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Production and Characterization of Monoclonal Antibodies to *Escherichia coli* (ATCC 8739)

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Escherichia coli causes intestinal and extraintestinal infections and has been an indicator of fecal pollution in water and food. BALB/c mouse was immunized by injection of somatic *E. coli* (ATCC 8739) cells to produce monoclonal antibodies. Splenocytes of mouse were fused with myeloma cells (Sp2/0-Ag14). Two hybridomas secreting monoclonal antibodies were established after being cloned. In SDS-PAGE analysis of *E. coli* antigens 37 protein profiles appeared from 14 kDa to 182 kDa. Western blot analysis using polyclonal antibodies demonstrated that protein antigens of 41 kDa, 38.2 kDa and 31.7 kDa were immunodominant. Monoclonal antibodies DY-CM1 and DY-CM2 recognized 31.7 kDa and 2.0 kDa antigens in Western blot analysis, respectely.

Escherichia coli causes intestinal and extraintestinal infections and has been an indicator of fecal pollution in water and food. *E. coli* is the most common gram-negative organism causing sepsis and meningitis in the neonatal period (6, 9). *E. coli* isolates responsible for intestinal infections can be differentiated into several categories designated as enterotoxigenic *E. coli*, enteropathogenic *E. coli*, entero- invasive *E. coli* and enterohemorrhagic *E. coli* (1, 13).

The presence of *E. coli* indicates that there might have been contamination from sewage and that *Salmonella* or other intestinal pathogens might be present (2). Monoclonal antibodies (mAbs) have become powerful tools for the analysis of antigenic substances when coupled with blotting techniques (4, 8).

This study was performed to produce monoclonal antibodies against O (somatic) antigen of *E. coli* (ATCC 8739) by hybridoma technology and to characterize them.

MATERIALS AND METHODS

Bacterial Strains and Animals

Escherichia coli (ATCC 8739) was obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). BALB/c mice were used for being immunized.

Preparation of Myeloma Cell Line

Myeloma Sp2/0-Ag14 cell lines were used as fusion partner and maintained in a CO_2 incubator at 37°C until used.

Media

The basal medium contained 11 mM D-glucose (Amresco Solon, Ohio, U.S.A.), 1.37 mM glutamic acid (Gibco, Grand Island, New York, U.S.A.), 24 mM sodium bicarbonate (Gibco), 25 mM HEPES (Gibco). Dulbecco's Modified Eagles Medium (DMEM: Gibco) was filtered by micropore size (0.22 μm) membrane, and stored at 4°C until used. 10% fetal bovine serum (Gibco) and 50 μg/ml gentamycin (Gibco) were added to the basal medium to make a complete medium.

Preparation of Immunogens

E. coli (ATCC 8739) was cultured in 10 ml of Brain Heart Infusion Broth (BHI: Difco, Detroit, Michigan, U.S.A.) at 37°C with shaking overnight. The growth of bacteria was checked by turbidity until its concentration reached up to 10^5~10^9 cells/ml ($O.D._{570\,nm}$= 1.5). In order to optimize bacterial growth, the 100 ml of Luria Bertani (LB: Difco Leb. Detroit, Michigan, U.S.A.) was added at 37°C with shaking for 18 hours. The cells harvested were boiled at 100°C for 2.5 hours and rediluted in saline solution with 0.3% of formalin to be used as immunogens and stored at 4°C.

Immunization of Mice

The 6 weeks old female BALB/C mice were used. Mice were injected intra-peritoneally with *E. coli* whole cells (10^9 cells/ml) and boosted at 3 weeks later. Mice were tail-bled on the 22nd day to determine

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the titer of anti-*E. coli* antibodies. The final tail injection was given intravenously with *E. coli* cells (0.1 ml) at three weeks after the second boosting. 10^9 cells/ml in Phosphate Buffered Saline (PBS) were given for every injection. Hyperimmune mice were sacrificed 3 days after the final booster, and spleen cells were removed to be fused with myeloma cells.

Preparation of Splenocytes

Spleen of the mouse was used as B lymphocyte for the cell fusion. When the cell fusion was performed, spleen was removed by aseptic technique from mouse's peritoneum and it was transformed to homogeneity on the wire mesh screen (0.45 μm) by plunger of syringe (5 cc), and washed with basal medium (20 ml) and centrifuged at 400×g for 10 min. They were washed with basal media twice.

Cell Fusion

Cell fusion was performed by Groth and Scheidegger (5) methods. Splenocytes and myeloma were mixed (10 : 1) into the 50 ml conical tube, and filled with basal media to 40 ml, and centrifuged at 400×g for 10 min. The supernatant was removed and 1 ml of polyethylene glycol 4000 (PEG 50% wt/vol: Gibco, Grand Island, New York, U.S.A.) was slowly added to the pellet of cells while gently flicking for 1 min and stayed for 1 min. The 20 ml of the basal media were slowly added to the cells with gently flicking for 4 min. These procedures were performed at 37°C in the water bath. The cell mixture was centrifuged at 300×g for 5 min and then the supernatant was removed. The thymocyte conditioned media (150 ml) were added and suspended to the cell mixture. The fused cells were dispensed with 1 ml into 24 well tissue culture plates and cultured at 37°C in a CO_2 incubator. After 24 hours of cell fusion, 1 ml of HAT media was added to the well.

Screening of Hybridoma by Indirect immunofluorescence Assay (IFA)

At 10 to 14 days post fusion, colonies of growing hybridoma clones were visible in 24-well tissue culture plates. Smears on glass slides were prepared from the culture of *E. coli* in PBS, air-dried, and fixed by heating. The smears were incubated for 30 min at 20°C with 100 μl of hybridoma supernatants, washed with PBS three times, incubated with 100 μl of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG+IgM+IgA (Cappel, West Chester) diluted to 1/1000 in PBS for 20 min. The cells were washed three times and dropped in PBS containing 10% (vol/vol.) glycerol. Immunofluorescence was evaluated with a fluorescence microscope (Olympus BH-2, Japan, × 1000).

Cloning of Hybridoma

Hybridomas producing monoclonal antibodies to *E. coli* were cloned by limiting dilution in 96-well plates to less than one cell per well (11). Cloning for cells, the wells with colony were rescreened and recloned. The cells were diluted to 1×10^7 cells/ml into complete media after being centrifuged at 400×g for 10 min. And the cells were dispensed into first 3 lanes of 96 well plates, which were previously added with 100 μl of thymocyte conditioned media, by 22 cells per well with its volume of 100 μl. And 4 ml of complete media were added into rest cells, which were dispensed into next 3 lanes with 100 μl of 4.4 cells per well. Next, 1.5 ml of complete media were added into last cells and they were dispensed into very last 2 lanes by 100 μl of 2.2 cells per well. All of the cells were cultured in 5% CO_2 incubator at 37°C.

Expanding and Freezing of Positive Clones

In the process of selection and cloning of hybridomas, positive wells had been identified and then the cells were transferred from the 24-well tissue culture to a 25 cm^2 flask and retransferred to a 75 cm^2 flask. Expanded cells were centrifuged at 400× g for 10 min. After washing twice with basal media, the pellets were diluted in the freezing medium (basal medium/40% FBS/10% DMSO: Sigma, St. Louis, U.S.A.) as to 1×10^7 cells per ml and kept to the cryotube (Corning, N.Y., U.S.A.) at -70°C deep freezer for 18 hours, and then stored at -185°C in a liquid nitrogen tank (Locator, JR., Thermolyne, Dubuque, Iowa, U.S.A.).

Determination of Isotype of Monoclonal Antibodies

The isotypes of mAbs were determined using mAb subisotyping kit (HYCNONE 98055) by enzyme linked immunosorbent assay (ELISA) sandwich method (7). The procedure was described briefly as follows; 96-well plate was coated with 100 μl/well of goat anti-mouse immunoglobulin for 18~24 hours at 4°C. After washing three times with PBS-surfactant, 50 μl of PBS-surfactant was added to each well and 50 μl of hybridoma supernatant to 6 wells and mouse serum diluted to 1 : 500 was used as a positive control. The plate was incubated at room temperature for one hour. After washing wells with PBS, 6 different kinds of goat anti-mouse Ig antiserum or PBS-surfactant 100 μl were added to the test wells, incubated for one hour at room temperature. After washing three times with PBS, were added 100 μl of goat anti-rabbit IgG peroxidase conjugate diluted to 1 : 4000 with PBS-surfactant to each well, and incubated the plate at room temperature for one hour. After washing three times with PBS, 100 μl substrate-chromophore were added to each well and added 25 μl 4 N NaOH or 50 μl 1 N HCl to stop reaction and optical density (O.D.)

was measured at 490 nm by THERMOmax microplate reader (Molecular Devices Corp. Menlo Park, California, U.S.A.).

Preparation of Cell Lysates

E. coli strains were grown in 200 ml of Luria-bertani (LB; Difco, Detroit, Michigan, U.S.A.) medium at 28°C for 18 hours and shaken at 60×g. The cultures were centrifuged for 20 min at 1,000×g, the cell pellets were washed twice in PBS (pH 7.2) and centrifuged for 20 min at 1000×g. The final pellets were suspended in 5 ml of lysis buffer containing 1 M NaCl and 0.01% Triton X-100 and kept at 4°C for 18 hours. The suspended pellets were disrupted by ultrasonicator (Braun-sonic 1510, South Sanfrancisco, Cal., U.S.A.) at an interval of 30 seconds between bursts for cooling. At this stage, the bacterial suspension was maintained on ice. After being centrifuged at 25,000×g at 4°C for 30 min, the protein contents of the soluble fractions were estimated by the BCA kit (Pierece, Rockford, Ill., U.S.A.) method. Soluble fractions were stored at -20°C.

SDS-PAGE Analysis of Sonicated *E. coli* Antigens

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out by the modification of the Laemmli method (10) in 12.5% separating gel and 5% stacking gel, using a micro slab gel electrophoresis system (SE 250-Might Small II, Hoefer, U.S.A.). Antigens (20-40 μg) of cell lysates were dissolved in 60 mM- Tris/HCl buffer (pH 6.8) containing 2% (W/v) SDS, 25% (V/V) glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue by heating at 100°C for 5 min. After electrophoresis, the gels were stained with Coomassie blue R-250 (Sigma, St., Louis, U.S.A.) for 20 min, or used for Western blot analysis. The standard molecular markers (Bio-Rad, U.S.A.) were used to myosin (200 kDa), β-galactosidase (116 kDa), rabbit muscle phosphorylase B (97. 4 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa) and bovine pancreases apotinin (6.5 kDa).

Western blot Analysis of *E. coli* Antigens using Monoclonal Antibodies

After electrophoresis, proteins were transferred to nitrocellulose sheets using an electro blotter (Hoefer, Sanfrancisco, Cali., U.S.A.) as described by Towbin (14). The buffer was 15.6 mM Tris (Sigma, St. Louis, U. S.A.) and 120 mM glycine (Sigma, St. Louis, U.S.A., pH 8.3) with a constant current applied for 90 min at 4°C. The gel was stained with Amido black (Sigma, St. Louis, U.S.A.) to confirm complete transfer of the proteins. The nitrocellulose was blocked with 3% BSA in Tris buffered saline (TBS; 10 mM Trizma base, 150 mM NaCl, pH 7.5) for 2 hours at 37°C and washed three times with TBS. The nitrocellulose was incubated with hybridoma supernatants for I hour at 37 °C. Then, horse radish peroxidase conjugated to goat anti-mouse IgG+IgM (Jackson Immuno-Research Lab, Inc., West Grove, Pa., U.S.A.) was diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS and incubated with the blots for 1 hour at 37°C. Following each step, unbound reagents were removed with TBS by three washes for 30 min. The blots were finally treated for 10 to 20 min at room temperature with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 μl H_2O_2 (30%) in 50 ml TBS. The developed blots were washed with distilled water and photographed.

Reactivities of Monoclonal Antibodies to *E. coli* using IFA

Monoclonal antibodies were reacted to 20 strains of *Enterobacteriaceae* using IFA and observed the distribution of *E. coli* antigens. Smears on glass slides were prepared from the culture of *E. coli* and the other strains in PBS, air-dried, and fixed by heating. The smears were incubated for 30 min at 20°C with hybridoma supernatants, washed with PBS twice, incubated with 100 μl of FITC conjugated goat anti-mouse IgG+IgM+IgA (Cappel, West Chester) diluted to 1/1000 in PBS for 20 min. The cells were washed twice again and resuspended in PBS containing 10% (vol/vol.) glycerol and 100 μl of o-phenylene- diamine per ml to prevent fading. Immunofluorescence was evaluated with a fluorescence microscope (Olympus BH-2, Japan, ×1000).

RESULTS AND DISCUSSION

Production of Monoclonal Antibodies

The fusion yielded hybridoma growth in a total 148 wells of 24-well cell culture plates. Of the 148 hybridomas, 105 were obtained (71%) from the fusion of immune BALB/c splenocytes with myeloma cells (SP2/0-Ag14) and 24 (23%) hybridomas produced monoclonal antibodies that reacted specifically with whole-cell antigens of *E. coli* (ATCC 8739), when assayed by indirect immunofluorescent microscopy. The two mAbs were selected for the characterization and named as DY-CM1 and DY-CM2, respectively.

Determination of Isotype of Monoclonal Antibodies

mAb isotypes were determined by a sandwich ELISA with a commercial isotyping kit (HYCLONE 98055). All mAbs of DY-CM1 and DY-CM2 were determined by the IgG2b class. Polyclonal antibodies contained IgM,

IgG1, IgG2a, IgG2b, and IgG3 class (Table 1).

SDS-PAGE Analysis of Sonicated *E. coli* Antigens

SDS-PAGE was used to analyze the protein profiles of soluble protein extracts of *E. coli*. As shown in Fig. 1, *E. coli* (ATCC 8739) had major antigens of 16.3, 18.5, 24.3, 27.5, 30.3, 35, 38.2, 41, 43, 46.2, 47.5, 53.1, 70.3, 86.6 and 100 kDa among 37 protein profiles. *E. coli* causes intestinal and extraintestinal infections and has been an indicator of fecal pollution in water and food. The aerobic plate count have been suggested bacteria such as coliforms, *E coli*, enterobacteriaceae, enterococci, pseudomonads, clostridia and staphylococci as indicator organisms (3). In the SDS-PAGE of coliform bacteria such as *E. coli, C. freundii, K. pneumoniae, E. aerogenes* and *E. cloaceae* strains showed similar in protein patterns (12). Kauffmann and Ørskov (16), reported that cross-reaction occurred between *E. coli* and *Klebsiella* O-antigen. Winkle *et al.* (15) reported that cross-reaction was occured between *Vibrio cholerae, E. coli, Salmonella* and *Citrobacter.*

Table 1. Determination of isotype of Monoclonal Antibodies by ELISA. mAb isotypes were determined by a sandwich ELISA. Optical Density (O. D.) was measured at 490 nm.

Isotypes	Monoclonal Antibodies to *E. coli*		Polyclonal Ab
	DY-CM1	DY-CM2	
IgM	0.052	0.046	0.247*
IgG1	0.051	0.047	0.111*
IgG2a	0.046	0.046	0.092*
IgG2b	0.167*	0.126*	0.147*
IgG3	0.049	0.045	0.121*
IgA	0.049	0.046	0.059

*: positive reaction.

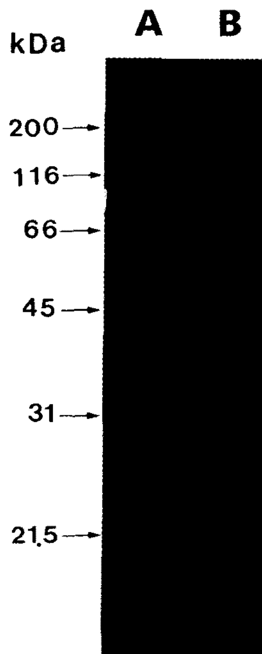

Fig. 1.SDS-PAGE analysis of total proteins of *E. coli*. SDS-PAGE was carried out by the modification of the Laemmli method (10) in 12.5% separating gel and 5% stacking gel, using a micro slab gel electrophoresis system.

The gel was stained with Coomassie blue R-250 for 20 min. and then destained at 18 hours. Lane A, Standard moecular markers; B, *Escherichia coli* (ATVCC 8739).

Western blot Analysis of *E. coli* Antigens using Polyclonal Antibodies

To determine the antigenic specificity of the polyclonal antibodies, Western blotting was performed with *E. coli* antigens. Western blot analysis demonstrated that 11.7, 12.6, 18.5, 20.1, 31.7, 36.8, 41, 56, 83.5 and 146 kDa antigens of *E. coli.* (ATCC 8739) were recognized by polyclonal antibodies. Among these antigens, 83.5, 41, 36.8, 31.7, 12.6 and 11.7 kDa were immunodominant (Fig. 2).

Western blot Analysis of *E. coli* Antigens using Monoclonal Antibodies

Two monoclonal antibodies, DY-CM1 and DY-CM 2, were established after being cloned. DY-CM1 recognized 31.7 kDa antigens of *E. coli*. DY-CM2 recognized antigens of 2.0 kDa and 53.1 kDa of *E. coli* (Fig. 3). Binding of mAbs to protein antigens of a strain showed that each protein held same kind of epitopes. Antigens observed in the Western blot analysis using mAbs did not appear in the analysis using pAbs.

Fig. 2. Western blot analysis of *E. coli* antigens using polyclonal antibody.

After SDS-PAGE, proteins were transferred to nitrocellulose sheets using an electro blotter as described by Towbin (14). Polyclonal antibody (anti-serum) was diluted to 1 : 100 in 0.5% (w/v) BSA/TBS solution. Horse radish peroxidase conjugated goat anti-mouse IgG+IgM was diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS. Lane A, *Escherichia coli* (ATCC 8739); B, standard molecular markers detected by Amido Black.

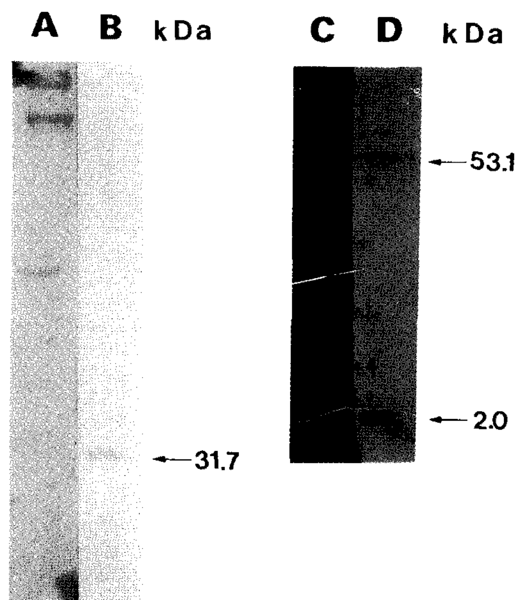

Fig. 3. Western blot analysis of *E. coli* antigens suing two monoclonal antibodies, DY-CM1 and DY-CM2.

After SDS-PAGE, proteins were transferred to nitrocellulose sheets using an electro blotter as described by Towbin (11). Horse radish peroxidase conjugated goat anti-mouse IgG+IgM was diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS. Lane A, standard molecular markers detected by Amido Black; B, *Escherichia coli* (ATCC 8739) using mAb DY-CM1; C, standard molecular markers; D, *Escherichia coli* (ATCC 8739) using mAb DY-CM2.

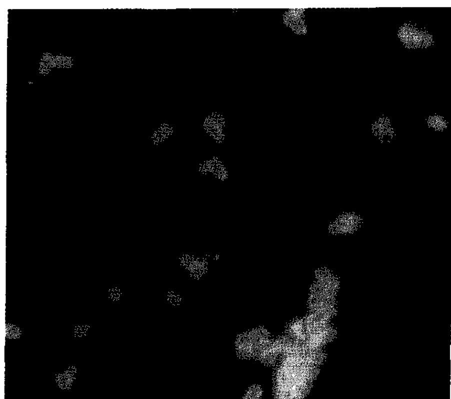

Fig. 4. Reactive appearances of monoclonal antibody (DY-CM1) with *E. coli* antigens by indirect IFA (×1000).

On the basis of this, two possibilities could be thought of; one is that the determinants are not recognized by pAbs, and the other is that the epitope may be denatured by the detergent such as SDS.

Reactivities of Monoclonal Antibodies to *E. coli* using IFA

Monoclonal antibodies were examined in indirect immunofluorescent assay (IFA) for the reactivity with *E. coli* (Fig. 4). Monoclonal antibodies, DY-CM1 and DY-CM2, reacted to *E. coli*. The epitopes recognized by mAbs appears to be the surface antigens of *E. coli*.

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