JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 679–684 Vol. 35, No. 3
0095-1137/97/$04.00+0

Production and Characterization of Monoclonal Antibodies Specific for the Lipopolysaccharide of *Escherichia coli* O157

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Received 5 August 1996/Returned for modification 4 October 1996/Accepted 2 December 1996

Identification of the O157 antigen is an essential part of the detection of *Escherichia coli* O157:H7, which is recognized as a major etiologic agent of hemorrhagic colitis. However, polyclonal antibodies produced against *E. coli* O157:H7 lipopolysaccharide (LPS) may react with several other bacteria including *Brucella abortus*, *Brucella melitensis*, *Yersinia enterocolitica* O9, *Escherichia hermannii*, and *Stenotrophomonas maltophilia*. We produced eight monoclonal antibodies (MAbs) specific for the LPS of *E. coli* O157. Western blots (immunoblots) of both the phenol phase (smooth) and the aqueous phase (rough) of hot phenol-water-purified LPS indicated that three of the MAbs were specific for the O antigen and five were reactive with the LPS core. The eight MAbs could be further differentiated by their reactivities to *Salmonella* O30 LPS (group N), which is reported to be identical to the *E. coli* O157 antigen. All eight MAbs reacted strongly to all of the 64 strains of *E. coli* O157 tested, which included 47 isolates of O157:H7 and 17 other O157 strains. None of the eight MAbs cross-reacted with any of the 38 other *E. coli* serotypes tested, which consisted of 29 different O-antigen serotypes, or with 38 strains (22 genera) of non-*E. coli* gram-negative enteric bacteria.

Escherichia coli O157:H7 has been identified as a major etiologic agent of hemorrhagic colitis (31), which is occasionally accompanied by serious complications such as hemolytic-uremic syndrome (18, 24) or thrombic thrombocytopenic purpura (7). Recent studies have shown that beef and dairy cattle are natural reservoirs of *E. coli* O157:H7 (2, 43) and that *E. coli* O157:H7 can be isolated from the feces of asymptomatic cattle (2, 22), from raw milk (2, 9), and from poultry, pork, and lamb (12, 30). However, undercooked ground beef is the major source of *E. coli* O157:H7 in food-borne outbreaks (8).

The *E. coli* O157:H7 serotype has traditionally been identified by agglutination tests (14, 21) or immunofluorescence assays (25) with polyclonal antibodies. However, use of polyclonal antisera may result in false-positive identification of *E. coli* O157 (3, 20). For example, polyclonal anti-O157 *E. coli* antisera may cross-react with *E. coli* O7 and O116 (13, 27). In addition, other bacteria possess cross-reacting epitopes which mimic epitopes on the lipopolysaccharide (LPS) of *E. coli* O157. These bacteria include *Escherichia hermannii* (3, 20, 26, 29), *Brucella abortus* (4, 26, 29, 35), *Brucella melitensis* (4, 26, 29), *Yersinia enterocolitica* serotype O9 (6, 29), *Stenotrophomonas maltophilia* (10), and *Citrobacter freundii* (1, 34).

Furthermore, the O antigen of *E. coli* O157 was reported to be identical to the O30 antigen of group N *Salmonella* (4, 5, 28, 29, 33). Perry et al. (29) reported the structure of the O antigen of *E. coli* O157 as an unbranched linear polysaccharide with a tetrasaccharide repeating unit (29). Group N *Salmonella* species have been divided into two subgroups, $O30_1$ and $O30_130_2$. The *Salmonella* $O30_1$ LPS is a repeating tetrasaccharide identical to the *E. coli* O157 LPS tetrasaccharide repeat (5, 28, 29, 33). The *Salmonella* $O30_130_2$ LPS contains a pentasaccharide repeating unit composed of a tetrasaccharide related to the tetrasaccharides of *Salmonella* $O30_1$ and *E. coli* O157 (5, 28, 29, 33), but it contains an additional hexose residue at a branch point in the pentasaccharide repeating unit (4, 5, 28).

Perry et al. (27) produced monoclonal antibodies (MAbs) specific for the O chains of *E. coli* O157 LPS. However, the characterization of these MAbs by Western blotting (immunoblotting) was not reported, and the MAbs were not made available for commercial or research use. This report describes the production and characterization of MAbs specific for the LPS of *E. coli* O157 and group N *Salmonella* for use as high-specificity diagnostic reagents. The MAbs may be obtained from the authors for research purposes on request.

MATERIALS AND METHODS

Bacteria and culture conditions. The MAbs were screened against 64 O157 *E. coli* strains (47 O157:H7 isolates), which included all strains available from the American Type Culture Collection (ATCC; Rockville, Md.). *E. coli* O157:H7 CDC EDL 933 (ATCC 43895) was used as the *E. coli* O157:H7 reference strain for MAb production and all experiments used to characterize the MAbs. An isolate (isolate 2886-75) from a patient with hemorrhagic colitis was provided by the *E. coli* Reference Center (ECRC; Pennsylvania State University, University Park). The remaining O157:H7 strains were isolated from bovine feces and were provided by the National Veterinary Services Laboratories (Ames, Iowa). Other bacteria used included 17 *E. coli* O157:non-H7 strains, 38 *E. coli* non-O157 strains (29 different O-antigen serotypes), and 38 non-*E. coli* gram-negative enteric bacterial strains (22 genera). A complete list of the bacterial species, serotypes, and isolates used is available from the authors on request.

All bacteria used for screening hybridomas were grown on Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) plates at 37°C overnight, with the following exceptions: *Pasteurella multocida* types A and D, *Pasteurella haemolytica*, *Actinobacillus suis*, and *Haemophilus somnus* were grown on blood agar plates (TSA plates supplemented with 5% whole sheep blood). For crude flagella preparations, *E. coli* O157:H7 CDC EDL 933 was grown in flagella broth consisting of 20 g of Bacto buffered peptone water (Difco Laboratories, Detroit, Mich.), 10 g of Bacto beef extract (Difco Laboratories), and 5 g of dextrose (BBL Microbiology Systems) per liter of water (15). For LPS extractions, *E. coli* O157:H7 CDC EDL 933 was grown in Luria broth (Difco Laboratories) containing tryptone and yeast extract. Broth cultures were grown at 37°C for 16 to 18 h with agitation.

LPS purification. The LPS was extracted from *E. coli* O157:H7 CDC EDL 933 by the hot phenol-water method described by Westphal and Jann (42), except that both the aqueous and phenol phases were used. The pellet (15,000 × *g*, 1 h) from a 500-ml culture was suspended in 10 ml of deionized water (72 to 75°C), vortexed for 20 s, and placed uncapped in a 72 to 75°C water bath. Ten milliliters

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of 88% liquid phenol (preheated to 72 to 75°C) was added, and the tube was vortexed and incubated for 15 min in the water bath. The tube was revortexed every few minutes during the 15-min incubation period and was then allowed to cool to room temperature. After centrifugation at 500 × *g* for 10 min, the upper aqueous layer was carefully removed and saved. The phenol-phase interface was heated to 72 to 75°C, and another 10 ml of water (72 to 75°C) was added and the process was repeated. The aqueous phases from the two extractions were combined and reheated to 72 to 75°C, 5 ml of 88% phenol (72 to 75°C) was added, the mixture was incubated for 15 min (72 to 75°C) with periodic vortexing, and the extraction process was repeated. The interface layer, which contained the precipitated protein, was discarded and the phenol phase was clarified by centrifugation (15,000 × *g*, 20 min). The aqueous and phenol phases were dialyzed extensively in water (4°C), until all traces of phenol were removed. The phenol phase was lyophilized and the LPS was precipitated from the aqueous phase with 6 volumes of 95% ethanol (containing 0.15 g of sodium acetate [Sigma Chemical Company, St. Louis, Mo.] per 50-ml volume) at −20°C overnight. The pellets were suspended in 1 ml of water and were stored at −20°C.

Antigen preparation for injection into mice. *E. coli* O157:H7 CDC EDL 933 was used for both the whole-cell and crude flagella antigen preparations. Bacteria for the whole-cell preparations were grown on TSA plates, suspended in water, heat killed (60°C, 1 h), sonicated (maximum setting for microprobe) for 2.5 min (on ice), rested for 5 min, and resonicated for 2.5 min (on ice). Protein concentrations were determined by the bicinchoninic acid (Pierce, Rockford, Ill.) method. The whole-cell sonicate preparations were diluted in adjuvant (Ribi Immunochem Research, Inc., Hamilton, Mont.) to a protein concentration of 50 μg/ml (equivalent to approximately 5×10^8 bacteria/ml). For the crude flagella preparations, live bacteria were pelleted by centrifugation at 15,000 × *g* for 1 h, homogenized in a Waring blender (5 min at maximum speed after cooling on ice), and centrifuged at 10,000 × *g* for 30 min. The flagella were pelleted from the supernatant by ultracentrifugation (100,000 × *g*, 3 h), and the crude preparation was diluted in Ribi adjuvant to a protein concentration of 250 μg/ml.

Injection of mice. BALB/c mice were purchased from Charles River Laboratories (Wilmington, Mass.), Harlan, Sprague, Dawley (Indianapolis, Ind.), and Hilltop Laboratory Animals, Incorporated (Scottdale, Pa.). Female mice (age, 6 to 10 weeks) were injected with antigen and Ribi adjuvant according to the adjuvant manufacturer's recommendation. The whole-cell preparations were used at 10 μg per injection, while crude flagella preparations were used at 50 μg per injection. Each injection consisted of 0.2 ml of antigen in Ribi adjuvant administered by intraperitoneal injection (0.1 ml on each side). Whole-cell preparations which were lethal to mice (presumably due to endotoxicity or residual Shiga toxins) were diluted twofold in Ribi adjuvant until all the mice (four to six mice per antigen preparation) survived. The mice received their second injection at 3 weeks and were subsequently injected every 2 weeks. Mice were sacrificed for fusions on the fourth day after their fourth to sixth injection.

Production of hybridomas. The methods used for the production and screening of hybridomas have been described previously (39–41). Briefly, microtiter plates were prepared for the fusion by adding 3×10^3 mouse peritoneal macrophages in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, N.Y.) supplemented with hypoxanthine-aminopterin-thymidine (ATCC), 10% hybridoma cloning factor (Sigma Chemical Company), and 15% heat-inactivated (56°C, 30 min) fetal bovine serum (Hyclone Laboratories, Logan, Utah) to each well on the day before the fusion. The plates (Costar; Costar Corporation, Cambridge, Mass.) were incubated in 8.5% CO_2 at 37°C. Hybridomas were produced by fusing unfractionated splenocytes with 2×10^8 Ag8 myeloma cells (X63Ag8.653; ATCC) with 50% polyethylene glycol (molecular weight, 1,450; Hybrimax; Sigma Chemical Company). The fusion mixture was distributed equally into 2,400 to 3,000 macrophage-containing wells per fusion.

Preparation of enzyme-linked immunosorbent assay (ELISA) plates for hybridoma screening. Polyvinyl chloride microtiter plates (Dynatech, Chantilly, Va.) were coated with bacteria by adding to each well 0.1 ml of a suspension of bacteria at 50 μg of protein/ml (equivalent to approximately 5×10^8 bacteria/ml) in 0.05 M carbonate buffer (pH 9.6). The plates were sealed with tape (Corning Glass Company, Corning, N.Y.), incubated for 4 h at 37°C, and stored at −20°C until use.

Screening of hybridomas. Hybridomas were screened by ELISA at 12 to 18 days postfusion against *E. coli* O157:H7 CDC EDL 933 (positive control) and enterotoxigenic *E. coli* O78:H11 H10407 (ATCC 35401), a randomly chosen negative control strain. Viable hybridomas, which were uniquely positive for O157:H7 and which showed no cross-reactivity to O78:H11, were expanded to 24-well plates (Costar) to which 5×10^4 to 1×10^5 mouse peritoneal macrophages had been added the day before. When cell growth covered the majority of the bottoms of the wells, the medium was screened against an additional 11 strains of non-O157:H7 *E. coli* or other gram-negative bacteria (prepared as described above), along with *E. coli* O157:H7 CDC EDL 933 (to ensure continued antibody production). Hybridomas secreting cross-reactive antibodies were eliminated, and those reacting only to O157:H7 were transferred to 75-ml flasks (Costar), from which the supernatants, after centrifugation at 1,000 × *g*, were used to test additional bacteria. The hybridomas of interest were subcloned by limiting dilution methods.

ELISA procedure for hybridoma screening. Plates previously coated with bacteria (as described above) were thawed and washed eight times with ELISA wash buffer (phosphate-buffered saline [PBS]–Tween–horse serum [PBS-T-HS]) consisting of fluorescent treponemal antibody (FTA) hemagglutination buffer (PBS; BBL Microbiology Systems) with 0.1% Tween 80 (Sigma Chemical Company) and 0.5% horse serum (Sigma Chemical Company). One hundred microliters of cultured hybridoma medium or controls consisting of uncultured medium, normal mouse serum, and immune serum from the fusion mouse (all sera were diluted at least 1:2,000 in PBS-T-HS) were added to the wells and the plates were incubated for 10 min at 37°C. The plates were washed eight times as described above, and 0.1 ml of anti-mouse immunoglobulin G (IgG) plus IgM (heavy and light chains)–horseradish peroxidase conjugate (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, Md.), diluted 1:2,000 in PBS-T-HS, was added to each well. After 10 min of incubation at 37°C, the plates were washed as described above, 0.1 ml of substrate was added to each well, and the plates were incubated at room temperature for 20 min. The substrate solution consisted of 3.125% hydrogen peroxide (Fisher Scientific, St. Louis, Mo.) and 0.5% 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (Sigma Chemical Company) in citrate buffer (pH 4.0; 50 mM citric acid; Sigma Chemical Company). The plates were read after 20 min at an optical density of 405 nm. An absorbance value of 0.1 or greater was considered positive and an absorbance value below 0.1 was considered negative by using undiluted cultured hybridoma supernatants.

Production of ascitic fluid. Retired breeder BALB/c mice were primed by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethyl pentadecane (Pristane; Sigma Chemical Company). After a 5- to 7-day rest period, 5×10^6 to 1×10^7 hybridoma cells in 1 ml of serum-free Dulbecco's modified Eagle medium were injected into each mouse by the intraperitoneal route. The ascitic fluid was collected 7 to 14 days after injection of the hybridoma cells. Cell debris and fibrin clots were removed by centrifugation (4,000 × *g*, 10 min), and the clarified ascitic fluid was stored at 4°C until all the ascitic fluid from a given hybridoma line was collected. The titers in the samples were determined separately and the samples were retested for specificity, pooled, aliquoted into 1-ml cryovials (Sarstedt, Incorporated, Princeton, N.J.), and stored at −70°C.

Isotyping and purification of immunoglobulins. The isotypes of the MAbs were determined with a subisotyping kit (Calbiochem, San Diego, Calif.) according to the manufacturer's instruction. Immunoglobulins of the IgG3 subisotype were purified with an Affi-Gel protein A MAPS II kit (Bio-Rad Laboratories, Inc., Hercules, Calif.), and immunoglobulins of the IgM isotype were purified with the ImmunoPure IgM purification kit (Pierce). The purified MAbs were dialyzed in FTA PBS with 0.02% sodium azide (Sigma Chemical Company) and were stored at −70°C.

SDS-PAGE and Western blotting. The Laemmli glycine system (19) was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gels to produce a more distinct ladder LPS banding pattern of the smooth (phenol-phase) LPS. The ProRanger (AT Biochem, Malvern, Pa.) tricine system was used with 16.5% gels for the rough (aqueous-phase) LPS. Working dilutions of the LPS preparations were determined empirically by using silver-stained gels (silver stain kit; Bio-Rad Laboratories, Inc.). Phenol-phase LPS was diluted 1:15 and aqueous-phase LPS was diluted 1:80 in electrophoresis sample buffer (19). The LPS was transferred to PolyScreen polyvinylidene difluoride transfer membranes (NEN Research Products, Boston, Mass.) by the protocol described by Towbin et al. (38).

Membranes were blocked overnight at 4°C with Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 7.5], 500 mM sodium chloride [Sigma Chemical Company]) with 5% nonfat dry milk (Carnation). Antibodies were diluted in TBS containing 1% milk. All ascitic fluid was diluted 1:200, and anti-mouse IgG plus IgM (heavy and light chains)–horseradish peroxidase conjugate (KPL) was diluted 1:750. Membranes were flooded with ascitic fluid and were incubated at 25°C for 1.5 h, while rocking. After washing, the conjugate was added and the mixture was incubated at 25°C for 1 h. Washing steps consisted of rinsing the membranes three times with distilled water and three times (5 min each, 25°C, while rocking) with TBS plus 0.05% Tween 20 (Sigma Chemical Company) without the addition of milk. Bands were visualized with diaminobenzidine (KPL) substrate, and the reaction was stopped by rinsing the membranes in distilled water.

Fluorescent-antibody microscopy. Microscope slides were coated with 10 μl of live bacteria (*E. coli* O157:H7 CDC EDL 933, *Citrobacter freundii*, or *Salmonella typhimurium*) harvested from TSA plates and were suspended in water. Slides were allowed to air dry before they were fixed in cold (−20°C) acetone for 10 min, air dried, and stored at −70°C. For the assay, the slides were thawed and allowed to dry at room temperature, and two fluorescent ink (MARC-TEX Corporation, Englewood, N.J.) circles were drawn on each slide to keep the liquid (MAbs and conjugate) from spreading on the slide and drying out during incubation. One hundred microliters of uncultured hybridoma medium was added to one of the circles on each slide as a negative control to indicate nonspecific binding of the conjugate, and 0.1 ml of spent culture medium containing the ELISA-positive MAbs was added to the other circle. After incubation at 37°C for 30 min in a moisture chamber, the slides were washed three times (3 min per wash) in FTA PBS. The slides were air dried, and 0.1 ml of anti-mouse IgG (heavy and light chains)–fluorescein isothiocyanate conjugate (KPL), diluted 1:40 in FTA PBS, with Evans blue counterstain (Sigma Chemical Company) added to a final concentration of 0.01%, was added to each well. The slides were again incubated for 30 min at 37°C in a moisture chamber, followed by washing, as before, with fresh FTA PBS and a final 2-min wash in distilled water. The slides were air dried, and coverslips (22 by 50 mm) were mounted by using a drop of buffered glycerol mounting medium (BBL Microbiology Systems) on each

TABLE 1. Reaction of anti-*E. coli* O157 MAbs with *E. coli* and *Salmonella* groups N and D by ELISA

MAb type and no.	Isotype	Reactivity[a]							
		E. coli O157:H7 CDC EDL 933	*E. coli* O157:H7 ECRC 2886-75	*E. coli* O55:H7 EQ5624-50[b]	*S. soerenga* group N $O30_1$[c]	*S. landau* group N $O30_1$	*S. urbana* group N $O30_130_2$[d]	*S. godesberg* group N $O30_130_2$	*S. dublin* group D
MAb to O antigen									
S5	IgG3	1.465	1.475	0.011	1.363	1.392	1.444	1.480	−0.001
12D6	IgG3	1.434	1.463	0.012	1.344	1.444	0.534	0.861	−0.005
13B3	IgG3	1.516	1.579	0.010	1.326	1.488	0.545	0.892	0.013
MAb to core antigen									
2G9	IgM	0.864	0.856	0.033	0.513	0.271	0.609	0.444	0.033
3B7	IgM	0.777	0.868	0.024	0.504	0.385	0.572	0.507	0.033
29F8	IgM	0.891	0.892	0.006	0.457	0.365	0.548	0.549	0.014
42C2	IgG3	1.066	1.038	−0.001	0.756	0.783	0.807	0.880	−0.001
46E9	IgM	0.776	0.860	0.003	0.421	0.431	0.431	0.583	0.016

[a] Values are optical densities at 405 nm and are averages for duplicate wells.
[b] Putative precursor to *E. coli* O157:H7.
[c] Reference strain $O30_1$ *Salmonella.*
[d] Reference strain $O30_130_2$ *Salmonella.*

circle. The slides were then examined and photographed with a ×100 oil immersion objective by using a Zeiss Axiophot UV microscope equipped with an Optronics DEI-470T camera with digital enhancement capabilities and a Sony color video printer (Hitschfel Optical Instruments, Inc., Independence, Mo.).

Direct ELISA. All four of the MAbs belonging to the immunoglobulin subisotype IgG3 were conjugated to horseradish peroxidase by the procedure described by Nakane and Kawaoi (23). Each conjugate, as well as a commercial anti-*E. coli* O157 conjugate (KPL), was used separately in a direct sandwich ELISA with all eight MAbs individually, as well as with unconjugated polyclonal anti-*E. coli* O157 antibodies (KPL), as coating antibodies, to determine the combination producing the most sensitive assay for detecting *E. coli* O157:H7. The plates were coated with crude ascites diluted in 0.05 M carbonate buffer (pH 9.6) as described above for coating the bacteria. Working dilutions for the coating antibodies and conjugates were determined empirically by standard block titration methods. The ELISA procedure used was the same as that described above for hybridoma screening.

Agglutination assay. The ability of the MAbs to agglutinate *E. coli* O157:H7, as well as $O30_1$ and $O30_130_2$ *Salmonella* strains (group N), was evaluated by the tube agglutination procedure provided by ECRC. Briefly, crude ascites were diluted 1:100 and 1:500 in phenol-saline (0.6% phenol). The bacteria were diluted in formal saline (0.6% formalin) to a cell density of 10^9 cells/ml. Tubes containing 0.5 ml of diluted MAb and 0.5 ml of diluted antigen were incubated in a 50°C water bath for 2 h and were then refrigerated overnight at 4°C.

RESULTS

Eight MAbs that were specific for the LPS of *E. coli* O157 were produced. All eight MAbs reacted with all 47 *E. coli* O157:H7 strains and 17 O157:non-H7 isolates. None of the MAbs cross-reacted with the 38 non-O157 *E. coli* strains. With the exception of group N *Salmonella* strains, none of the MAbs reacted with any of the 38 non-*E. coli* gram-negative enteric bacterial strains (22 genera) tested. Although actual absorbance readings varied slightly between cultured hybridoma samples due to variations in antibody concentrations within the various MAb preparations, the average absorbance for six of the MAbs for all *E. coli* O157 strains tested was greater than 1.0 (data not shown). MAbs 29F8 and 3B7 produced slightly lower but clearly positive average absorbance readings of 0.8 and 0.6, respectively. The average absorbance for all eight MAbs for all non-O157 *E. coli* strains and all non-*E. coli* gram-negative bacteria (except for group N *Salmonella* strains) was less than 0.025, demonstrating the absolute specificity for *E. coli* O157.

The MAbs could be further characterized by their reactivities with four strains (two subgroups) of group N *Salmonella*: subgroup $O30_1$ (*S. landau* and *S. soerenga*) and subgroup $O30_130_2$ (*S. urbana* and *S. godesberg*) (Table 1). MAbs S5, 42C2, and 46E9 reacted equally well with all four strains, whereas MAbs 12D6 and 13B3 reacted strongly with subgroup $O30_1$ but significantly less with subgroup $O30_130_2$, with *S. urbana* being less reactive than *S. godesberg*. MAbs 3B7 and 29F8 reacted equally well with three of the four strains (*S. soerenga*, *S. urbana*, and *S. godesberg*), but less with *S. landau*. MAb 2G9 reacted the strongest with *S. urbana* and *S. soerenga* and less with *S. godesberg*, and the reaction with *S. landau* was weak.

The core antigen and O-antigen specificities of the MAbs were determined by their reactivities with both the phenol and aqueous phases of hot phenol-water-purified LPS on Western blots (Fig. 1). MAbs 13B3, 12D6, and S5 were determined to react with the *E. coli* O157 O antigen because they produced the characteristic ladder banding pattern on phenol-phase (smooth) LPS (Fig. 1), while they did not react with the core bands in the 12- to 16-kDa region of the aqueous-phase (rough) LPS (Fig. 1). However, these O-antigen-specific MAbs produced some minor bands in the 43-kDa region of the aqueous phase. The lack of banding in this region with MAb S5 was determined to be due to low antibody concentration (data not shown). MAbs 2G9, 46E9, 29F8, and 3B7 were determined to react with the LPS core antigen on the basis of their reactivities with the 12- to 16-kDa bands of the aqueous-phase LPS. MAb 42C2 also displayed core antigen specificity, reacting with all three bands in the 12- to 16-kDa region of aqueous-phase LPS, in addition to higher-molecular-weight bands. The reactivity of MAb 42C2 was specific for *E. coli* O157 and *Salmonella* $O30_1$. MAbs 2G9, 46E9, 29F8, and 3B7 were also core antigen specific but did not react strongly with higher-molecular-weight LPS.

The MAbs were evaluated for their diagnostic potential as reagents for immunofluorescent assays by using slides coated with *E. coli* O157:H7 CDC EDL 933, *C. freundii*, or *S. typhimurium*. All of the MAbs produced bright fluorescence on slides coated with *E. coli* O157:H7, while they showed no reactivity or background fluorescence on slides coated with *C. freundii* or *S. typhimurium* (data not shown). The reaction of *E. coli* O157 O-antigen-specific MAb 13B3 demonstrated that nearly all of the bacteria in a given field of view were expressing the O157 antigen.

The sensitivities of the MAbs for detecting *E. coli* O157 were compared with those of commercially available anti-O157 polyclonal antibodies (KPL) in a capture ELISA format. All eight MAbs were tested individually as capture (coating) anti-

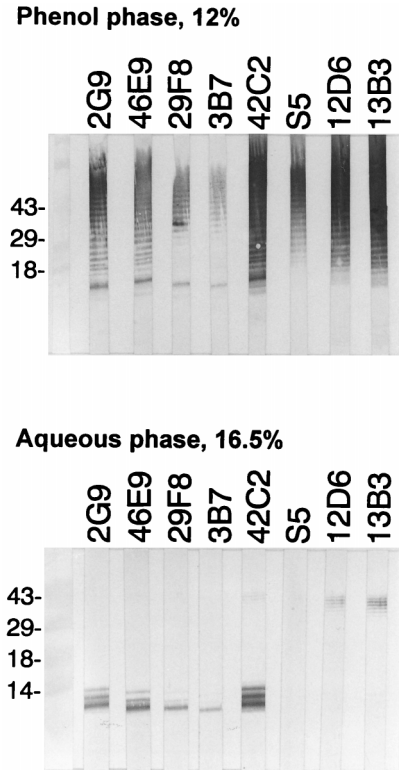

FIG. 1. Western blot of MAbs on hot phenol-water-purified LPS from *E. coli* O157:H7 CDC EDL 933. Results for the phenol phase from a 12% gel and the aqueous phase from a 16.5% gel are presented.

bodies (as were the polyclonal antibodies) by using the four IgG3 MAbs 13B3, 12D6, S5, and 42C2 conjugated to horseradish peroxidase, as well as the polyclonal conjugate (KPL). MAb 13B3 used as both the coating antibodies (crude ascites diluted 1:3,000) and conjugate (diluted 1:2,000) provided the most sensitive MAb-based ELISA in which 1.85×10^4 (1.85×10^5/ml) *E. coli* O157 cells could readily be detected, which was also the detection limit when the polyclonal antibodies were used as both coating antibodies (diluted 1:2,000) and conjugate (diluted 1:2,000) (data not shown). Use of various combinations of antibodies in a polyclonal-monoclonal ELISA system did not increase the sensitivity of the assay.

All of the MAbs were tested for their ability to agglutinate *E. coli* O157:H7 CDC EDL 933 and both subgroups of group N *Salmonella* by a tube agglutination assay (data not shown). Both MAbs 12D6 and 13B3 produced a +4 reaction for *E. coli* O157:H7, in which the agglutination had the appearance of fine granules with total clearing of the background. The MAb 12D6 also agglutinated both $O30_1$ (+3 reaction) and $O30_130_2$ (+2 reaction), whereas MAb 13B3 agglutinated $O30_1$ (+3 reaction) but not $O30_130_2$. MAb S5 did not agglutinate O157:H7, but it did agglutinate both $O30_1$ (+3 reaction) and $O30_130_2$ (+3 reaction). None of the MAbs with core antigen specificity, including MAb 42C2, were able to produce agglutination.

DISCUSSION

The data presented here demonstrate the specificity of eight MAbs for the LPS of *E. coli* O157 (and group N *Salmonella* strains). Three of the MAbs with core antigen specificity (MAbs 2G9, 3B7, and 29F8) originated from a mouse immunized with a whole-cell sonicate of *E. coli* O157:H7, and all three are of the IgM isotype. The remaining five MAbs, including the three MAbs with O-chain specificity, originated from a mouse immunized with a crude flagella preparation. The flagella preparation was intended for the production of MAbs specific for the H7 flagella antigen and was estimated to be at least 95% pure flagella on the basis of SDS-PAGE with Coomassie brilliant blue staining (which does not stain LPS) (data not shown). MAbs S5, 12D6, 13B3, and 42C2 were all of the immunoglobulin subisotype IgG3, and MAb 46E9 belonged to the IgM isotype. The fact that all eight of the MAbs were of either the IgM or the IgG3 isotype supports the report that the anti-O-antigen and anti-core antigen antibody response to LPS is restricted to the Ly-1 B-cell lineage in BALB/c mice and that Ly-1 B cells preferentially secrete the IgM and IgG3 isotypes (32).

The original protocol for LPS purification by Westphal and Jann (42), as well as a modification of the technique by Johnson and Perry (16), which incorporated the use of proteases and nucleases prior to extraction with phenol, both disregarded the phenol phase and subsequently isolated the LPS from the aqueous phase only. Perry et al. (29) later determined the structure of the *E. coli* O157 O chain following the same protocol, but they used the LPS isolated from the phenol phase for structure determination. The Western blots of the *E. coli* O157 LPS-specific MAbs in the phenol phase (Fig. 1A) and aqueous phase (Fig. 1B) show that nearly all of the O chains were located in the phenol phase. The only O chains present in the aqueous phase were the minor bands at about 43 kDa, which is in agreement with the data reported by Dodds et al. (11), who used polyclonal anti-*E. coli* O157 LPS antibodies. Visualization of the aqueous-phase core antigen banding required 16.5% acrylamide gels because the low-molecular-weight bands ran off of the 12% acrylamide gels ahead of the dye front.

If the UV light microscope used for the detection of *E. coli* O157 does not possess digital enhancement capabilities, the core antigen-specific MAbs would be preferred over the O-antigen-specific MAbs in the fluorescent-antibody assay. LPS was easily separated from the bacteria during sample preparation, especially if the organisms were either heat killed or previously frozen. The free LPS was spread all over the slide when the smears were prepared, and the high number of available O-antigen epitopes per molecule of smooth LPS, compared with the number of core epitopes, resulted in a "starry night" effect of extremely high (although antigen-specific) background (data not shown). The addition of Evans blue counterstain to the conjugate enabled all of the bacteria on the slide to be easily visualized by light microscopy, which allowed the reactivities of the MAbs to be determined on an individual bacterium basis. It was also an important control measure to ensure that the lack of antibody reactivity to *C. freundii* or *S. typhimurium* was not the result of all of the bacteria being removed from the slide by the washing technique used to remove the unbound conjugate. The bright reaction of MAb 13B3 for *E. coli* O157:H7 demonstrated that the fluorescent-antibody assay would be the method of choice for the detection of *E. coli* O157 present in low numbers within a high concentration of non-O157 bacteria, because each bacterium was so strikingly positive. There have been no reports of the use of MAb-based fluorescent-antibody assays for the detection of *E. coli* O157; however, the use of polyclonal antibodies has been reported. Tortorello and colleagues (36, 37) used an antibody-direct epifluorescent filter technique to enumerate *E. coli* O157:H7 in milk and apple juice (36) and in ground beef (37). Park et al. (25) used immunofluorescence to identify *E. coli* O157:H7 directly from fecal smears and detected *E. coli*

O157 in all the specimens from which *E. coli* O157 was recovered by culture.

The capture ELISA was the most rapid assay method for the identification of *E. coli* O157, with results determined in 45 min to 1 h. However, the expected increase in assay sensitivity when the MAb-based ELISA was compared with the polyclonal ELISA was not achieved. This was probably a result of the huge number of O-chain repeats per bacterium, as visualized by the fluorescent-antibody assay and because there are potentially several different epitopes on the smooth LPS that could be bound by polyclonal antibodies.

All the MAbs were tested for their ability to agglutinate *E. coli* O157 because, despite the insensitivity of agglutination assay systems in general, most laboratories continue to rely on latex agglutination tests for the identification of *E. coli* O157 (25, 34). Two of the three O-antigen-specific MAbs (MAbs 13B3 and 12D6) agglutinated *E. coli* O157 equally well and could be adapted to a more rapid agglutination assay system requiring fewer MAbs than the method described here. The failure of all of the core antigen-specific MAbs to agglutinate bacteria was probably due to steric hindrance by the O chains on the smooth LPS.

None of the eight MAbs reacted with *E. hermannii*, *B. abortus*, *B. melitensis*, *Y. enterocolitica* serotype O9, *S. maltophilia*, or *C. freundii*, bacteria known to be cross-reactive with polyclonal antibodies to the LPS of *E. coli* O157. Bundle et al. (4), Caroff et al. (6), and Perry and colleagues (26, 29) reported that the cross-reactive epitopes in the LPS O chains of *E. coli* O157, *B. abortus*, and *B. melitensis* are probably 1,2-substituted *N*-acyl derivatives of 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues and that the cross-reactive epitopes on the LPS of *Y. enterocolitica* serotype O9 and *S. maltophilia* are probably *N*-acetyl derivatives of 4-amino-4,6-dideoxy-α-D-mannopyranosyl residues. Lack of reactivity of anti-core LPS MAbs would be expected since the cross-reacting epitopes supposedly reside on the O chains. For the same reason, we were surprised that all of the MAbs presumed to be core specific cross-reacted with group N *Salmonella* strains. The fact that the three O-chain-specific MAbs reacted differently to $O30_1$ and $O30_130_2$ strains of group N *Salmonella* and did not cross-react with *Brucella*, *Yersinia*, or *S. maltophilia* alludes to the location of their epitopes on the O-chain tetramers. Bundle et al. (4, 5) and Perry et al. (28, 29) reported that the differences in the tetrasaccharide repeating of $O30_1$ *Salmonella* and the pentasaccharide repeating unit of $O30_130_2$ *Salmonella* is that the 4-acetamide-4,6-dideoxy-α-D-mannopyranosyl contains a 2-O-substituted residue in the $O30_130_2$ LPS. The decreased reactivity of MAbs 12D6 and 13B3 to *S. urbana* and *S. godesberg* suggests that their epitope specificity is at or proximal to the substitution site on the 4-acetamide-4,6-dideoxy-α-D-mannopyranosyl unit and that the additional hexose in the pentasaccharide repeating unit blocks or sterically hinders the binding of MAbs 12D6 and 13B3 to the pentasaccharide repeating units. The fact that MAb S5 reacts equally to all four strains of group N *Salmonella* suggests that its epitope specificity is distal to the substitution site on the 4-acetamide-4,6-dideoxy-α-D-mannopyranosyl unit and that the epitope specificity of MAb S5 is different from those of MAbs 12D6 and 13B3.

The ability to differentiate *E. coli* O157:H7 from *E. coli* O157:non-H7 strains is important because human isolates of *E. coli* O157 possessing H antigens other than H7, such as O157:H45, O157:H39, O157:H19, and O157:H43, which may produce heat-labile enterotoxin, have not been implicated as etiologic agents of human hemorrhagic colitis or hemolytic-uremic syndrome (17). Detection of the O157 antigen is a major step in the identification of *E. coli* O157:H7. The MAbs described here provide a valuable tool for the rapid detection of *E. coli* O157, although these MAbs alone are insufficient for distinguishing O157:H7 isolates from other *E. coli* isolates possessing the O157 antigen.

ACKNOWLEDGMENTS

We thank Richard Wilson (ECRC) for graciously providing the *E. coli* reference strains; Tammy Sorensen, Nancy Ferrell, Ron Mlejnek, and Sandy Fryda-Bradley for excellent technical assistance; and Joan Rosch for careful preparation of the manuscript.

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