INFECTION AND IMMUNITY, Feb. 1993, p. 559–564
0019-9567/93/020559-06$02.00/0

Vol. 61, No. 2

Suppression of Lymphocyte and Neutrophil Functions by *Pseudomonas aeruginosa* Mucoid Exopolysaccharide (Alginate): Reversal by Physicochemical, Alginase, and Specific Monoclonal Antibody Treatments

G. T. MAI,[1] W. K. SEOW,[1] G. B. PIER,[2] J. G. McCORMACK,[1] AND Y. H. THONG[1]*

Immunobiology Laboratory, Departments of Child Health, Dentistry, and Medicine, University of Queensland, Mater Public Hospital, South Brisbane, Australia 4101,[1] and Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115[2]

Received 17 August 1992/Accepted 10 November 1992

The mucoid exopolysaccharide (MEP or alginate) of *Pseudomonas aeruginosa* is thought to be a virulence factor for this organism by virtue of its ability to suppress local host defense mechanisms. We purified MEP from clinical isolates of mucoid *P. aeruginosa*, subjected it to degradation by ultrasonication, heat, alkali, and alginase, and reacted it with monoclonal antibodies specific for MEP epitopes. Partial reversal or complete abrogation of the inhibitory effects of alginate on human neutrophil random migration, chemotaxis, and hexose monophosphate shunt activity and lymphocyte transformation were observed following most of these treatments. Physicochemical analysis of degraded MEP revealed a positive correlation between changes in molecular size and viscosity and loss of biological properties. The biological properties of MEP were also shown to be dependent on the structural integrity of the *O*-acetyl groups substituted for the mannuronic acid residues. The results show that the capacity of MEP to suppress neutrophil and lymphocyte functions is dependent on its acetyl content and the physical properties of large size and viscosity and may provide part of the explanation for the propensity of mucoid *P. aeruginosa* to persist in the airways of patients with cystic fibrosis. These findings highlight the important role of MEP as one of the virulence factors in the pathogenesis of inflammatory damage and subsequent pulmonary destruction in cystic fibrosis.

Pseudomonas aeruginosa, a ubiquitous gram-negative aerobic bacillus found in soil and water habitats, is an important cause of morbidity and mortality in patients with cancer or burns and in immunocompromised patients (4, 9, 20, 26). While nonmucoid strains of *P. aeruginosa* are generally responsible for these opportunistic infections, mucoid strains are usually isolated from patients with cystic fibrosis (CF), in whom their emergence in the respiratory tract heralds the onset of inexorable pulmonary deterioration (12, 14, 17, 18, 28, 34). The reasons for the ability of mucoid *P. aeruginosa* to persist in CF patients is unclear, but at least two explanations, which are not mutually exclusive, have been forwarded. One explanation concerns the propensity of mucoid *P. aeruginosa* to adhere to the respiratory tract epithelium and mucin of CF patients (6, 24, 31, 32).

Another explanation is centered on the antiphagocytic and immunosuppressive properties of the mucoid exopolysaccharide (MEP), also known as alginate, which is involved in the evasion of host defense in the respiratory tract. Although this contention is supported by a number of studies (2, 15, 19, 23, 25, 27, 33, 36, 38, 39), it is possible that contaminants in some MEP preparations are responsible for the laboratory observations. Moreover, only a minority of the strains studied by Baltimore and Mitchell (2) showed decreased phagocytosis of the mucoid compared with the nonmucoid revertant bacteria. Furthermore, while some studies have implicated a relationship between the chemical composition, molecular size, and viscosity of MEP and its biological activity (38), these studies have not examined this effect directly on neutrophils and lymphocytes. In this study, we sought to answer this question by subjecting MEP to degradation by physicochemical methods and an alginase enzyme and by reacting it with specific monoclonal antibodies (MAbs).

MATERIALS AND METHODS

Purification of MEP. The three clinical isolates of mucoid *P. aeruginosa* used, IRL-1, IRL-2, and IRL-3, were originally obtained from Martyn Tilse and Theo Mollee of the Department of Microbiology, Mater Public Hospital, South Brisbane, Australia (22, 23). The isolates were stored in aliquots in Protect Preservers (Sigma, St. Louis, Mo.) at −70°C by the method of Feltham et al. (10). For MEP production, a stock culture was first plated overnight on blood agar and then streaked heavily on MacConkey agar plates modified by the addition of 3% (vol/vol) glycerol to enhance MEP secretion (8). After 4 days of culturing at 25°C, surface growth was removed and placed in 25 volumes of sterile 0.9% saline, and the suspension was stirred until a uniform appearance was observed. The suspension was centrifuged at 20,000 × *g* for 60 min, and the supernatant was recentrifuged at the same speed for another 2 h. After removal, the supernatant was treated with the slow addition of 3 volumes of 95% ethanol. The precipitate was recovered by centrifugation at 3,000 × *g* for 30 min and subjected to treatment with 95% ethanol twice and pure ethanol once. Further purification of MEP (7) was accomplished by dissolution in 0.1 M Tris buffer (pH 7.4) at a concentration of 5 mg/ml and the addition of 1 mg of pronase (Sigma) per ml. After incubation at 37°C for 48 h, the mixture was dialyzed for 17 h against two changes of 40 volumes of distilled water and centrifuged at 20,000 × *g* for 2 h to remove formal

* Corresponding author.

precipitates. The supernatant was removed, and sodium acetate was added to a final concentration of 0.5%, after which the supernatant was treated with 3 volumes of 95% ethanol to precipitate alginate. After further treatments with 80%, 95%, and pure ethanol and ether, the supernatant was dried in ether for the experiments. MEP so prepared was checked for protein and nucleic acid contamination by measuring the A_{280} and A_{260}, respectively, with a spectrophotometer (7), and the contamination was found to be <0.5% for both. Lipopolysaccharide contamination was found to be <0.01% by the *Limulus* amebocyte assay. This highly purified MEP was used at a final concentration of either 3 μg/ml for neutrophil locomotion and lymphocyte transformation experiments or 30 μg/ml for neutrophil hexose monophosphate (HMP) shunt experiments (23).

Physical degradation. Physical degradation of the purified MEP was accomplished by ultrasonication in an ice bath for 30 min with a Branson sonicator at setting no. 6 or by heating of MEP in a boiling water bath (100°C) for 30 min. Degradation of MEP was verified by the detection of changes in viscosity and size. The viscosity of the MEP solution (120 μg/ml) was measured in a viscosity meter (Contraves, Zurich, Switzerland) by use of low-shear setting 30. The molecular size was determined by introducing samples in 0.2 M ammonium carbonate buffer (pH 8.6) into a Sepharose CL-4B column (1.6 by 100 cm). The column flow rate was 30 ml/h. The void volume was measured by elution of blue dextran 2000, and the bed volume was measured by elution of acetone. The proportional elution volume was calculated by standard practice (*Gel Filtration Theory and Practice*; Pharmacia Fine Chemicals).

Chemical degradation. Chemical degradation of the purified MEP was accomplished with 1 and 2 N NaOH (60 min, 25°C), after which the pH was readjusted to 7.4 with 1 N HCl. Degradation was verified by acetate determination via gas chromatography. Acetate content was measured with samples hydrolyzed in 0.1 M NH_4OH at 95°C for 5 h. The samples contained isovaleric acid as an internal standard at a sample/internal standard ratio of 10:1. After hydrolysis, 2 to 5 μl of the samples was applied to a Nukol fused-silica capillary column (15 m by 0.53 mm [inner diameter]; Supelco Inc., Bellefonte, Pa.) in a Hewlett-Packard 5880 gas chromatograph. The carrier gas was hydrogen at a flow rate of 20 ml/min, and detection was done by flame ionization. The response to acetic and isovaleric acids was determined by injection of known quantities of standards. This response was used to calculate the milligrams of acetate per milligram of alginic acid sample.

Preparation of alginase. *Bacillus circulans* was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 15518), and grown in alginate-yeast extract medium at 26°C with vigorous aeration (7, 16). After 3 to 4 days, the medium was centrifuged at 8,000 × *g* and 4°C for 20 min, and the supernatant was removed and concentrated 10-fold with Centriprep-10 (Amicon). Alginase was precipitated from the concentrate by the addition of ammonium sulfate (70% saturation), and the precipitate was collected by centrifugation at 20,000 × *g* for 30 min at 4°C. It was dissolved in 10 mM Tris hydrochloride (pH 7.4) and dialyzed overnight against Tris hydrochloride.

This crude preparation of alginase was further purified by chromatography in a DEAE-Sephacel column (2.5 by 20 cm; Sigma). The alginase was eluted with Tris buffer containing an NaCl gradient (0.05 to 0.5 M), and the fractions were collected, dialyzed against distilled water overnight, and lyophilized by the method of Eftekhar and Speert (7).

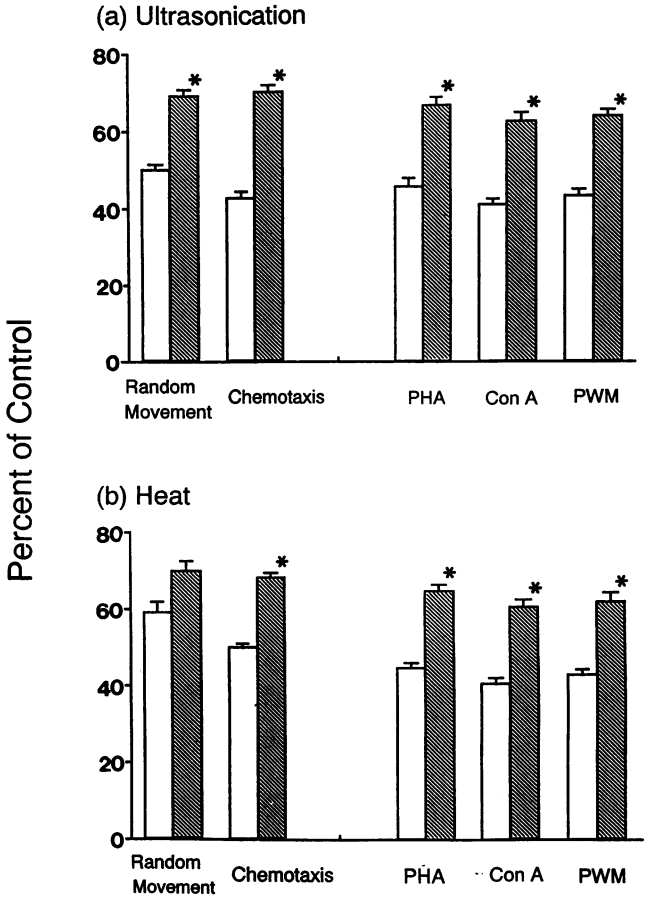

FIG. 1. Reversal by treatment with ultrasonication (a) and heat (b) of the inhibitory effects of MEP on neutrophil locomotion and lymphocyte transformation by phytohemagglutinin (PHA), concanavalin A (Con A), and pokeweed mitogen (PWM). Symbols: □, native alginate; ▨, treated alginate. Results represent the mean ± standard error of the mean for three experiments, each performed in triplicate. Similar results were obtained with alginate derived from two other clinical isolates. *, $P < 0.05$.

The specific activity of the alginase was quantified by the periodate-thiobarbiturate assay (43). In brief, 0.5 μl of enzyme was added to 0.5 ml of sodium alginate (2.5 mg/ml) at room temperature. Samples of 50 μl were removed at 10-min intervals for up to 60 min and added to 0.25 ml of 0.25 N HIO_4 in 0.125 N H_2SO_4. After 20 min at room temperature with shaking, 0.5 ml of 2% sodium arsenite in 0.5 N HCl was added, and the mixture was allowed to stand for 2 min; then, 2 ml of 0.3% thiobarbituric acid (pH 2.0) was added, and the mixture was stirred and heated to 100°C for 10 min. On cooling, the optical density was measured at 549 nm in an LKB spectrophotometer. One unit of alginase activity was defined as the amount of enzyme required to liberate 1 nmol of β-formylpyruvate per min per ml (0.01 μmol produces an increase in the optical density of 0.29). The protein concentration was determined by the method of Lowry et al. (21), so that alginase activity could be expressed as units per milligram of protein. The enzyme concentration was adjusted to 500 U/ml for the experiments.

Preparation of MAbs. MAbs were prepared against *P. aeruginosa* MEP in mice by standard methods. In brief, animals were immunized with whole bacteria (10^8 CFU per

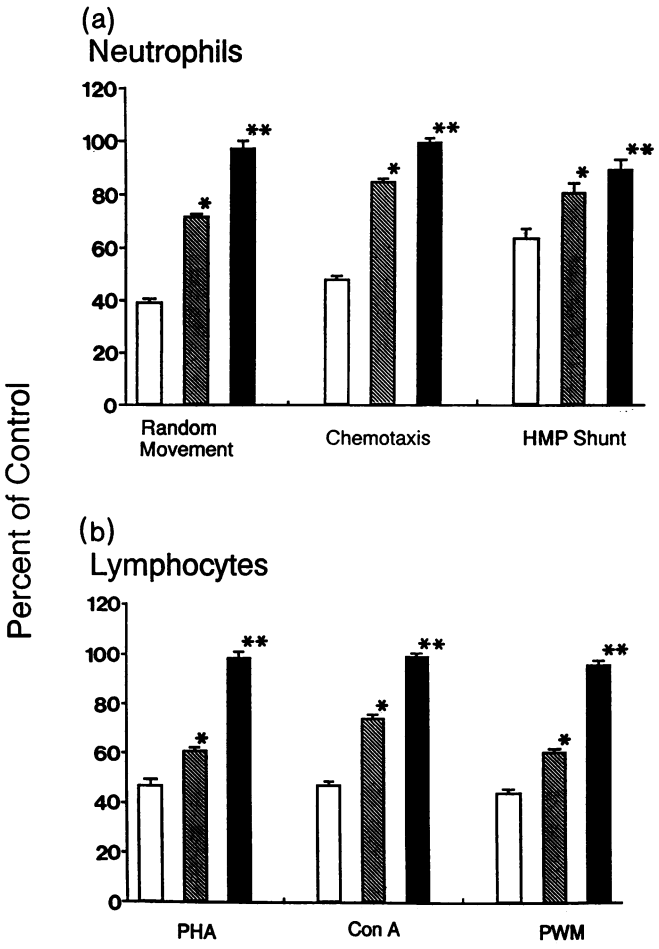

FIG. 2. Reversal of the inhibitory effects of MEP on neutrophil function (a) and lymphocyte transformation (b) by degradation with sodium hydroxide. Symbols: □, native alginate; ▨, alginate degraded with 1 N NaOH; ■, alginate degraded with 2 N NaOH. Results represent the mean ± standard error of the mean for three experiments, each performed in triplicate. Similar results were obtained with alginate derived from two other clinical isolates. *, $P < 0.05$; **, $P < 0.001$. Abbreviations are as defined in the legend to Fig. 1.

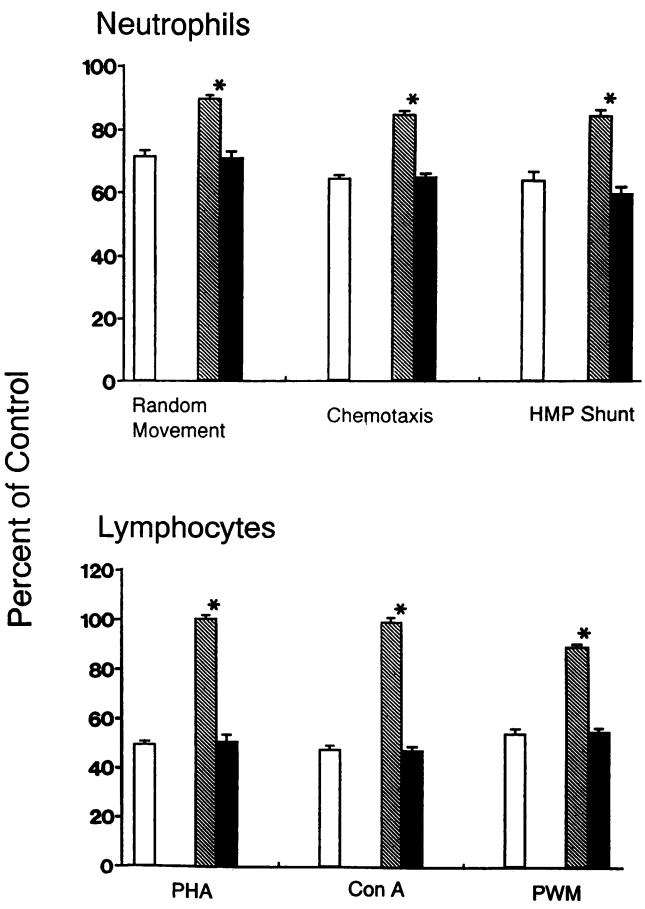

FIG. 3. Reversal of the inhibitory effects of MEP (Fig. 2) by degradation with alginase. Symbols: □, native alginate; ▨, alginate treated with native alginase; ■, alginate treated with heat-inactivated alginase. Results represent the mean ± standard error of the mean for three experiments, each performed in triplicate. Similar results were obtained with alginate derived from two other clinical isolates. *, $P < 0.001$. Abbreviations are as defined in the legend to Fig. 1.

animal) or purified MEP (50 μg per mouse) at multiple times. Spleen cells were recovered 3 days after the final immunization and fused with myeloma cell lines (SP/2 for 8/5/31.3 and P3X for M/K/16.2) by use of polyethylene glycol. Supernatants were screened for antibodies in an enzyme-linked immunosorbent assay (ELISA) (5), and positive wells were cloned twice by limiting dilution. Wells were re-screened in the ELISA, and the hybridoma cells were transformed to progressively larger cell culture volumes to produce sufficient amounts for antibody testing in an opsonophagocytic assay (1). The antibodies were then classified as opsonic or nonopsonic on the basis of this in vitro activity. They were stored as culture supernatants at −70°C until use. The two selected for use in these experiments were designated 8/5/31.3 and M/K/16.2. The former mediates opsonic killing of mucoid *P. aeruginosa* in the presence of human neutrophils and complement, while the latter does not mediate opsonic killing and binds to a different epitope on the MEP antigen. Both MAbs are of the immunoglobulin G2b class. A dilution of 1:10 was used for these MAb culture supernatants in these experiments. These MAbs have been well characterized in previous studies (29, 30, 35).

Isolation of lymphocytes and neutrophils. About 30 to 50 ml of blood was obtained from healthy donors and layered onto Mono-Poly Resolving Medium (Flow Laboratories, McLean, Va.) (11). Centrifugation at 600 × *g* for 30 min resulted in the formation of two leukocyte bands at the interface. Mononuclear cells from the top band were washed twice and resuspended in RPMI 1640 medium for the experiments. The second band, containing neutrophils of >97% purity (11), was removed, washed twice, and resuspended in medium 199 or Dulbecco's phosphate-buffered saline for the experiments.

Neutrophil locomotion. The movement of neutrophils under agarose was monitored as previously described (37). In Brief, 3 ml of double-strength medium 199 containing 20% heat-inactivated fetal calf serum was mixed with 3 ml of 2% agarose solution, and the mixture was poured into tissue culture plates (60 by 15 mm). Wells 2 mm in diameter and 2.5 mm apart were punched in sets of three. The middle well received 5 μl of neutrophil suspension (with or without

MEP) at a cell concentration of 4×10^7/ml, the outer well received a chemoattractant, and the inner well received medium 199 only. The chemoattractant was made by incubating 10^7 *Candida albicans* cells with 2 ml of fresh human serum for 30 min at 37°C, centrifuging the mixture at 1,000 × *g* for 10 min, and removing the activated serum for use. The agarose plates were kept in a 5% CO_2–air atmosphere with high humidity for 2 h, and the distances moved towards the inner well (random movement) and the outer well (chemotaxis) were measured with the aid of an eyepiece grid in an inverted microscope. All experiments were performed in triplicate.

HMP shunt activity. Stimulated neutrophils metabolize glucose via the HMP shunt to generate oxygen-derived free radicals. HMP shunt activity was quantified as the conversion of ^{14}C-1-glucose to $^{14}CO_2$ (42). In brief, 2×10^6 neutrophils per ml in Dulbecco's phosphate-buffered saline was treated with 0.1 μg of PMA per ml in a total volume of 1 ml at 37°C for 45 min. Then, 1 N hydrochloric acid was added to drive out any dissolved $^{14}CO_2$, which was captured in 5 N sodium hydroxide and quantitated in a scintillation spectrophotometer. All experiments were performed in triplicate.

Mitogen-induced lymphocyte transformation. The lectins phytohemagglutinin A, pokeweed mitogen, and concanavalin A were purchased from Wellcome, Sydney, Australia; GIBCO, Grand Island, N.Y.; and Calbiochem-Behring, Sydney, Australia, respectively. They were used at optimal concentrations of 0.5 μg/ml, 1/100 dilution, and 12.5 μg/ml, respectively. To each microtiter well was delivered 0.1 ml of RPMI 1640 medium containing 2×10^6 lymphocytes per ml, 0.5 ml of lectin, and 0.5 ml of alginate or medium only as previously described (41). The microtiter trays were kept at 37°C in a 5% CO_2–air atmosphere and high humidity for 72 h. At 6 h prior to harvesting, 1 μCi of ^{3}H-thymidine was added to each culture. The cultures were aspirated onto glass fiber filter paper with a Skatron harvester, and ^{3}H-thymidine uptake was determined by liquid scintillation spectroscopy. All experiments were performed in triplicate.

Statistical analyses. The one-way analysis of variance, the Newman-Keuls test for multiple comparisons, and the Pearson correlation test were used for statistical analyses.

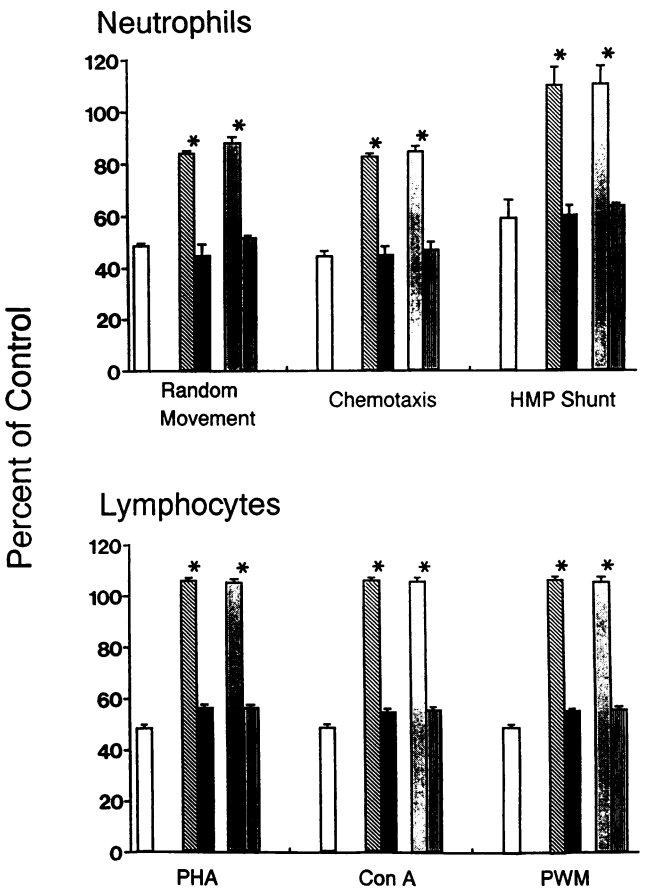

FIG. 4. Reversal by treatment with specific MAbs of the inhibitory effects of MEP (Fig. 2). Symbols: □, native alginate; ▨, alginate treated with native MAb 8/5/31.3; ■, alginate treated with heat-inactivated MAb 8/5/31.3; ▧, alginate treated with native MAb M/K/16.2; ▥, alginate treated with heat-inactivated MAb M/K/16.2. Results represent the mean ± standard error of the mean for three experiments, each performed in triplicate. Similar results were obtained with alginate derived from two other clinical isolates. *, $P < 0.001$. Abbreviations are as defined in the legend to Fig. 1.

RESULTS

Effect of physical degradation. We first subjected MEP to degradation by ultrasonication in an ice bath for 30 min with the probe tip of the Branson sonicator set at 6. As shown in Fig. 1a, ultrasonicated MEP had fewer inhibitory effects on neutrophil locomotion and lymphocyte transformation than did native MEP.

Similarly, heating of MEP at 100°C for 30 min resulted in the partial abrogation of its inhibitory effects on neutrophil locomotion and lymphocyte transformation, as shown in Fig. 1b.

Effect of chemical degradation. We next subjected MEP to chemical treatment with either 1 or 2 N NaOH for 60 min at room temperature, after which the pH was readjusted to 7.4 with 1 N HCl. The results (Fig. 2) showed a significant stepwise abrogation of the inhibitory activity of MEP by NaOH degradation. The acetate level following treatment with 1 N NaOH was not determined, but there was likely only a partial removal of acetate from this sample, inasmuch as it retained some inhibitory capacity with regard to neutrophil and lymphocyte functions (Fig. 2). Complete removal of acetate by 2 N NaOH, as documented by gas chromatographic analysis, resulted in complete abrogation of the inhibitory activity of MEP. Such treatment also reduced the viscosity of MEP from 3.5 to 0.9 cP. In addition, this treatment resulted in only a small change in the molecular size, as reflected in a broadening of the peak of MEP eluting from the sizing column and a resultant slight downward shift of the proportional elution volume. However, <25% of MEP shifted downward in molecular size.

Effect of alginase. The alginase enzyme derived from *B. circulans* ATCC 1558 was used to degrade MEP for the next set of experiments. After incubation of MEP with alginase (300 U/ml) for 30 min at 37°C, the mixture was added to either neutrophil or lymphocyte cultures. Controls contained MEP treated with heat-inactivated enzyme (60°C for 2 h). The results (Fig. 3) showed that specific degradation of MEP by the native but not the heat-inactivated enzyme significantly reduced the inhibitory activity of MEP.

Effect of MAbs. In the next set of experiments, we used two MAbs directed against epitopes on the MEP molecule; one was opsonic and the other was nonopsonic. Both MAbs were able to reverse the inhibitory effects of MEP (Fig. 4). In

TABLE 1. Correlation analysis of the molecular size, viscosity, and biological activity of native and degraded MEP[a]

MEP	Molecular size		Viscosity (cP)	Biological activity (% inhibition of:)	
	Proportional elution vol	Mol wt		Neutrophil chemotaxis	Lymphocyte transformation
Native	0.025	>2,000,000	3.5	48.3	54.2
Heated	0.175	687,000	1.6	38.4	38.3
Ultrasonicated	0.425	115,000	1.1	27.4	33.0
Alginase treated	0.500	67,000	0.8	13.0	3.1

[a] *r* values were as follows: size versus viscosity, 0.995; size versus chemotaxis, 0.875; size versus transformation, 0.806; viscosity versus chemotaxis, 0.882; and viscosity versus transformation, 0.833.

contrast, heat-inactivated (60°C for 2 h) MAbs had no effect on the inhibitory activity of MEP (Fig. 4).

Size, viscosity, and biological activity. Finally, we performed a correlation analysis of the biological activity, molecular size, and viscosity of MEP treated with heat, ultrasonication, and alginase. The results (Table 1) showed a significant correlation among all these variables; i.e., the larger the size, the higher the viscosity, and the greater the inhibitory effects of MEP on neutrophil chemotaxis and lymphocyte transformation.

DISCUSSION

P. aeruginosa MEP is an acetylated polymer of β-D-mannuronic and α-L-guluronic acids. The two uronic acids of MEP are arranged in different ways within the large alginate molecule to form block structures (8, 12, 34). The relative proportions of the two uronic acids and the presence of *O*-acetyl groups are major determinants of its physicochemical properties and its resistance to degradation. The ability of MEP to form a gel with water is postulated as a mechanism of protecting the bacterium from the periodic cycles of dehydration in its aquatic habitat (12, 14, 28, 34). Another biological role for MEP may be to enhance the capacity of the bacterium to adhere to substrates in its habitat (21a). Thus, in the evolutionary context, the superb adaptation of mucoid *P. aeruginosa* to the respiratory tracts of CF patients may be regarded as a form of biological serendipity.

The results of the present study show that purified *P. aeruginosa* MEP is partially degraded by physical factors such as ultrasonication and heat and that this degradation results in a decrease in its capacity to inhibit neutrophil and lymphocyte responses. It is more susceptible to degradation by alkali in the form of sodium hydroxide, which has been shown to cause deacetylation of the exopolysaccharide (34, 38, 40). Our data also show that the biological effects of MEP can be altered by degradation with the alginase enzyme derived from *B. circulans*. This enzyme acts on mannuronic residues and *O*-acetyl groups to alter the block structures of the exopolysaccharide (7, 16). In addition, our data show that MAbs raised against MEP epitopes (29, 30, 35) are able to block the inhibitory effects of MEP on neutrophil and lymphocyte responses. Furthermore, heat inactivation of the alginase enzyme and the two MAbs prevented the destruction of the inhibitory effects of alginate. Similar results were obtained with purified MEP derived from two other clinical isolates of *P. aeruginosa* (data not shown). These results suggest that the capacity of MEP to suppress neutrophil and lymphocyte functions is specific to the molecule itself and is not due to contaminants. The correlation between the biological properties of MEP and its viscosity and molecular size confirms and extends the work of Simpson et al. (38) and Garner et al. (13) and is consistent with a recent report by Bayer et al. (3).

Taken together, the data from this and other studies provide a plausible explanation for the propensity of mucoid *P. aeruginosa* to persist in the respiratory tracts of CF patients. A suggested mechanism for the pathogenesis of pulmonary tissue injury involves the change to a mucoid *P. aeruginosa* phenotype, which allows the bacteria to evade phagocytic killing and multiply in great profusion within the lumen of the airways. Since MEP is confined to the airway lumen, general immunological responses within the lung parenchyma and circulation remain intact, so that phagocytic cells continue to enter the lung, being drawn by bacteria and host-derived chemotactic molecules. The accumulation of large quantities of pseudomonal toxins, enzymes, and metabolites in the airway lumen and their subsequent diffusion result in damage to the adjacent lung parenchyma. The diffusion of phagocyte-derived toxic enzymes and free radicals into the lung parenchyma causes additional inflammatory damage. However, when the phagocytic cells finally reach the airways, they are unable to eliminate the MEP-coated bacteria, which continue to multiply in great profusion to maintain the vicious cycle. This sequence of events may account for the inexorable deterioration of pulmonary function in CF patients colonized by mucoid strains of *P. aeruginosa*.

In conclusion, these studies provide further insights into the pathogenesis of persistent colonization by mucoid strains of *P. aeruginosa* and subsequent progressive pulmonary destruction. They also highlight the important role of MEP in this multifactorial process and endorse the development of therapeutic and preventive measures aimed at this substance as a strategy worthy of consideration. In this regard, alginate lyase has been suggested by Russell and Gacesa (34) as a therapeutic agent, alginase has been successfully used in the treatment of experimental endocarditis in rabbits (3), and alginate as a vaccine is now undergoing clinical trials. Further studies along these lines will indicate whether this strategy is correct.

ACKNOWLEDGMENTS

This work was supported in part by the John P. Kelly Research Foundation (Mater Public Hospital), the Cystic Fibrosis Association (Australia), the Mayne Bequest Fund (University of Queensland), and the National Institutes of Health (grant AI 22806).

REFERENCES

1. **Ames, P., D. DesJardins, and G. B. Pier.** 1985. Opsonophagocytic killing activity of rabbit antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide. Infect. Immun. **49**:281–285.
2. **Baltimore, R. S., and M. Mitchell.** 1980. Immunologic investigations of mucoid strains of *Pseudomonas aeruginosa*: comparison of susceptibility to opsonic antibody in mucoid and non-

mucoid strains. J. Infect. Dis. **141**:238–247.
3. **Bayer, A. S., S. Parks, M. C. Ramos, C. C. Nasty, F. Eftekhar, and N. L. Schiller.** 1992. Effects of alginase on the natural history and antibiotic therapy of experimental endocarditis caused by mucoid *Pseudomonas aeruginosa*. Infect. Immun. **60**:3979–3985.
4. **Bergan, T.** 1981. Pathogenetic factors of *Pseudomonas aeruginosa*. Scand. J. Infect. Dis. **29**(Suppl.):7–12.
5. **Bryan, L. E., A. Kureishi, and H. R. Rabin.** 1982. Detection of antibodies to *Pseudomonas aeruginosa* alginate extracellular polysaccharide in animals and cystic fibrosis patients by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **18**:276–282.
6. **Doig, P., N. R. Smith, T. Todd, and R. T. Irvin.** 1987. Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. Infect. Immun. **55**:1517–1522.
7. **Eftekhar, F., and D. P. Speert.** 1988. Alginase treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocyte-derived macrophages. Infect. Immun. **56**: 2788–2793.
8. **Evans, L. R., and A. Linker.** 1973. Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol. **116**:915–924.
9. **Feigin, R. D., and W. T. Shearer.** 1975. Opportunistic infection in children. Parts I to III. J. Pediatr. **87**:507–514, 677–694, 852–866.
10. **Feltham, P. K., A. K. Power, P. A. Pell, and P. A. Sneath.** 1978. A simple method for storage of bacteria at −76 degrees C. J. Appl. Bacteriol. **44**:313–316.
11. **Ferrante, A., and Y. H. Thong.** 1980. Optimal conditions for the simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood. J. Immunol. Methods **36**:108–117.
12. **Gacesa, P., and N. J. Russell (ed.).** 1990. Pseudomonas infection and alginates: biochemistry, genetics and pathology. Chapman & Hall, Ltd., London.
13. **Garner, C. V., D. DesJardins, and G. B. Pier.** 1990. Immunogenic properties of *Pseudomonas aeruginosa* mucoid exopolysaccharide. Infect. Immun. **58**:1835–1842.
14. **Govan, J. R. W., and G. S. Harris.** 1986. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. Microbiol. Sci. **3**:302–308.
15. **Grasso, R. J., R. Ganguly, and J. F. Breen.** 1984. Inhibition of yeast phagocytosis in macrophage cultures treated with slime polysaccharide purified from *Pseudomonas aeruginosa*. J. Leukocyte Biol. **36**:771–774.
16. **Hansen, J. B., R. S. Doubet, and J. Ram.** 1984. Alginase enzyme production by *Bacillus circulans*. Appl. Environ. Microbiol. **47**:704–709.
17. **Hoiby, N., V. Andersen, and G. Bendixen.** 1975. *Pseudomonas aeruginosa* infections in cystic fibrosis. Acta Pathol. Microbiol. Scand. **83**:459–468.
18. **Kubesch, P., M. Linger, D. Grothües, M. Wehsling, and B. Tümmler.** 1988. Strategies of *Pseudomonas aeruginosa* to colonize and to persist in the cystic fibrosis lung. Scand. J. Gastroenterol. **23**(Suppl. 143):77–80.
19. **Learn, D. B., E. P. Brestel, and S. Settharama.** 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. Infect. Immun. **55**:1813–1818.
20. **Liu, P. V.** 1976. Biology of *Pseudomonas aeruginosa*. Hosp. Pract. **82**:139–147.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
21a. **Mai, G. T., et al.** Submitted for publication.
22. **Mai, G. T., W. K. Seow, J. G. McCormack, and Y. H. Thong.** 1989. Direct activation of human polymorphonuclear leukocytes by *Pseudomonas aeruginosa*. Int. Arch. Allergy Appl. Immunol. **90**:358–363.
23. **Mai, G. T., W. K. Seow, J. G. McCormack, and Y. H. Thong.** 1990. In-vitro immunosuppressive and antiphagocytic properties of the exopolysaccharide of mucoid strains of *Pseudomonas aeruginosa*. Int. Arch. Allergy Appl. Immunol. **92**:105–112.
24. **Marcus, H., and N. R. Baker.** 1985. Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. Infect. Immun. **47**:723–729.
25. **Meshulam, T., N. Obedeanu, D. Morzback, and J. D. Sobel.** 1984. Phagocytosis of mucoid and non-mucoid strains of *Pseudomonas aeruginosa*. Clin. Immunol. Immunopathol. **32**: 151–165.
26. **Neu, H. C.** 1983. The role of *Pseudomonas aeruginosa* in infections. J. Antimicrob. Chemother. **11**(Suppl. B):1–13.
27. **Oliver, A. M., and D. M. Weir.** 1983. Inhibition of bacterial binding to mouse macrophages by pseudomonas alginate. J. Clin. Lab. Immunol. **10**:221–224.
28. **Pier, G.** 1985. Pulmonary disease associated with *Pseudomonas aeruginosa* in cystic fibrosis: current status of the host-bacterium interaction. J. Infect. Dis. **151**:575–580.
29. **Pier, G. B., M. Grout, and D. DesJardins.** 1991. Complement deposition by antibodies to *Pseudomonas aeruginosa* mucoid exopolysaccharide (MEP) and by non-MEP specific opsonins. J. Immunol. **147**:1869–1876.
30. **Pier, G. B., G. J. Small, and H. B. Warren.** 1990. Protection against mucoid *Pseudomonas aeruginosa* in rodent models of endobronchial infection. Science **249**:537–540.
31. **Ramphal, R., C. Guay, and G. B. Pier.** 1987. *Pseudomonas aeruginosa* adhesins for tracheobronchial mucin. Infect. Immun. **55**:600–603.
32. **Ramphal, R., and G. Pier.** 1985. Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. Infect. Immun. **47**:1–4.
33. **Ruhen, R. W., P. G. Holt, and J. M. Papadimitriou.** 1980. Antiphagocytic effect of *Pseudomonas aeruginosa* exopolysaccharide. J. Clin. Pathol. **33**:1221–1222.
34. **Russell, N. J., and P. Gacesa.** 1988. Chemistry and biology of the alginate of mucoid strains of *Pseudomonas aeruginosa* in cystic fibrosis. Mol. Aspects Med. **10**:1–91.
35. **Schreiber, J. R., G. B. Pier, M. Grout, K. Nixon, and M. Patawaran.** 1991. Induction of opsonic antibodies to *Pseudomonas aeruginosa* mucoid exopolysaccharide by an anti-idiotype monoclonal antibody. J. Infect. Dis. **164**:507–514.
36. **Schwarzmann, S., and J. R. Boring III.** 1971. Antiphagocytic effect of slime for a mucoid strain of *Pseudomonas aeruginosa*. Infect. Immun. **3**:762–767.
37. **Seow, W. K., A. Ferrante, S. Y. Li, and Y. H. Thong.** 1988. Anti-phagocytic and anti-oxidant properties of the plant alkaloid tetrandrine. Int. Arch. Allergy Appl. Immunol. **85**:404–409.
38. **Simpson, J. A., S. E. Smith, and R. T. Dean.** 1988. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. J. Gen. Microbiol. **134**:29–36.
39. **Simpson, J. A., S. E. Smith, and R. T. Dean.** 1989. Scavenging by alginate of free radicals released by macrophages. J. Free Radicals Biol. Med. **6**:347–353.
40. **Smith, S. E., and J. A. Simpson.** 1990. The contribution of *Pseudomonas* alginate to evasion of host defence, p. 135–159. *In* P. Gacesa and N. K. Russell (ed.), Pseudomonas infection and alginates: biochemistry, genetics and pathology. Chapman & Hall, Ltd., London.
41. **Thong, Y. H., and A. Ferrante.** 1979. Inhibition of mitogen-induced human lymphocyte proliferative responses by tetracycline analogues. Clin. Exp. Immunol. **35**:443–446.
42. **Thong, Y. H., and V. Rencis.** 1977. Bilirubin inhibits hexose-monophosphate shunt activity of phagocytosing neutrophils. Acta Paediatr. Scand. **66**:757–759.
43. **Weissbach, A., and J. Hurwitz.** 1959. The formation of 2-keto-3-deoxyheptonoic acid in extracts of *Escherichia coli* B. I. Identification. J. Biol. Chem. **234**:705–709.