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Antibody Specific for *Escherichia coli* J5 Cross-Reacts to Various Degrees with an *Escherichia coli* Clinical Isolate Grown for Different Lengths of Time

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Rabbit antiserum raised against the rough mutant of *Escherichia coli* O111:B4, designated J5, was examined for cross-reactivity to an *E. coli* clinical isolate (A2385). In whole-cell enzyme-linked immunosorbent assays, J5 antiserum reacted to a greater extent with A2385 grown for 5 h than with the same bacteria grown for 19 h, while the homologous antiserum reacted similarly with bacteria grown for different lengths of time. J5 antiserum reacted to the greatest extent with lipopolysaccharide (LPS) from A2385 grown for up to 10 h, and reactivity greatly diminished thereafter; homologous antiserum showed no difference in reaction over time. LPS from smooth bacteria grown for 19 h showed no reaction with J5 antiserum in immunoblots, while LPS from A2385 grown for 5 or 10 h showed a positive reaction. Little or no difference among the three LPS samples could be seen when homologous antiserum was used. Mice vaccinated with J5 LPS before lethal challenge with live A2385 were protected from this challenge, whereas most nonimmunized mice died. Toxicity tests in mice showed LPS from A2385 grown for 19 h to be twice as lethal as LPS from A2385 grown for 3 h. Mice vaccinated with J5 LPS were protected to a greater extent when challenged with a lethal dose of LPS from A2385 grown for 3 h than when challenged with LPS from A2385 grown for 19 h. The results reported here may explain the means by which J5 vaccination (active or passive) sometimes protects against heterologous challenge.

The relative ineffectiveness of treating gram-negative infections with antibiotics has led to the exploration of immunological approaches to treatment. In fact, antibiotics can sometimes exacerbate the consequences of gram-negative infection by causing the release of endotoxin from bacteria (24). Endotoxin is responsible for many or most of the deleterious effects of gram-negative infections. As an endogenous part of the gram-negative cell wall, endotoxin comes in contact with the surrounding cells and body fluids of the host. Endotoxin, or lipopolysaccharide (LPS), is composed of three main parts: the outermost O polysaccharide, the core oligosaccharide, and the innermost lipid A. The O polysaccharide is composed of a number of identical repeating subunits and is highly variable among gram-negative organisms, while the core oligosaccharide and, to a greater extent, lipid A are more conserved structures among the various gram-negative bacteria. The conserved nature of these inner moieties has been the basis for an intensive search for a cross-protective vaccine with rough mutants which lack the O polysaccharide and sections of the core oligosaccharide (3, 17). The J5 mutant of *Escherichia coli* O111:B4 (8) has been used in a number of in vitro and in vivo experiments and has been somewhat successful in protecting against heterologous challenge (4, 6, 18, 26, 27). Ziegler et al. have shown that antiserum produced in healthy volunteers after injection of J5 boiled cells can protect bacteremic patients from death, as compared to nonimmunized controls (27). The Re mutant of *Salmonella minnesota* 595 has been used successfully in cross-protective studies (12–14). However, contradictory results have also been reported when these organisms were used as cross-protective vaccines (12, 20).

The means by which J5 antiserum exerts its protective effects has been the subject of much study. When the opsonic activity of J5 antiserum has been examined, both positive and negative results have been demonstrated (7, 24, 26). Antiserum to J5 may directly neutralize the toxic effects of LPS by binding to or sterically inhibiting endotoxin (27). In vitro studies have shown that antiserum to J5 binds to heterologous LPS or whole bacteria to various degrees (19, 21, 22). Antiserum to J5 may bind to live bacteria early in infection at a time when the core epitopes may be most exposed (27).

It is this last possibility that was examined in this study. A smooth *E. coli* clinical isolate from a mastitic cow was grown in batch cultures for different lengths of time. The ability of antiserum specific for J5 to cross-react with both whole bacteria and isolated LPS was examined. The in vivo ability of vaccination with J5 LPS to protect against challenge with live organisms or with LPS extracted from bacteria grown for different lengths of time was also examined.

MATERIALS AND METHODS

Bacteria. *E. coli* J5, the Rc mutant of strain O111:B4, was supplied by Lynette Corbeil, University of Washington, Seattle. *E. coli* A2385 was a clinical isolate obtained from a mastitic cow. Serotyping was performed by Richard Wilson, Pennsylvania State University, University Park. A2385 possesses the antigen O2a.

Antiserum. Bacteria for immunization were grown overnight at 37°C in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). The bacteria were pelleted by centrifugation, washed three times, suspended in 0.85% NaCl, and boiled for 2.5 h. The bacteria were diluted in 0.85% NaCl to 70% transmittance at 610 nm (5).

Rabbits were injected with the respective vaccines intradermally (1.0 ml) with bacteria emulsified (1:1) in Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.) on days 0 and 21. Rabbits received intramuscular injections (1.0 ml) of the vaccines without adjuvant on days

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40, 42, 44, and 47. Rabbits were exsanguinated on day 54. Blood was allowed to clot at room temperature, and serum was collected and stored at −70°C.

Antiserum adsorption. J5 antiserum was adsorbed with human erythrocytes coated with alkali-treated J5 LPS. Packed, sensitized erythrocytes were added to J5 antiserum at a ratio of 10 ml of antiserum per ml of sensitized cells and incubated at 25°C for 1 h. Cells were separated by centrifugation, and adsorption was repeated twice (14, 18). Depletion of J5 antibodies was measured by enzyme-linked immunosorbent assay (ELISA) with J5 LPS as the coating antigen.

Whole-cell ELISAs. The bacteria were grown for 5 or 19 h in Trypticase soy broth, harvested by centrifugation, and washed three times with 0.85% NaCl. The bacteria were then suspended in carbonate-bicarbonate buffer (0.06 M; pH 9.6) to 90% transmittance at 610 nm with further dilutions also made in carbonate buffer. Samples (0.1 ml) were placed in wells of 96-well microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated in a 37°C water bath for 60 min. Plates were then washed three times with phosphate-buffered saline containing 0.5% Tween 20 (PBS-T). Dilutions (in PBS-T) of rabbit antiserum (0.1 ml) were added to the wells and incubated in a 37°C water bath for 30 min. The plates were washed as before, and 0.1 ml of goat anti-rabbit immunoglobulin G peroxidase conjugate (Cappel Laboratories, West Chester, Pa.) diluted 1:2,000 in PBS-T was added to the wells and incubated for 20 min in a 37°C water bath. Plates were washed again, and 0.1 ml of the substrate, 2,2-azinodi-(3-ethylbenzthiazoline sulfonic acid) (pH 6.0), was added and incubated for 10 min at room temperature, after which the stopping reagent (0.1 M hydrofluoric acid; pH 3.3) was added. Plates were immediately read (at 405 nm) on a Minireader II (Dynatech).

LPS ELISAs. LPS samples were optimally diluted (1:320) in carbonate-bicarbonate buffer and placed (0.1 ml portions) into Immulon-2 96-well plates (Dynatech). Plates were incubated at 37°C for 4 h and then incubated overnight at 4°C. The plates were washed three times with PBS-T. Antiserum was diluted as described above, and 0.1-ml samples were placed in the wells. After a 30-min incubation in a 37°C water bath, plates were washed three times with PBS-T. Goat anti-rabbit immunoglobulin G peroxidase conjugate (0.1 ml) diluted 1:2,000 in PBS-T was placed in the wells and incubated for 20 min in a 37°C water bath. After the final wash, the substrate and stopping reagent were added as described above, and plates were read on the Minireader II.

LPS extraction. *E. coli* (both strains) was grown overnight or at designated time intervals in Trypticase soy broth, harvested by centrifugation, and washed once in deionized water and once in 95% ethanol. The bacteria were then suspended in acetone and dried overnight. J5 LPS was extracted by the phenol-chloroform-petroleum ether method of Galanos et al. (10), while the A2385 LPS was extracted by the hot phenol-water method (9). LPS samples were standardized by the 2-keto-3-deoxyoctulosonic acid assay (15).

SDS-polyacrylamide gel electrophoresis. Samples of A2385 LPS from 5-, 10-, 15-, and 19-h growth were standardized by 2-keto-3-deoxyoctulosonic acid measurements to approximately 35 μg/ml. Gels were prepared by the method of Laemmli (16). A 12% acrylamide running gel and 4% stacking gel were used. Samples were diluted 1:1 in Rosenbusch buffer (without 2-mercaptoethanol) and heated at 100°C for 10 min. The Tris-glycine running buffer contained 0.1% sodium dodecyl sulfate (SDS). Samples (50 μl) were added to the gel slots, and electrophoresis was run at 16 mA until the dye front was approximately 2 cm from the bottom of the gel.

Immunoblotting. Gels containing LPS samples were placed in 500 ml of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) (23) overnight on an orbital shaker. Transfer to nitrocellulose (Bio-Rad Laboratories, Richmond, Calif) was accomplished with the Bio-Rad Trans-Blot Cell; electroblotting was done for 3 h at 70 V. The nitrocellulose sheet was soaked in Tris-buffered saline (TBS) for 10 min and agitated overnight in blocking solution (3% gelatin in TBS). Rabbit anti-J5 antiserum (1:50) and rabbit anti-A2385 antiserum (1:100) were diluted in antibody buffer (1% gelatin in TBS), and 15 ml of each of these was incubated with the nitrocellulose sheets for 1.5 h. Nitrocellulose sheets were washed in TBS containing 0.5% Tween 20. Goat anti-rabbit immunoglobulin G peroxidase conjugate (Bio-Rad), diluted 1:3,000 in antibody buffer (15 ml, total), was added to the sheets and incubated for 1.5 h at room temperature. Sheets were washed again in TBS containing 0.5% Tween 20. The substrate (0.015% H_2O_2, 0.05% 4-chloro-1-naphthol) was added to the sheets and incubated until color development was sufficient. The developed sheets were washed in deionized water and air dried.

Animal studies. All mice used were of the Swiss/Webster strain (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and were approximately 6 to 8 weeks old when used. The lethal dose of *E. coli* A2385 was determined by growing bacteria for 18 h at 37°C in Trypticase soy broth. Dilutions and plate counts showed the number of bacteria to be approximately 9×10^8 CFU/ml. Mice received 1.0-ml injections (intraperitoneal) of a dilution (in 0.85% NaCl) of A2385, mixed 1:1 with 5% hog gastric mucin (type 1701-W; Wilson Laboratories, Chicago, Ill.). Mortality was recorded at 48 h.

Active protection against live A2385 with the J5 vaccine consisted of two 0.5-ml immunizations on days 0 and 7 with a dilution (in deionized water) of J5 LPS that gave an A_{610} of 1.2. The mice were challenged with various dilutions of an 18-h culture of A2385 (1:1 with hog gastric mucin) on day 14. Control mice were given 0.5 ml of deionized water on days 0 and 7 and then were similarly challenged. All injections were given intraperitoneally.

Lethality of A2385 preparations was assessed by giving groups of five mice various doses intraperitoneal; in 0.5 ml of deionized water) of LPS from A2385 grown for 3 or 19 h. Mortality was recorded at 48 h.

To assess the protective value of J5 LPS vaccination against A2385 LPS, mice were immunized with J5 vaccine prepared as described above. Groups of 10 mice were immunized on days 0 and 14. Control mice again were given 0.5 ml of deionized water. The mice were challenged on day 21 with the 100% lethal dose of LPS from A2385 grown for 3 or 19 h. Mortality was recorded at 72 h.

RESULTS

Whole-cell ELISAs. Table 1 shows the results of the ELISA with A2385 whole cells as the coating antigen. The initial antigen concentration gave a transmittance of 90% at 610 nm, and dilutions were made from this. The titer derived was the highest dilution giving an optical density of more than 0.5. The homologous (anti-A2385) antiserum gave similar readings with bacteria grown for 5 or 19 h (Table 1). However, the antiserum specific for J5 was much more reactive with bacteria grown for 5 h, showing little (or no) reactivity with A2385 whole cells grown for 19 h.

TABLE 1. Endpoint titers against *E. coli* A2385 whole cells[a]

Incubation time (h)	Endpoint titer (±SD) with:	
	Anti-J5	Anti-A2385
19	20 (5.77)	163,840 (125,135)
	20	81,920
	10	327,680
5[b]	640 (562)	81,920
	160	327,680
	1,280	163,840

[a] Endpoint, optical density of >0.5. A_{610} for antigen dilutions was 0.0548.

LPS ELISAs. A2385 LPS (from 3-, 5-, 10-, 15-, and 19-h bacterial growth) was used as the coating antigen in ELISAs and assayed against rabbit antiserum raised against A2385 or J5 boiled cells (Fig. 1). As with the whole-cell ELISAs, the homologous antiserum showed little difference in reaction to the LPS taken from bacteria grown for the five lengths of time. However, J5 antiserum reacted to a greater extent with LPS from the 3- and 5-h growth periods than with LPS from bacteria grown for 15 or 19 h. J5 antiserum reactivity peaked with LPS from bacteria grown for 10 h. With J5 LPS as the coating antigen, J5 antiserum showed a positive reaction compared with preimmune serum. J5 antiserum adsorbed with J5 LPS-sensitized erythrocytes was about 50% depleted of J5 antibody according to ELISA (data not shown).

With LPS from A2385 grown for different lengths of time as the coating antigen, the adsorbed J5 antiserum showed a diminished response to each of these antigens compared with the response of the unadsorbed serum, but followed a trend similar to that of the unadsorbed serum (Fig. 1).

Immunoblotting. A2385 samples (after 5, 10, 15 and 19 h of bacterial growth) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets, and reacted with rabbit antiserum specific for A2385 boiled cells (Fig. 2), J5 boiled cells, or J5 boiled cells adsorbed with J5 LPS-sensitized erythrocytes (Fig. 3). A2385 antiserum reacted similarly with LPS from A2385 grown for different lengths of time (Fig. 2). However, J5 antiserum had the greatest reactivity with LPS from A2385 grown for 5 or 10 h, only slight reactivity with LPS from A2385 grown for 15 h, and no reactivity with LPS from A2385 grown for 19 h (Fig. 3). Adsorbed J5 antiserum still showed some reactivity with LPS from A2385 grown for 5 or 10 h. Immunoblots were repeated with equal amounts (dry weight) of LPS, and similar results were observed (data not shown). It is important to note that antiserum specific for J5 boiled cells can react (as shown by immunoblot analysis) with high-molecular-weight A2385 LPS (i.e., those LPS moieties containing a number of O subunits).

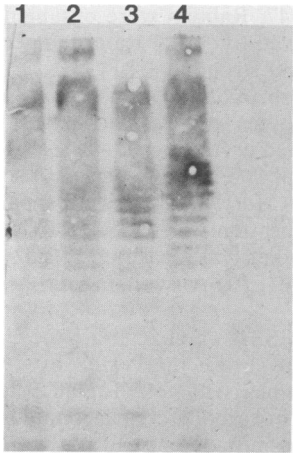

FIG. 2. Immunoblot analysis of A2385 LPS extracted from bacteria grown for 5 h (lane 1), 10 h (lane 2), 15 h (lane 3), or 19 h (lane 4). The immunoblot was developed with a 1:100 dilution of A2385 antiserum.

Animal studies. Dilutions were made (in 0.85% NaCl) of a culture of A2385 grown for 18 h, mixed 1:1 with hog gastric mucin, and injected intraperitoneally into groups of four or five mice. Mice vaccinated previously with J5 LPS showed a greater degree of protection than mice given deionized water (control) injections (Table 2).

Lethality tests with LPS from A2385 grown for 3 or 19 h showed that half as much LPS from A2385 grown for 19 h was needed to achieve complete lethality compared with the amount of LPS needed from A2385 grown for 3 h (Table 3).

Groups of 10 mice, immunized with J5 LPS or given

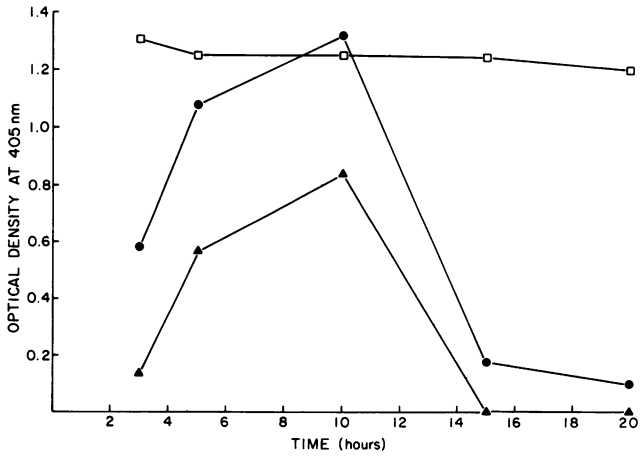

FIG. 1. ELISA results with LPS from A2385 grown for different lengths of time as the coating antigen. Antisera were a 1:10,000 dilution of anti-A2385 (□), a 1:80 dilution of anti-J5 (●), and a 1:80 dilution of anti-J5 adsorbed with J5 LPS-coated erythrocytes (▲).

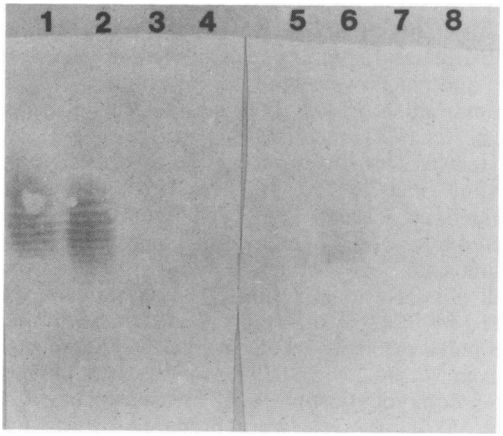

FIG. 3. Immunbolt analysis of A2385 LPS extracted from bacteria grown for 5 h (lanes 1 and 5), 10 h (lanes 2 and 6), 15 h (lanes 3 and 7), or 19 h (lanes 4 and 8). Lanes 1 through 4 were developed with a 1:50 dilution of J5 antiserum; lanes 5 through 8 were developed with a 1:50 dilution of J5 antiserum adsorbed with J5 LPS-coated erythrocytes.

TABLE 2. Active immunization with J5 LPS against challenge with *E. coli* A2385

No. of organisms injected[a]	Mouse survival ratio[b] (no. dead/no. challenged)	
	Controls[c]	J5 vaccinates
16,000	5/5	3/5
8,000	4/5	3/5
1,000	4/5	1/4
75	4/5	1/4

[a] At 48 h postchallenge.
[b] Determined from CFU.
[c] Injected with 0.5 ml of deionized H_2O.

deionized water (controls), were challenged (intraperitoneally) with the 100% lethal dose of LPS from A2385 grown for 3 or 19 h. Immunized mice challenged with LPS from A2385 grown for 3 h were protected to a greater degree (80% survival) than immunized mice challenged with LPS from A2385 grown for 19 h (10% survival) or control mice challenged with LPS taken from A2385 grown for 3 or 19 h (20 and 0% survival, respectively).

DISCUSSION

The use of rough mutants of gram-negative bacteria as cross-protective vaccines has gained much attention in recent years. The encouraging results reported by Ziegler et al. and other researchers (4, 6, 18, 26, 27) with the J5 mutant of *E. coli* O111:B4 have led to a search for the basis of the cross-protective immunity induced by J5. The conserved nature of the core oligosaccharide among gram-negative organisms is the most obvious reason for the cross-reactivity of antiserum specific for J5. However, in vitro investigation of the ability of J5 antiserum to bind to whole (heterologous) bacteria or to the isolated LPS has given conflicting results (19, 21, 22). Alternatively, vaccination with rough bacteria or LPS may lead to the production of specific antibody to the heterologous organism because of the mitogenic activity of LPS (1, 14, 22). Some reports have also suggested that LPS may be neutralized by endogenous components of plasma (25) or serum (2). However, if cross-reacting antibody is the protective factor, the exact nature of the cross-protection must be reconciled with the existing reports, since both positive and negative results have been found in in vitro cross-reactivity assays (19, 21, 22). It is possible that variable findings are the result of different methods of bacterial growth, extraction procedures, assay methods, etc.

LPS derived from a smooth *E. coli* strain was shown, after SDS-polyacrylamide gel electrophoresis, to separate into more than 40 components depending on the number of O polysaccharide units (11). The protective effect seen after active or passive immunization with J5 may be the result of antibody binding to bacteria at a time during infection when core epitopes (which J5 antibody recognizes) are most exposed (27). It is this theory which is explored in this report.

TABLE 3. Lethality of LPS from A2385 grown for different lengths of time

LPS dosage (mg)	Mouse survival ratio[a] (no. dead/no. challenged) with LPS from A2385 grown for:	
	3 h	19 h
1.0	2/5	2/5
1.25	ND[b]	5/5
1.50	ND	5/5
2.00	2/5	ND
2.30	3/4	ND
2.50	5/5	ND

[a] At 48 h postchallenge.
[b] ND, Not done.

The results reported here suggest that there may be a change in the LPS and, possibly, in other components of the outer membrane of *E. coli* A2385 as the bacteria grow. As shown by immunoblot analysis (Fig. 3), an epitope(s) recognized by J5 antiserum was lost or was inaccessible after 19 h of growth. Silver stains of the three LPS samples done after SDS-polyacrylamide gel electrophoresis showed little difference in the characteristic LPS bands (data not shown), although there may be a difference in the relative amounts of each of the LPS species.

Although mitogenic stimulation of specific antibody cannot be ruled out, the finding that adsorption of J5 antiserum with J5 LPS-sensitized erythrocytes decreases the reactivity of this antiserum with A2385 LPS is evidence for the presence of cross-reactive antibody.

The ability of vaccination with J5 LPS to protect mice from a lethal dose of LPS from bacteria grown for 3 h but not from LPS from bacteria grown for 19 h suggests that protection from live challenge may result because antibody specific for core epitopes can bind to smooth organisms at a time during infection when these determinants are most exposed. This means of protection may also explain the protection seen in other in vivo studies. Whether this protection from live challenge results from direct neutralization of endotoxin or from opsonization and subsequent phagocytosis is unknown. The phenomenon described here may be used as a basis for speculation that active immunization, whereby the antibody is present before the invading bacteria are, may be more effective than passive administration of specific immunoglobulins at a time when the invaders are more mature.

The phenomenon of binding to smooth bacteria or isolated LPS by antiserum to J5 may extend to other gram-negative organisms as well. Experiments which have shown no cross-reactivity between J5 antiserum and other heterologous bacteria may have had different outcomes if the organisms used had been grown for shorter periods.

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ADDENDUM IN PROOF

Rabbits vaccinated with J5 boiled cells were also given intramuscular injections of J5 LPS (approximately 25 μg per injection) on days 42, 44, and 47.

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