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Opsonophagocytic Killing Activity of Rabbit Antibody to *Pseudomonas aeruginosa* Mucoid Exopolysaccharide

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We used an in vitro opsonophagocytic killing assay to measure the functional activity of antibody directed at the mucoid exopolysaccharide (MEP) antigen expressed by *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. Rabbit antibodies raised to purified MEP were able to mediate phagocytic killing in the presence of human peripheral blood leukocytes and a low level (final concentration, 0.3%) of fresh normal human serum as a complement source. No bacterial killing was observed when peripheral blood leukocytes, antiserum, or complement was omitted. Specificity of the antibody for the MEP antigen was shown by adsorption and inhibition assays. Affinity-purified antibody to MEP also mediated phagocytic killing. These data indicate that antiphagocytic properties attributable to MEP can be overcome by specific antibody.

Chronic pulmonary colonization of cystic fibrosis (CF) patients with mucoid exopolysaccharide (MEP)-producing strains of *Pseudomonas aeruginosa* is a well-known phenomenon (3, 4, 8). This colonization probably contributes to the destructive lung process that causes death in many of these patients. A number of recent reports have demonstrated the presence of a high antibody titer to MEP in the sera of chronically colonized CF patients (2, 13, 17). Despite this humoral immune response, CF patients are unable to eliminate lung colonization by *P. aeruginosa*. Therefore, it is possible that antibody to MEP is unable to mediate the killing of mucoid *P. aeruginosa*.

One problem in studying the biological activity of antibody to MEP is that most strains of mucoid *P. aeruginosa* are serum sensitive because they produce only a rough type of lipopolysaccharide (LPS) (7). The killing of these strains occurs in the presence of low levels (≤5.0%) of fresh normal human serum (FNHS). This killing appears to be mediated by the alternative pathway of complement (11), although some investigators have presented evidence that antibody and the classical complement pathway kill mucoid *P. aeruginosa* (1). Therefore, to study the biological activity of antibody to a particular antigen made by mucoid *P. aeruginosa*, one must control for the role of complement in killing these bacteria.

Protective immunity against *P. aeruginosa* is thought to be phagocytic killing mediated by peripheral blood leukocytes (PBL) and antibody (19). When immune serum was used, complement augmented killing when the antibody was human immunoglobulin G (IgG), had no affect on killing mediated by human IgA, and was absolutely required for killing mediated by human IgM (14). In nonimmune human serum, complement appeared essential for opsonization (9), but killing was not investigated. However, in these studies strains were used that were serum resistant and had a smooth type of LPS. Since mucoid *P. aeruginosa* strains are often serum sensitive, lack LPS side chains, and express a copious amount of MEP, we were interested in the interactions among antibody to MEP, PBL, and complement in killing these strains. We used an in vitro opsonophagocytic assay to measure the killing activity of rabbit antibody raised to purified MEP. A critical factor in this assay is the use of a low concentration (0.3%) of FNHS as a complement source. This concentration of FNHS is below the bactericidal level for serum-sensitive strains. This report describes the role of rabbit antibody to MEP in opsonophagocytic killing of mucoid *P. aeruginosa* in conjunction with PBL and complement.

MATERIALS AND METHODS

Bacterial strains. Mucoid strains of *P. aeruginosa* used in these studies were clinical isolates obtained from the Microbiology Laboratory, Children's Hospital Medical Center, Boston, Mass., through the courtesy of Don Goldmann and Ann MaCone. Three of these strains, *P. aeruginosa* 1, 258, and 2192, previously were described in the context of isolation and characterization of purified MEP (13). All strains were nontypeable and serum sensitive (>90% killed in the presence of ≤10% FNHS).

Antigens. Purified MEP from strains 1, 258, and 2192 were prepared as previously described (13), as was carbodiimide-reduced MEP. Analysis of the MEP used in these studies by quantitative gas-liquid chromatography indicated that >99.5% of the material could be accounted for as the known uronic acid components, mannuronic and guluronic acid.

Antisera. Rabbit antisera were raised to the purified MEP antigens as previously described (13). Pre-immune sera, designated normal rabbit sera, were obtained in all cases. Levels of antibody (micrograms per milliliter) to *P. aeruginosa* LPS antigens in these sera have been reported previously (12). To remove antibody to antigens other than MEP, sera were adsorbed with Formalin-killed and lyophilized nonmucoid revertant *P. aeruginosa* cells, as previously described (13). The number of nonmucoid revertant cells used was 10 times the amount of lyophilized mucoid *P. aeruginosa* cells needed to remove all passive hemagglutinating antibody to MEP.

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Affinity-purified antibody to MEP. Purified MEP was coupled to epoxy-activated Sepharose 4B (Sigma Chemical Co., St. Louis, Mo.) according to the instructions of the manufacturer. The addition of a small amount of radiolabeled MEP to the reaction mixture allowed us to calculate that 0.3 mg of MEP was bound for each ml of gel. The gel was poured into a 10-cm^3 syringe barrel for the use in the column mode. Rabbit antisera (0.5 ml) to MEP were applied to the gel, and unbound material was eluted with phosphate-buffered saline (0.1 M phosphate, 0.15 M NaCl [pH 7.2]). The material binding to the gel was eluted with 0.1 M glycine hydrochloride (pH 2.5). The eluted material was immediately neutralized with 0.5 M Tris (pH 8.5), dialyzed against phosphate-buffered saline for 24 h, and then stored in the presence of 1% bovine serum albumin (BSA) at −20°C. Serological activity against MEP was confirmed in the passive hemagglutination assay. The material that had bound to the affinity gel gave a precipitin line in immunodiffusion against goat antiserum to rabbit IgG that was identical to that of purified rabbit IgG.

Serology. Determinations of antibody levels to MEP in sera were done by our previously described passive hemagglutination assay (13).

Opsonophagocytosis assay. The opsonophagocytosis assay was a modification of previously described opsonic assays (10, 18). The growth from overnight (37°C) plates of *P. aeruginosa* strains was used to inoculate 10 ml of tryptic soy broth in screw cap tubes (16 by 125 mm). The concentration of bacteria was adjusted to give an optical density at 650 nm of 0.1 in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). The bacteria were then grown at 37°C for 2 to 3 h by tumbling the tubes end over end on a rotator until an optical density at 650 nm of 0.4 was obtained. The cells were pelleted by centrifugation, suspended in 10 ml of minimal essential medium containing 1% BSA (MEM-BSA), and diluted 1:100 in MEM-BSA for use. This gave an approximate concentration of 2×10^7 bacteria per ml. Human PBL were obtained from laboratory donors who gave informed consent, by the dextran sedimentation method previously described (10). PBL were used at a final concentration of 2×10^7 per ml. The complement source was FNHS obtained from laboratory volunteers, prepared, and stored as previously described to maintain complement activity (11). For the opsonic assay, 26 μl of thawed FNHS was added to 1.974 ml of MEM-BSA. Complement activity was eliminated by heating antisera at 56°C for 30 min.

To test for opsonophagocytic killing, 100 μl of each of the above components (bacteria, PBL, complement, and antibody diluted in MEM-BSA) were mixed in sterile microfuge tubes. Shortly thereafter (time 0) a 25-μl portion was removed, added to 225 μl of deionized water to lyse the PBL, then further diluted in 10-fold steps in saline, and plated for bacterial enumeration as described elsewhere (10). Control tubes were run with each assay by omitting antibody, complement, or PBL and substituting 100 μl of MEM-BSA. The tubes then were placed at 37°C and tumbled end over end for 2 h. At this time, a 25-μl portion was removed, diluted in deionized water and saline as described above, and plated for bacterial counts. The plates were incubated overnight at 30°C, and CFU were counted the next day. The percent kill was calculated as: percent kill = {[1 − (CFU surviving at 2 h)]/(CFU added at time 0)} × 100.

For some of the experiments, we opsonized the bacteria (2×10^6 in 100 μl of MEM-BSA) with an equal volume of the antibody source at 4°C for 60 min and then washed the bacteria twice in MEM-BSA before the addition of PBL and complement dilutions (18). The assay then proceeded as described above.

TABLE 1. Determination of the opsonic activity in immune rabbit antiserum raised to *P. aeruginosa* 2191 MEP

Addition to 2×10^6 bacteria	% Kill[a] at 120 min
1:2,000 heated serum +:	
0.3% complement (C')	−10
2×10^6 PBL	−5
2×10^6 PBL + 0.3% C'	92
2×10^6 PBL + 0.3% heated C'	3
2×10^6 PBL and 0.3% C' (no serum)	−12
1:2 heated pre-immune serum + 2×10^6 PBL + 0.3% C'	0

[a] Negative numbers indicate growth during the experiment.

Statistical analysis. A two-sample *t* test was used to compare the distribution of the percent kill values in different experimental groups. A confidence interval of 95% was chosen, and an attained significance level of 0.05 (*P* value) was used as a cutoff value for significance. Calculations were done on a Digital VAX computer (Digital Equipment Co., Maynard, Mass.) by using the Minitab software system (15).

RESULTS

Antibody levels to MEP in sera used. The passive hemagglutination titers against MEP of the sera used in this study have been reported previously (Table 3 of reference 12). Reciprocal high-titer (1:128) hemagglutination activity was found in antisera raised to MEP from strains 1 and 2192, whereas the antibody titer in these sera to strain 258 MEP was lower (1:8). Antisera to strain 258 MEP had a high hemagglutination titer to the homologous antigen (1:128) but lower titers to MEP from strains 1 and 2192 (1:8 and 1:32, respectively).

Opsonophagocytic killing of strain 2192. Utilizing rabbit antisera raised to strain 2192 MEP, we determined the conditions that resulted in phagocytic killing after 2 h. The experiment was repeated three times and typical results are shown in Table 1. At antiserum dilutions as high as 1:2,000 we could kill >90% of the input inoculum in the presence of PBL and complement (final concentration, 0.3%). There was no phagocytic killing when the antibody, PBL, or complement was omitted. Heating the complement source (56°C, 30 min) also eliminated killing activity. Preimmune serum did not mediate any killing in conjunction with PBL and complement. Thus, phagocytic killing of this normally serum-sensitive strain (11) could proceed in the presence of antibody, PBL, and a complement concentration below the bactericidal level.

Specificity of the antibody for MEP. Pier and Elcock (12) have shown that rabbit antisera raised to MEP contain high-titer binding and opsonic antibodies directed against *P. aeruginosa* serotype antigens. Although mucoid *P. aeruginosa* 2192 expresses little to no serotype antigen (11), we needed to show that phagocytic killing was due to antibody specific to MEP. One way to do this is to show that there is little effect on the opsonic titer after adsorption of serum with nonmucoid revertant cells, which presumably would remove antibody to all antigenic determinants except MEP. The opsonic killing titer (serum dilution at which >70% of the input inoculum is killed) was determined in

rabbit immune serum after adsorption with strain 2192 *P. aeruginosa* mucoid and nonmucoid revertant cells. To eliminate any anticomplementary factors in the adsorbed sera, we opsonized the bacteria with the antibody source and then washed the cells before adding them to the PBL and complement as described by Verhoef et al. (18). Results from this experiment showed that adsorption of the antiserum with the mucoid cells reduced the opsonic titer to the preimmune level, whereas adsorption with the nonmucoid revertant cells did not change the opsonic titer from that seen in Table 1. Since the sera were adsorbed with 10 times the amount of nonmucoid cells as mucoid cells without any effect on the opsonic titer, it appeared that the antibody to MEP was mediating the phagocytic killing.

Heterologous and homologous opsonic titers in antisera to MEP. We next determined the heterologous and homologous opsonic titers in antisera raised to MEP isolated from three different strains of mucoid *P. aeruginosa* (Table 2). Reciprocal high-titer opsonic activities were found in antisera raised to strain 1 and 2192 MEP, whereas the opsonic titers of these two sera against strain 258 were lower. Antisera raised to strain 258 MEP had a high level of opsonic activity directed against homologous bacteria, and moderate titers directed against strains 1 and 2192. All of these titers were at least eightfold higher than those determined for preimmune sera. Thus, immunization of rabbits with three different MEP preparations resulted in moderate to high levels of opsonic antibody directed at both homologous and heterologous mucoid *P. aeruginosa*.

To extend these findings, we performed experiments that more thoroughly investigated antibody specificity for MEP and phagocytic killing of heterologous strains. In these experiments we tested whether rabbit antiserum raised to strain 2192 MEP could kill heterologous strains of mucoid *P. aeruginosa* and whether the killing activity was inhibited by incubation of the serum with purified MEP before opsonization of bacteria. A 1:50 dilution of serum was incubated at 4°C for 60 min with an equal volume of 2192 MEP (0.5 mg/ml) in MEM-BSA, with MEM-BSA only, or with MEM-BSA containing a 0.5 mg/ml amount of a heterologous polysaccharide, the high-molecular-weight polysaccharide isolated from the Fisher immunotype 1 strain of *P. aeruginosa* (10). The inhibited sera were then added to PBL, complement, and a variety of mucoid *P. aeruginosa* strains. The results (Table 3) showed that incubation of antiserum to strain 2192 MEP with the purified antigen before the addition of the bacteria significantly ($P < 0.001$) reduced phagocytic killing. Uninhibited serum, or serum inhibited with a heterologous antigen, had high levels of phagocytic killing. To ensure that the inhibited sera did not contain anticomplementary activity, a control was run in which inhibited sera were used to opsonize bacteria at 4°C for 30 min and the bacteria were washed and then added to the PBL and complement. Again, high levels of opsonic killing were seen in uninhibited serum and serum inhibited with a heterologous polysaccharide, whereas MEP-inhibited serum lost its opsonic activity.

Specificity was tested in another way by using affinity-purified antibody to MEP to opsonize mucoid *P. aeruginosa* strains for phagocytic killing. In addition, we tested whether opsonization by this antibody could be inhibited by purified MEP and carbodiimide-reduced MEP. The latter material lacks serological activity because the carbodiimide treatment specifically reduces the uronic acid constituents of MEP to hexoses. In these experiments the affinity antibody mediated the killing of the heterologous strains (Table 4).

TABLE 2. Opsonic titers of rabbit antisera raised to purified MEP from various *P. aeruginosa* strains

Target mucoid strain	Titer[a] of antisera raised to purified MEP isolated from:			
	2192	1	258	NRS[b]
2192	1,024	1,024	64	2
1	1,024	2,048	512	2
258	16	32	1,024	2

[a] Represents reciprocal of serum dilution at which >70% killing of the target mucoid strain was observed.
[b] NRS, Preimmune normal rabbit serum.

TABLE 3. Specificity of the opsonophagocytic killing of mucoid strains of *P. aeruginosa* by antibody raised to purified MEP isolated from strain 2192

Mucoid strain	% Kill[a] in the presence of MEP serum +:		
	Buffer[b]	2192 MEP[c]	IT-1 PS[c,d]
2192	82	11	79
1	97	45	97
221	61	−9	59
258	70	0	55
264	75	16	83
832	82	26	92
2314	92	−3	89
Mean[e]	80 ± 12	10 ± 18[f]	79 ± 16

[a] Negative numbers indicate growth during the experiment.
[b] A 1:50 dilution of antiserum was mixed with an equal volume of the indicated inhibitor at 4°C for 60 min before the addition to bacteria, PBL, and complement.
[c] Inhibitors at 0.5 mg/ml in MEM-BSA.
[d] High-molecular-weight polysaccharide from the Fisher immunotype 1 strain of *P. aeruginosa*.
[e] Average ± standard deviation of the percent kill.
[f] $P < 0.001$ compared with uninhibited serum by a two-sample *t* test.

TABLE 4. Opsonophagocytic killing of *P. aeruginosa* strains by affinity-purified antibody specific to MEP isolated from strain 2192

Mucoid strain	% Kill[a] in the presence of affinity antibody +:		
	Buffer[b]	2192 MEP[c]	Carbodiimide-treated 2192 MEP[c,d]
2192	90	12	84
1	87	0	97
221	73	2	81
258	59	0	62
264	88	18	74
832	74	−3	77
2314	99	7	88
Mean[e]	83 ± 15	5 ± 8[f]	80 ± 11

[a] Negative numbers indicate growth during the experiment.
[b] The affinity antibody was mixed with an equal volume of the indicated inhibitor at 4°C for 60 min before the addition to bacteria, PBL, and complement.
[c] Inhibitors were used at a concentration of 0.5 mg/ml.
[d] Carbodiimide-treated 2192 MEP has been chemically modified so that the carboxyl groups of the uronic acid constituents are reduced. This material is serologically inactive (4).
[e] Average ± standard deviation of the percent kill.
[f] $P < 0.001$ compared with uninhibited serum by a two-sample *t* test.

This killing was significantly ($P < 0.001$) inhibited by purified MEP but was not significantly affected by carbodiimide-reduced MEP.

DISCUSSION

Studying bacterial pathogenesis is greatly facilitated by an in vitro assay that measures the functional activity of an immunological effector mechanism. We adapted the opsonophagocytic assay to study the biological activity of antibody raised to the MEP antigen of *P. aeruginosa*. In the presence of a low level of human complement and PBL, rabbit antisera to purified MEP had high-titer opsonic killing activity. Opsonic titers remained moderate to high when antisera raised to three different MEP antigens were tested against homologous and heterologous bacterial strains. When either a 1:100 dilution of antiserum raised to one of these MEP antigens (strain 2192) or an affinity-purified antibody to MEP was tested against a variety of mucoid *P. aeruginosa* strains, high levels of killing also were observed. Specificity of the opsonic antibody for MEP was shown by both adsorption and inhibition experiments, and care was taken to exclude interference with complement activity as a reason for our results.

The low level of complement used in this assay was found to be effective and necessary for opsonic killing of mucoid *P. aeruginosa*. We chose to use this low amount of complement for both practical and theoretical reasons. In practice, most mucoid strains of *P. aeruginosa* are readily killed by concentrations of FNHS as low as 1%. Strain 2192 is usually killed at a concentration of 1.25 to 2.5% FNHS. Thus, the final complement concentration in our phagocytic assay had to be lower than the bactericidal concentration. In theory, complement levels in the lungs of CF patients are probably below the bactericidal levels for mucoid *P. aeruginosa* strains, or CF patients would not be colonized with serum-sensitive strains. Interestingly, the complement requirement for phagocytic killing by antibody to MEP is different than that for antibody raised to the *P. aeruginosa* serotype antigen located on the LPS polysaccharide side chain (13, 14). In those studies, complement potentiated the IgG-mediated killing and was not an absolute requirement. Rabbit antibodies to MEP, which are also principally IgG (unpublished data), are unable to mediate opsonic killing in the absence of added complement. If human antibody to MEP also has a complement requirement for phagocytic killing, then the low availability of active complement in the lungs of CF patients could account, in part, for the inability of these patients to handle *P. aeruginosa* colonization. In contrast, the fact that a complement concentration of 1/10 that required for bactericidal killing is effective in promoting antibody-mediated phagocytic killing suggests that opsonic antibodies may be better able to protect CF patients. If this is true, then one would predict that the antibody to MEP in the lungs of colonized CF patients is of a non-complement-fixing isotype or that the antibodies are degraded to Fab and $(Fab)_2$ fragments as has been suggested by Fick et al. (5, 6). We currently are using the opsonophagocytic assay described here to test this hypothesis.

In performing the phagocytic killing assays we paid particular attention to the potential role of anticomplementary factors in affecting the outcome of our experiments. Mucoid exopolysaccharide has been reported to activate complement (D. P. Speert, Y. Kim, and R. Grandy, Program Abstr. 21st Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 349, 1981), but we have been unable to confirm these results with our highly purified preparations. Because of the low levels of complement used in our assays, a small amount of anticomplementary activity could interfere with these assays. Whenever there was a possibility of anticomplementary products being introduced into the reaction mixtures, we washed them away before the addition of complement. This practice assured that phagocytic killing in our adsorption and inhibition assays required MEP-specific antibody.

A major concern throughout this study was the specificity of the opsonic antibody for MEP. Pier and Elcock (12) have shown that immunization of rabbits with MEP nonspecifically elicits opsonic antibody to a variety of *P. aeruginosa* LPS antigens. To ensure that the opsonic activity described here was specific for MEP, first, we only used nontypable, serum-sensitive strains which express little or no LPS O side chains (7). Second, we adsorbed the antisera with nonmucoid revertant cells and could demonstrate no loss of opsonic titer. Presumably, the nonmucoid revertant cells express all of the antigens found on mucoid cells except MEP. This may be only a quantitative difference, however, since it has been shown that immunization of rabbits with the nonmucoid revertant of strain 2192 elicits antibody to MEP (13). Third, we used highly purified MEP (>99.5% of the MEP is identified as uronic acid) as both an immunogen and inhibitor of opsonization. Thus, any contaminant would have to be highly immunogenic at low doses and be able to inhibit opsonization at nanogram concentrations. Fourth, we used affinity-purified antibody to confirm the specificity of the opsonic activity for MEP. Again, any contaminant would need to bind to the affinity column to a sufficient degree to allow for affinity purification of an antibody directed against the contaminant. Furthermore, epoxy-activated Sepharose specifically binds hydroxyl groups such as those found in MEP. Finally, our studies with purified MEP to inhibit opsonization confirm the specificity of the antibody. When the inhibiting MEP was treated with carbodiimide, a procedure which specifically reduces the uronic acid constituents of MEP to hexoses, there was no interference with opsonization. This can be correlated with our previous demonstration of loss of serological activity of MEP after carbodiimide treatment (13).

The ability of antibody to MEP to function as an opsonin for phagocytic killing has not been previously demonstrated. Mucoid exopolysaccharide has been credited with a variety of roles in the pathogenesis of disease associated with mucoid *P. aeruginosa* colonization of CF patients. One of these roles is the ability of MEP to be antiphagocytic (16), although these data are questionable because the experiments did not use purified MEP. Nonetheless, overcoming any possible pathogenic role of MEP by antibody could be an important protective mechanism. We currently are investigating the functional activity of antibody to MEP found in high titer in CF patients.

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