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Development of an Enzyme-Linked Immunosorbent Assay for Studying *Pseudomonas aeruginosa* Cell Surface Antigens

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An enzyme-linked immunosorbent assay for the measurement of antibodies directed against *Pseudomonas aeruginosa* cell surface antigens was developed. Formalin-killed whole cells of *P. aeruginosa*, adsorbed to polystyrene acrylic copolymer cuvettes, were used as immobilized antigens. Antisera to *P. aeruginosa* mucoid strain 144M and to its spontaneous nonmucoid derivative, 144NM, were raised in rabbits by immunization with Formalin-killed bacteria. By using this enzyme-linked immunosorbent assay, anti-144M serum was found to have a ca. 10-fold-higher antibody titer to 144M than did anti-144NM serum, suggesting that 144M may have either immunogenic determinants not present on 144NM or perhaps simply more antigenic determinants. In contrast, anti-144M and anti-144NM immune sera were found to have nearly identical antibody titers to 144NM, suggesting that these strains share many determinants. Anti-*P. aeruginosa* immune serum was found to contain *Pseudomonas*-specific antibodies as well as antibodies which cross-reacted with other gram-negative bacteria. Finally, absorption studies demonstrated that this assay can detect both LPS and non-LPS surface-exposed antigenic determinants. Thus, this whole bacterial cell enzyme-linked immunosorbent assay should prove useful in monitoring patient sera and secretions for potentially protective immunoglobulins directed at *P. aeruginosa* cell surface antigens.

Pseudomonas aeruginosa is a ubiquitous, gram-negative saprophytic organism. Although normal healthy individuals are rarely infected by members of the genus *Pseudomonas*, individuals with burns, neoplastic disease, granulocytopenia, immunological deficiencies, or cystic fibrosis (CF) or patients who have received intensive immunosuppressive therapy for connective tissue disorders or as a consequence of organ transplantation are particularly susceptible to *Pseudomonas* infection (1).

Humoral antibody titers to *P. aeruginosa* have been detected by a number of different assays and techniques, most of which utilize either bacterial products or sonicated bacterial cells as antigens (see reference 11 for review). Studies on sera of CF patients with chronic *P. aeruginosa* respiratory tract infections have demonstrated the presence of humoral antibodies to the extracellular enzymes alkaline protease, elastase, and exotoxin A, using a radioimmunoassay (4, 16) or an enzyme-linked immunosorbent assay (ELISA) (15). Using crossed immunoelectrophoresis and sonicated bacterial cells, Hoiby and Axelsen (12) detected as many as 22 precipitins in CF patient sera but did not demonstrate protective antibodies. Crossed immunoelectrophoresis has also been used to detect antibodies to a "common antigen" from *P. aeruginosa* (13) and, more recently, to lipopolysaccharide (LPS) and to three outer membrane proteins F, H_2, and I (17). However, to measure antibodies necessary to promote *Pseudomonas* clearance by the host, an assay which could determine antibody titers to bacterial surface-exposed antigenic determinants on intact bacteria might be more appropriate.

Although antibodies against whole *Pseudomonas* organisms have been assayed by techniques such as bacterial agglutination or indirect immunofluorescence (see reference 11 for review), a whole bacterial cell ELISA is much more sensitive and quantitative. The use of whole organisms avoids the complications of bacterial antigen purification techniques. In addition, the antigenic orientation of the isolated components may not mimic that observed in the normal state. The whole bacterial cell ELISA technique offers the advantage of measuring antibodies directed to surface-exposed antigenic determinants, both LPS and proteins, when presented in the same steric arrangement and epitope density observed by the host. Therefore, it should more accurately reflect the presence of antibodies which might serve a protective function in vivo. Several investigators have successfully utilized a whole bacterial cell ELISA to quantitate humoral antibody levels to various bacterial cell surface antigens (3, 9, 14). This study examines the use of a whole bacterial cell ELISA to measure humoral antibody levels to *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A *P. aeruginosa* mucoid strain, 144M (obtained from the sputum of a patient with CF), and its spontaneous nonmucoid derivative, 144NM, were maintained on brain heart infusion (BHI) agar (BBL Microbiology Systems, Cockeysville, Md.) at 37°C in 5% CO_2 and transferred every 18 to 24 h. Both 144M and 144NM colonial phenotypes were stable on BHI agar. *Klebsiella pneumoniae* (ATCC 13883) and *Serratia marcescens* (ATCC 274) were obtained from the American Type Culture Collection, Rockville, Md., whereas a common laboratory strain of *Escherichia coli* (origin unknown) was obtained from the Microbiology Prep Facility, Biology Department, University of California, Riverside.

Rabbit antisera. Antisera to 144M and 144NM were prepared in rabbits, using Formalin-treated cells. Each strain was grown overnight on BHI agar at 37°C in 5% CO_2, swabbed into 0.01 M phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was then resuspended in PBS, and the concentration was adjusted spectrophotometrically (at 550 nm) to ca. 10^9 CFU/ml with PBS. (To confirm the bacterial cell number, a

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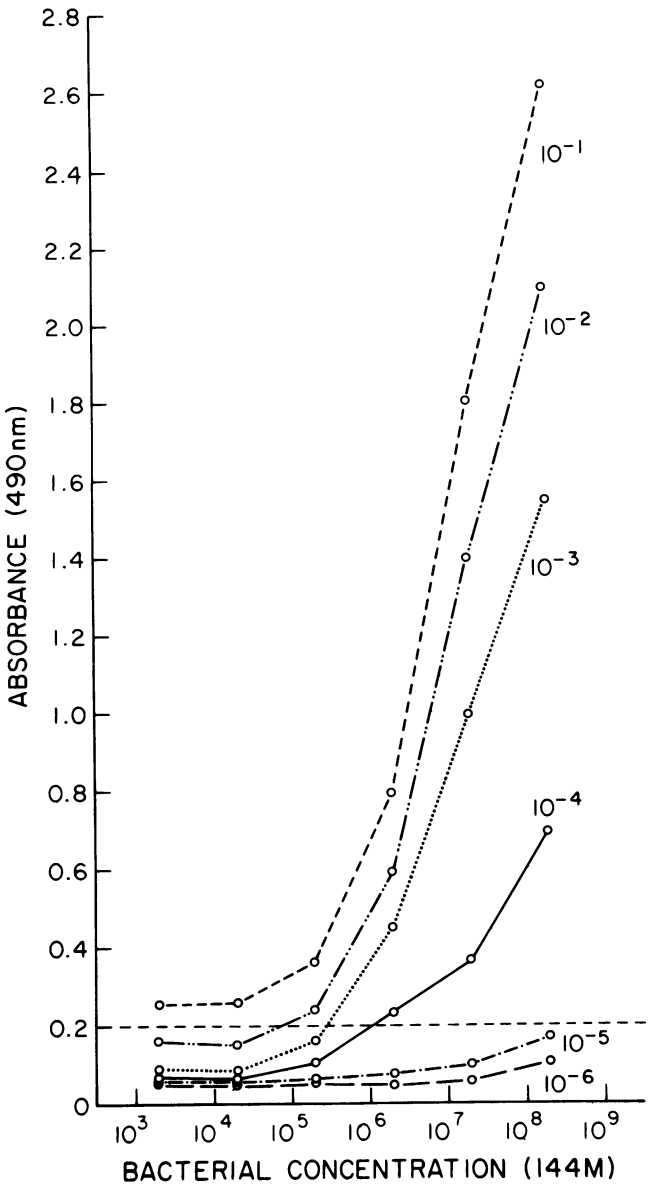

FIG. 1. Comparison of the antibody titer in anti-144M immune serum when various concentrations of *P. aeruginosa* 144M were used as the antigen. Each curve represents a separate antiserum dilution (as indicated in the figure), and each point represents the average of six determinations (three separate experiments, each done in duplicate).

sample of the cell suspension was removed, serially diluted in PBS, plated onto BHI agar, and incubated overnight at 37°C in 5% CO_2, and the number of CFU per milliliter was determined.) Formalin was added to the cell suspension to a final concentration of 0.3% formaldehyde, and the cell suspension was maintained at 4°C overnight. The cells were pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, washed twice, and resuspended in PBS to a final concentration of 10^9 CFU/ml. The bacterial suspension was mixed with an equal volume of Freund incomplete adjuvant, and a total of 10^9 CFU was inoculated intramuscularly in 1.0-ml volumes at two sites. A subsequent injection of 10^9 CFU was given in the same manner 2 weeks later. Rabbits were bled 2 weeks after the booster, and the immune sera were collected, heated at 56°C for 30 min, and stored at −70°C in small portions.

ELISA. An ELISA for the detection of antibody directed against cell surface antigens of *P. aeruginosa* was developed based on the principles pioneered by Engvall et al. (6–8). Disposable cuvettes (Cuvette-Paks; Gilford Diagnostics, Cleveland, Ohio) were used as the solid support. Each bacterial strain was grown overnight on BHI agar plates at 37°C in 5% CO_2, swabbed into PBS, and centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was resuspended in PBS to a concentration of ca. 10^9 CFU/ml and Formalin-killed as described above. After the second wash, the Formalin-treated bacteria were resuspended to a final concentration of 10^9 CFU/ml in coating buffer, pH 9.6 (1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ per liter of aqueous solution). To each cuvette was added 200 μl of cell suspension (or in some experiments, 200 μl of purified LPS at a concentration of 100 μg/ml of distilled water), followed by overnight incubation at 4°C. Coated cuvettes were then stored at 4°C and used within a week.

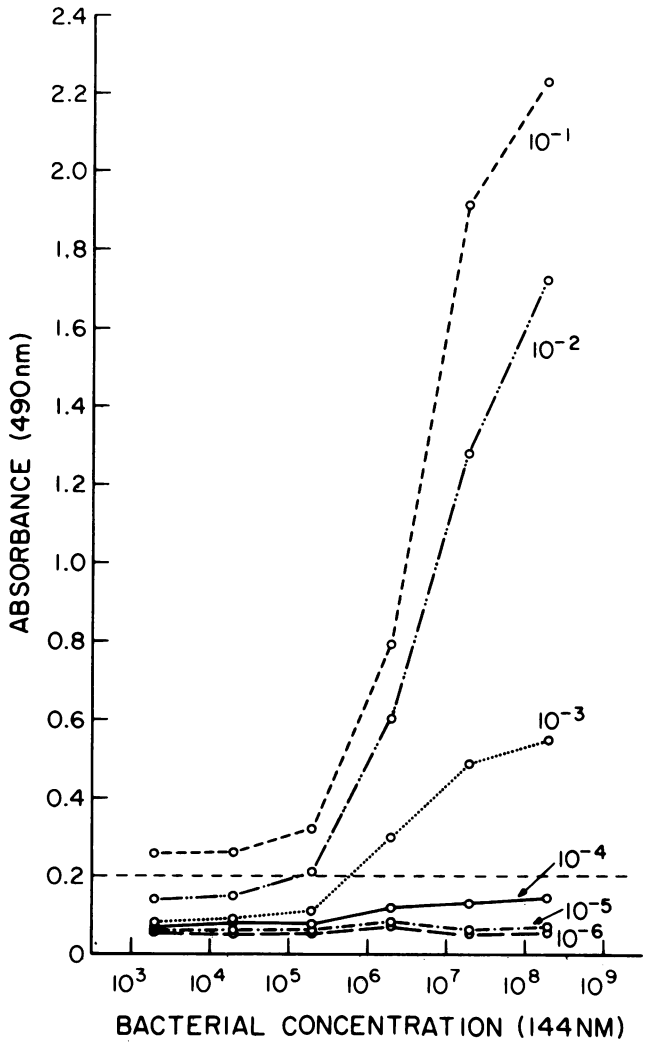

FIG. 2. Comparison of the antibody titer in anti-144NM immune serum when various concentrations of *P. aeruginosa* 144NM were used as the antigen. Each curve represents a separate antiserum dilution (as indicated in the figure), and each point represents the average of six determinations (three separate experiments, each done in duplicate).

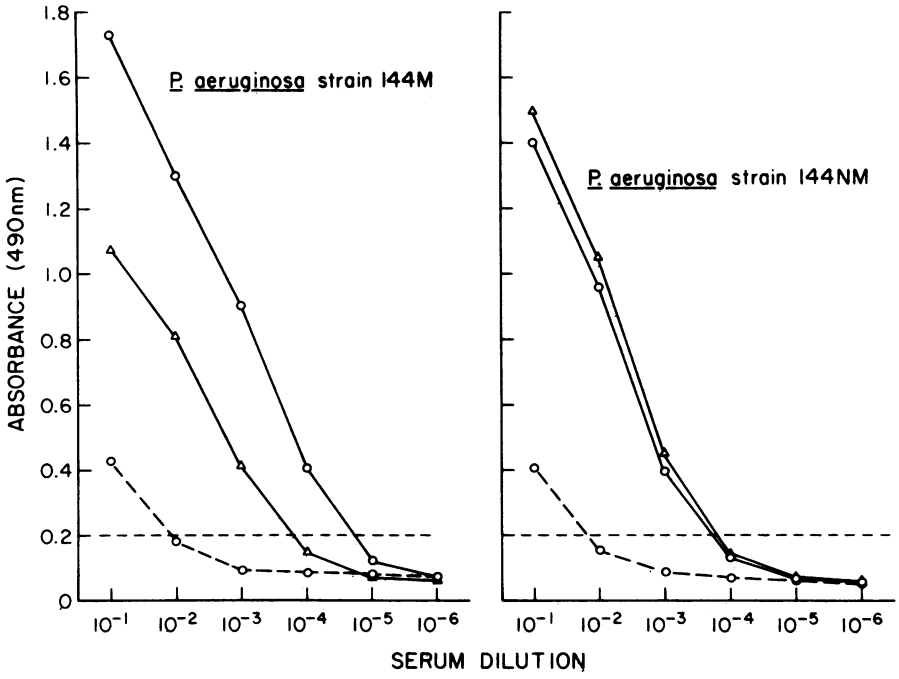

FIG. 3. Comparison of the antibody titrations of anti-144M immune, anti-144NM immune, and nonimmune sera for *P. aeruginosa* 144M and 144NM. The values for anti-144M immune serum, using 144M as the antigen, represent the average of 12 determinations (six separate experiments, each done in duplicate). All other values represent the average of six determinations (three separate experiments, each done in duplicate). Symbols: O—O, anti-144M immune serum; Δ—Δ, anti-144NM immune serum; O- -O, nonimmune control serum.

Coated cuvettes were washed three times with 0.1% Tween in PBS, pH 7.4 ([per liter of aqueous solution] 8.0 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KCl, 1.0 ml of Tween 20), subsequently referred to as PBS-Tween. The cuvettes were filled with PBS-Tween for 5 min between each wash. To each cuvette was added 200 μl of serially diluted rabbit antiserum or, as a control, serially diluted 5% lysozyme (Sigma Chemical Co., St. Louis, Mo.). (Dilutions were prepared in PBS-Tween.) The cuvettes were covered and incubated in a moist chamber for 2 h at 37°C and then washed three times with PBS-Tween, with 5 min of soaking between each wash. Donkey anti-rabbit immunoglobulin [$F(ab')_2$] conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, Ill.) was prepared as a reagent daily. Preliminary experiments determined that a 1:1,000 dilution of stock solution with PBS-Tween was optimal. After adding 200 μl of anti-rabbit immunoglobulin [$F(ab')_2$)] peroxidase-linked reagent to each coated cuvette plus two uncoated cuvettes (blanks), the cuvettes were covered and incubated in a moist chamber for 2 h at 37°C. The cuvettes were then washed three times with PBS-Tween, with 5 min of soaking between each wash. After adding 200 μl of substrate solution, pH 5.0, containing 10 mg of *o*-phenylenediamine, 75 μl of 3% H_2O_2, and 25 ml of citrate phosphate buffer (24.3 ml of 0.1 M citric acid–25.7 ml of 0.2 M sodium phosphate–50 ml of distilled water) to each coated cuvette and to each of the two blanks, the cuvettes were incubated for 20 min in the dark at room temperature. Afterwards, 100 μl of 4 N H_2SO_4 was added to each cuvette to stop the reaction. Absorption at 490 nm was measured with a Gilford EIA Manual Reader (Gilford Diagnostics). The two controls, i.e., the cuvettes containing either the serially diluted lysozyme or the reagent blanks, consistently produced absorbance values of ≤0.1. Absorbance readings greater than or equal to twice this background value (i.e., ≥0.2) were considered positive.

LPS isolation. *P. aeruginosa* 144M was grown overnight at 37°C, with shaking in 500-ml BHI broth lots; a total of 12 liters was harvested by centrifugation at 10,000 × *g* for 10 min at 4°C and washed twice with saline; and the pellet was resuspended in distilled water and lyophilized. LPS was isolated from 144M by a recently described protocol of Darveau and Hancock (5).

Absorption of antisera. Bacteria were grown overnight on BHI agar, swabbed into PBS, pelleted by centrifugation at 10,000 × *g* for 10 min at 4°C, washed twice with saline, and resuspended in PBS to a concentration of 10^9 bacteria per ml. Formalin was added to the cell suspension to a final concentration of 0.3% formaldehyde, and the cell suspension was incubated at room temperature for 30 min. A total of 100 ml of Formalin-treated bacteria (10^{11} bacteria) was then centrifuged, washed twice with saline, resuspended in 7 ml of PBS, and added to 1 ml of undiluted immune rabbit serum. The mixture was incubated at 37°C for 1 h with gentle tumbling, and the cells were removed by centrifugation at 20,000 × *g* for 30 min at 4°C. The supernatant was then used to resuspend a second pellet of bacteria (10^{11} bacteria), and this mixture was incubated overnight at 4°C with tumbling. The absorbed serum was clarified by centrifugation at 20,000 × *g* for 30 min at 4°C, followed by 0.45-μm sterile filtration. The absorbed serum was then stored at −70°C in small portions until ready for use.

Immune antiserum was absorbed with purified LPS by mixing sera with an equal volume of LPS solution (5 mg of LPS per ml of distilled water). This mixture was tumbled at 4°C for 24 h. The absorbed serum was clarified by centrifuga-

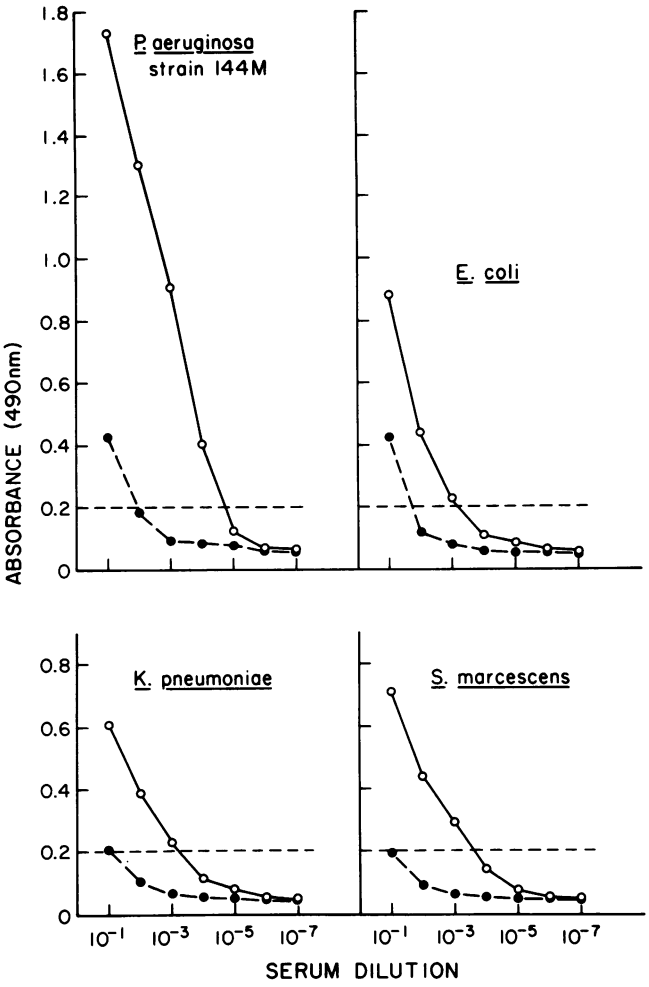

FIG. 4. Cross-reactions between *P. aeruginosa* 144M and three other gram-negative bacteria. Each strain was used at a concentration of 2×10^7 Formalin-treated bacteria added per cuvette. Values for *P. aeruginosa* 144M and anti-144 immune serum represent the average of 12 determinations (six separate experiments, each done in duplicate). All other values represent the average of six determinations (three separate experiments, each done in duplicate). Symbols: ○, anti-144M immune serum; ●, nonimmune control serum.

tion at 45,000 × *g* for 30 min at 4°C, followed by 0.45-μm sterile filtration, and the serum was stored at −70°C in small portions until ready for use.

RESULTS

Preliminary studies established that Formalin-killed bacterial cells of *P. aeruginosa* physically attach to polystyrene acrylic copolymer cuvettes in a manner which allows their use as antigens in an ELISA. The results of a checkerboard-type titration, using various concentrations of Formalin-treated 144M (Fig. 1) or 144NM (Fig. 2) and various dilutions of their respective immune antisera are shown. A positive sample was classified as one which gave an absorbance value of ≥0.2 optical density at 490 nm units (essentially greater than or equal to twice that observed in control cuvettes). Although maximal absorbance values were obtained when cuvettes were coated with 2×10^8 CFU, excellent results were also obtained when 2×10^7 CFU was used to coat the ELISA wells. This concentration required fewer cells, produced readable absorbance values for serum dilutions of 10^{-1} to 10^{-4} for 144M (10^{-1} to 10^{-3} for 144NM) and the same relative antibody titers for 144M (10^{-4}) and 144NM (10^{-3}) as did 2×10^8 CFU per well, and occurred within a reasonable substrate reaction time (20 min). Therefore, all further experiments were performed with 2×10^7 CFU per well.

Antiserum raised against either 144M or 144NM produced the highest absorbance values when reacted with its homologous antigen (Fig. 3). Anti-144M immune serum was found to have a ca. 10-fold-higher antibody titer to 144M than did anti-144NM immune serum. This might suggest that 144M has immunogenic determinants not present on 144NM or simply a greater number of antigenic determinants than 144NM. In contrast, both anti-144NM and anti-144M immune sera were equally effective against 144NM, suggesting that these strains share many determinants. As expected, nonimmune control serum gave low absorbance readings, all considered to be negative except for that at a 10^{-1} dilution, which could reflect trace amounts of cross-reacting antibodies to common gram-negative bacterial antigens.

To examine the specificity of immune serum prepared against *P. aeruginosa* 144M, the antibody titer of anti-144M immune serum for several gram-negative bacteria was determined. In this experiment cuvettes were coated with 2×10^7 CFU of *P. aeruginosa* 144M, *E. coli*, *K. pneumoniae*, or *S. marcescens*. Anti-144M immune serum had elevated levels of antibodies directed to determinants present on all four strains, when compared with levels of nonimmune control serum (Fig. 4). This implies that at least some antigens were shared on these four gram-negative microorganisms. When the absolute titers of anti-144M immune serum for each strain were determined, the antibody titer of anti-144M immune serum was at least 10-fold greater for 144M than for any of the three other strains (Table 1) and 812-fold greater than that of nonimmune serum for 144M.

To determine whether this whole cell ELISA can detect antibodies to both LPS and non-LPS surface antigens, anti-144M immune serum was absorbed with either whole 144M organisms or purified 144M LPS. Absorption with whole 144M bacteria removed most antibodies directed at either whole organisms or isolated LPS (Table 2). Specific absorption with 144M LPS similarly reduced most antibodies directed against LPS but only reduced the anti-144M immune titer to 144M whole cells by one half, which suggests that antibody to surface components other than LPS can be detected by this assay.

DISCUSSION

The purpose of this project was to develop an ELISA which could be used to detect and quantitate humoral antibodies directed against cell surface antigens of *P. aeruginosa*. The major advantage of this method is the ability to

TABLE 1. Cross-reactivity of anti-144M immune serum with strain 144M and three heterologous gram-negative strains

Bacterium	Immune titer[a]	Nonimmune titer[a]	Immune titer/nonimmune titer
P. aeruginosa 144M	6.5×10^4	8.0×10^1	812.5
S. marcescens	4.0×10^3	5.0×10^1	80
K. pneumoniae	2.0×10^3	1.0×10^1	200
E. coli	1.6×10^3	1.0×10^1	160

[a] ELISA titers are expressed as the reciprocal of the dilution of serum which gave an absorbance reading of 0.2 and are derived from the data presented in Fig. 4.

TABLE 2. Absorption of anti-144M immune serum with *P. aeruginosa* 144M whole cells or LPS

Anti-144M immune serum	Titer[a]	
	144M ELISA[b]	LPS ELISA[c]
Unabsorbed	65,536	131,072
Absorbed with 144M whole cells[d]	8,192	4,096
Absorbed with 144M LPS[d]	32,768	4,096

[a] ELISA titers are expressed as the reciprocal of the dilution of serum which gave an absorbance reading of 0.2 and represent the average of six determinations (three separate experiments, each done in duplicate).

[b] Cuvettes were coated with 2×10^7 Formalin-killed *P. aeruginosa* 144M.

[c] Cuvettes were coated with 20 μg of isolated LPS from *P. aeruginosa* 144M.

[d] Absorptions were performed as described in the text.

use intact organisms as the antigenic target. We believe that this method best recreates the orientation and steric arrangement of all outer membrane components of *P. aeruginosa* seen by the host and to which protective antibodies produced by the host would have to be directed.

Quantitative titers were easily obtained by using a Gilford EIA Manual Reader. Initially, the combination of antigen-serum dilutions which gave the best separation between high and low samples and low negative values was evaluated. For our purposes, coating the cuvettes with 2×10^7 Formalin-treated CFU of *P. aeruginosa* gave excellent, reproducible results. The antibody titer was then determined to be the dilution of serum producing an absorbance value of 0.2 optical density at 490 nm units.

Rabbits immunized with Formalin-treated, intact *P. aeruginosa* produced elevated antibody titers to the organism (as compared with the titers of nonimmune rabbits), indicating that the immunocompetent host produces antibodies to *P. aeruginosa* structures exposed on the bacterial surface. Antiserum raised against either 144M or 144NM produced the highest absorbance values when combined with its homologous antigen. Furthermore, anti-144M immune serum was found to have a ca. 10-fold-higher antibody titer to 144M than did anti-144NM immune serum. This would suggest that 144M has immunogenic determinants not present on 144NM, in agreement with results seen with bactericidal and opsonophagocytic assays described previously (2). Alternatively, 144M may have similar but quantitatively more immunogenic determinants than 144NM. In contrast, both anti-144NM and anti-144M immune sera were equally effective against 144NM, suggesting that these strains share many determinants. Again, these results are in concert with our earlier findings (2).

Anti-*P. aeruginosa* 144M immune serum was found to cross-react with strains of *E. coli*, *K. pneumoniae*, and *S. marcescens*. These results demonstrate that *P. aeruginosa* shares antigens in common with other gram-negative bacteria, perhaps the common antigen described by Høiby et al. (13) and Sompolinsky et al. (18, 19). This ELISA can also discern noncommon antigens, however, since the immune titer was always greater for *P. aeruginosa* than for the other gram-negative bacteria.

Although various immunodiagnostic assays have been described to quantitate antibodies to *P. aeruginosa* LPS, outer membrane proteins, or extracellular products, this whole cell ELISA enables one to monitor the antibody titer to surface-exposed antigenic determinants on intact organisms. As shown here, this includes antibodies to both LPS and non-LPS surface components. In an earlier study (N. L. Schiller, D. R. Hackley, and A. Morrison, Curr. Microbiol., in press), we determined that 144M LPS is primarily of the "short" or "rough" LPS type, which agrees with the observations of Hancock et al. (10), who determined that LPSs of strains isolated from patients with CF are often rough LPSs. Although the LPS from 144M is immunogenic, it is not the only major antigenic determinant on 144M, since absorption of anti-144M immune serum with 144M LPS removed only 50% of the antibodies directed to 144M whole bacteria. This result argues for a closer look at other immunogenic surface components, such as outer membrane proteins, for potential candidate vaccine agents for CF patients, an argument recently voiced by Lam et al. (17).

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