

Longitudinal Study of Antibody Response to Lipopolysaccharides during Chronic *Pseudomonas aeruginosa* Lung Infection in Cystic Fibrosis

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Antibodies to *Pseudomonas aeruginosa* from 10 cystic fibrosis patients with chronic *P. aeruginosa* lung infections were quantitatively and qualitatively analyzed. The development of specific antibodies in patient serum was evaluated in a longitudinal study (1972 to 1987). The concentrations and specificities of immunoglobulin G (IgG) and IgM antibodies to purified lipopolysaccharides (LPS) from clinical isolates of *P. aeruginosa* and to a variety of other gram-negative bacteria were studied by immunoblotting and enzyme-linked immunosorbent assay techniques. Results were compared with the number of immunoprecipitates to *P. aeruginosa* whole-cell extracts detected by crossed immunoelectrophoresis. IgG, but not IgM, anti-*Pseudomonas* LPS concentrations increased significantly at the onset of chronic infection and continued to increase during the course of the infection. There was a good positive correlation between the concentration of IgG anti-*Pseudomonas* LPS antibodies and the number of crossed-immunoelectrophoresis precipitins. The increases in IgG anti-LPS antibody concentrations were much higher to *Pseudomonas* LPS than to other LPSs. Binding studies demonstrated an increase in binding of IgG anti-*Pseudomonas* LPS during infection, whereas the binding of other anti-LPS antibodies decreased. Immunoblotting studies confirmed that antibodies reacted strongly with *Pseudomonas* LPS and weakly with *Escherichia coli* core-lipid A. The specificity of the reaction with *Pseudomonas* LPS increased with the duration of infection. It is concluded that anti-LPS response in cystic fibrosis patients during chronic *P. aeruginosa* infection demonstrates a marked increase in IgG anti-*Pseudomonas* LPS antibody concentration, specificity, and affinity. The anti-LPS enzyme-linked immunosorbent assay is proposed as a routine test to diagnose and to follow the course of chronic *P. aeruginosa* lung infection in patients with cystic fibrosis.

Chronic lung infection with *Pseudomonas aeruginosa* is the major factor which determines the severity of illness and mortality in cystic fibrosis (CF) patients (28). The change from initial colonization to chronic invasive infection is commonly considered a sign of poor prognosis (13). At the Danish CF center, a diagnosis of chronic *P. aeruginosa* lung infection is an indication to begin intensive treatment with antibiotics. Although antibiotics rarely eradicate the organisms, this treatment regimen improves the lung function, well-being, and long-term survival of the patient (S. S. Pedersen, T. Jensen, N. Høiby, C. Koch, and C. W. Flensburg, *Acta Paediatr. Scand.*, in press). It is therefore important to evaluate the development of immunological responses against this pathogen to identify early signs of infection and the appropriate time to begin antibiotic treatment.

The antigenic structure of *P. aeruginosa* is complex, and as many as 64 antigens have been described by using quantitative immunoelectrophoretic methods (13). The determination of precipitating antibodies to whole-cell extracts of *P. aeruginosa* by crossed immunoelectrophoresis (CIE) can be used to diagnose the onset of infection and to follow its course. The number of precipitins increases with the time and the severity of infection (12, 13).

We have focused on the lipopolysaccharide (LPS) antigen

of the outer membrane of the gram-negative cell wall. LPS is the most biologically active molecule in gram-negative bacteria and is responsible for many of the symptoms and pathophysiological characteristics of gram-negative infections (27). Antibodies to LPS have been shown to protect against endotoxic reactions, and the development of an anti-LPS response may play an important role in preventing the spread of infection (7, 23, 30).

We have previously shown that common antigenic determinants reside in the core-lipid A part of *P. aeruginosa* LPS in clinical isolates from CF and that these determinants are accessible in LPS from polyagglutinable (agglutinated by more than one antiserum) strains because of a deficiency in the O polysaccharides (3). Because of the cross-reactive properties of this polyagglutinable LPS, it is a potentially useful tool in diagnosis and evaluation of *P. aeruginosa* infections. Since polyagglutinable strains are selected to cause chronic infections in CF (11, 28), the LPS of such clinical isolates may be of special relevance in evaluating chronic *P. aeruginosa* infection.

In the present study we have developed sensitive quantitative enzyme-linked immunosorbent assay (ELISA) methods for determining human immunoglobulin G (IgG) and IgM antibodies to purified LPS from typable and polyagglutinable *P. aeruginosa* strains. The humoral responses to these *P. aeruginosa* LPSs as well as to LPS from other gram-negative bacteria were investigated in CF patients in a longitudinal

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TABLE 1. *P. aeruginosa* strains isolated from CF patients with chronic pulmonary infections

Strain	O serotype	Phage type	Phenotype
1118	3	7,21,F7,M4, col 2, col 11+	Nonmucoid
174	9	68,109,352,F8,M4, col 2, col 11	Mucoid
170	3/9	21,48,68,109,352,1214, F3,M4, col 11+	Mucoid
14580/83	3/4/6/9/10	21,188	Nonmucoid
15704/83	3/4/6/9/10	11,13,14,31+	Nonmucoid

study. The LPS response was also followed by immunoblotting and compared with the increase in numbers of precipitating antibodies (precipitins) against *P. aeruginosa* whole-cell sonicate as measured by CIE.

MATERIALS AND METHODS

Patients. Diagnosis of CF was based on accepted criteria, including abnormal sweat electrolyte levels in repeated tests, characteristic lip biopsy, exocrine pancreatic insufficiency, and altered pulmonary function (28). After diagnosis of CF, the patients were monitored during monthly visits to the Danish CF center at Rigshospitalet, where clinical data and bacteriology of lung infections were recorded. The beginning of chronic *P. aeruginosa* lung infection was defined as the point at which the bacteria had been detected in every sputum sample for at least 6 months and serum contained more than one immunoprecipitate (precipitin) formed by antibodies to *P. aeruginosa* whole-cell extract in CIE (12). Colonized patients were those with consistent or intermittent growth of *P. aeruginosa* in sputum but with ≤ 1 precipitins band. During the period of the study, all patients were infected with polyagglutinable strains of *P. aeruginosa* involving O group 3 (O:3), O:6, O:9, or O:10. In one case, a polyagglutinable O:2/5 strain was also isolated. The most frequent typable strains isolated were O:3 (8 of 10 patients), O:9 (6 of 10 patients) and O:6 (4 of 10 patients). O:1, O:4, and O:7 strains were isolated occasionally, and an O:11 strain was found in one sputum sample. This is in accordance with earlier investigations of CF patients in Denmark (16, 29).

The antibody response to LPS was determined in two groups of CF patients. In the first group were 20 noncolonized CF patients without precipitins detectable by CIE. In the second were 10 patients with consistent growth of *P. aeruginosa* and with ≥ 15 precipitins. Serum samples obtained during periods of from 8 to 16 years from the second group of patients were then examined in a longitudinal study.

Control samples. Human serum samples obtained from 40 healthy children from 0 to 14 years old and from 1,000 healthy adult blood donors from 18 to 65 years old without any history of *P. aeruginosa* infection were examined for different kinds of anti-LPS antibodies.

LPS antigens. *P. aeruginosa* strains were isolated from sputum of CF patients with chronic pulmonary infections (Table 1). LPS from strains 1118, 174, 14580/83, and 15704/83 was extracted by the hot phenol-water method (27), and LPS from strain 170 was extracted by the phenol-chloroform-petroleum-ether method (9). LPS was purified by successive ultracentrifugation steps and converted to the uniform salt forms by electrodialysis (8). The chemical composition and the immunochemical characterization of these LPSs are reported in detail elsewhere (3). LPS from strains 1118 and 170 was used in ELISA for quantitative study of anti-*Pseudomonas* antibody response in CF patients.

LPS extracted by hot phenol-water (Sigma Chemical Co., St. Louis, Mo.) was obtained from 11 other bacteria: *Escherichia coli* O26:B6, *E. coli* O55:B5, *E. coli* O111:B4, *E. coli* O127:B8, *E. coli* O128:B12, *Salmonella minnesota*, *S. typhosa*, *S. typhimurium*, *S. enteritidis*, *S. abortus equi*, and *Serratia marcescens*. A mixture of these 11 LPSs (10 μ g of each per ml) in 0.04 M sodium phosphate-0.1 M NaCl, pH 7.4 (phosphate-buffered saline [PBS]) was used in ELISA to quantitate IgG and IgM antibody responses in CF patients to LPS from gram-negative bacteria other than *P. aeruginosa*.

LPS preparations from *Shigella flexneri*, *Klebsiella pneumoniae*, and *P. aeruginosa* strain F-D 1, O:6, were obtained from List Inc. (Campbell, Calif.). Rough LPS from *E. coli* Ra and *S. minnesota* R60 (Ra) was extracted by the phenol-chloroform-petroleum-ether method. These LPSs were used in immunoblotting with LPSs from the other strains to study the specificity of anti-LPS antibodies from CF patients.

***Pseudomonas* standard antigens.** Water-soluble antigens from a pool of *P. aeruginosa* strains representing all 17 serotypes of the International Antigenic Typing Scheme were prepared by sonication as previously described (12) and used as an standard antigen preparation for CIE (12, 13).

ELISA for IgG and IgM anti-LPS antibodies. Polystyrene microdilution plates (96-well; Immunoplate I; Nunc A/S, Roskilde, Denmark) were incubated with 1 μ g of *P. aeruginosa* O:3 LPS per 100 μ l in PBS or 1 μ g of *P. aeruginosa* O:3/9 LPS per 100 μ l PBS containing 0.02 M MgCl₂. Increasing coating concentrations of the *P. aeruginosa* LPSs resulted in increasing anti-LPS antibody binding. The presence of MgCl₂ at an optimal concentration of 0.02 M in the coating buffer significantly improved the adsorption of the polysaccharide-deficient O:3/9 LPS. We did not find a maximal coating concentration of LPS when concentrations up to 100 μ g/ml of each LPS were investigated, indicating a high binding capacity of polystyrene for LPS. A coating concentration of 1 μ g of *P. aeruginosa* LPS per 100 μ l resulted in a convenient A₄₉₂ and was chosen for reasons of economy. By following these coating procedures, the plates could be stored at 4°C with the coating solution remaining in the wells and were usable for at least 6 months.

For assay of antibodies to LPS from gram-negative bacteria other than *P. aeruginosa*, some plates were coated with 100 μ l of the LPS pool. The plates were stored at 4°C for at least 72 h and were used within 1 month. The plates were washed with saline containing 0.1% Tween-20 and incubated overnight at 20°C with test serum samples diluted in 100 μ l of PBS plus 0.1% Tween-20. Samples were assayed in two different dilutions. After being washed, the plates were incubated for 1 h at room temperature with peroxidase-conjugated rabbit anti-human IgG (DAKO A/S, Copenhagen, Denmark) diluted 1:15,000 in PBS-Tween (100 μ l) for assay of IgG anti-LPS. Peroxidase-conjugated rabbit anti-human IgM (DAKO A/S) diluted 1:2,000 in PBS-Tween (100 μ l) was used for assay of IgM anti-LPS antibodies. The plates were washed and incubated with 100 μ l of enzyme substrate (12 mg of *o*-phenylenediamine [Pitman-Moore Inc., Washington Crossing, N.J.] in 18 ml of distilled water plus 7.5 μ l of hydrogen peroxide 30% [vol/vol]) per well. The color reaction was stopped after 30 min by the addition of 150 μ l of sulfuric acid (2.5 M) to each well. A₄₉₂ of the wells was measured with a 620-nm reference by using a photometer (Kontron SLT-210, Zürich, Switzerland). The specificity of the anti-LPS ELISA was demonstrated by absorption experiments (5). Samples were applied in two dilutions to confirm parallelism with the IgG or the IgM standard curve. Serum samples with high anti-LPS antibody concen-

trations (absorbance above 1.800) were further diluted (10-fold) and assayed again. Although the serum incubations were deemed sufficiently complete within 2 h, an overnight incubation was selected for practical reasons.

Standard-free quantitation of anti-LPS antibodies. The principle of standard-free quantitation (4) was used to convert ELISA absorption values into milligrams of specific anti-LPS antibodies per liter. Double-antibody sandwich ELISAs for total IgG (6) and total IgM (1) were carried out to create standard curves of IgG and IgM by using a standardized human serum (Seronom 103, Nycomed, Oslo, Norway). The ELISAs for total IgG and IgM were performed simultaneously with the anti-LPS ELISAs with identical buffers, conjugates, and assay conditions. The different ELISAs were performed on the same microdilution plates in a combined design (4). The concentrations of specific IgG or IgM anti-LPS antibodies were then read on the simultaneously performed total IgG or IgM standard curves.

To measure residual immunoreactivity in the samples after one complete plasma incubation, the contents of each well were transferred to an identically coated plate and re-assayed. The ratio between absorbances in the second and first plates was calculated for each well. This ratio was used as an estimate of the individual residual immunoreactivity. The results obtained were corrected for differences in antibody binding measured in the anti-immunoglobulin-coated parts of the plate and in the LPS-coated parts as described elsewhere (4).

Precipitating antibodies against *P. aeruginosa*. *P. aeruginosa* precipitins were detected by CIE as previously described (12). Briefly, in 1% agarose gels (1% Indubiose A 37; L'Industrie Biologique Francaise S.A.), 2 μ l of the standard antigen preparation containing 22 g of protein per liter was applied to the well, and first dimension separation was carried out at 10 V/cm for 1 h. Second dimension separation was performed for 18 h at 1 to 2 V/cm into 1% agarose containing 15 μ l of patient serum per cm². Tris-barbital buffer (pH 8.6; ionic strength 0.02) was used. The immunoprecipitates were stained with Coomassie brilliant blue (Sigma), and the number of precipitin peaks was counted.

SDS-PAGE and immunoblotting. LPS preparations were treated for 5 min at 100°C in 0.05 M Tris hydrochloride (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% sucrose, and 0.01% bromophenol blue and fractionated on a SDS-polyacrylamide gel containing 5% and 14% acrylamide in the stacking and the separating gels, respectively. Polyacrylamide gel electrophoresis (PAGE) was carried out at 12 mA in the stacking gel and 25 mA in the separating gel until the tracking dye had run 10 cm. Gels were silver stained (26), or the samples were transferred to nitrocellulose paper (19) (Schleicher and Schuell, Dassel, Federal Republic of Germany) in a vertical electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.) at 60 V for 6 h (3). The transfer buffer contained 192 mM glycine, 25 mM Tris, and 20% methanol, pH 8.3. After blocking 2 h in a solution containing 50 mM Tris, 100 mM sodium chloride, and 1% gelatin, pH 7.4 (TSG), the nitrocellulose was incubated 2 h at 22°C in patient serum diluted 1:10,000 in TSG. The nitrocellulose was washed three times in Tris saline and incubated for 1 h at 20°C in peroxidase-labeled rabbit anti-human IgG (DAKO) diluted 1:500 in TSG. The sheets were again washed three times, and the color was developed in 10 mM Tris, 0.9% saline containing 0.003% (wt/vol) 4-chloro-1-naphthol, and 0.05% (vol/vol) H₂O₂. The color reaction was stopped with water after 5 min.

TABLE 2. Percentage of antibody bound after completion of the first serum incubation in anti-LPS ELISAs

Antibodies to LPS prepn ^a	% Antibody bound by ^b :	
	CF (-P) noncolonized	CF (+P) late chronic
IgG O:3 Pa	23 \pm 2	49 \pm 3
O:3/9 Pa	27 \pm 2	48 \pm 3
LPS pool	43 \pm 4	33 \pm 5
IgM O:3 Pa	50 \pm 2	46 \pm 3
O:3/9 Pa	46 \pm 3	56 \pm 4
LPS pool	23 \pm 3	34 \pm 2

^a Pa, *P. aeruginosa*; LPS pool, pool of 11 LPSs from gram-negative bacteria other than *P. aeruginosa*.

^b Medians \pm standard errors from serum of 10 CF patients. -P, No precipitins against *P. aeruginosa* whole-cell extracts detectable in CIE; +P, >15 precipitins detectable in CIE; noncolonized, no bacteria grown from sputum; late chronic, *P. aeruginosa* grown constantly from sputum and >1 precipitins detectable in CIE for 3 to 15 years.

RESULTS

Prevalence of anti-LPS antibodies. The ELISA for IgG and IgM antibodies to the LPS pool as well as the indirect standardization method have been described earlier (4, 5). The distribution of anti-LPS IgG and IgM concentrations among healthy individuals and CF patients was determined (see Table 3). In healthy children, the anti-LPS IgG and IgM concentrations were highest against the LPS pool and lowest against the *P. aeruginosa* O:3 LPS. The IgG anti-LPS antibody concentration was constant throughout childhood, although the IgM anti-LPS antibody concentration increased with age. The percentages of the specific anti-LPS antibodies bound after one complete plasma incubation for CF patients with and without precipitins are given in Table 2. Binding of anti-*Pseudomonas* LPS antibodies increased in CF patients with chronic infection compared with noninfected CF patients, whereas the IgG anti-pool LPS antibody binding decreased (Table 2).

Patients with chronic *P. aeruginosa* lung infections showed extremely high concentrations of IgG antibodies to both *P. aeruginosa* LPSs (IgG antibody concentrations up to 2,300 mg/liter were measured as shown in Fig. 1) and a less pronounced increase in IgG antibodies to the LPS pool (up to 270 mg/liter). The difference in anti-*Pseudomonas* LPS IgG concentrations between chronically infected CF patients (CF ++P) and noncolonized CF patients (CF -P) or healthy controls was significant ($P < 0.001$). In contrast, no significant difference in anti-LPS IgM concentration could be demonstrated between chronically infected and noncolonized CF patients; however, this concentration was higher in both cases than that of healthy controls.

Antibody concentrations increased markedly in the early stages of infection (Table 3). The median anti-O:3/9 LPS IgG concentration in eight CF patients at the early state of chronic infection was 104 mg/liter, compared with 38 mg/liter in the same patients before a diagnosis of chronic infection. These differences in anti-O:3/9 LPS IgG concentrations in the paired samples were significant ($P < 0.01$). Serum samples from noncolonized CF children which showed no precipitins to *P. aeruginosa* antigens in CIE (CF -P) nevertheless gave significantly higher concentrations of IgG anti-*Pseudomonas* LPS antibodies in ELISA than serum samples from healthy controls did (Table 3). The IgG anti-LPS antibody concentration was also raised in five out of eight noncolonized CF infants (ages, 6 to 18 months), compared with age-matched controls (data not shown).

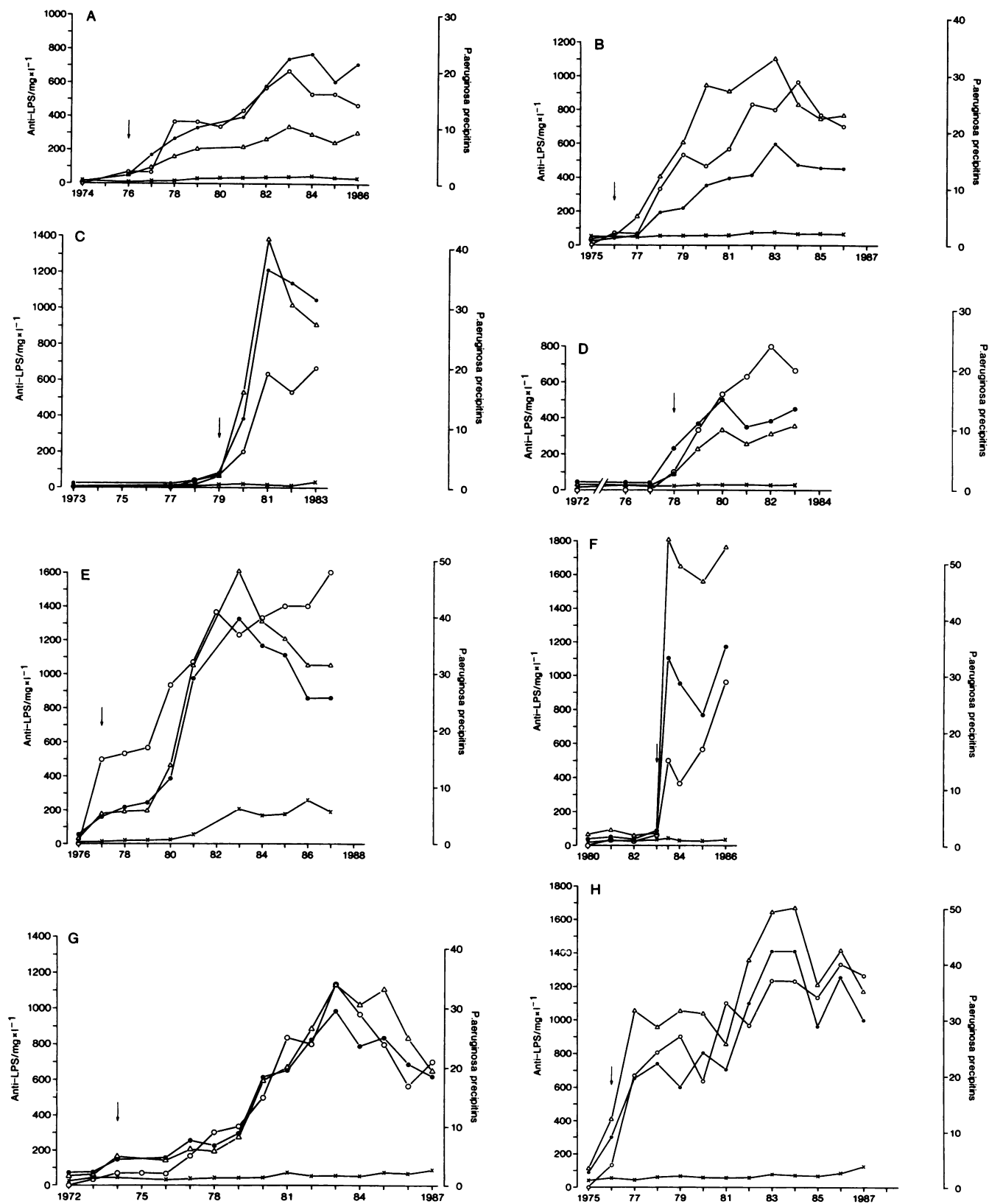


FIG. 1. Longitudinal study (1972 to 1987) of IgG antibodies to LPS from O:3/9 *P. aeruginosa* (●), O:3 *P. aeruginosa* (Δ), and 11 different gram-negative bacteria (×) as well as numbers of precipitins (○) to *P. aeruginosa* standard antigens in serum from CF patients 1 through 8 (A through H, respectively), determined by ELISA and CIE. The arrow indicates onset of chronic *P. aeruginosa* lung infection.

TABLE 3. Prevalence of anti-LPS IgG and IgM concentrations in CF patients and healthy controls

Patient group ^a	Age ^b	No. tested	Concn of IgG against ^c :			Concn of IgM against:		
			O:3 Pa	O:3/9 Pa	LPS pool	O:3 Pa	O:3/9 Pa	LPS pool
Control	0-1	13	3 (1-6)	4 (2-12)	9 (4-19)	1 (1-3)	3 (1-8)	11 (3-32)
	1-2	12	4 (2-8)	6 (2-16)	7 (3-23)	3 (3-4)	9 (5-21)	22 (14-35)
	2-14	15	4 (2-22)	6 (3-14)	8 (3-21)	5 (2-11)	15 (5-33)	17 (12-25)
	18-65	1,000	7	11	12	10	14	23
CF (-P) noncolonized	8 (5-19)	20	19 (3-202)	23 (7-280)	19 (5-87)	10 (3-42)	14 (5-133)	13 (7-96)
CF (-P) colonized	6 (0-11)	8	36 (12-114)	38 (21-96)	34 (4-40)	16 (3-37)	16 (5-20)	34 (4-40)
CF (>1 P) early chronic	7 (1-12)	8	84 (50-418)	104 (42-299)	30 (15-79)	13 (7-29)	11 (9-19)	30 (15-79)
CF (++P) late chronic	22 (12-27)	10	985 (327-1,786)	986 (458-1,898)	70 (26-198)	33 (15-166)	30 (15-109)	69 (12-282)

^a CF (-P), CF patients without precipitins detectable in CIE; CF (>1 P), patients with 1 to 15 precipitins; CF (++P), patients with 15 to 48 precipitins; noncolonized, no bacteria grown from sputum taken at monthly visits to the CF center; colonized, *P. aeruginosa* grown intermittently from sputum; chronic, *P. aeruginosa* grown constantly from sputum for at least 6 months and >1 precipitins detectable in CIE; early, first serum sample containing >1 precipitins; late, chronic infection for 3 to 15 years.

^b Median (range) in years.

^c Median (range) in milligrams per liter. Pa, *P. aeruginosa*; LPS pool, pool of 11 LPSs from gram-negative bacteria other than *P. aeruginosa*.

Reproducibility of ELISA. The relative standard intra- and interassay deviations and the plate-to-plate variation at different days for assay of anti-*Pseudomonas* LPS IgG (range, 17 to 2,616 µg/ml) in 19 serum samples with low ($n = 7$), medium ($n = 5$), and high ($n = 7$) antibody concentrations were 5, 9, and 10%, respectively.

Development of antibody response during chronic infection. Figure 1 shows the results of a longitudinal study (1972 to 1987) of anti-LPS antibodies and antibodies to whole-cell extracts determined by quantitative ELISA and CIE in CF patients during chronic *P. aeruginosa* lung infection. Chronic *P. aeruginosa* lung infection developed during the observation period in eight patients (Fig. 1). Two patients (patients 9 and 10) were already infected when they entered the study. An increase in anti-*Pseudomonas* LPS IgG concentration was observed within the year after onset of chronic infection when an immune response to whole-cell antigens was also established (>1 precipitin). After the initial rise in production of IgG antibody to *P. aeruginosa* LPS, the concentration of specific anti-*Pseudomonas* LPS continued to increase in all 10 patients, whereas the concentration of antibodies to LPS from other gram-negative bacteria remained constant. This increase could be either fast (e.g., in patients 3 and 6) or slow (e.g., in patient 1). In the cases of patients 3 and 6, this increase in speed of anti-LPS immune response was associated with a change in the phenotype of the infecting strain from nonmucoid to mucoid. The profile

of IgG anti-*P. aeruginosa* LPS O:3/9 or O:3 concentration paralleled that of numbers of precipitins against whole-cell antigens in all cases. The correlation coefficients of IgG anti-O:3/9 LPS and anti-O:3 LPS antibodies to the number of precipitins detectable in CIE are given for each of the 10 patients in Table 4.

The concentration of the IgM anti-LPS antibodies to these two *Pseudomonas* LPSs as well as to the LPS pool was elevated compared with concentrations in healthy controls (Table 3) but was constant throughout the course of the chronic infection (data not shown).

SDS-PAGE and immunoblotting. The silver-stained profiles of LPS from six *P. aeruginosa* strains and six other gram-negative bacteria, including two rough strains, are shown in Fig. 2. Only faint bands of high-molecular-weight (M_w) polysaccharides appeared in LPS from the polyagglutinable *P. aeruginosa* strains which are relatively poorly O-substituted (3, 11). Whereas few or no slow-migrating LPS molecules were seen in the silver-stained profiles of the polyagglutinable *P. aeruginosa* strains, the use of hyperimmune CF serum in immunoblotting revealed the presence of bands with higher molecular weight in the O-antigenic region in strains 170 and 14580/83 (Fig. 3, lanes 4 and 6). No high- M_w bands were seen by silver-stain in the SDS-PAGE of the rough *Salmonella* and *E. coli* LPS. In both silver-stained gels and immunoblots, the other LPSs showed a

TABLE 4. Correlation between IgG anti-*Pseudomonas* LPS (O:3/9 and O:3) concentrations and numbers of CIE precipitins in CF patients during chronic lung infection (1972 to 1987)

Patient no.	Correlation coefficient for ^a :	
	Anti-Pa O:3/9	Anti-Pa O:3
1	0.93	0.96
2	0.93	0.93
3	0.98	0.93
4	0.86	0.89
5	0.86	0.88
6	0.91	0.88
7	0.96	0.94
8	0.88	0.88
9	0.82	0.71
10	0.80	0.85

^a Anti-Pa O:3/9 and anti-Pa O:3, IgG anti-*P. aeruginosa* O:3/9 and O:3 antibodies, respectively.

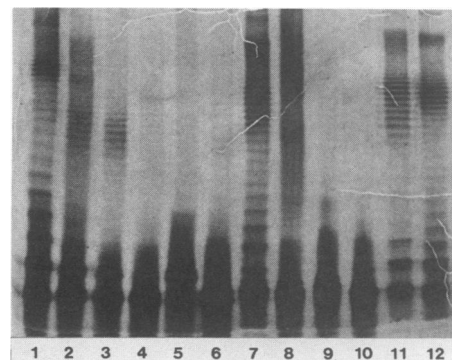


FIG. 2. SDS-PAGE and silver stain of LPS from *P. aeruginosa* strains F-D 1, 1118, 174, 170, 15704/83, and 14580/83; *Shigella sonnei*; *K. pneumoniae*; *S. minnesota* R60; and *E. coli* Ra, O111:B4, and O55:B5 (lanes 1 to 12, respectively).

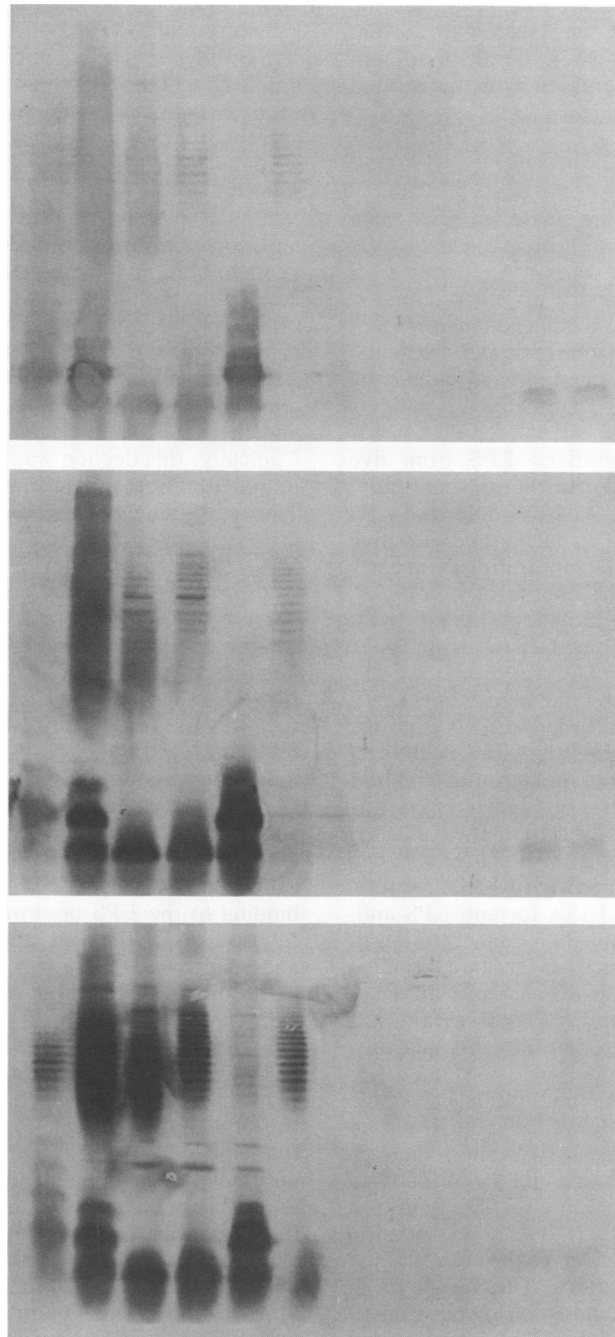


FIG. 3. Immunoblotting of serum (1:10,000) from patient 7 showing the reaction of IgG antibodies to 12 LPSs (described in the legend to Fig. 2) at onset of chronic *P. aeruginosa* lung infection (1974) (top) at development of maximal concentrations of anti-*Pseudomonas* LPS antibody concentration (1983) (middle), and at the time of the most recent serum sample obtained (1987) (bottom).

ladder pattern characteristic of smooth LPS, indicating a mixture of LPS molecules of repetitive antigenic nature.

In a control experiment, high- and low- M_w components of all LPSs could be visualized by a polyspecific anti-LPS IgG preparation (Nordimmun anti-LPS; Nordisk Gentofte A/S, Gentofte, Denmark) obtained from a pool of blood donors with high anti-LPS concentrations (5; data not shown). Serum samples from chronically infected CF patients were reacted with an immunoblot of the 12 LPSs shown in Fig. 2 at three stages of infection: at onset, at maximal anti-LPS antibody concentration, and at the time of the most recent

serum sample available. An example (from patient 7) is shown in Fig. 3. A faint reaction occurred with all *P. aeruginosa* LPSs and two bands of low M_w in *E. coli* LPS (O111:B4 and O55:B5) at the onset of chronic infection. At this early stage, this serum sample (diluted 1:10,000) showed no response to low-molecular-weight material in strain 14580/83. As the chronic infection proceeded, more antigenic determinants of intermediate and high molecular weights were recognized in the *P. aeruginosa* LPSs and the reaction, judged by the density of color, became more intense. However, the antibody binding to low-molecular-

weight components of *E. coli* LPS decreased as the chronic infection proceeded. This pattern was seen with serum samples from all 10 patients, even when the absolute concentration of specific antibodies decreased as demonstrated in patient 7 (Fig. 1).

DISCUSSION

A number of serological tests using single antigens, such as the extracellular enzymes alkaline protease, elastase (2, 10, 18), and phospholipase C (10); exotoxin A (10, 18); LPS (22); proteins F, H₂, and I (20); and a protein "common antigen" (14) have been described and have added important information about the humoral response in CF patients. However, none of these tests has been able to detect the antibody response in all *P. aeruginosa*-infected CF patients (2, 10, 12, 14).

In an earlier study, we characterized LPS from five clinical isolates of *P. aeruginosa* (3). In the present study, the use of two of these purified and well-characterized LPS preparations in ELISA resulted in a specific and sensitive assay which was able to differentiate colonization from early signs of infection and to follow the course of chronic *P. aeruginosa* lung infection in all the CF patients investigated.

Since ELISA measures the combined effect of antibody concentration and affinity, the standard curve method has rarely been used to produce results in absolute units (e.g., specific-antibody concentration). In order to quantitate the actual concentration of anti-LPS antibodies irrespective of affinity and to circumvent the need for standard IgG and IgM anti-LPS antibodies, the standard-free indirect method was used. The results of the present study demonstrate that the concentration of human antibodies to *P. aeruginosa* LPS can be estimated by a combination of sandwich ELISA for total immunoglobulins and an indirect ELISA for anti-LPS antibodies. However, to calculate an absolute concentration, it is necessary to correct for differences in antibody-binding ratios ($1 - [\text{absorbance of plate II}/\text{absorbance of plate I}]$) of the antibodies. The exact interpretation of binding ratios has not been investigated, but it is likely that they are measures of antibody affinity.

Using the anti-LPS ELISA, it was possible to determine early stages of *P. aeruginosa* infection (Table 3). The concentrations of IgG anti-*Pseudomonas* LPS antibodies increased with time in all CF patients examined and followed the numbers of precipitins detectable in CIE. These in turn reflect the severity of the infection (12). The noncorrelation of IgM anti-LPS antibody concentrations to the course of the infection may reflect its chronic nature. The variations in concentration of IgG and IgM in acute *P. aeruginosa* infections and during acute exacerbations in the chronic lung infection in CF might differ and are currently being investigated.

To follow the course of the chronic *P. aeruginosa* infections in CF patients, we chose LPS from a polyagglutinable strain (strain 174 O:3/9), because common antigenic sites are exposed in the LPS of this strain (3). In addition, CF patients infected with *P. aeruginosa* are expected to harbor polyagglutinable strains (11, 16, 28, 29). In fact, all the patients in this study were infected by polyagglutinable *P. aeruginosa* strains involving the reactions with O:3, O:6, O:9, and O:10 antisera. It is interesting that the response in ELISA to the type-specific strain 1118 O:3 resembled the response to the polyagglutinable O:3/9 LPS and to the number of precipitins (Fig. 1). In some of these cases, the reason could be infections with an O:3 strain, which was also the most

common typable isolate in this study (8 of 10 patients) and in previous studies (16, 29). Alternatively, the LPS immunization could give rise to a polyclonal increase of other anti-LPS antibodies (25). The steep increase in anti-LPS antibodies seen in patients 3 and 6 was not related to changes in serotype but to a change from a nonmucoïd to a mucoïd strain. The significance of this finding is not known at present.

All CF patients showed a rapid increase in the numbers and concentrations of specific antibodies during the first years of chronic lung infection (Fig. 1). In most patients, the concentration of anti-LPS antibodies as well as the number of precipitins continued to increase or remained steady. This is in accordance with earlier observations of precipitins (12). In one patient (no. 7), both the number of precipitins and the concentration of anti-*Pseudomonas* LPS IgG reached a peak and then started to decline over a 4-year period (Fig. 1). Clinically, this decline was associated with an improvement in lung function, a gain in weight, and improved well-being. The significance of this observation will be investigated in a greater number of patients.

A high concentration of antibodies to the pool of LPSs from gram-negative bacteria other than *P. aeruginosa* appeared during the chronic lung infections. However, the antigenic sites involved in the antibody binding were seen by immunoblotting to be only in the low- M_w bands of the *E. coli* LPS containing the core-lipid A region (Fig. 3). This observation could be explained by infections with *E. coli* and/or by cross-reacting anti-*Pseudomonas* antibodies, since common antigenic sites reside in this region of the LPSs. In the later stages of infection, the antibody reaction to the *E. coli* antigens decreased in the immunoblotting, whereas the binding to the *Pseudomonas* LPSs seemed to increase (Fig. 3). The measured decrease in the percentage of IgG antibody binding to the LPS pool in ELISA and the increase in the IgG anti-*Pseudomonas* antibody binding (Table 2) are in accordance with these observations and may reflect changes in antibody affinities occurring during a long period of antigenic stimulation with *P. aeruginosa* LPS.

Immunoblotting showed that the IgG anti-LPS antibodies in CF patients bound primarily to *P. aeruginosa* LPS. The number of bands increased with time even when the concentrations of anti-*Pseudomonas* LPS antibodies decreased (patient 7). This possibly indicates a continuous increase in antibody specificity to antigenic sites of low immunogenicity. This is the same phenomenon seen in hyperimmunization of rabbits. It is interesting that while SDS-PAGE showed a rough appearance of LPS from the polyagglutinable strains, the immunoblotting revealed the presence of faint bands in the O-antigenic region. This may indicate material that is immunoreactive when serum from the hyperimmune CF patients is used but that is not well visualized by the silver-staining technique.

The antibody response was directed to LPS from both typable and polyagglutinable *P. aeruginosa* strains. The continuous presence of LPS antigens in this chronic infection appears to result in an extreme antibody response with increases in concentration, specificity, and affinity. The high concentration of anti-LPS antibodies in CF patients may play an important role in protecting against systemic endotoxemia (23). The anti-LPS antibodies may also prevent the systemic spread of infection (23, 30) although the O-deficient *P. aeruginosa* strains are unlikely to cause bacteremia because most of these strains are sensitive to complement lysis (15). The extremely high concentrations of anti-LPS antibodies found in these patients also support the idea that

terminal CF is an immune disease (17). This immune disease may involve LPS-anti-LPS immune complexes. The effects of anti-LPS antibodies in the circulation and locally in the lungs of CF patients require further studies.

The results demonstrate that LPS is a strong immunogen which can be used as a well-defined single antigen in sensitive methods like ELISA and immunoblotting to diagnose and follow the course of chronic *P. aeruginosa* lung infection in CF patients. An ELISA for IgG antibodies to purified LPS from the most common clinical isolate of *P. aeruginosa* or to the LPS from O-deficient polyagglutinable strains in which common LPS antigens are exposed is recommended for this purpose. The anti-LPS ELISA is an alternative to CIE or the ELISA using mixed *P. aeruginosa* whole-cell extracts previously described in studies from our laboratory (13, 21). In the present ELISA, the antigen was defined and is of considerable relevance because of the high biological activity and the toxic properties of LPS-endotoxin. Other advantages of the anti-LPS ELISA are the ease of handling a large number of samples, the higher sensitivity, and the ability to distinguish the different classes of antibodies. These properties could be useful in future diagnosis and research on the antibody response in CF.

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