Human Immune Response to Pseudomonas aeruginosa Mucoid Exopolysaccharide (Alginate) Vaccine

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Chronic lung infection with mucoid Pseudomonas aeruginosa is the major pathologic feature of cystic fibrosis. Previous studies suggested that a failure to produce opsonic antibody to the mucoid exopolysaccharide (MEP; also called alginate) capsule is associated with the maintenance of chronic bacterial infection. Provision of MEP-specific opsonic antibodies has therapeutic potential. To evaluate the ability of MEP to elicit opsonic antibodies, humans were immunized with two lots of MEP vaccine that differed principally in molecular size. Lot 2 had a larger average MEP polymer size. Both vaccines were well tolerated, but lot 1 was poorly immunogenic, inducing long-lived opsonic antibodies in only 2 of 28 vaccinates given doses of 10 to 150 μg. In contrast, at the optimal dose of 100 μg, lot 2 elicited long-lived opsonic antibodies in 80 to 90% of the vaccinates. The antibodies elicited by both lots enhanced deposition of C3 onto mucoid P. aeruginosa cells and mediated opsonic killing of heterologous mucoid strains expressing distinct MEP antigens. These results indicate that the polymers of MEP with the largest molecular sizes safely elicit opsonic antibodies in a sufficiently large proportion of vaccinates to permit studies of active and passive immunization of cystic fibrosis patients against infection with mucoid P. aeruginosa.

Chronic respiratory tract infections with strains of Pseudomonas aeruginosa elaborating large quantities of mucoid exopolysaccharide (MEP; also called alginate) constitute the major pathologic factor in chronic lung disease and death in patients with cystic fibrosis (CF) (7, 8, 11). Although little is known about the mechanisms that enable P. aeruginosa to initiate infection in CF patients, this ability is likely related to disruptions in the physiology and chemistry of the respiratory mucus of CF patients and the expression of adhesins for epithelial cells and mucus by P. aeruginosa (12, 26, 28, 29, 32). The strains initiating infection are phenotypically distinct from those that emerge to cause chronic infection (19). Specifically, initially infecting strains tend to elaborate a smooth lipopolysaccharide (LPS) containing long O side chains, and they are resistant to the bactericidal effects of serum (19). In contrast, chronically colonizing strains undergo phase variations that result in the emergence of LPS-rough, serum-sensitive strains producing copious amounts of MEP. An intense immune response to multiple bacterial antigens, including MEP, ensues (5, 15, 16, 21, 25, 33), but the antibodies produced during infection are clearly ineffective at eliminating the bacteria. Previous work has suggested that the inability of CF patients to effectively deal with chronic P. aeruginosa infection is associated with the production of antibodies to MEP that fail to mediate opsonic killing of the bacteria in vitro (2, 22). A few older CF patients (>12 years) not chronically colonized have been described; these individuals possess MEP-specific serum antibodies that do mediate in vitro opsonic killing. Protection of rodents against chronic endobronchial infections with mucoid P. aeruginosa was found to be mediated by opsonic—but not nonopsonic—MEP-specific antibodies (23). Although CF patients do develop high titers of opsonic antibodies to P. aeruginosa during infection, these antibodies are directed not at MEP but at bacterial surface antigens located below the MEP capsular layer (22). The non-MEP-specific opsonins deposit C3b and C3bi below the MEP layer (20), where they may be unable to interact with complement receptors on polymorphonuclear leukocytes, particularly in vivo, where the bacteria have been shown by numerous studies to grow in MEP-encased microcolonies (4, 14, 35). Indeed, recent studies support this idea: antibodies in sera from chronically colonized CF patients have a markedly reduced ability to mediate opsonic killing of mucoid P. aeruginosa growing as a biofilm in vitro (18).

The infrequency with which MEP-specific opsonic antibody is detected in sera from chronically infected CF patients appears not to be related to the underlying genetic condition but rather to be characteristic of humans in general. We and others have documented moderate titers of naturally occurring antibodies to MEP among persons without CF (5, 21, 36), but these antibodies are nearly universally nonopsonic (22, 23). In mice, preexisting nonopsonic antibody to MEP prevents the production of MEP-specific opsonic antibodies when an appropriate dose of MEP antigen is subsequently administered (9). This effect has been associated with the ability of the MEP antigen and nonopsonic antibodies to form an immune complex; this complex binds to Fc receptors on the surfaces of T cells that can mediate cytotoxic killing of hybridoma cells secreting MEP-specific opsonic antibody (24). Similar immune complexes in the sera of colonized CF patients sensitized activated human peripheral-blood T cells for cytotoxic activity against the hybridoma cells (24).

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From these results, we hypothesize that the progression of chronic lung infections with mucoid *P. aeruginosa* in CF patients is due to a lack of production of MEP-specific opsonins. To test this hypothesis, one could provide these opsonins to patients and determine their effect on infection and disease. To this end, a source of MEP-specific opsonins suitable for clinical use is needed. In this study we describe the abilities of two preparations of MEP antigen to elicit opsonic antibodies in healthy human subjects, and we document other parameters characteristic of the human immune response to purified MEP antigen.

(Portions of this work were presented in abstract form at the 1988 meeting of the American Federation for Clinical Research, the 1988 annual North American Cystic Fibrosis Conference, the 1990 annual meeting of the American Society for Microbiology, the 11th International Cystic Fibrosis Congress in 1992, and the 1992 annual North American Cystic Fibrosis Conference.)

**MATERIALS AND METHODS**

**Bacterial strains.** A clinical isolate of mucoid *P. aeruginosa*, strain 2192, was employed in the preparation of MEP antigen and the evaluation of immune responses in the opsonophagocytic assay. Additional clinical isolates of mucoid *P. aeruginosa* were obtained courtesy of the microbiology laboratory at Children's Hospital in Boston from Anne MaCone and Donald Goldmann. Strain FRD 1, a clinical isolate from a CF patient, and strain FRD 462, a chemical mutant that produces a MEP antigen composed solely of mannnuronic acid residues (6), were kindly provided by D. Ohman, University of Tennessee, Memphis.

**Vaccine antigens.** Two lots of MEP vaccine (human lots 1 and 2) were prepared from mucoid *P. aeruginosa* strain 2192. Human lot 1 was the same as the MEP lot 1 antigen described previously (9); human lot 2 was comparable to but distinct from the MEP lot 2 antigen described previously (9). Human lot 2 was prepared as described for MEP lot 2 (9). Human lots 1 and 2 differed with regard to the medium used to grow the bacteria for vaccine production and with regard to molecular size (9) but were otherwise comparable.

Vaccines for human use were commercially bottled (Bellmore Laboratories, Hampstead, Md.) and commercially evaluated for safety and pyrogenicity in animals and for endotoxin content (South Mountain Laboratories, Orange, N.J.) to comply with the requirements of the U.S. Food and Drug Administration. Before being administered to humans, all vaccines were evaluated for immunogenicity in mice, as described elsewhere (9).

**Immunization studies.** Healthy volunteers (of both sexes; >18 years of age) gave informed consent according to institutional review board-approved protocols. Women were supplied with home pregnancy tests to self-administer before vaccination. All volunteers underwent physical examinations and had histories taken to rule out factors necessitating exclusion. Individuals given human lot 2 antigen had one blood sample drawn 24 to 48 h before vaccination and another drawn 48 h after vaccination; part of each sample was submitted to a clinical laboratory for a complete blood count. Blood (20 to 100 ml) was also obtained at 7, 14, 21, and 28 days after vaccination from most volunteers. Additional blood samples were collected from available participants at 35 and 42 days and at 2, 4, 6, and 12 months and from some participants at 24 months. Serum was stored at -20°C.

Vaccine was administered either subcutaneously or intramuscularly into the upper arm in a 0.5-ml dose, with sterile saline as the vehicle for injection. Reactions to the vaccine were assessed by interviews with the volunteers at 20 to 45 min, 24 h, and 48 h after vaccination. The local and systemic responses described in these interviews were graded on a subjective scale of 0 to 3, with the respective scores representing absent, mild, moderate, and severe reactions. In addition, vaccines were instructed to take their temperatures 2 to 3 times a day during this period and were provided with logs in which to record any reactions thought to be associated with immunization.

**Immunologic analysis.** The opsonophagocytic assay and the inhibition of opsonophagocytosis were performed as described previously (2, 9, 22). Briefly, equal volumes of dextran-purified human leukocytes (2 × 10⁶ per tube) were mixed with 2 × 10⁶ mucoid *P. aeruginosa* cells in the log phase of growth; a 1:15 dilution of fresh, normal human serum as a complement source; and various dilutions of the test sera that had been heat inactivated (at 56°C for 30 min). Aliquots for bacterial enumeration were removed from some tubes at time 0 and from all tubes after incubation for 90 min at 37°C with end-over-end tumbling. Titers were expressed as reciprocals of the highest serum dilutions in which ≥50% of the bacteria were killed. Killing was calculated by the following formula: percent bacteria killed = 100 - ([CFU in sample of preimmunization serum/CFU in sample of preimmunization serum] × 100]. Samples of preimmunization sera never showed any reduction in bacterial numbers compared with controls lacking any serum. One exception involved sera from vaccinated human lot 2 in the dose-response study; the percentage of bacteria killed was calculated by using the surviving CFU in tubes containing a standard, nonimmune serum.

For measurement of immunoglobulin G (IgG) responses, an enzyme-linked immunosorbent assay (ELISA) (5, 19) employing serum samples from individuals given human MEP vaccine lot 2 was used; this specific antigen was used to sensitize the plates. Titers were expressed as reciprocals of the highest dilutions producing readings threefold above the background. Deposition of C3 onto mucoid *P. aeruginosa* 2192 cells was studied as described elsewhere (20), except that the purified C3 (Diamedix Corp., Miami, Fla.) was labeled with [125I] by means of the Iodobeads reagent (Pierce Chemicals, Rockford, Ill.). The biologic activity of the labeled C3 was confirmed by measurement of its ability to restore lysis of antibody-sensitized sheep erythrocytes by C3-depleted human serum (Diamedix).

**Derivation of antibodies from transformed peripheral-blood leukocytes.** For the separation of human peripheral-blood T and B cells, rosetting of 2-aminoethyl-isothiouronium bromide-treated sheep erythrocytes with the T cells was followed by Ficoll-Hypaque density-gradient separation (13). The B-cell-enriched band was recovered, washed three times with RPMI tissue culture medium, and then infected with a supernatant of the B95-8 marmoset cell line (ATCC CRL 1612) containing Epstein-Barr virus (EBV) (37). Cultures were grown in complete RPMI 1640 medium supplemented with 10% fetal calf serum, streptomycin (1 mg/ml), and penicillin (1 mg/ml) at 37°C in 5% CO₂. Supernatants were harvested after 2 to 3 weeks of culture. Ammonium sulfate was added to 50% of saturation to precipitate antibodies, which were then recovered by centrifugation, redissolved in water, and dialyzed against phosphate-buffered saline. The final antibody solutions were adjusted to a constant concentration factor based upon the initial amount of cell supernatant used in preparing the antibodies. When supernatants from EBV-transformed cells were tested in the opsonophagocytic assay, controls containing only the supernatant and bacteria were run to ensure that no
bacterial death resulted from the presence of residual antibiotics derived from the culture medium.

Statistical analyses. The significance of differences in ELISA and opsonic titers between preimmunization and postimmunization serum samples and that of differences in titer among samples tested in the opsonophagocytic inhibition assays were determined by using a Wilcoxon signed-rank test; that of differences in titer among individuals immunized with different doses of the vaccine was determined by the Kruskal-Wallis test; and that of differences in titer between groups receiving different vaccine doses was determined by the Dunn procedure of the Kruskal-Wallis test (30). Differences in C3 deposition and in levels of opsonic killing of heterologous strains were identified by analysis of variance (ANOVA); the Scheffe F test was used to determine the significance levels of these differences. Most of the calculations described above were performed on a Macintosh II computer with the StatView SE+ graphics software program (Abacus Concepts, Inc., Berkeley, Calif.).

RESULTS

Properties of MEP vaccines. The chemical composition, molecular size, and immunogenicity in mice of human MEP vaccine lot 1 have been reported previously (9); this preparation was referred to as lot 1 in the previous report. Briefly, lot 1 vaccine was a polymer with a \( M_w \) of 0.26, and it was composed of \( \geq 99.9\% \) uronic acid, with a 4:1 ratio of mannuronic to guluronic acid residues. It elicited opsonic antibodies in mice at doses of 1 and 10 \( \mu \)g per mouse, but it elicited only nonopsonic antibodies at doses of \( \geq 20 \) \( \mu \)g per mouse. Human MEP vaccine lot 2 was similar—with only slight differences—to a nonhuman version (referred to as lot 2) also described previously (9). Human MEP vaccine lot 2 was composed of 82% mannuronic acid and 18% guluronic acid, it had a \( M_w \) of 0.102 on a column (1.6 by 100 cm) of Sepharose CL4B, and it contained <1% protein or nucleic acid. Both human vaccine lots contained 4.0% acetate by weight. Human MEP vaccine lot 2 had an immunogenicity profile in mice similar to that previously reported for nonhuman lot 2 (9), as measured by increases in antibody titers (detected by ELISA) and in opsonic titers (detected by a phagocytosis assay). Thus, human lot 2 induced a \( \geq \)fourfold rise in ELISA titers and opsonic titers at doses of 1 to 50 \( \mu \)g per mouse. Human MEP vaccine lots 1 and 2 both passed all U.S. Food and Drug Administration-mandated evaluations for pyrogenicity, endotoxin content, and general safety in mice and guinea pigs.

Response to vaccination with human MEP vaccine lot 1. Twenty-eight individuals (16 men and 12 women) were vaccinated subcutaneously at least once with MEP human vaccine lot 1 in doses of 10 to 150 \( \mu \)g. Booster doses of 100 \( \mu \)g were given to eight individuals 2 to 6 weeks after their initial immunization; in addition, three individuals given initial doses of 100 \( \mu \)g received booster doses of 150 \( \mu \)g 4 weeks later. The booster doses had no effect on antibody responses and elicited reactions comparable to those to the initial doses. The vaccine was well tolerated, and the adverse reactions documented were typical of those seen with other bacterial polysaccharide vaccines (3, 10, 17). These reactions included erythema at the site of injection (diameters of 1 to 3 cm noted at 24 h) in the case of 1 of 5 vaccines given 10 \( \mu \)g, 2 of 5 vaccines given 50 \( \mu \)g, 6 of 11 vaccines given 100 \( \mu \)g, and 5 of 7 vaccines given 150 \( \mu \)g. A total of 20 of the 28 vaccines experienced mild tenderness at the injection site up to 48 h after immunization, but this reaction appeared to be independent of the dose. No additional toxicity was associated with booster doses. One individual had systemic symptoms—fever and headache—that commenced 48 h after immunization, but these symptoms were judged to be likely due to a viral syndrome rather than the vaccine.

The development of opsonic antibodies to mucoid \( P. \) aeruginosa after vaccination is shown in Table 1. Overall, the response was rather disappointing, in that only six individuals (five men and one woman) mounted an opsonic response (killing of \( \geq 50\% \) of the bacteria compared with survival in the preimmunization sample) that was still detectable more than 5 weeks after immunization and only two individuals maintained their responses for \( \geq 6 \) months. Both of these individuals developed peak titers of 32, while the remaining four responders had only low titers (range, 4 to 16). Five of the six responders received initial doses of 100 \( \mu \)g. One of the responders producing antibodies for \( \geq 6 \) months did not develop detectable antibodies until 35 days after vaccination.

Many of the vaccinates had responses that were detectable in the first 1 to 3 weeks after vaccination (Table 1). These antibody responses could not be augmented by booster doses and thus appeared to be transient. The six individuals who responded for \( \geq 35 \) days did have increased deposition of the third component of complement (C3) onto mucoid \( P. \) aeruginosa cells, as previously reported (20). In addition, immune sera from these six vaccinates had opsonic antibody titers comparable to those against the vaccine strain in tests with four heterologous strains of mucoid \( P. \) aeruginosa expressing MEP antigens with viral ratios of the two constituent monosaccharides, mannuronic acid and guluronic acid (data not shown). At the time of these studies, no available ELISA or other technique could measure increases in levels of binding antibodies after immunization, primarily because all preimmunization sera had high levels of binding—but nonopsonic—antibodies that interfered with our ability to detect increases resulting from immunization. Later, when a reproducible ELISA was developed for use with human vaccine lot 2 (see below), insufficient amounts of preimmunization sera from these vaccinates were available for study.

Because of the high proportion of early, transient responses to vaccination, we prepared transformed B cells from peripheral-blood samples obtained from eight individuals before and 7 to 10 days after immunization, and we analyzed these cells for the production of opsonic antibodies. As shown in Fig. 1, only one of eight individuals had a low level of opsonic activity in supernatants of transformed B cells obtained before immunization, while at 1 week after immunization all eight individuals had peripheral-blood B cells that could be transformed with EBV to produce opsonic antibodies in vitro. Peripheral-blood B cells were not obtained later than 10 days after immunization, since, even when an individual responds to a vaccine, antibody-producing cells are not usually found in the periphery by 10 days after antigen exposure (1).

### Table 1. Human immune response to MEP vaccine lot 1

<table>
<thead>
<tr>
<th>Vaccine dose (( \mu )g)</th>
<th>No. of vaccinates</th>
<th>No. of vaccinates responding* with opsonic antibody at the following times after vaccination:</th>
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<td></td>
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<td>Days 7–21</td>
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<td>150</td>
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* Fourfold or greater increase in opsonic antibody titer of serum compared with that of preimmunization serum; titers ranged from 4 to 32.
Response to immunization with 100 μg of human MEP vaccine lot 2. Studies with naive mice (9) indicated that human MEP vaccine lot 1 elicited opsonic antibodies at doses of 1 to 10 μg per mouse but that it elicited only nonopsonic antibodies at doses of 50 μg per mouse. In animals previously immunized with 50 μg of MEP, a booster dose of 1 to 10 μg of human lot 1 vaccine failed to elicit opsonic antibodies (9). Thus, the results with these mice are like those with human vaccines: in the presence of preexisting nonopsonic antibodies (either induced in mice or occurring naturally in humans), human vaccine lot 1 only poorly elicits opsonic antibodies. However, a vaccine composed of only the polymers of MEP with the largest molecular sizes elicited opsonic antibodies in mice with preexisting nonopsonic antibodies (9). We thus prepared human MEP vaccine lot 2 and administered it initially to 20 individuals in a single 100-μg dose and later to 50 individuals—five groups of 10 given doses of 1, 10, 50, 100, and 300 μg, respectively—to determine whether this preparation elicited a better immune response than lot 1.

Among the 20 individuals given the initial 100-μg dose, there were 10 men and 10 women; five volunteers of each sex received the vaccine subcutaneously, and five of each sex received it intramuscularly. The subjects ranged in age from 20 through 49 years, and apart from moderate allergies in some instances, they were all in good health, with all vital signs and almost all complete blood count values within normal ranges. The rates of reaction did not differ with the route of administration. Since all reported reactions were characterized as mild, results for local reactions were pooled for all 20 vaccines. At 20 min after vaccination, seven subjects (35%) had redness at the injection site, one (5%) had induration, two (10%) had pain, six (30%) had local tenderness, two (10%) reported a mild burning sensation, and four (20%) reported warmth of the injection site. By 24 h only two vaccines (10%) had mild pain and tenderness, and just one vaccinee reported mild pain at 48 h. No systemic reactions (malaise, myalgia, headache, fever, nausea, or vomiting) were reported at any time after vaccination, and no clinically significant abnormalities in blood cell counts or compositions were detected at 48 h after injection.

The IgG and opsonic antibody responses measured at 28 days after immunization are shown in Fig. 2. No IgM or IgA responses were detected (data not shown). Overall, 12 (60%) of 20 vaccines responded with a ≥fourfold increase in IgG titer (P < 0.001, Wilcoxon signed-rank test for the entire group; P values for subgroups are shown in Fig. 2A), and 7 vaccines (35%) had a ≥fourfold rise in opsonic antibody titer (P = 0.01, Wilcoxon signed-rank test for the entire group; P values for subgroups are shown in Fig. 2B). There were some discrepancies between the IgG antibody response measured by ELISA and that measured by the opsonophageptosis assay, suggesting that some vaccines had produced nonopsonic IgG antibodies. The group responding with opsonic antibodies was composed of six men and one woman. Five of these individuals had been vaccinated intramuscularly, and two had been vaccinated subcutaneously; four of five men immunized intramuscularly responded with opsonic antibodies. Most individuals...
responded within 14 days. In six of the seven volunteers available for study 1 and 2 years after immunization, titers remained elevated over preimmunization levels. Deposition of C3 onto mucoid *P. aeruginosa* cells was increased for six of seven vaccinees responding with opsonic antibody (the mean increase over the preimmunization level of C3 molecules bound per CFU of mucoid *P. aeruginosa* ± the standard error of the mean was 2,737 ± 1,203; *P* = 0.01, paired *t* test, two-sided); this result confirmed previous reports (20) that this measurement coincides with increases in opsonic antibody levels. The increase in C3 deposition for the nonresponders was not significant, and that for the entire group was of borderline significance (*P* = 0.058, paired *t* test, two-sided).

The specificity of antibodies was evaluated by means of inhibition assays wherein serum samples were incubated with either purified MEP (100 μg/ml) or an irrelevant polysaccharide from *P. aeruginosa* before determination of the percentage of bacteria killed in the opsonophagocytosis assay. As shown in Fig. 3, the sera exhibited significantly reduced killing activity (*P* < 0.001, paired *t* test, two-sided) if incubated with MEP before the opsonic assay but not if incubated with a serologically unrelated polysaccharide (*P* = 0.37, paired *t* test, two-sided).

The ability of the antibodies elicited in immune serum to kill heterologous strains of mucoid *P. aeruginosa* containing various ratios of mannnuronic and guluronic acid monosaccharides in their MEP antigens is shown in Fig. 4. The rates of killing for strains FRD 1 and 258 did not differ significantly from that for the vaccine strain 2192 (*P* > 0.05, ANOVA). The killing of mutant strain FRD 462, which contains no guluronic acid residues in its MEP, was slightly but significantly reduced (*P* = 0.05, ANOVA, Scheffe *F* test). Since all naturally occurring strains of mucoid *P. aeruginosa* analyzed to date contain some guluronic acid in their MEP antigens and since no strain with >50% guluronic acid residues has been described (31, 34), the ability of antibodies elicited by vaccination with MEP from strain 2192 to kill mucoid *P. aeruginosa* strains expressing either low or high levels of guluronic acid indicates a potentially broad specificity of these opsonic antibodies for a large number of clinical isolates of mucoid *P. aeruginosa*.

**Response to doses of 1 to 300 μg of human MEP vaccine lot**

2. Fifty men were divided into five groups of 10 subjects each, and each group was given a single intramuscular injection of 1, 10, 50, 100, or 300 μg of vaccine. The vaccine was well tolerated, with all reported reactions judged to be mild. Of the 50 vaccinees, 15 had tenderness at the injection site, 2 had erythema, and 2 experienced a temperature rise to 37.5°C at some point during the first 48 h. Of the 15 vaccinees with tenderness, 11 received doses of 100 or 300 μg. All reactions subsided within 48 h. There were deviations from the normal ranges in <5% of the hematologic values among some subjects, with approximately the same number of measurements deviating from the normal ranges in the case of preimmunization blood samples as deviated from the normal ranges in the case of postimmunization blood samples. This indicated that out-of-range values for postimmunization blood samples were not due to vaccination but rather that they occurred at a rate comparable to that seen when this set of measurements is taken for blood samples from 50 humans. There was neither a pattern or consistent change in any particular hematologic value in the case of postimmunization samples, and none of the values outside the normal ranges were judged to be clinically significant (by HCM). Similar data accrued for serum chemistry studies and urinalyses of samples provided by these volunteers. One subject had an increase in atypical lymphocyte level from 0 to 20/mm² after vaccination, but this change was thought to be due to a subacute viral infection.

The IgG antibody response to the vaccine measured by ELISA is shown in Fig. 5 as a single point determination for the serum samples obtained from all 50 individuals at 28 days after vaccination. All subjects had blood drawn 7, 14, 21, and 28 days after vaccination as well as on additional days up to 1 year. Most serum samples were tested by ELISAs utilizing dilutions between 1:100 and 1:25,600. The data shown in Fig. 5 are representative of the general response observed. Clearly, the responses were concentrated among recipients of 100 or 300 μg of MEP vaccine. Nine of 10 individuals given 100 μg and 4 of 10 given 300 μg showed increased IgG antibody binding to MEP at 28 days after vaccination. Responders maintained increased antibody levels for at least 1 year after vaccination. The ability to detect antibody by ELISA for the
vaccinates immunized with human lot 2 vaccine (as opposed to those given human lot 1) was ascribed to use of the polymers with larger molecular sizes for sensitization of the ELISA plates. Use of smaller polymers (similar to human lot 1) for sensitization of ELISA plates did not result in detectable differences in antibody binding in the case of these or other serum samples.

The opsonic responses measured at 28 days after vaccination with human MEP vaccine lot 2 at doses of 1 to 300 μg are shown in Fig. 6. Titers of opsonic antibody did not increase among vaccinates given 1 or 10 μg (data not shown); in contrast, three recipients of 50 μg, nine recipients of 100 μg, and four recipients of 300 μg responded to vaccination by producing opsonizing antibodies ($P < 0.001$, Kruskal-Wallis analysis). Titers in the group given 100 μg were significantly higher ($P < 0.05$, Dunn procedure of Kruskal-Wallis analysis) than those in the groups given 1, 10, or 50 μg. In general, the opsonic response paralleled the ELISA response, except among individuals given the 50-μg dose. Two recipients of 50 μg (vaccinates 28 and 29) developed modest opsonic responses in the absence of a detectable ELISA response. Otherwise, all recipients of 100 or 300 μg who had increased binding to MEP as determined by ELISA also had increases in opsonic titer.

Patterns of deposition of C3 onto mucoid P. aeruginosa cells are shown in Fig. 7. Vaccinates given 100 μg had significantly higher levels of C3 deposition ($P < 0.05$, ANOVA, Scheffé $F$ test) than did those given other doses. Some individuals who failed to respond to the vaccine with opsonic antibody nonetheless had levels of C3 deposition comparable to or greater than those of individuals who did respond. We believe that this is likely due to deposition of C3 onto the bacterial surface in a manner or form in which it is unable to interact with complement receptors on phagocytes, as previously documented for certain nonopsonic sera in this system (20).

**FIG. 5.** ELISA readings for IgG antibodies in sera obtained before and 28 days after immunization of 50 volunteers with the indicated doses of human MEP vaccine lot 2. Readings are for a single dilution (1:250) of serum. Bars represent the means of duplicate determinations. A450, absorbance at 450 nm.

**FIG. 6.** Titers of opsonic antibody in sera obtained 28 days after immunization from recipients of 50, 100, or 300 μg of human MEP vaccine lot 2. Points represent the means of quadruplicate determinations. Titers for the group given 100 μg were significantly higher ($P < 0.05$) than those for the groups given 1 or 10 μg (data not shown) or 50 μg.

**FIG. 7.** Deposition of C3 onto mucoid P. aeruginosa cells by antibodies in sera obtained 28 days after vaccination with the indicated doses of human MEP vaccine lot 2. Each point represents the mean of duplicate determinations; the solid boxes with error bars represent the means and standard errors for the different dosage groups. The group receiving 100 μg had a significantly higher level of C3 deposition ($P < 0.05$, ANOVA) than any other groups.

**DISCUSSION**

Comparison of two preparations of MEP vaccine derived from mucoid P. aeruginosa strain 2192 demonstrated that both were safe for use with humans but that each had a different immunogenicity profile. Consistent with previous findings with mice (9) was the observation that a vaccine composed of only the polymers of MEP with the largest molecular sizes was more immunogenic in humans than a vaccine composed of polymers with a smaller average size. The poor immunogenicity of the smaller polymers in humans also correlated with a similar finding with mice, wherein animals immunized so that they produced only nonopsonic antibodies to MEP failed to produce opsonic antibodies when they received a booster dose of antigen that readily elicits opsonic antibodies in naive animals (9). This phenomenon may have been evident in humans, because it appears that most individuals have preexisting nonopsonic antibody to MEP (5, 21, 36) similar to that induced in mice by immunization with certain doses of MEP (9). We
also demonstrated that opsonic antibody responses were usually accompanied by increases in levels of IgG binding antibodies, as measured by ELISA, and in deposition of C3 onto mucoid P. aeruginosa cells, but there were also some individuals who had increases in binding antibody levels or in levels of C3 deposition that were not associated with opsonic killing activity. We believe that these discrepancies are due to the complexity of the system, wherein nonopsonic antibodies that bind to MEP may have been elicited in some vaccinates, while some sera contained antibodies capable of depositing C3 onto mucoid P. aeruginosa cells, where it could not promote uptake and killing by phagocytes. In spite of these discrepancies, our results establish an initial set of criteria for the composition and immunogenic properties of MEP vaccine that will allow further evaluation of its potential in active and passive therapy of chronic P. aeruginosa infection in CF patients.

Despite a poor overall response to human MEP vaccine lot 1 (as measured by increases in long-term titers of opsonic antibody to mucoid P. aeruginosa), most vaccinates had a transient response within the first 3 weeks that could not be augmented by booster doses. This phenomenon was confirmed by EBV transformation of peripheral-blood B cells obtained from eight vaccinates 7 to 10 days after immunization. All eight of the transformed cell lines produced opsonic antibodies, whereas no transformed B cells that had been obtained from these volunteers before immunization produced these antibodies. Since seven of these eight vaccinates exhibited transient serum immune responses to human lot 1 vaccine, while only one vaccinate had a response that lasted 6 months, it appears that the lot 1 vaccine initially stimulated B cells that produce opsonic antibodies but that either these cells developed only into short-lived plasma cells that could not be renewed, or memory cells were not established. The basis for the transient immune response might reside in the ability of preexisting nonopsonic antibodies to form immune complexes with the MEP vaccine antigen; these complexes can sensitize Fc receptor-positive cytotoxic T cells, which could potentially kill the B cells that produce opsonic antibody. We have recently documented the occurrence of this phenomenon in mice (24) and have also shown that sera from CF patients contain immune complexes capable of sensitizing concanavalin A-activated human peripheral-blood T cells, which can kill the murine hybridoma cells secreting MEP-specific opsonic antibodies (24). In this experimental system, the polymers of MEP with larger molecular sizes are less efficient than smaller polymers in promoting T-cell-mediated killing of target hybridoma cells (unpublished observation). This observation suggests that the larger polymers may interact with membrane-bound antibody on B cells in a manner that renders these cells less susceptible to cytotoxic activity.

In both the study with human MEP vaccine lot 1 and the initial study of human MEP vaccine lot 2 (with 20 individuals given a single 100-μg dose), men made up the majority of the responders. We do not know whether this is a general phenomenon, since these sample sizes are too small for any substantive conclusions. However, MEP vaccine will first be administered to volunteer plasma donors in order to produce a hyperimmune intravenous IgG preparation that can be used for evaluation of the therapeutic efficacy of these antibodies (given via passive infusion) in colonized CF patients. Since the vast majority of plasma donors are men, a preferential response of males to this vaccine would not be problematic in these initial clinical evaluations. However, if there are indications of potential protective efficacy of the vaccine against chronic infection with P. aeruginosa if administered to uncodo-

ized CF patients, then it will be critical to assess gender-associated differences in responses.

In the first portion of the study with human lot 2 vaccine, four of five men immunized intramuscularly responded with opsonic antibodies, whereas only two of five men immunized subcutaneously responded. While these sample sizes are obviously too small for an evaluation of the significance of this observation, the fact that reactions to administration by the two routes were comparable led us to use the intramuscular route in dose-response studies. Human lot 1 vaccine was given only by the subcutaneous route; this point may have accounted, in part, for the poor immune response to this preparation. While we cannot completely exclude this explanation for the difference between the immune responses to lots 1 and 2, the difference in molecular size appears to be more critical; specifically, the responses of these volunteers strongly parallel those of mice (9), in the case of which all vaccines were given by the same route. Moreover, opsonic antibody elicited by human lot 2 vaccine lasted for at least 24 months in all vaccinates available for study, while only two recipients of human lot 1 vaccine had long-term antibody responses.

MEP expressed by P. aeruginosa is composed of two constituent monosaccharides: α-l-guluronic acid and β-d-mannuronic acid, the latter sugar being partially acetylated. However, different mucoid strains produce MEP antigens with variable mannuronic acid-to-guluronic acid ratios (~0.1:1 to close to 1:1) (31, 34), and so it was important to determine that antibodies elicited by one preparation were able to opsonize different mucoid P. aeruginosa strains expressing different MEP antigens. We found that the MEP from strain 2192, composed of about a 4:1 ratio of mannuronic acid to guluronic acid, elicited human antibodies that mediated opsonic killing of a clinical isolate whose MEP contained 55% mannuronic acid (1.2:1 mannurionic acid-to-guluronic acid ratio) and a clinical isolate whose MEP contained 90% mannuronic acid (9:1 mannuronic acid-to-guluronic acid ratio). In addition, the laboratory-derived mutant strain FRD 462 produces an MEP antigen composed solely of mannuronic acid. Although there was slightly less killing of strain FRD 462 by antibodies from seven vaccinates, as shown in Fig. 5 (P = 0.05), most of the vaccinates’ sera efficiently mediated opsonic killing of this strain. This finding implies that the epitope recognized by some populations of MEP-specific opsonic antibodies is composed solely of mannuronic acid residues, the major constituent of most MEP antigens.

In conclusion, we have demonstrated that purified MEP from mucoid P. aeruginosa strain 2192 is safe and immunogenic in human adults when the polymers with the largest molecular sizes are used for the vaccine. The optimal dose appears to be 100 μg, and the immune response is maintained for up to 2 years. A third lot of MEP vaccine for human studies has been administered to plasma donors in order to prepare hyperimmune intravenous IgG material for eventual clinical evaluation with CF patients (27). Previous work has indicated that almost all CF patients fail to respond to chronic infection with mucoid P. aeruginosa by producing opsonic antibodies to MEP, while some CF patients who survive into the second to fourth decades of life without chronic infection do have MEP-specific opsonic antibodies in serum (22). If the progression of chronic P. aeruginosa lung infections in CF patients is due to the lack of MEP-specific opsonic antibodies, then provision of these antibodies by active or passive immunization should theoretically help to halt progression. The demonstration here of the properties of an MEP vaccine that are needed to elicit these antibodies in humans will allow further evaluation of this hypothesis.
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REFERENCES


