124): 21 (1972).
14. Markestad, T.: Plasma concentrations of 1,25-dihydroxyvitamin D, 24,25-dihydroxyvitamin D, and 25,26-dihydroxyvitamin D in the first year of life. J. Clin. Endocrinol. Metab., *57:* 755 (1983).
15. Markestad, T., Aksnes, L., Finne, P. H., and Aarskog, D.: Plasma concentrations of vitamin D metabolites in premature infants. Pediatr. Res., (in press).
16. Milner, R. D. G. and Woodhead, J. S.: Parathyroid hormone secretion during exchange transfusion. Arch. Dis. Child., *50:* 298 (1975).
17. Omdahl, J. L., Allen, R. C., and Eaton, R. P.: Computer modeling of vitamin D metabolism. In: Anthony W. Norman *et al.*, eds.: Vitamin D, basic research and its clinical application. pp. 515–21 (Walter de Gruyter, Berlin-New York, 1979).
18. Schedewie, H. K., Odell, W. D., Fisher, D. A., Krutzik, S. R., Dodge, M., Cousins, L., and Fiser, W. P.: Parathormone and perinatal calcium homeostasis. Pediatr. Res., *13:* 1 (1979).
19. Seino, Y., Ishida, M., Yamaoka, K., Ishii, T., Hiejima, T., Ikehara, C., Tanaka, Y., Matsuda, S., Shimotsuji, T., Yabuuchi, H., Morimoto, S., and Onishi, T.: Serum calcium regulating hormones in the perinatal period. Calcif. Tissue Int., *34:* 131 (1982).
20. Snodgrass, G. J. A. I., Stimmler, L., Went, J., Abrams, M. E., and Will, E. J.: Interrelations of plasma calcium in organic phosphate, magnesium, and protein over the first week of life. Arch. Dis. Child., *48:* 279 (1973).
21. Steichen, J. J., Tsang, R. C., Gratton, T. L., Hamstra, A., and DeLuca, H. F.: Vitamin D homeostasis in the perinatal period. N. Engl. J. Med., *302:* 315 (1980).
22. Tsang, R. C. and Erenberg, A. P.: Calcium homeostasis in the newborn—the calciotropic hormones: parathyroid hormone, calcitonin, and vitamin D. In: Mors, A. J., ed.: Pediatric update. pp. 193–241 (Blackwell Scientific Publications, Oxford-Boston, 1980).
23. Valaes, T.: Bilirubin distribution and dynamics of bilirubin removal by exchange transfusion. Acta Paediatr. Scand., *52* (suppl. 149): 604 (1963).
24. Valaes, T. and Hyte, M.: Effect of exchange transfusion on bilirubin binding. Pediatrics, *59:* 881 (1977).
25. Weisman, Y., Sapir, R., Haell, A., and Edelstein, S.: Maternal-perinatal interrelationships of vitamin D metabolism in rats. Biochim. Biophys. Acta, *428:* 388 (1976).
26. Requests for reprints should be addressed to: Dr. T. Markestad, Department of Pediatrics, N-5016 Haukeland sykehus, Bergen, Norway.
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Antibody to *Pseudomonas Aeruginosa* Mucoid Exopolysaccharide and to Sodium Alginate in Cystic Fibrosis Serum

DAVID P. SPEERT,[33] DANIEL LAWTON, AND LUCY M. MUTHARIA

Division of Infectious Diseases, [D.P.S., D.L.] British Columbia Children's Hospital and the Departments of Pediatrics [D.P.S.] and Microbiology [D.P.S. and L.M.M.], University of British Columbia, Vancouver, British Columbia, Canada

Summary

Antibodies in cystic fibrosis (CF) sera to *Pseudomonas aeruginosa* mucoid exopolysaccharide and to sodium alginate (a polysaccharide from seaweed chemically similar to mucoid exopolysaccharide) were measured in sera of CF patients to determine if the exopolysaccharide is immunogenic. An enzyme-linked immunosorbent assay was used to test sera from 26 CF patients (18 colonized with pseudomonas and eight non-colonized) and 26 healthy controls. CF patients colonized with pseudomonas had more antibody to mucoid exopolysaccharide ($P = 0.0008$) and to sodium alginate ($P = 0.0008$) than did non-colonized CF patients. Virtually none was found in healthy controls. Duration of colonization was correlated with the level of antibody to sodium alginate ($P = 0.003$) but not with antibody to mucoid exopolysaccharide. Mucoid exopolysaccharide is immunogenic in patients with CF.

Abbreviations

CF, cystic fibrosis
ELISA, enzyme-linked immunosorbent assay
MEP, mucoid exopolysaccharide
OD, optical density
PA, *Pseudomonas aeruginosa*
PBS, phosphate-buffered saline
PBS/FBS, phosphate-buffered saline with 1% fetal bovine serum

In most older patients with CF oropharyngeal colonization and pulmonary infection with mucoid PA ultimately occurs (5, 14, 26, 30) and may be associated with an adverse prognosis (14). Mucoid PA elaborates a copious amount of an exopolysaccharide (closely resembling alginic acid from seaweed) that is responsible for its growth in the mucoid form (9). Colonization with mucoid PA once established usually persists, but the pathogenic role for PA and its mucoid exopolysaccharide (MEP) is poorly understood. We used a sensitive enzyme-linked immunosorbent assay to measure antibody to MEP and to sodium alginate in CF sera in order to determine if MEP is immunogenic. Antibodies to both MEP and to sodium alginate were found in CF sera; the level of antibody to sodium alginate was correlated with duration of colonization with mucoid PA.

MATERIALS AND METHODS

Subjects. Sera from 26 patients with CF and 26 healthy adults (obtained from the Vancouver Red Cross) were tested for antibody to MEP and to sodium alginate. After informed consent was obtained, venous blood was drawn from all subjects, and allowed to clot at room temperature for 45 min. The serum was frozen at −70°C until used. Eighteen CF subjects (age, 3–24 yr; mean, 12.5 yr) were colonized with PA, but eight CF subjects (age 6 mo to 26 yr; mean, 7.5 yr) had always been free of PA in throat and sputum cultures. Disease severity was assessed using

the scoring system of Shwachman and Kulczycki (28). Duration of colonization was determined for each patient from the date on which PA was first grown from a throat or sputum culture.

Mucoid exopolysaccharide. A modification of the method of Evans and Linker was used for purification of MEP (9). A mucoid PA strain (P-1 M) from a patient with CF was grown on tryptose agar plates (without added blood) at 30°C for 72 h. Bacteria were gently scraped into normal saline and stirred until uniform. This suspension was repeatedly centrifuged at 20,000 × *g* until a pellet was no longer seen. Three volumes of 95% ethanol were added to the supernatant fluid, and the precipitated MEP was washed repeatedly with ethanol and lyophilized. The MEP preparation contained less than 5% protein by Lowry determination (17), and less than 2% nucleic acid (absorbance of light at 260 and 280 nm). There was 1.2 μg endotoxin-like activity/mg as assessed by the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA) using a PA lipopolysaccharide standard (List Biological Laboratories, Campbell, CA). Sodium alginate was purchased from Sigma Laboratories (St. Louis, MO).

ELISA. A modification of the method of Hancock *et al.* (11) was used. MEP (50 μg/ml) or sodium alginate (200 μg/ml) in carbonate-bicarbonate buffer (pH 9.6) was incubated for 18 h at 4°C in flat-bottomed well polyvinylchloride microtiter plates (Dynatech, Alexandria, VA). The plates were washed three times with PBS, pH 9.6 with 0.05% polysorbate 80 and then with PBS. PBS/FBS was added to the wells for 1 h at 37°C, to block non-specific binding of antibody to the plates. Test sera were then added to the wells for 1 h at 37°C. After the 1-h period, plates were washed and PBS/FBS again added. The plates were incubated at 37°C for 30 min and then removed. Alkaline-phosphate-conjugated anti-human IgG (Sigma) was added for 1 h at 37°C and washed out with PBS. Alkaline phosphatase substrate (Sigma) was then added and incubated at room temperature in the dark until a routine positive control (crude mucoid PA extracellular material with a standard CF serum) reached an OD of 1.1 at 405 nm, whereupon test wells were read. The OD measurements for the positive control always exceeded those of the CF sera because the control was run with a crude pseudomonas extract, which was not as pure as the MEP preparation used to test the CF sera. OD was determined in a Titertek Multiskan (Flow Laboratories, Helsinki). All assays were run in triplicate. Results represent the averages of determinations on 2 separate days and were highly reproducible. Positive and negative controls were included on each plate and outside walls were not used to avoid "edge effect."

Statistical methods. Correlation was analyzed using linear regression (8) and groups were compared using the median test (22) because the variances of the two populations (CF colonized *versus* CF non-colonized) were so dissimilar.

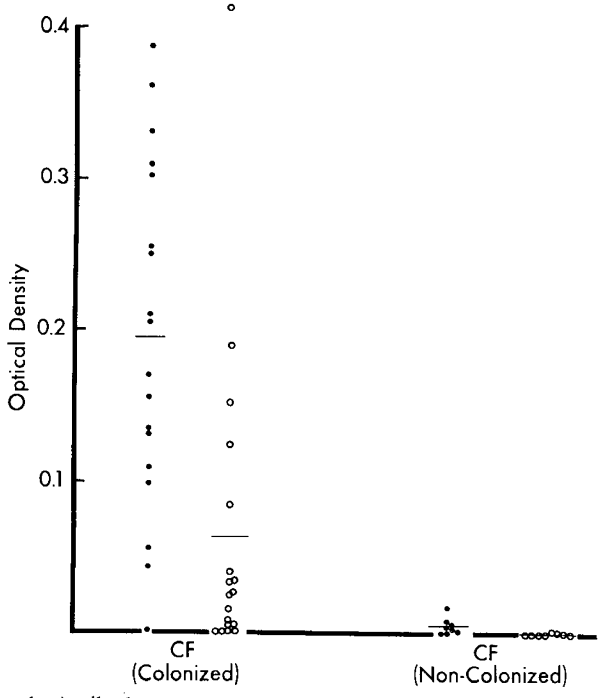

Fig. 1. Antibody to *P. aeruginosa* mucoid exopolysaccharide (*closed circles*) and to sodium alginate (*open circles*) in cystic fibrosis patients. Patients colonized with PA had significantly more antibody to each of the two polysaccharides ($P = 0.0008$) than did those non-colonized.

RESULTS

Preliminary experiments established that 50 μg/ml MEP and 200 μg/ml sodium alginate incubated at 4°C for 18 h produced optimal antigen binding to the microtiter plates. Coating with higher concentrations of the antigens led to non-specific color reactions with serum pooled from five healthy laboratory volunteers. A serum dilution of 1:32 gave the maximal color reaction with the antibody-positive sera; this dilution was therefore used to test all sera.

Figure 1 shows that the 18 CF patients colonized with PA had significantly more antibody to MEP (OD, 0.195 ± 0.114) than did the eight non-colonized patients (OD, 0.005 ± 0.005) ($P = 0.0008$). Likewise with sodium alginate (Fig. 1), CF patients colonized with PA had more antibody (OD, 0.064 ± 0.103) than did non-colonized patients (OD, 0.001 ± 0.001) ($P = 0.0008$). Normal adults had negligible antibody to MEP (OD, 0.007 ± 0.009) and to sodium alginate (OD, 0.008 ± 0.017). One 7-yr-old girl colonized with *Pseudomonas cepacia* did not have detectable antibodies to MEP (OD, 0.002) or to sodium alginate (OD, 0.003). A 26-yr-old man with CF had never been colonized with PA and also had no detectable antibodies to the polysaccharides (MEP OD, 0.017 and sodium alginate OD, 0).

Duration of colonization with PA was correlated ($r = 0.75$, $P = 0.003$) with the level of antibody to sodium alginate but not to MEP ($r = 0.32$, $P = 0.183$). Disease severity, as assessed by Shwachman score, was not significantly correlated with the level of antibody to sodium alginate or to MEP. There was also not a significant correlation between levels of antibody to MEP and to sodium alginate.

DISCUSSION

The MEP from CF pseudomonas isolates has been chemically well characterized (9) but its role in CF pulmonary disease remains poorly understood. It is a heteropolymer composed of mannuronic and guluronic acids with *o*-acetyl groups, has a molecular weight of greater than 100,000 daltons and forms a viscous gel in solution (9). This property of high viscosity, which is enhanced in the presence of calcium (unpublished observation), may interfere mechanically with clearance of bacteria from the lungs of CF patients infected with mucoid PA. Except for a lack of *o*-acetyl groups, alginic acid from seaweed is identical to MEP (9).

P. aeruginosa elaborates several different polysaccharides and their terminology has been very confusing (2, 9, 24). MEP appears to be unique to mucoid strains (which are rarely recovered from non-CF sources); it has been referred to as pseudomonas "alginate," "glycocalyx," and "slime." Confusion arises because the term "slime" has been used to describe the extracellular substance from both mucoid (9) and from non-mucoid strains (2, 24). The term MEP is used herein to clearly distinguish it from the "slime" polysaccharide from non-CF strains of *P. aeruginosa.*

The MEP is one of several extracellular products of PA for which a potential pathogenetic role in CF lung infections has been suggested. Other pseudomonas products to which antibody has been found in CF sera include protease (15), exotoxin A (15), crude pseudomonas sonicate (12), and cell envelope proteins (10). Because our MEP preparation contained small amounts of protein and lipopolysaccharide, the antibody we

measured may have been directed in part against these impurities. The presence of antibody to sodium alginate in the CF serum suggests that much of the antibody we measured with the MEP preparation was directed against the polysaccharide moiety. Our data further suggest that mucoid PA produce a polysaccharide in the CF respiratory tract that closely resembles that which is elaborated *in vitro*.

Other attempts at measuring antibody to MEP in CF patients have yielded conflicting results. Hoiby and co-workers (13) failed to demonstrate antibody, but Doggett and Harrison (7) found antibody to the "capsular polysaccharide" using a hemagglutination assay (6). The antibody they measured may have been directed against other pseudomonas products, such as outer membrane proteins, exotoxin or protease because their antigen preparation was crude in nature.

Many questions remain regarding the role of mucoid PA in CF pulmonary disease (20). It is apparent that CF patients mount an antibody response to the MEP but that still does not establish its pathogenic role. Analyses of postmortem lung sections from patients with CF who died reveal microcolonies encased in a meshwork of fibers thought to represent MEP (16). It has been suggested that this mucoid substance serves as a simple physical barrier to clearance by pulmonary phagocytes (16). MEP has been shown to interfere with phagocytosis of pseudomonas by polymorphonuclear leukocytes (29). Baltimore and Mitchell (1) have suggested that mucoid PA strains resist phagocytosis by concealing their opsonic cell wall determinants. Although it is clear that mucoid PA can persist in the CF lung once colonization has occurred, the means by which these bacteria cause disease is at present unclear.

The role of antibody to MEP in the CF pulmonary disease process remains to be determined. It probably is not an effective opsonic antibody, as it is directed against an extracellular product rather than a constituent of the bacterial surface. A growing body of evidence suggests that pulmonary damage in CF may be in part an immune-mediated phenomenon. Antibody to pseudomonas products may in fact potentiate rather than modulate disease either in an Arthus reaction (31) or by formation of circulating immune complexes (4, 20). Immune complexes have in fact been found in CF sera (3, 18, 19, 23) and sputum (27) and levels may (23) or may not (19) be correlated with disease severity. Immunization with pseudomonas vaccine does not influence the course of disease in CF patients already colonized with PA (25). Furthermore, hypogammaglobulinemic CF patients appear to have less severe disease than do those with normal or elevated serum immunoglobulin (21). Further investigation is required before it can be determined if antibody to the pseudomonas MEP plays a role in CF pathogenesis by potentiating pulmonary damage.

ADDENDUM

Since submission of this manuscript, two other groups have made similar observations about the immunogenicity of mucoid exopolysaccharide.

1. Pier, G. B., Matthews, W. J., Jr., and Eardley, D. D.: Immunochemical characterization of the mucoid exopolysaccharide of *Pseudomonas aeruginosa*. J. Infect. Dis., *147:* 494 (1983).
2. Byran, L. E., Kureishi, A., and Rabin, H. R.: 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 (1983).

REFERENCES AND NOTES

1. Baltimore, R. S. and Mitchell, M.: 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 (1980).
2. Bartell, P. F., Orr, T. E. and Chudio, B.: Purification and chemical composition of the protective slime antigen of *Pseudomonas aeruginosa*. Infect. Immun., *2:* 543 (1970).
3. Berdischewsky, M., Pollack, M., Young, L. S., Chia, D., Osher, A. B., and Barnett, E. V.: Circulating immune complexes in cystic fibrosis, Pediatr. Res., *14:* 830 (1980).
4. Brentjens, J. R., O'Connell, D. W., Pawlowski, I. B., Hsu, K. C., and Andres, G. A.: Experimental immune complex disease of the lung, J. Exp. Med., *140:* 105 (1974).
5. di Sant'Agnese, P. A. and Davis, P. B.: Research in Cystic Fibrosis. N. Engl. J. Med., *295:* 597 (1976).
6. Doggett, R. G., Harrison, G. M., Stillwell, R. N., and Wallis, E. S.: Enzymatic action on the capsular material produced by *Pseudomonas aeruginosa* of cystic fibrosis origin. J. Bacteriol., *89:* 476 (1965).
7. Doggett, R. G. and Harrison, G. M.: *Pseudomonas aeruginosa*: immune status in patients with cystic fibrosis. Infect. Immun., *6:* 628 (1972).
8. Draper, N. R. and Smith, H. Jr.: Applied Regression Analysis. 2nd Ed. (John Wiley and Sons, New York, 1981).
9. Evans, L. R. and Linker A.: Production and characterization of the slime polysaccharide of *Pseudomonas aeruginosa*. J. Bacteriol., *116:* 915 (1973).
10. Fernandes, P. B., Kim, C., Cundy, K. R., and Huang, N. N.: Antibodies to cell envelope proteins of *Pseudomonas aeruginosa* in cystic fibrosis patients. Infect. Immun., *33:* 527 (1981).
11. Hancock, R. E. W., Wieczorek, A. A., Mutharia, L. M., and Poole, K.: Monoclonal antibodies against *Pseudomonas aeruginosa* outer membrane antigens: isolation and characterization. Infect. Immun., *37:* 166 (1982).
12. Høiby, N. and Axelsen, N. H.: Identification and quantitation of precipitins against *Pseudomonas aeruginosa* in patients with cystic fibrosis by means of crossed immunoelectrophoresis with intermediate gel. Acta. Path. Microbiol. Scand. Sect. B, *81:* 298 (1973).
13. Høiby, N., Andersen, V. and Bendixen, G.: *Pseudomonas aeruginosa* infection in cystic fibrosis, Acta. Pathol. Microbiol. Scand. Sect. C, *83:* 459 (1975).
14. Høiby, N., Flensborg, E. W., Beck, B., Friis, B., Jacobsen, S. V., and Jacobsen, L.: *Pseudomonas aeruginosa* infection in cystic fibrosis. Scand J. Resp. Dis., *58:* 65 (1977).
15. Klinger, J. D., Straus, D. C., Hilton, C. B., and Bass, J. A.: Antibodies to proteases and exotoxin A of *Pseudomonas aeruginosa* in patients with cystic fibrosis: demonstration by radioimmunoassay. J. Infect. Dis., *138:* 49 (1978).
16. Lam, J., Chan, R., Lam, K., and Costerton, J. W.: Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun., *28:* 546 (1980).
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with folin phenol. J. Biol. Chem., *193:* 265 (1951).
18. McFarlane, H., Holzel, A., Brenchley, P., Allan, J. D., Wallwork, J. C., Singer, B. E., and Worsley, B.: Immune complexes in cystic fibrosis. Br. Med. J., *1:* 423 (1975).
19. Manthei, U., Taussig, L. M., Beckerman, R. C., and Strunk, R. C.: Circulating immune complexes in cystic fibrosis. Am. Rev. Resp. Dis., *126:* 253 (1982).
20. Marks, M. I.: The pathogenesis and treatment of pulmonary infections in patients with cystic fibrosis. J. Pediatr., *98:* 173 (1981).
21. Matthews, W. J., Jr., Williams, M., Oliphint, B., Geha, R., and Colten, H. R.: Hypogammaglobulinemia in patients with cystic fibrosis. N. Engl. J. Med., *302:* 245 (1980).
22. Mood, A. M., Graybill, F. A. and Boes, D. C.: Introduction to the Theory of Statistics. 3rd ed. pp. 521–522 (McGraw Hill Book Company, New York, 1974).
23. Moss, R. B., Hsu, Y. and Lewiston, N. J.: ^{125}I-Clq-binding and specific antibodies as indicators of pulmonary disease activity in cystic fibrosis. J. Pediatr., *99:* 215 (1981).
24. Pier, G. B., Sidberry, H. F., Zolyomi, S. and Sadoff, J. C.: Isolation and characterization of a high-molecular-weight polysaccharide from the slime of *Pseudomonas aeruginosa*. Infect. Immun., *22:* 908 (1978).
25. Pennington, J. E., Reynolds, H. Y., Wood, R. E., Robinson, R. A., and Levine, A. S.: Use of a *Pseudomonas aeruginosa* vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med., *58:* 629 (1975).
26. Reynolds, H. Y., Levine, A. S., Wood, R. E., Zierdt, C. H., Dale, D. C., and Pennington, J. E.: *Pseudomonas aeruginosa* infections: persisting problems and current research to find new therapies. Ann. Int. Med., *82:* 819 (1975).
27. Schiotz, P. O., Nielsen, H., Høiby, N., Olikmann, G., and Svehag, S.-E.: Immune complexes in the sputum of patients with cystic fibrosis suffering from chronic *Pseudomonas aeruginosa* lung infection. Acta. Path. Microbiol. Scand. Sect. C, *86:* 37 (1978).
28. Shwachman, H. and Kulczycki, L. L.: Long-term study of one hundred five patients with cystic fibrosis. Am. J. Dis. Child., *96:* 6 (1958).
29. Schwarzmann, S. and Boring, J. R.: Antiphagocytic effect of slime from a mucoid strain of *Pseudomonas aeruginosa*. Infect. Immun., *3:* 762 (1971).
30. Wood, R. E., Boat, T. F. and Doershuk, C. F.: Cystic Fibrosis. Am. Rev. Resp. Dis., *113:* 833 (1976).
31. Zavala, D. C., Rhodes, M. L., Richerson, H. B., and Oskvif, R.: Light and immunofluorescent study of the Arthus reaction in the rabbit lung, J. Allergy Clin. Immunol., *56:* 450 (1975).

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33. Requests for reprints should be addressed to: Dr. David P. Speert, Division of Infectious Diseases, British Columbia Children's Hospital, 4480 Oak Street, Vancouver, B.C., Canada V6H 3V4.

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