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Pseudomonas aeruginosa Lipopolysaccharide Binds Galectin-3 and Other Human Corneal Epithelial Proteins

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The aim of this study was to test whether galectin-3 is present in human corneal epithelium and whether lipopolysaccharide (LPS) purified from *Pseudomonas aeruginosa* ATCC 19660 binds to this animal lectin and/or to another human corneal epithelial protein(s) (HCEP) and to confirm which component of LPS (inner or outer core or lipid A) is important in bacterial binding by using the eye in organ culture. LPS isolated and purified from *P. aeruginosa* ATCC 19660 and a commercial LPS (serotype 10) differed in polyacrylamide gel analysis but bound similarly to blotted HCEP. Binding was determined to be a receptor-ligand type of interaction by the solid-phase assay, because it was both specific and saturable. Several LPS binding proteins in HCEP were identified by an overlay method. Western blotting with antibody against galectin-3 revealed the presence of this protein in both freshly isolated and cultured transformed human corneal epithelium. Binding inhibition assays showed that antibody specific for the outer core region of LPS and an anti-galectin antibody significantly inhibited bacterial binding in vitro. These data provide further evidence that LPS is an important adhesin of *P. aeruginosa*, that it binds to protein receptor molecules in HCEP, that one of the LPS binding proteins is galectin-3, and that the outer core portion of the molecule appears to be critical for LPS binding to the eye.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that poses a major threat to immunocompromised individuals such as patients with cancer, burn wounds, or cystic fibrosis (55). Also, it produces a fulminating, highly destructive corneal infection in humans which may result in decreased visual acuity or blindness (33). *Pseudomonas* keratitis causes rapid liquefactive destruction of the human cornea and is observed with increasing frequency following the use of extended-wear soft contact lenses (8, 21). The keratitis produced by the bacterium is difficult to treat, progresses rapidly, and often leads to extensive corneal scarring and the need for transplantation (12, 33).

For invasiveness and keratitis to occur, *Pseudomonas*, like many other microbes, must first adhere to host tissue (6). It is generally believed that this is the initial step necessary in the development of an infection at a mucosal surface and that it involves the specific adherence of bacteria to the involved epithelial cells (41, 48). The mechanisms by which *P. aeruginosa* adhere to host tissue, in particular to the corneal epithelium, are not completely understood. Analysis of the mechanisms of *P. aeruginosa* adhesion to the mouse cornea have revealed that receptor-adhesin interactions are multifactoral and require corneal scarification in the adult animal (24, 47) but not in the immature animal (24). As well as age (22), the lipase sensitivity (23), the presence of sialic acid on gangliosides (52), and the nature of glycosidic linkages (30) all modulate the adherence of *P. aeruginosa*. In addition, we recently have demonstrated that bacterial exoproducts such as alkaline protease and elastase significantly increase the binding of *P. aeruginosa* to mouse corneal epithelium in vitro (18). *P. aeruginosa* also is unique among opportunistic pathogens in that it produces a large number of other potential virulence factors such as toxin A, exoenzyme S, phospholipase C, hemolysin, alginate, and lipopolysaccharide (LPS), which also potentially contribute to ocular pathogenesis (27, 35, 38, 57). A previous study from this laboratory has shown that pili and LPS are adhesins of *P. aeruginosa* which bind to the glycolipid asialo GM_1 (17). We also have shown that pili of *P. aeruginosa* bind to several mouse epithelial glycoproteins (52) and human epithelial glycoproteins (61) and that three of the human (62) and four of the mouse (25) glycoproteins belong to the intermediate filament class of cytoskeletal proteins. These studies are prototypical of the present investigation, which continues to elucidate the role of virulence factors in the pathogenesis of ocular *P. aeruginosa* infection.

LPS is the major component of the outer membrane of *P. aeruginosa* and is a well-known virulence factor which promotes infection by interference with the host immune response (9, 56). The principal target cell for LPS appears to be the macrophage, which displays numerous acute (minutes) and long-term (hours) responses to LPS, including increased phosphatidylinositol turnover (44, 64), increased arachidonic acid release and eicosanoid formation (31), priming (1), and the synthesis of tumor necrosis factor (7), and interleukin-1 (29). LPS also triggers a wide range of other cellular responses in mammalian cells. The exposure of eukaryotic cells to LPS at the picogram-per-milliliter level may cause changes in shape, metabolism, or gene expression (58). Recently, Zaidi et al. (63) have reported that the outer core region of LPS is a ligand for corneal cell binding and ingestion of *P. aeruginosa*. Their results suggest that the outer core with a terminal glucose residue is necessary for maximal association and entry of *P. aeruginosa* into corneal cells. In addition, interest in various LPS binding proteins is growing (5). Galectins, previously known as S-type or s-Lac lectins, are a family of soluble animal lectins with affinity for terminal β-galactose residues (4, 5). Detailed functions of galectins are not known, but they are presumed to function in important biological processes, such as modulating cell-cell and cell-matrix interactions (3). The results of Mey et

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al. (39) demonstrate that bacterial LPS interacts with galectin-3 at two independent sites. Several other LPS binding molecules also have been described; these include phosphatidylinositol-anchored CD14 (60), which is involved in LPS induction of tumor necrosis factor, and the CD11b/CD18 integrin, (59), both expressed on monocytes. In addition, the scavenger receptor for low-density lipoprotein on macrophages is involved in LPS clearance from plasma (20). Despite all this information about the function of LPS, no study has examined human ocular epithelium for LPS-specific binding proteins such as galectin. Hence, the purpose of this study was to isolate LPS from *P. aeruginosa* ATCC 19660, to study its binding to human corneal epithelium, to begin to characterize the receptor protein(s), and to use an antibody inhibition assay to confirm which region of LPS is of biological significance for binding to cornea.

MATERIALS AND METHODS

Bacterial cell culture conditions for isolation of LPS. *P. aeruginosa* (American Type Culture Collection [ATCC], Rockville, Md.) ATCC 19660 was used to inoculate broth medium containing 5% peptone and 0.25% tryptic soy broth (Difco Laboratories, Detroit, Mich.) (24). Approximately 320 ml of medium was added per 1-liter flask. Cultures were grown on a rotary shaker at 37°C for 18 h. The bacteria from 30 to 40 flasks were pelleted by centrifugation at 7,000 × *g* for 20 min at 4°C, and the pellet was used to isolate LPS.

Isolation, purification, and characterization of LPS. The method of Darveau and Hancock (10) was used to isolate and purify LPS from *P. aeruginosa* ATCC 19660. LPS was characterized by polyacrylamide gel electrophoresis by the method of Apicella et al. (2) with a 12% polyacrylamide gel. LPS was solubilized in Laemmli sample buffer (without sodium dodecyl sulfate [SDS]) at 1.0 mg/ml and loaded onto the gel (10 μg/lane). The gel was run at 10 mA through a stacking gel, and the current was then raised to 15 mA. After electrophoresis, the bands were visualized by a silver-staining method (54) to determine sample purity.

Binding of biotin-labeled LPS in a solid-phase binding assay. Purified LPS (*P. aeruginosa* ATCC 19660) and LPS serotype 10 (Sigma; prepared from *P. aeruginosa* ATCC 27316) were labeled with biotin by the procedure of Prasadarao et al. (42). The biotin derivative NHS-LC-biotin (Pierce, Rockford, Ill.) was dissolved in phosphate-buffered saline (PBS) (2 mg/ml). Similarly, the purified LPS from strain ATCC 19660 or Sigma was dissolved in PBS at 2.0 mg/ml. Equal volumes of each were mixed, and the mixtures were incubated for 60 min on ice, centrifuged at 6,000 × *g* for 20 min, and washed with PBS to remove free biotin.

A solid-phase binding assay was used to test the binding interaction between biotin-labeled LPS and HCEP (from human corneal epithelium obtained from the National Disease Research Interchange, Philadelphia, Pa.) as described previously (17, 62). For the assay, a 96-well plate (Immulon II; Dynatech Laboratories, Inc., Chantilly, Va.) was coated with solubilized HCEP (0.5 μg/well) overnight at 4°C. Control wells were coated with Tris-buffered saline (TBS) only. The next day, the plate was inverted quickly to remove excess buffer, and nonspecific binding was blocked with 3% bovine serum albumin (BSA) in TBS. The plate was washed three times with TBS and incubated with 50 μl of biotin-labeled LPS (5 μg/well) for 2 h at 37°C. For competition experiments, the wells were treated with a 10-fold excess of unlabeled LPS 30 min before the addition of biotin-labeled LPS. After incubation, the wells were again washed three times. The wells were treated with avidin-peroxidase (1:1,000 dilution) for 30 min at 37°C. After the wells were washed, the amount of biotin-labeled LPS bound was determined, after color development with orthophenylene diamine (0.4 mg/ml) in 0.1 M citric-phosphate buffer (pH 8.0)–0.005% H_2O_2, by measuring the optical density at 492 nm.

Saturation binding curve of biotin-labeled LPS to HCEP. We also tested LPS from strain ATCC 19660 for saturability. The saturation curve was generated as previously described (17, 62). In this experiment, each well was coated with a known amount of HCEP (0.5 μg/well) overnight at 4°C. The next day, excess buffer was removed and nonspecific sites were blocked with 3% BSA in TBS. After being washed, the wells were incubated with nine different concentrations of biotin-labeled LPS (ranging from 0.048 to 12.5 μg/well) for 3 h at room temperature (RT). In some wells, an excess of unlabeled LPS was added 30 min before the addition of biotin-labeled LPS. After incubation, the wells were washed, treated with peroxidase-conjugated streptavidin, and processed for color development. Finally, after measurement of the optical density at 492 nm, the specific amount of biotin-labeled LPS was calculated by subtracting nonspecific from total binding values. Scatchard analysis of these data was done with a nonlinear curve-fitting program (BDATA; EMF Software, Knoxville, Tenn.). The data were analyzed for either one or multiple populations of binding sites, together with statistical analysis comparing "goodness for fit" between a one- or two-affinity-state model.

Separation of HCEP by SDS-PAGE and analysis of LPS binding by the overlay assay. HCEP were prepared as described recently (16, 62). HCEP were separated by SDS-polyacrylamide gel electrophoresis (PAGE) with 10% polyacrylamide gels as described by Laemmli (32). HCEP (7.0 μg) were loaded in each lane and electrophoresed at a constant current of 20 mA/gel (Mini Protean II Dual Slab Cell; Bio-Rad, Richmond, Calif.). R-galectin was loaded at 200 ng/lane and run similarly. After separation, the proteins were transferred to a nitrocellulose membrane (Bio-Rad) at a constant voltage of 100 V for 1 h at 4°C (Mini Trans Blot Electrophoresis Transfer Cell; Bio-Rad) by the method of Towbin et al. (53). After the transfer, nonspecific binding sites were blocked with 1% BSA in PBS for 1 h at RT. After blocking, blots were washed three times with PBS at RT. The blots were incubated with biotin-labeled LPS (50 μg/ml in PBS) for 3 h at 37°C. Control blots were treated similarly, except that they were incubated with unlabeled LPS. After incubation, the blots were washed three times with PBS and incubated for 30 min at RT with streptavidin conjugated with peroxidase (4 μg/ml in PBS). Finally, the blots were washed three times with PBS and processed for visualization of protein bands. The bands were visualized by color development with 0.1% diaminobenzidine in 1% aqueous imidazole solution–0.02% hydrogen peroxide as described by Prasadarao et al. (42). For reprobing of blots, the method of Kaufmann et al. (28), which allows removal of the signal while preserving the originally mobilized polypeptides, was used. Briefly, these blots were incubated in buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM 2-mercaptoethanol) at 55°C for 30 min and washed with PBS, and nonspecific sites were blocked (described above) before incubation with anti-galectin or an irrelevant rat anti-mouse CD8 antibody (described below).

Cell culture and passage of transformed human corneal epithelial cells. Transformed human corneal epithelial cells (courtesy of Sherry Ward, The Gillette Co., Gaithersburg, Md.) were maintained in keratinocyte growth medium (KGM) (no. CC 3111; Clonetics, San Diego, Calif.) supplemented with bovine pituitary extract (30 μg/ml), insulin (5 μg/ml), human epidermal growth factor (0.1 ng/ml), hydrocortisone (0.5 μg/ml), and 10% fetal bovine serum. Cell cultures at 60 to 80% confluency were used for passage. The cells were collected in a centrifuge tube and centrifuged for 5 min at 7,000 × *g* at RT. The pellet was resuspended in KGM, seeded at a density of 1.0×10^4 cells/cm^2, and incubated in a humidified incubator overnight at 37°C under 5% CO_2. The cultures were fed twice weekly with KGM. For immunofluorescence staining (described below), the cells were grown on uncoated 22-mm^2 glass coverslips to a density of approximately 10^7 cells/ml in KGM as described above.

Extraction of proteins from transformed human corneal epithelial cells. When the cultures reached 90% confluency, they were processed for protein extraction. The cells were scraped from cultures dishes, and the proteins were extracted with Laemmli sample buffer without 2-mercaptoethanol. Protein concentrations were determined with a bicinchoninic acid reagent (Pierce), and 2-mercaptoethanol and dye were added for protein separation by SDS-PAGE (32).

Western blot analysis of galectin-3 in HCEP. HCEP or a cell extract from cultured transformed human corneal epithelial cells was separated on by SDS-PAGE (10% polyacrylamide) (32). A 10-μg portion of each HCEP sample was loaded per lane. Human r-galectin-3 (37) (a gift of H. Leffler, University of California, San Francisco, Calif.), loaded at 1 μg/lane, was used as a positive control. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (53). Nonspecific binding sites were blocked with 3% BSA in TBS for 1 h at RT. The blots then were washed twice with TBS and incubated with rat anti-mouse Mac-2 monoclonal antibody MAb (1:100 dilution) (a gift of H. Leffler; clone M 3/38 immunoglobulin G2a [IgG2a] isotype; ATCC) in TBS–0.1% BSA for 2 h at RT. The negative control blot was incubated with an irrelevant rat anti-mouse $CD8^+$ T-cell MAb (ATCC clone GK 2.43) of the IgG2b isotype. After incubation, the blots were washed six times with TBS. After being washed, the blots were incubated with goat anti-rat IgG conjugated to horseradish peroxidase (1:12,000 dilution; Amersham, Arlington Heights, Ill.) in TBS–0.1% BSA for 1 h at RT. The blots were washed again as above, and the bands were visualized by chemiluminescence as specified by the manufacturer (Amersham).

Immunofluorescence analysis. Coverslips of cultured transformed human corneal epithelium were removed from the culture medium, and nonspecific binding was blocked for 45 min in PBS containing 1% BSA, 5% FBS, and 0.3% Tween 20 (Sigma). The coverslips were rinsed, dried, and incubated for 1 h with the primary anti-galectin MAb (1:10). They were rinsed in blocking solution (as above) and then with 0.05 M Tris-HCl, dried, and incubated with a fluorescein-conjugated goat anti-rat IgG antibody (1:30) (Cappel Research Products, Durham, N.C.) for 1 h. They were rinsed, dried, and mounted in 90% glycerol containing 1% PBS and 0.1% *p*-phenylenediamine. Controls were similarly treated with an irrelevant antibody (anti-HLA DR-5, clone SFR3-DR5; IgG2b isotype [ATCC]), or the specific primary antibody was omitted. The coverslips were photographed with a Zeiss Axiophot fluorescence microscope at similar exposure times (60 s).

Binding analysis of *P. aeruginosa* to scarified corneal epithelium. The mice were anesthetized with isoflurane (Anaquest, Madison, Wis.) and killed immediately by cervical dislocation. Afterward, the animals were placed beneath a ×40 stereoscopic microscope, and three 1-mm incisions to the center of both corneas were made with a sterile 25-5/8-gauge needle. Following scarification, the eyes were enucleated with sterile scissors and placed into sterilized prepared culture

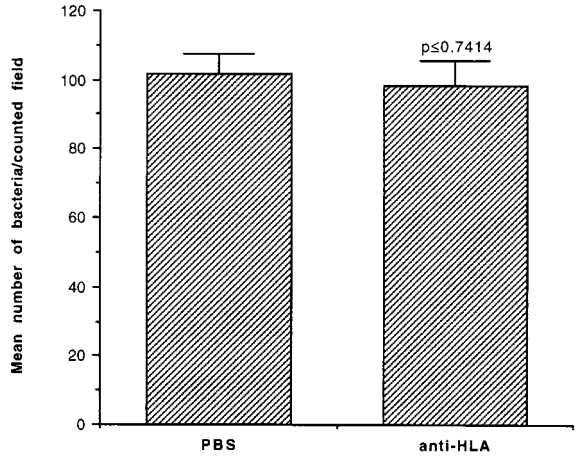

FIG. 1. Comparison of the binding of *P. aeruginosa* to mouse corneal epithelium in the presence of PBS and an irrelevant MAb. Eyes with scarified corneas were incubated for 60 min at 37°C with 5 μl of a bacterial suspension (5.0×10^7 CFU) in PBS or with 500 μg of anti-HLA DR5 antibody per ml. SEM was used to quantitate bacteria adherent to the cornea and showed no difference between the two treatments. The experiment was performed in triplicate, and typical data shown are the mean ± standard error of the mean.

wells containing 4 to 5 ml of minimal essential medium with Earle's salts, L-glutamine, and sodium bicarbonate (pH 7.5; Gibco Laboratories, Grand Island, N.Y.) at 37°C. The corneal surface, oriented superiorly in the wells, was covered with a thin layer of minimal essential medium. A 5-μl bacterial cell suspension containing 5.0×10^7 CFU of *P. aeruginosa* was topically delivered onto the surface of each eye in culture via a calibrated micropipette (Cole-Parmer, Chicago, Ill.) with a sterile disposable tip. All the eyes were immediately placed in a CO_2 incubator with a water jacket (American Scientific Products, McGaw Park, Ill.) at 37°C under 5% CO_2 and cultured for 60 min. The eyes were then rinsed vigorously in PBS, fixed, dehydrated, and critical point dried for scanning electron microscopy (SEM) (22).

Specimen preparation for SEM. Following incubation with bacteria, the cultured eyes were vigorously rinsed with PBS (pH 7.5) to remove nonadherent organisms and immediately fixed at 4°C in a fixative containing 2.0% osmium tetroxide, 2.5% glutaraldehyde, and 0.2 M phosphate buffer (pH 7.5) (1:1:1) for a total of 3 h with one change to fresh fixative after 1.5 h. The eyes were dehydrated through graded ethanol concentrations to 100% and critical point dried in an AutoSamdri 814 critical point drier (Tousimis Research Laboratories, Rockville, Md.) with liquid CO_2 as the transition fluid. The eyes, with the cornea superiorly oriented, were mounted on aluminum specimen stubs with colloidal silver paint suspended in methanol (Tousimis). A thin layer of gold (30 nm) was evaporated onto the surface of each specimen with a Pelco SC5 sputter coater (Ted Pella, Redding, Calif.). The eyes were viewed with a JEOL JSM-840 A SEM microscope (JEOL Ltd., Tokyo, Japan).

Quantitation of bacteria. Adherent bacteria were counted 60 min after ocular application, by using SEM procedures described previously (22). For each test, five randomly selected fields were photographed from each group consisting of three eyes, and the negatives were photographically enlarged to a total magnification of ×6,000. Counts were done with a square cut to measure 80 mm^2 at ×6,000, and the data are expressed as the mean number of bacteria bound per counted field.

Effect of anti-LPS or anti-galectin antibodies on the binding of *P. aeruginosa* to mouse corneal epithelium. MAbs prepared against the outer core, lipid A, and inner core regions from a rough *P. aeruginosa* strain, strain AK 1401 (11) (a gift of Joseph Lam, University of Guelph, Guelph, Ontario, Canada), or against galectin-3 were used in binding inhibition assays. When tested for agglutinating capacity by previously described methods (51), none of the antibodies (500 μg/ml each) agglutinated strain ATCC 19660 after 5 h of incubation at room temperature (data not shown). To determine whether any of the antibodies inhibited the binding of *P. aeruginosa* to mouse corneal epithelium, each antibody was mixed with 2.0×10^{10} CFU of *P. aeruginosa* ATCC 19660 per ml at a ratio of 1:1 so that when a 5-μl mixture of antibody with bacteria was topically applied to the surface of the mouse eye, the final concentration of bacteria was 5.0×10^7 CFU. The final concentrations of antibodies tested (500 and 250 μg/ml) were selected based on experience (17). PBS and an irrelevant MAb, anti-HLA-DR5, were used as controls. No differences in binding between PBS or this irrelevant antibody (Fig. 1) were seen, as in previous studies (17, 18). The eyes were incubated for 60 min at 37°C in a CO_2 incubator. Then they were washed with PBS and processed for SEM as described above, and the number of bacteria bound to the cornea was counted. The data were analyzed statistically by Student's *t* test at $P \leq 0.05$.

RESULTS

Isolation and purification of LPS from *P. aeruginosa* ATCC 19660. To perform the binding studies with *P. aeruginosa* ATCC 19660, we isolated and purified LPS from this strain. Since in a previous study (17) we had used commercially available LPS of *P. aeruginosa* serotype 10 (Sigma) in binding inhibition assays, we first compared LPS from these two strains. LPS from *P. aeruginosa* ATCC 19660 (isolated and purified) and commercially obtained LPS serotype 10 were separated on a 12% polyacrylamide gel, and the bands were identified by silver staining. The results are presented in Fig. 2. Qualitatively, the major patterns of the LPS bands of these two strains were different in that serotype 10 LPS lacked the higher-molecular-weight subunits seen in the LPS from strain ATCC 19660.

Identification of LPS binding proteins in human corneal epithelium. We further identified LPS binding proteins in a total extract from human corneal epithelium and simultaneously tested LPS binding to r-galectin-3. HCEP and r-galectin-3 were subjected to SDS-PAGE (10% polyacrylamide) and then transferred to a nitrocellulose membrane. The blots were incubated with biotin-labeled LPS and then treated with streptavidin to identify LPS binding proteins. Figure 3 (lane 2) represents a Coomassie blue-stained gel showing HCEP. Figure 3 (lane 3) represents the LPS binding pattern of *P. aeruginosa* ATCC 19660. There are five major proteins ranging in molecular mass from approximately 18 to 66 kDa that stained positively (darker) and several minor protein bands that were less intensely stained. Figure 3 (lane 4) represents a reprobed blot (lane 3) which was stripped of signal and then incubated similarly with the anti-galectin or an irrelevant rat anti-mouse CD8 MAb. Staining of a doublet band at 31 to 32 kDa was seen only with the specific MAb. Figure 3 (lane 5) represents LPS binding to r-galectin-3 and shows that this band migrates approximately with the 31-kDa standard and with similarly migrating bands in lanes 3 and 4. It is clear from Fig. 3 (lane 6) that when a similar blot was incubated with unlabeled LPS instead of biotin-labeled LPS, the reactive bands were not visualized, indicating that the proteins shown in lane 3 are LPS binding proteins. Serotype 10 LPS bound similarly to HCEP (data not shown).

Solid-phase assay of the binding of biotin-labeled LPS to HCEP. We investigated the binding of biotin-labeled LPS from

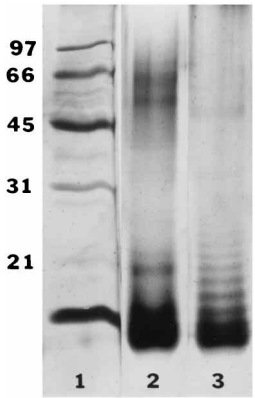

FIG. 2. Comparison of LPS prepared from *P. aeruginosa* ATCC 19660 with serotype 10 (Sigma) on a polyacrylamide gel stained with silver nitrate. LPS from each preparation was separated on the gel, and the LPS bands were detected by silver staining. Lanes: 1, low-molecular-weight markers (in thousands); 2, LPS from ATCC 19660; 3, LPS from serotype 10.

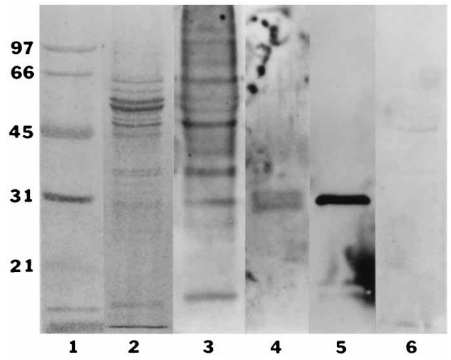

FIG. 3. *P. aeruginosa* ATCC 19660 LPS binding proteins in a HCEP overlay assay. HCEP (7 μg/lane) and human r-galectin-3 (200 ng/lane) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated with biotin-labeled LPS, followed by treatment with streptavidin conjugated with peroxidase, and the bands were detected by color development. Lanes: 1, low-molecular-weight markers (in thousands); 2, Coomassie blue-stained HCEP; 3, LPS binding proteins detected with biotin-labeled LPS from strain ATCC 19660; 4, protein (from lane 3) reprobed with anti-galectin antibody; 5, r-galectin-3 overlaid with biotin-labeled LPS; 6, negative control blot incubated with unlabeled LPS.

P. aeruginosa ATCC 19660 to HCEP in a solid-phase binding assay and also examined whether the binding is a receptor-adhesin type interaction. The results presented in Fig. 4 revealed that biotin-labeled LPS binds to HCEP and that the binding was significantly greater than the control value ($P \leq 0.0001$). These results also reveal that in competition experiments, when the binding of biotin-labeled LPS to HCEP was studied in the presence of a 10-fold excess of unlabeled LPS, it was reduced to around the control value, suggesting that the binding interaction of LPS with HCEP was a receptor-ligand type of interaction.

We tested if the binding of LPS to HCEP was saturable. In a solid-phase binding assay, the amount of HCEP was kept constant (0.5 μg/well) while nine different increasing concentrations of biotin-labeled LPS were added to the wells and the binding was studied. The results depicted in Fig. 5 shows that as the concentration of biotin-labeled LPS was increased, the binding was also increased, and that at very high concentrations of biotin-labeled LPS, there was no further increase in the binding, indicating that all binding sites were saturated. A Scatchard plot (Fig. 6) was generated from these saturation binding data. A linear plot was obtained, suggesting that binding occurred predominantly to one binding site.

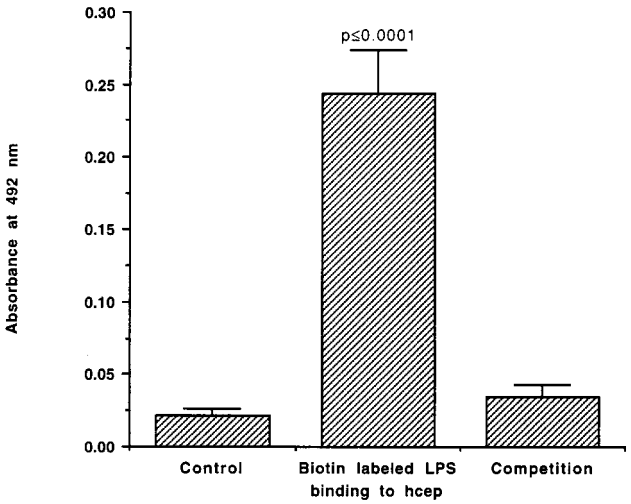

FIG. 4. Binding of biotin-labeled LPS to HCEP in a solid-phase binding assay. HCEP-coated wells (0.5 μg/well) were incubated with biotin-labeled LPS or labeled LPS in the presence of a 10-fold excess of unlabeled LPS. Control wells (only TBS in the wells) also were incubated with biotin-labeled LPS. The amount of LPS bound to HCEP-coated wells was significantly reduced by addition of unlabeled LPS. The experiment was performed in triplicate, and typical data shown are the mean ± standard error of the mean.

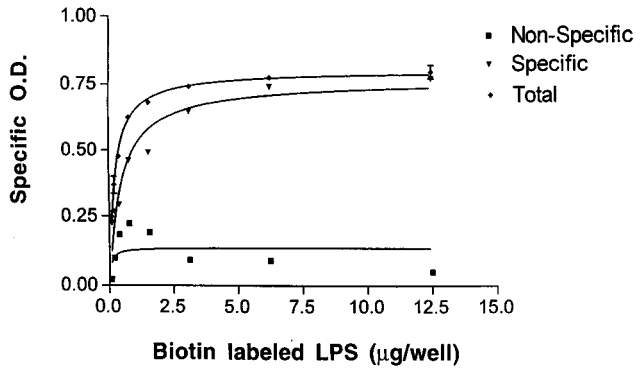

FIG. 5. Saturation curve illustrating the binding of biotin-labeled LPS to HCEP receptors in a solid-phase binding assay. Increased concentrations of biotin-labeled LPS were added to the HCEP-coated (0.5 μg/ml) wells, and binding was studied. For nonspecific binding, the wells were treated with a 10-fold excess of unlabeled LPS, and finally the specific binding was calculated. Specific binding data are shown and represent the mean ± standard error of the mean. O.D., optical density.

Identification of galectin-3 in fresh and cultured HCEP. Western blotting with anti-galectin MAb allowed the identification of galectin-3 in HCEP and in a cell extract from cultured transformed human corneal epithelium (Fig. 7, lanes 3 and 4). Positive bands were similar to the lower band of the positive control, i.e., human r-galectin-3 (lane 2). A duplicate blot, when treated similarly with a nonspecific irrelevant antibody (rat anti-mouse CD8), was negative (lane 5). Immunofluorescence analysis of transformed HCEP grown onto a coverslip revealed strong positive staining in cell preparations incubated with the Mac-2 antibody (Fig. 8A), while similarly run control coverslips in which the anti-galectin MAb was either substituted with an irrelevant anti-HLA DR5 antibody (data not shown) or omitted (Fig. 8B), were negative.

SEM binding inhibition assay. Eyes in culture were incubated with *P. aeruginosa* ATCC 19660 in the presence of MAbs

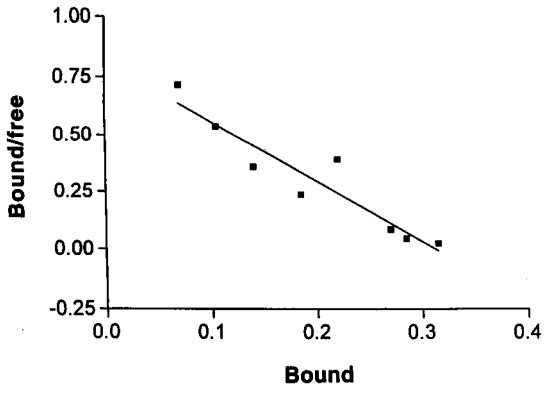

FIG. 6. Scatchard plot. Data from the saturation binding curve (Fig. 5) were fit into a Scatchard plot. A linear curve was generated and is shown.

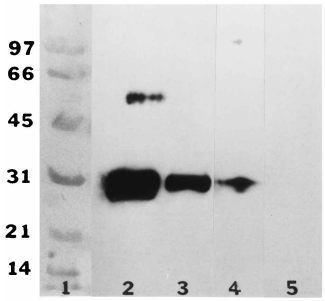

FIG. 7. Localization of galectin-3 in HCEP and a human corneal epithelial cell extract by Western blotting. Freshly isolated HCEP and proteins extracted from transformed cultured human corneal epithelial cells (10 μg each) were separated by SDS-PAGE (10% polyacrylamide). Then the proteins were transferred to a nitrocellulose membrane and subjected to Western blot analysis by using the Mac-2 antibody and chemiluminescence. Lanes: 1, low-molecular-weight markers (in thousands); 2, human r-galectin-3; 3, HCEP; 4, cultured cell extract from human corneal epithelium; 5, HCEP-negative control (nonspecific anti-CD8 primary antibody).

against LPS lipid A, outer core or inner core of strain AK 1401, and galectin-3. MAb against the LPS outer core region significantly ($P \leq 0.0001$) blocked the binding of *P. aeruginosa* ATCC 19660 to scarified corneas in whole-eye culture at either 500 μg/ml (Fig. 9A) or 250 μg/ml (data not shown). Binding was inhibited by approximately 50% by the MAb against the outer core region of LPS (500 μg/ml) compared to binding in

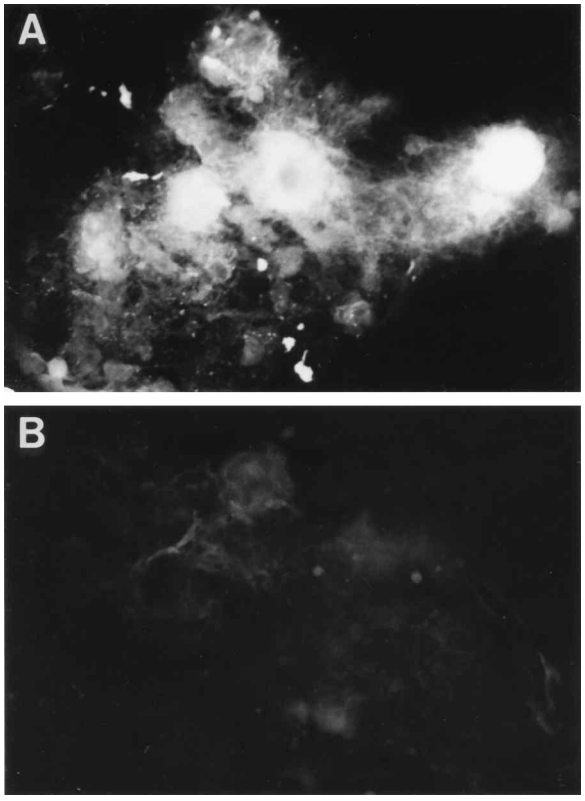

FIG. 8. Immunofluorescence analysis. Transformed human corneal epithelial cells grown on coverslips were immunostained with the Mac-2 antibody. (A) Strongly positive fluorescent staining is seen on cells grown on coverslips that were incubated with this antibody. (B) Coverslips incubated similarly with an irrelevant anti-HLA DR5 MAb (data not shown) or with omission of the primary specific antibody were negative for Mac-2 staining. Magnification, ×400.

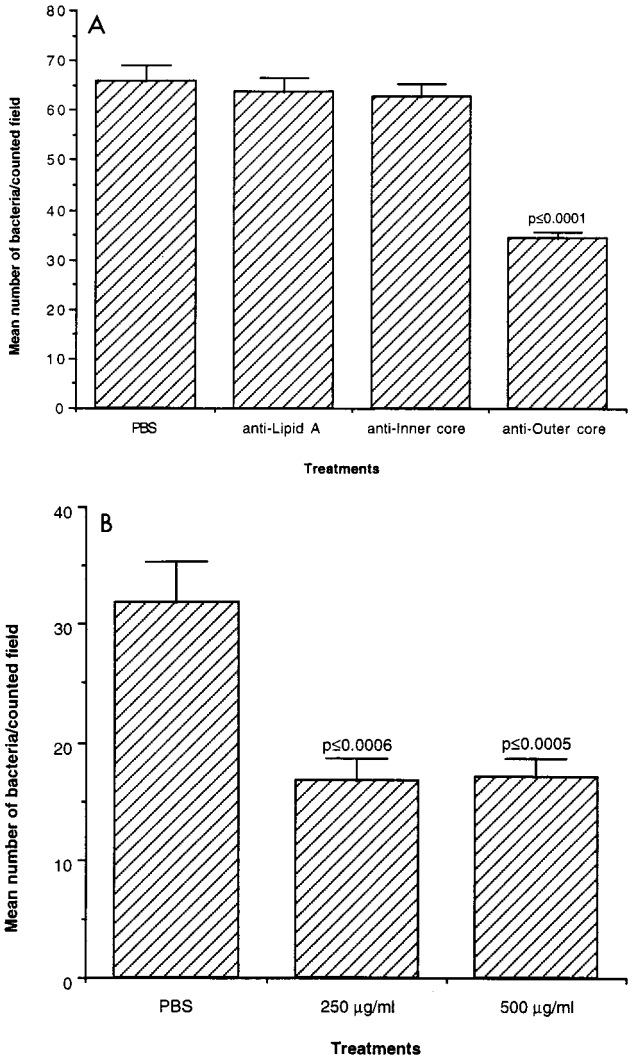

FIG. 9. In vitro binding inhibition of *P. aeruginosa* to mouse cornea. Eyes with scarified corneas were incubated with bacteria in the presence of antibody. Control eyes were incubated similarly with a bacterial suspension mixed with an irrelevant anti-HLA DR5 MAb (data not shown) or PBS. After a 60-min incubation at 37°C, the samples were processed for SEM. Antibody against the outer core region of LPS (A) and anti-galectin antibody (B) significantly decreased bacterial binding. The experiments were performed in triplicate, and typical data shown are the mean ± standard error of the mean.

the presence of a similar concentration of an irrelevant anti-HLA DR5 antibody (data not shown) or PBS. MAb against galectin-3 (Fig. 9B) also significantly inhibited bacterial binding by 54 and 51%, respectively, at 500 μg/ml ($P \leq 0.0005$) or 250 μg/ml ($P \leq 0.0006$) compared to binding in the presence of the irrelevant anti-HLA DR5 antibody at similar concentration (data not shown) or PBS.

DISCUSSION

Bacterial LPS is a potent physiological agent that affects a variety of animal cells. Almost all eukaryotic cells exhibit a change in shape, metabolism, or gene expression upon exposure to minute amounts of LPS (55). The fact that minute quantities of LPS can activate a large number of processes in target cells suggests the existence of an amplification system such as that used by signal transduction pathways of hormones

and other extracellular modulators. This observation has prompted many investigators to search for LPS receptors (36, 40, 46). Moreover, in a previous study (17), we were the first to demonstrate that LPS is an adhesin of *P. aeruginosa* which interacts with lipid receptors in the eye. For the above study, binding inhibition of *P. aeruginosa* ATCC 19660 to mouse corneal epithelium was performed successfully with a non-serotype-matched, commercially available LPS (*P. aeruginosa* serotype 10 [Sigma]). In this study, we isolated and purified LPS from *P. aeruginosa* ATCC 19660 and then studied the binding of both LPS to HCEP.

The LPS isolated and purified from *P. aeruginosa* ATCC 19660 was different from the LPS obtained commercially (*P. aeruginosa* serotype 10 [Sigma]) on silver-stained polyacrylamide gels. This variation was expected, because strain differences occur and also some minor differences can occur during the purification procedure due to the relatively labile linkages at the 2-keto-3-deoxyoctonic acid–lipid A band, ester-linked fatty acids, and the 4′-phosphoryl group (26, 45, 49). The purified LPS from *P. aeruginosa* ATCC 19660 and serotype 10 was labeled with biotin, and LPS binding proteins in HCEP were identified by an overlay assay procedure on nitrocellulose blots. Several bands, including one at approximately 31 kDa, reacted positively and stained similarly for both LPS. The molecular masses of the major darkly stained LPS-interactive proteins ranged from approximately 18 to 66 kDa, and other faint bands exhibited different intensities on the blot. Stripping and reprobing of this overlaid HCEP blot with anti-galectin antibody revealed a doublet band at approximately 31 to 32 kDa. In addition, r-galectin-3 run on the same gel and blotted to nitrocellulose migrated at approximately 31 kDa and bound biotin-labeled LPS. These data strongly suggest that this 31- to 32-kDa protein in HCEP is an LPS receptor. Recently, Proctor et al. (43) reported that at least four distinct mammalian membrane proteins can bind LPS. These include CD14 (55 kDa), CD18 (95 kDa), a 70- to 73-kDa protein, and the acetyl low-density lipoprotein receptor. Similarly, Halling et al. (19) examined the LPS binding proteins on human peripheral blood cell populations. They identified a major 73-kDa LPS binding protein which is similar to a previously described LPS binding protein identified on mouse splenic lymphocytes, macrophages, and peripheral blood mononuclear cells from a variety of mammalian species. Recently (14), it was shown that this 70- to 73-kDa protein is cell-bound albumin, which is present on many cells grown in the presence of serum- or albumin-containing medium but not present when the medium is free of any albumin or serum. In other work (19), three other major LPS binding proteins of 50, 31, and 20 kDa in lymphocyte and monocyte populations and a minor LPS binding protein of approximately 38 kDa in both cell types also were identified. Thus, binding studies with labeled LPS or its lipid A portion from several different laboratories using similar methods have shown that several proteins are candidates for LPS receptors (15). The ability of LPS to bind multiple proteins in HCEP is comparable to the above findings.

The results discussed so far, including results from other investigations, indicate that the binding of LPS to HCEP is a receptor-ligand type of interaction. Moreover, Lei et al. (34) in their study of the identification of LPS binding sites on murine splenocytes, observed that LPS binding proteins were membrane localized and that the receptors were saturable. Furthermore, competitive inhibition studies indicated that the binding of LPS could be significantly reduced by homologous and heterologous underivatized LPS (34). We also tested and found that the binding of LPS to HCEP was specific and further tested whether the HCEP identified as receptor proteins for LPS fulfill other requirements of a receptor, such as competition and saturation. A solid-phase binding assay revealed that binding of biotin-labeled LPS to HCEP was significantly higher than the control levels obtained when no HCEP were present and that binding of biotin-labeled LPS was reduced almost to the control level when binding was carried out in the presence of an excess of unlabeled LPS. Further, our results show that binding of biotin-labeled LPS to HCEP was saturable. Scatchard analysis of the saturation binding data suggest that in HCEP there is predominant binding of LPS to one site. These data are somewhat surprising, but hypothetically might be explained if LPS binding to these proteins involved the recognition of a common carbohydrate site.

Galectin-3, a protein of approximately 31 kDa, was identified in freshly isolated human corneal epithelium and is expressed in a transformed human corneal epithelial cell line. Binding studies with human r-galectin-3 confirm that LPS binds to the S-lac lectin and that this interaction can be inhibited by antibody to the LPS outer core or anti-galectin antibody. Our results demonstrate that LPS and anti-galectin antibody bind to the same protein in HCEP and that the protein is galectin-3. Furthermore, our data show that an approximately 31-kDa LPS binding protein in HCEP and r-galectin-3 have a similar migration pattern on SDS-PAGE, providing confirmatory evidence that galectin-3 is present in HCEP.

In summary, we have isolated and purified LPS from *P. aeruginosa* ATCC 19660, identified and begun characterization of the LPS binding proteins in HCEP, showed for the first time that galectin-3 is present in human cornea and is a corneal LPS binding protein, showed that anti-galectin MAb substantially (50%) inhibits in vitro bacterial binding, and confirmed that the outer core portion of LPS is important for binding to the cornea. Collectively, this information provides a better understanding of the mechanism(s) of bacterium-host interactions pertinent to human corneal epithelium, which should prove useful in the development of agonists and/or antagonists for LPS (galectin) receptors as adjunctive therapy for *P. aeruginosa* keratitis (32, 39).

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