA-FLA conjugate and the PMA-flagellin mixture was high, with titers of >250,000 by 35 days post-initiation of the immunization (Fig. 2B). The PMA-flagellin mix did, however, elicit small but significantly higher levels of IgG titers to type a flagellin. Similar results, but lower total levels of antibodies, were observed in preliminary studies in mice that received 0.3 or 1 μg of PMA conjugated to or mixed with type a flagellin (data not shown). When examining the proportions of mouse IgG antibody isotypes elicited, we found only low levels of IgG2a, while IgG1 antibodies accounted for >80% of the binding activity (data not shown).

After immunizing three different rabbits with either type a flagellin, PMA, or the PMA-FLA conjugate in adjuvants, antibody titers to those antigens were high (Table 1), indicating that the adjuvants used with the rabbits promoted the immunogenicity of the purified PMA in contrast to the immune response of mice that received a milder adjuvant. Antibody to PMA-FLA showed reactivity to PMA similar to that of the antiserum raised to unconjugated PMA. The antiserum to PMA-FLA also reacted with type a flagellin, but to a lower level than the antiserum raised to type a flagellin alone.

**Motility inhibition assays.** To determine the functional activity of the antisera raised either to *P. aeruginosa* type a flagellin or to the PMA-FLA conjugate, we evaluated the ability of the antisera to inhibit the motility of *P. aeruginosa* type a flagellin strains PAK, N6, and N13 and type b flagellin strain

TABLE 1. Titers of antibodies to type a flagellin or to PMA in rabbit sera raised to type a flagellin, PMA or PMA-FLA

| Serum raised to: | Titer to indicated target antigen | |
|---|---|---|
| | Type a flagellin | PMA |
| Type a flagellin | 920,000[a] | N/A |
| PMA | N/A[b] | 112,635 |
| PMA-FLA | 204,320 | 130,916 |

[a] Titer calculated as the serum dilution giving a final optical density value of 0 using least-squares linear regression.
[b] N/A, not available.

TABLE 2. Estimated dilution of serum needed to mediate opsonophagocytic killing of 50% ($EC_{50}$) of the target bacterial strain

| Target strain | Titer (95% confidence interval) mediating $EC_{50}$ in antiserum raised to: | | |
|---|---|---|---|
| | Flagellin | PMA | PMA-FLA |
| PAK (type a) | 10 (6–17) | 14 (6–32) | 5 (2–13) |
| N6 (type a) | <4[a] | 7 (3–16) | <4 |
| N13 (type a) | <4 | <4 | <4 |
| PA01 (type b) | 6 (3–12) | 16 (9–28) | <4 |
| FRD1 | N/A | 545 (273–1,088) | 382 (275–529) |
| 2192 | N/A | 34 (21–56) | 36 (18–70) |
| 8050 | N/A | 15 (11–22) | 13 (8–22) |
| FRD1 Δ*algD* | N/A | <4 | <4 |

[a] Opsonic killing of <30% in the 1:4 serum dilution was considered to be within the range of the controls lacking an essential component needed for opsonization and/or killing, and thus titers are reported as less than 4.

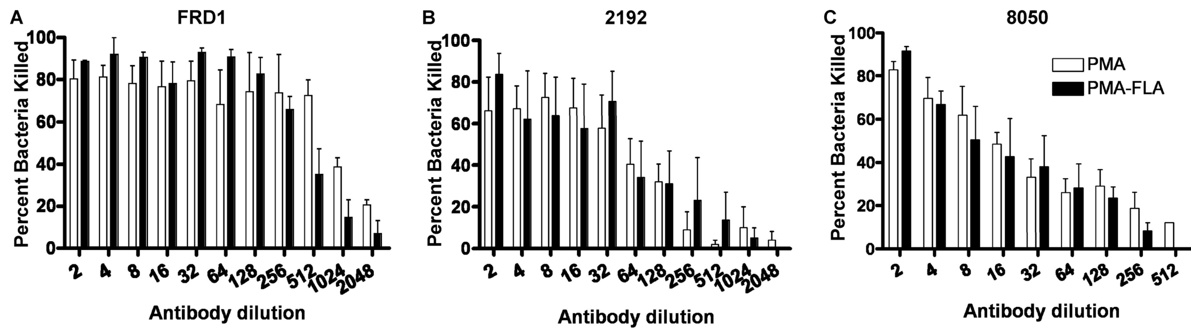


FIG. 3. Phagocyte-dependent killing activity of rabbit antibody to PMA or to PMA-FLA conjugate vaccine against *P. aeruginosa* mucoid strains. (A) *P. aeruginosa* strain FRD1. (B) *P. aeruginosa* strain 2192. (C) *P. aeruginosa* strain 8050. Bars represent means of quadruplicate-hextuplet determinations, and error bars represent standard errors of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons, and results showed to be not significantly different ($P > 0.05$).

PAO1. Before this assay was performed, the flagellin type of the clinical isolates from CF patients was identified by PCR as described previously (8). In the motility inhibition assays, NRS was used as a negative control. At a serum dilution of 1:10, the rabbit antiserum to flagellin was a better inhibitor of the motility of strain PAK than was the rabbit antiserum raised to the PMA-FLA conjugate. The antisera either to flagellin or to the PMA-FLA conjugate were comparable in their abilities to inhibit the motility of *P. aeruginosa* type a flagellin strains N6 and N13. Both antisera also showed some cross-inhibition of the motility of flagellin type b strain PAO1 (data not shown).

**Opsonic killing activity of antisera raised to either PMA, PMA-FLA, or type a flagellin against *P. aeruginosa*.** In an opsonophagocytic killing assay using antibodies raised to PMA, PMA-FLA conjugate, or type a flagellin, we determined the overall activity and estimated the serum dilution mediating killing of 50% of the bacterial cells ($EC_{50}$) (Table 2; Fig. 3 and 4). When testing the opsonic killing activity of the antisera against *P. aeruginosa* mucoid strains (Fig. 3A to C), antisera either to PMA or to PMA-FLA were active in opsonic killing against strains FRD1 ($EC_{50}$ = 545 and 382 respectively) and 2192 ($EC_{50}$ = 34 and 36 respectively), while they had a modest opsonic killing activity against mucoid strain 8050, with $EC_{50}$ values of 15 and 13, respectively. Antiserum to type a flagellin was not tested against these nonmotile mucoid strains. The specificity of the antisera for alginate was shown by testing the opsonic killing activity against strain FRD1 Δ*algD*, which cannot produce alginate. No opsonic killing was promoted by

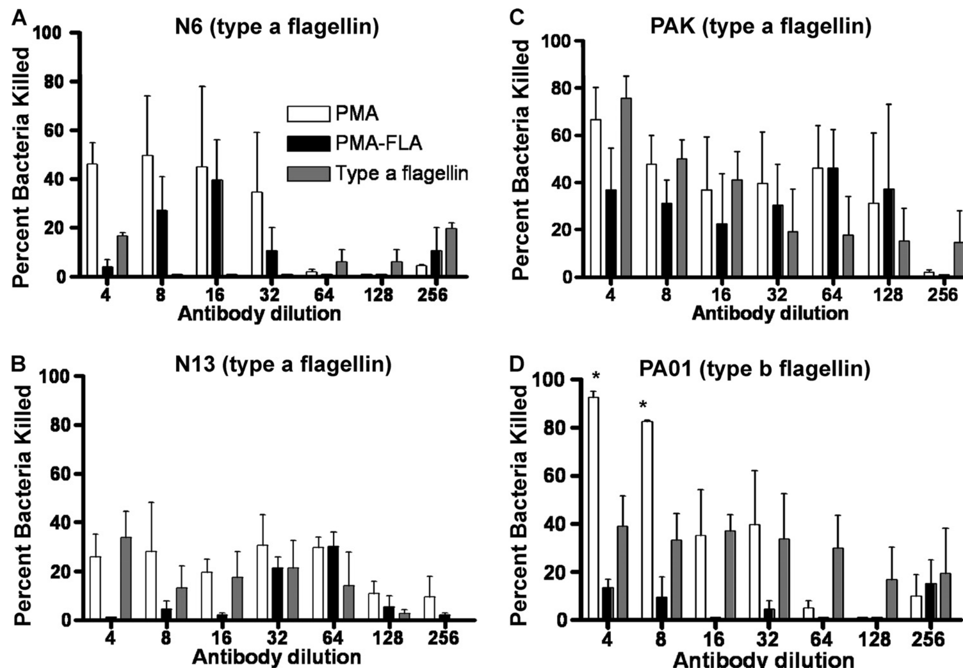


FIG. 4. Phagocyte-dependent killing activity of rabbit antibody to PMA, PMA-FLA, and type a flagellin against *P. aeruginosa* nonmucoid strains. (A) *P. aeruginosa* type a flagellin strain N6. (B) *P. aeruginosa* type a flagellin strain N13. (C) *P. aeruginosa* type a flagellin strain PAK. (D) *P. aeruginosa* type b flagellin strain PAO1. Bars represent means of duplicate-quadruplicate determinations, and error bars represent standard errors of the mean (SEM). Statistical analysis was performed by two-way analysis of variance (ANOVA) using the Bonferroni correction for multiple comparisons. *, $P < 0.05$.

antisera to PMA or to PMA-FLA against this strain (Table 2). When testing the antibody to PMA, PMA-FLA, or type a flagellin against nonmucoid *P. aeruginosa* strains, the three antisera showed similar patterns of opsonic killing, promoting little to no killing of type a flagellin strains N6, N13, and PAK or of type b flagellin strain PAO1 (Table 2; Fig. 4A to D). Mouse sera raised to PMA-FLA conjugate also mediated the opsonic killing of *P. aeruginosa* mucoid strains 2192 and FRD1 but not the nonmucoid strain PAK (data not shown). These patterns of opsonic killing are the same as those observed with other *P. aeruginosa* alginate-based vaccines wherein activity is generated to mucoid but not nonmucoid strains (15, 33, 35, 47).

**Promotion of pulmonary clearance of mucoid *P. aeruginosa* strains.** We used a clearance model to evaluate the efficacy of the antisera to PMA or PMA-FLA for effects on mucoid *P. aeruginosa* strains (Fig. 5A to C), since most mucoid isolates do not cause a lethal lung infection except at very high inocula, due to the fact they are LPS rough strains with a poor ability to disseminate systemically. Also, other models of mucoid *P. aeruginosa* infection such as embedding the organisms in agar or alginate beads are limited due to the poor ability of most mucoid strains to establish infections in mice by using these techniques (33). Intranasal delivery of antisera raised to the PMA-FLA conjugate 48 and 24 h before i.n. infection significantly enhanced the clearance of *P. aeruginosa* mucoid strains 2192, FRD1, and 8050 after 4 h of infection compared with mice given antisera to PMA or control NRS. The 4-h time period postinfection was chosen, as clearance mediated by a monoclonal antibody to alginate showed similar results at promoting clearance of mucoid strains at 4 and 18 h, but better than at 2 h after infection in mice (33). Antisera to the PMA-FLA conjugate yielded a 71.5% reduction in the CFU of strain 2192 ($P = 0.0008$ versus NRS), an 80% reduction in the CFU of strain FRD1 ($P = 0.0021$ versus NRS), and an 85.5% reduction in the CFU of strain 8050 ($P = 0.0032$ versus NRS) compared with control NRS. When comparing the CFU recovered from the lungs of mice given antisera to PMA with those recovered from control mice given NRS, the antiserum to PMA reduced bacterial burdens by 39%, 39.5%, and 23.5% after challenge with strains 2192, FRD1, and 8050, respectively (*P* values of 0.045, 0.0751, and 0.1045 versus NRS, respectively).

**Protection against acute pneumonia by nonmucoid clinical isolates.** As nonmucoid, flagellum-positive, LPS smooth isolates of *P. aeruginosa* are relatively virulent in an acute lung infection model (2), we evaluated whether the antisera raised to the PMA-FLA conjugate or PMA (Fig. 6A to D) or type a flagellin (Fig. 7A to D) were protective against these strains *in vivo*. Passive immunization with antibody raised to the PMA-FLA conjugate vaccine resulted in protection against lethal pneumonia due to *P. aeruginosa* strains PAK (77.7% survival) (Fig. 6A), N13 (31.3% survival) (Fig. 6B), and PAO1 $ExoU^+$ (85.3% survival) (Fig. 6D), and these levels of survival were all significantly greater than that in mice given either antibody raised to PMA alone or NRS. The antiserum to the PMA-FLA conjugate resulted in 65% survival of mice challenged with *P. aeruginosa* strain N6 (Fig. 6C), which was significantly greater ($P = 0.03$) than survival in mice given antibody to PMA but not mice given NRS, indicating a low efficacy of these

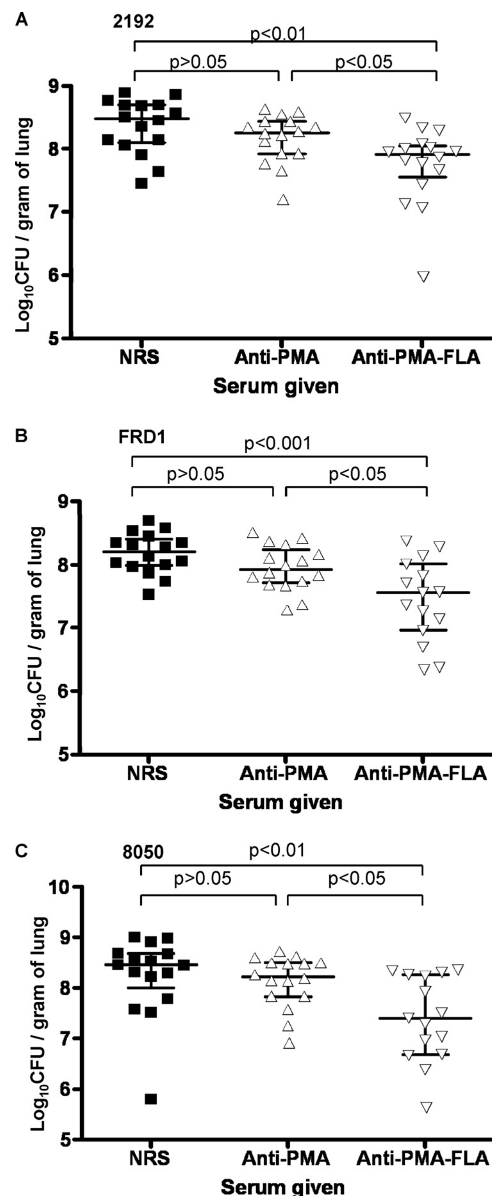


FIG. 5. Pulmonary clearance of *P. aeruginosa* mucoid strains from lungs of infected mice by antisera to PMA and PMA-FLA. (A) Clearance of strain 2192 ($\approx 6.65 \times 10^7$ CFU/mouse) 4 h after infection (anti-PMA-FLA versus anti-PMA, $P < 0.05$; anti-PMA-FLA versus NRS, $P < 0.01$; anti-PMA versus NRS, $P > 0.05$). (B) Clearance of strain FRD1 ($\approx 3.175 \times 10^7$ CFU/mouse) 4 h after infection (anti-PMA-FLA versus anti-PMA, $P < 0.05$; anti-PMA-FLA versus NRS, $P < 0.001$; anti-PMA versus NRS, $P > 0.05$). (C) Clearance of strain 8050 ($\approx 3.175 \times 10^7$ CFU/mouse) 4 h after infection (anti-PMA-FLA versus anti-PMA, $P < 0.05$; anti-PMA-FLA versus NRS, $P < 0.01$; anti-PMA versus NRS, $P > 0.05$). Values for *P* were determined by one-way analysis of variance (ANOVA) with Bartlett's test for equal variances and Tukey's multiple comparison test.

antibodies against this strain at the challenge dose used. Antisera raised to purified PMA did not protect against any of the nonmucoid strains (Fig. 6A to D) when compared to mice receiving NRS.

Antibody to type a flagellin provided for 47.8% survival of mice infected with strain PAK ($P = 0.0002$ versus the NRS

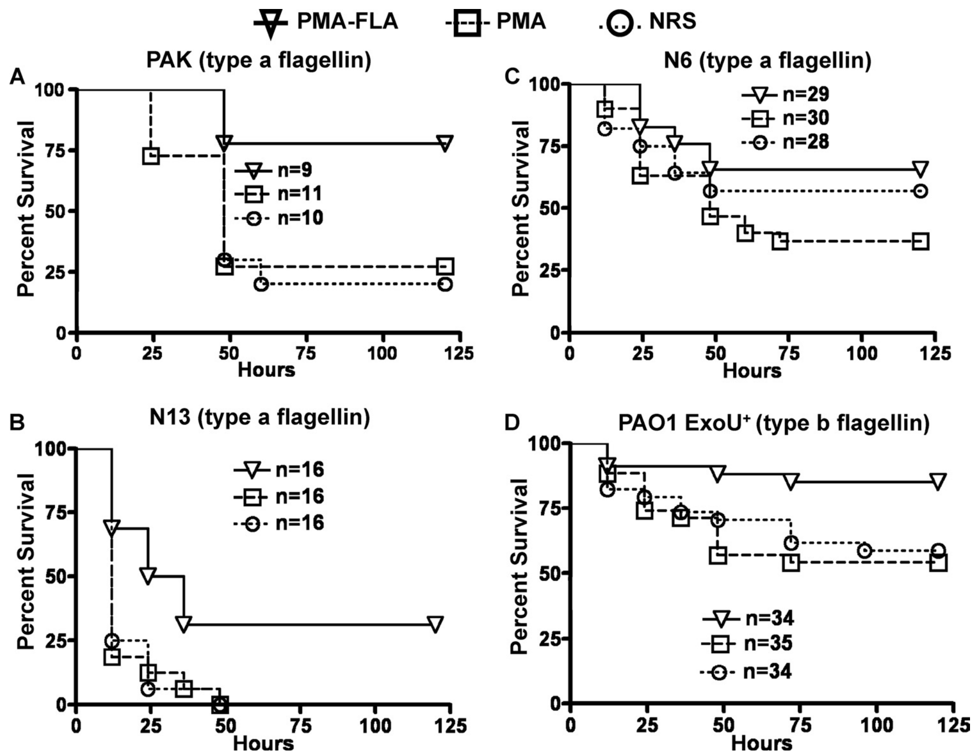


FIG. 6. Survival of mice passively immunized with antibody to PMA or PMA-FLA. (A) Mice challenged with *P. aeruginosa* type a strain PAK ($\approx 5 \times 10^7$ CFU/mouse; log-rank test: PMA-FLA versus PMA, $P = 0.021$; PMA-FLA versus NRS, $P = 0.0141$; PMA versus NRS, $P = 0.6714$; median survival: PMA-FLA, undefined; PMA or NRS, 48 h). (B) Mice challenged with *P. aeruginosa* type a strain N13 ($\approx 1.27 \times 10^7$ CFU/mouse; log-rank test: PMA-FLA versus PMA, $P = 0.0034$; PMA-FLA versus NRS, $P = 0.0024$; PMA versus NRS, $P = 0.9349$; median survival: PMA-FLA, 30 h; PMA or NRS, 12 h). (C) Mice challenged with *P. aeruginosa* type a strain N6 ($\approx 2.66 \times 10^7$ CFU/mouse; log-rank test: PMA-FLA versus PMA, $P = 0.0293$; PMA-FLA versus NRS, $P = 0.4009$; PMA versus NRS, $P = 0.2330$; median survival: PMA-FLA and NRS, undefined; PMA, 48 h). (D) Mice challenged with *P. aeruginosa* type b strain PAO1 $ExoU^+$ ($\approx 5 \times 10^7$ CFU/mouse; log-rank test: PMA-FLA versus PMA, $P = 0.0062$; PMA-FLA versus NRS, $P = 0.0168$; PMA versus NRS, $P = 0.6980$; median survival: undefined for either group).

group) (Fig. 7A) and 61.9% survival against infection with strain N13 ($P = 0.0337$ versus the NRS group) (Fig. 7B). There was no protection, however, against type a flagellin *P. aeruginosa* strain N6 (Fig. 7C) or against flagellin type b strain PAO1 $ExoU^+$ (Fig. 7D).

**Immune serum inhibition of *P. aeruginosa*-flagellin mediated TLR5 activation.** We next compared the ability of the flagellin component of the PMA-FLA conjugate to activate TLR5 with that of purified flagellin, using equal amounts of flagellin in the assays, and also evaluated whether antibody elicited by the PMA-FLA conjugate vaccine or type a flagellin alone inhibited TLR5 activation (Fig. 8). Unconjugated flagellin activated the cells to a higher level than did the flagellin component of the PMA-FLA conjugate at every concentration tested (Fig. 8A). This suggests that some of the epitopes in the flagellin protein responsible for TLR5 activation could have been modified or hidden by the conjugation. When antibodies to flagellin or PMA-FLA were used to inhibit TLR5 activation by purified *P. aeruginosa* flagellin, antibody to purified flagellin neutralized TLR5 activation, as has been previously described (8), whereas antisera to PMA-FLA were not capable of inhibiting the activation of TLR5 (Fig. 8B). Thus, conjugation of flagellin to PMA did not result in antibody that could interfere with the innate immune response to flagellin and make hosts potentially more susceptible to infection.

## DISCUSSION

Many studies have indicated the importance of the prevention of acquisition of nonmucoid *P. aeruginosa* and its subsequent conversion to the mucoid phenotype in the CF lung (12, 16, 21, 42, 49). In this regard, we found that a conjugate vaccine containing PMA, targeting a conserved antigenic epitope on the alginate antigen, along with type a flagellin as a vaccine carrier protein, showed protective efficacy against both mucoid and nonmucoid *P. aeruginosa* lung infection. Conjugation of PMA to flagellin enhanced its immunogenicity without eliciting antibodies that inhibit TLR5, indicating that desirable protective antibodies were elicited but not at the price of possibly inhibiting an important and conserved mechanism of innate immune resistance to pathogens (6).

In mice, the PMA-FLA conjugate vaccine was more effective at eliciting antibody to PMA than was the PMA-flagellin mixture, and although the IgG titers to PMA obtained from sera of rabbits immunized with either the PMA-FLA conjugate or PMA alone were similar, the amount of PMA required to obtained this same response was more than 10 times higher than the amount of PMA contained in the conjugate. Also, the results showing that unconjugated PMA was highly immunogenic in rabbits were not surprising, as such high-molecular-weight polysaccharides injected along with strong adjuvants

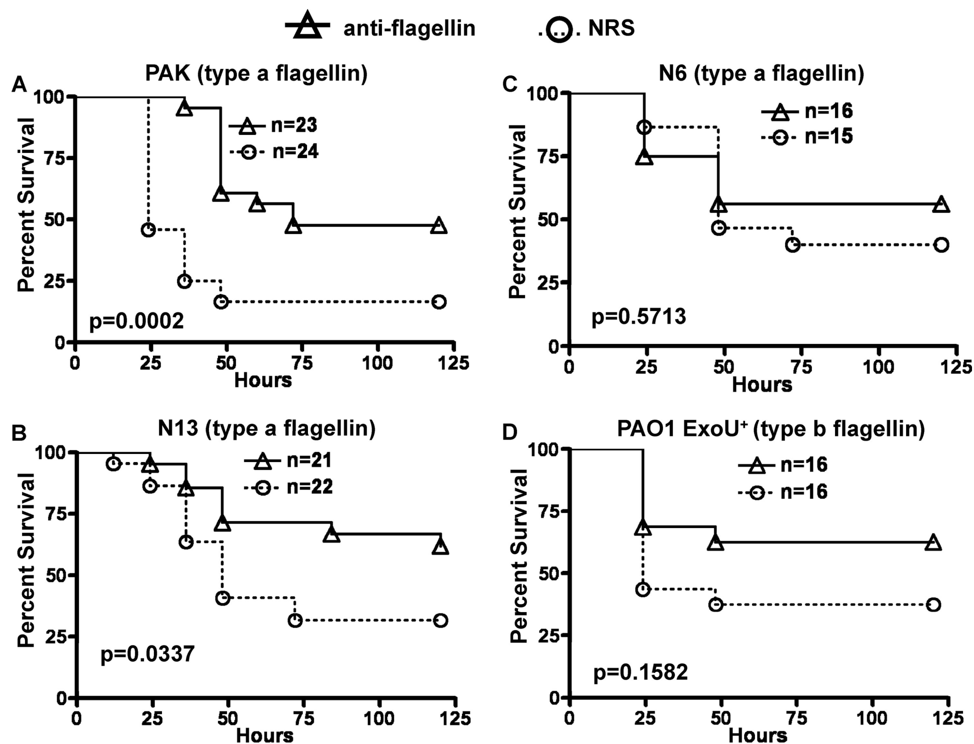


FIG. 7. Survival of mice passively immunized with antibody to type a flagellin. (A) Mice challenged with *P. aeruginosa* type a strain PAK ($\approx 4.125 \times 10^7$ CFU/mouse; log-rank test: anti-flagellin versus NRS, $P = 0.0002$; median survival: anti-flagellin, 72 h; NRS, 24 h). (B) Mice challenged with *P. aeruginosa* type a strain N13 ($\approx 5.27 \times 10^6$ CFU/mouse; log-rank test: anti-flagellin versus NRS, $P = 0.0337$; median survival: anti-flagellin, undefined; NRS, 48 h). (C) Mice challenged with *P. aeruginosa* type a strain N6 ($\approx 3 \times 10^7$ CFU/mouse; log-rank test: anti-flagellin versus NRS, $P = 0.5713$; median survival: anti-flagellin, undefined; NRS, 48 h). (D) Mice challenged with *P. aeruginosa* type b strain PAO1 ExoU$^+$ ($\approx 8.9 \times 10^6$ CFU/mouse; log-rank test: anti-flagellin versus NRS, $P = 0.1582$; median survival: anti-flagellin, undefined; NRS, 24 h).

are immunogenic on their own, as documented previously for other *P. aeruginosa* alginates (15, 35).

The immunization of mice and rabbits with the PMA-FLA conjugate induced a significant humoral immune response to flagellin, but in this case it was clearly lower than the one induced by the PMA-flagellin mix in mice or with flagellin when given alone to rabbits. Thus, the conjugation process might have modified some epitopes in the flagellin protein or reduced its immunogenicity overall. However, conjugated flagellin appears to retain sufficient immunogenicity to induce functional antibodies, as demonstrated in the motility inhibition assays, where antibody to the PMA-FLA conjugate had comparable activity at inhibiting motility of three type a and one type b flagellin *P. aeruginosa* strains as did that of the antibody to purified flagellin. This suggests that the flagellin component of the PMA-FLA conjugate provides sufficient immune responses to inhibit an important aspect of *P. aeruginosa* pathogenesis. The finding of some cross-reactivity between antibody to type a flagellin and a type b flagellin strain was not unexpected, as amino acid segments of the two flagellin types are partially similar, as previously described (8).

When we evaluated the effects of rabbit antibody to PMA-FLA and PMA on opsonic killing of mucoid *P. aeruginosa in vitro*, we found that antibody to PMA alone was able to promote opsonic killing of three mucoid CF clinical isolates, as were antibodies raised by the PMA-FLA conjugate. Specificity of antisera to PMA and PMA-FLA for alginate was shown by its inability to promote opsonic killing of an *algD* mutant strain of FRD1. Previously published data demonstrated that immunization with native *P. aeruginosa* alginate can induce opsonic antibodies to mucoid *P. aeruginosa* (15, 20, 35), and other groups found that conjugation of alginate to carrier proteins significantly increased the opsonic killing activity of mucoid strains (10, 20, 47). Likely, the conjugation of PMA to flagellin enhanced its immunogenicity, as lower doses of the conjugate were needed compared with the purified carbohydrate antigen alone in order to induce opsonic killing antibody.

The finding that antibody to flagellin, PMA, or the PMA-FLA conjugate had little to no opsonic killing activity against nonmucoid strains of *P. aeruginosa* was not unexpected. It has been previously described that antibody to flagellin is not opsonic against nonmucoid *P. aeruginosa* strains (8), and results with polyclonal rabbit antibodies to conjugated alginate vaccines that promoted opsonic killing of mucoid strains also showed poor killing of nonmucoid strains (47). These findings suggest that although many nonmucoid strains have been shown to produce low levels of alginate *in vitro* (3, 36), the level of alginate expression may not be sufficient for it to serve as a target for opsonic killing by polyclonal serum. In contrast, a fully human monoclonal antibody to *P. aeruginosa* alginate has demonstrated opsonic killing of nonmucoid CF clinical isolates (33), and its specificity was shown to be toward the PMA epitopes used in the PMA-FLA conjugate vaccine. However, it is not known if the PMA-FLA conjugate generated a high

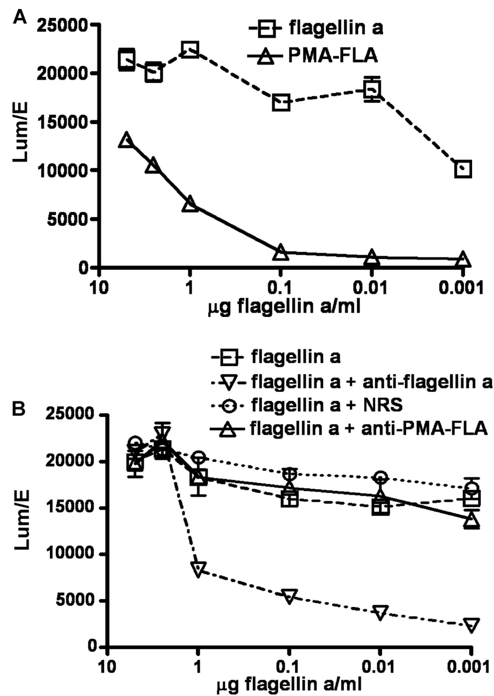


FIG. 8. Inhibition of *P. aeruginosa* flagellin-mediated activation of TLR5 by antisera to flagellin or PMA-flagellin conjugate vaccine. (A) Activation of TLR5 by *P. aeruginosa* type a flagellin or PMA-FLA conjugate vaccine. (B) Effect of antibody to type a flagellin or PMA-FLA conjugate on TLR5 activation. The points are the results of triplicate determinations, and error bars represent standard errors of the mean (SEM). Normal rabbit serum (NRS) was also included for comparison.

population of antibodies with the specificity for the uronic acid on the C-6 carbon of mannuronic acid, as was shown for the more broadly opsonic monoclonal antibody to alginate.

The results showing that antibody to flagellin protects against some nonmucoid, homologous type a flagellin *P. aeruginosa* strains in the mouse pneumonia model of infection were unexpected based on prior results using flagellin as a vaccine (8), wherein this antigen was poorly protective. Yu et al. (51) have recently shown that immunization with flagellin protected mice against *P. aeruginosa* lung infection, but they used active immunization, which may provide better results than passive immunization, and they used native, not recombinant, flagellin. Native flagellin, in contrast with the recombinant flagellin used in our studies, retains glycan groups, which stimulate an inflammatory response (41) that could contribute to the protection they observed. Instead, our prior results with antibody to recombinant flagellin showed no protection against acute *P. aeruginosa* lung infection with nonmucoid strains in mice (8). A possible explanation for this discordance could be the different route used to passively immunize the challenged mice (i.n. versus i.p. used previously), as it has been previously described that in order to achieve sufficient levels of a human monoclonal antibody to alginate within the lungs of mice, the antibody had to be delivered i.n. Our results with antibody to flagellin also differ from the ones obtained after immunization with a flagellin DNA vaccine that protected against heterologous but not homologous *P. aeruginosa* lung infection, suggesting that the DNA vaccine may be superior at promoting cross-protective antibody compared to the flagellin protein or that active immunization with a DNA vaccine may be superior to passive antibody transfer at promoting protection against infection (45).

Given the role that flagellin has in innate immunity through activation of TLR5 (18), it was critical to evaluate the consequences of immunization with PMA-FLA on TLR5 activation. Because chronic *P. aeruginosa* airway infection and the accompanying inflammatory responses are clearly the major clinical problems for CF patients today (23), it has been proposed that inhibition of TLR5 may reduce the damaging inflammatory response generated by the immune response triggered following exposure to *P. aeruginosa* (7). In addition, it has been demonstrated that the recognition of either lipoproteins or lipopolysaccharide by TLR2 and TLR4, or flagellin by TLR5, is sufficient to activate TLR-dependent signaling and control *P. aeruginosa* in the murine lung (41, 43), suggesting that if TLR5 activation is blocked, *P. aeruginosa* could still be recognized by TLR2 and TLR4. However, it has also been reported that the TLR5 mRNA expression is increased in CF airway epithelial cells and, as a consequence, these cells almost exclusively rely upon TLR5 to sense *P. aeruginosa* through its flagellin protein (7). Other studies have shown that TLR5-deficient mice are more susceptible to challenge with *P. aeruginosa* (28, 45), showing an inoculum-dependent defect in bacterial clearance associated with dysregulated early cytokine responses and delayed accumulation of bronchoalveolar neutrophils, suggesting that TLR5 plays an important role in the early innate immune response to *P. aeruginosa* (28). Taken together, it seems that blocking of TLR5 activation may impede the induction of protective immunity against *P. aeruginosa* and may increase the risk of acquiring infections from other flagellated bacteria that activate TLR5.

When testing the activation of TLR5 by PMA-FLA, we could demonstrate that conjugation of flagellin to PMA prevented the induction of antibody that could interfere with the innate immune response to flagellin. Saha et al. (45) established that antibody responses directed against the TLR5 activation domain of the flagellin protein hinder the induction of protective immunity and that modifying that domain to prevent the stimulation of those antibodies improves the host's ability to generate a protective immune response against *P. aeruginosa* (45). This suggests that during the conjugation process, the TLR5 activation domain of flagellin could have been modified or obscured and thus not immunogenic.

In summary, we have synthesized and characterized a PMA-FLA conjugate vaccine that elicited high titers of specific antibodies to PMA and flagellin that were able to protect mice against mucoid and nonmucoid strains of *P. aeruginosa* without interfering with TLR5-mediated immunity. Immunization of CF patients early in life with PMA-FLA conjugate vaccine may prevent the initial *P. aeruginosa* colonization and may also lower the incidence of chronic mucoid *P. aeruginosa* infection. However, it may be necessary to include other *P. aeruginosa* flagellin types in a future vaccine preparation, since some strains of *P. aeruginosa* expressing flagellin antigens not included in the PMA-FLA conjugate vaccine may arise, as has been previously described (12).

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