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Purification, Pore-Forming Ability, and Antigenic Relatedness of the Major Outer Membrane Protein of *Shigella dysenteriae* Type 1

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The major outer membrane protein (MOMP), the most abundant outer membrane protein, was purified to homogeneity from *Shigella dysenteriae* type 1. The purification method involved selective extraction of MOMP with sodium dodecyl sulfate in the presence of 0.4 M sodium chloride followed by size exclusion chromatography with Sephacryl S-200 HR. MOMP was found to form hydrophilic diffusion pores by incorporation into artificial liposome vesicles composed of egg yolk phosphatidylcholine and dicetylphosphate, indicating that MOMP of *S. dysenteriae* type 1 exhibited significant porin activity. However, the liposomes containing heat-denatured MOMP were barely active. The molecular weight of MOMP found by size exclusion chromatography was 130,000, and in sodium dodecyl sulfate–10% polyacrylamide gel it moved as an oligomer of 78,000 molecular weight. Upon boiling, fully dissociated monomers of 38,000 molecular weight were seen for *S. dysenteriae* type 1. However, among the four *Shigella* spp., the monomeric MOMP generated upon boiling ranged from 38,000 to 35,000 in molecular weight. Antibody raised in BALB/c mice immunized with MOMP of *S. dysenteriae* type 1 reacted strongly with purified MOMP of *S. dysenteriae* type 1 in an enzyme-linked immunosorbent assay (ELISA). The antibody reacted with whole-cell preparations of *S. dysenteriae* type 1 in an ELISA, suggesting that MOMP possessed surface components. Moreover, MOMP could be visualized on the bacterial surface by immunoelectron microscopy with anti-MOMP antibody. *S. dysenteriae* type 1 MOMP-specific immunoglobulin eluted from MOMP bound to a nitrocellulose membrane was found to cross-react with MOMP preparations of *S. flexneri*, *S. boydii*, and *S. sonnei*, indicating that MOMPs were antigenically related among *Shigella* species. The strong immunogenicity, surface exposure, and antigenic relatedness make MOMP of *Shigella* species an immunologically significant macromolecule for study.

The outer membrane of gram-negative bacteria harbors some major proteins (major outer membrane proteins [MOMPs]) which are called porins because of their role in cellular permeability (2, 27, 33). The structural (7, 17, 47), chemical (35, 43), physiological (3, 16), and immunological (18, 28, 42) aspects of porins of enteric bacteria have been studied. Porins form large, open, water-filled channels that nonspecifically mediate the passive penetration of ions and small, hydrophilic molecules up to an exclusion limit of about 600 Da. In addition to their pore-forming activity, bacterial porins serve as receptors for bacteriophages and bacteriocins, and in the case of pathogenic bacteria, they also appear to be targets of the immune system (11, 12, 46). Porins of several gram-negative bacteria have been found to exhibit high immunogenicity (18, 49) and to be exposed on the cell surface (22, 45). These features make them attractive as vaccine candidates (13, 19).

Shigella spp., a group of nonmotile, gram-negative, enteric bacteria, cause bacillary dysentery in humans (14, 20) by a multistep invasive process. Following infection, the organisms invade colonic epithelial cells (23), multiply (37), cause cell death (6), and spread intra- and intercellularly (4), resulting in extensive degeneration of the epithelium (29). In developing countries, the disease thrives due to poor sanitation and socioeconomic conditions, and it is one of the predominant causes of infant mortality (21). The clinical management of the disease has become difficult due to the rapid emergence of drug-resistant strains of *Shigella* spp. (39). Therefore, it is desirable to study bacterial components that have vaccine potential. In addition to involvement in pathogenesis (5, 34), the cell surface antigens, which include lipopolysaccharide (LPS) and outer membrane proteins (OMPs), can apparently induce protective immunity in hosts. Convalescence from shigellosis has shown induction of serotype-specific immunity mediated by anti-LPS antibody (25, 26). Immunization of guinea pigs and rabbits with OMPs of *Shigella* spp. protected the animals against keratoconjunctivitis (1). Moreover, the surface polypeptides coded by the virulence plasmid of *Shigella* spp. generated good immune response in humans (15) and experimentally infected monkeys (34). The study of OMP is, therefore, important in view of its possible role in conferring immunity to shigellosis. Since porins of several gram-negative bacteria such as *Salmonella typhi* (19) and *Pseudomonas aeruginosa* (13) showed promise as potential vaccines, immunological studies of the MOMP of *Shigella* spp. could be significant for development of a vaccine. The partially purified MOMP of *Shigella* spp. was found to share several properties with porins, namely, resistance to denaturation by sodium dodecyl sulfate (SDS) and insensitivity to trypsin digestion (36). The aim of this study was to purify the MOMP of *Shigella dysenteriae* type 1 and determine its pore-forming ability. Antigenicity and antigenic relatedness among MOMPs of *Shigella* spp. were also studied.

MATERIALS AND METHODS

Bacterial strains and culture. *S. dysenteriae* type 1 (N-120), *S. flexneri* type 2a (N-117), *S. boydii* type 11 (N-228), and *S. sonnei* (N-238) were isolated from stool samples collected from

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patients admitted to the Infectious Diseases Hospital, Calcutta, India. The virulence of *Shigella* spp. was assessed routinely by the Sereny test (38). The organisms were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) for 16 h at 37°C. Cells were harvested by centrifugation at 10,000 × *g* for 10 min and washed twice with phosphate-buffered saline (PBS; pH 7.2).

Purification of *S. dysenteriae* type 1 MOMP. The harvested cells were suspended in 50 ml of PBS (pH 7.2) and disrupted with an ultrasonic disintegrator (MSE) at 4°C, applying 20 cycles of 30 s each. The undisrupted cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was centrifuged at 100,000 × *g* for 1 h to obtain the envelope fraction. The envelope fraction was suspended in 50 ml of 1% sodium lauroyl sarcosine (Sigma Chemical Co., St. Louis, Mo.) in PBS, and the suspension was gently stirred at 25°C for 30 min to solubilize the inner membrane (10) and centrifuged at 100,000 × *g* for 1 h. The pellet obtained was composed of OMP.

MOMP was purified from OMP according to the method of Nikaido (31). The OMP fraction was suspended in 12 ml of 2% SDS (Sigma) in 10 mM Tris-HCl, pH 7.7. The suspension was then kept at 37°C for 1 h with shaking, which was followed by centrifugation at 100,000 × *g* for 30 min at 25°C. This step was repeated to remove SDS-soluble proteins. The pellet was extracted at 37°C for 2 h in 6 ml of 50 mM Tris-HCl (pH 7.7)–0.4 M NaCl–1% SDS–5 mM EDTA–3 mM NaN_3 (extraction buffer). The suspension obtained was centrifuged at 100,000 × *g* for 1 h at 25°C. The supernatant was concentrated and applied to a Sephacryl S-200 HR column (1.6 by 90 cm) preequilibrated with the extraction buffer. Proteins were eluted at a flow rate of 9.5 ml/h with the extraction buffer, and the A_{280} of the eluent was recorded. The peak fractions containing the MOMP were electrophoresed, pooled, and dialyzed. Protein was estimated by the bicinchoninic acid method (41), using bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin (BSA) as the standard.

Preparation and estimation of LPS. The LPS was extracted from acetone-dried cells of *S. dysenteriae* type 1 by the phenol-water extraction method (48). Total carbohydrate content of the LPS was estimated by the phenol-sulfuric acid method (9).

SDS-polyacrylamide gel electrophoresis (PAGE). MOMP was electrophoresed in 0.1% SDS–10% polyacrylamide gel according to the method of Laemmli (24), and bands were visualized by Coomassie brilliant blue staining. Protein standards (Pharmacia) were run in parallel for determination of the molecular weights of MOMP and OMP.

Assay for pore-forming activity of MOMP. Pore-forming activity was measured by the liposome-swelling method (32). Briefly, egg yolk phosphatidylcholine (2.6 μmol; Sigma) and dicetylphosphate (0.1 μmol; Sigma) were mixed, sonicated, and dried in vacuo to form a film at the bottom of a test tube to which test fractions were added. Liposomes were prepared by dissolving MOMP and the phospholipid mixture in Tris-HCl (pH 7.4), drying the mixture under reduced pressure, and then suspending the mixture in 0.6 ml of 15% dextran 40,000 (Sigma) in 5 mM Tris-HCl (pH 7.4). Portions (20 μl) of these liposomes and 0.6 ml of a 30 mM solution of sugars, such as arabinose, glucose, sucrose, raffinose or stachyose (Sigma), in 5 mM Tris-HCl (pH 7.4) were mixed rapidly in a cuvette, and the A_{400} was recorded. The rate of penetration of the sugars was determined by the initial rate of swelling of the liposome membranes added with the isotonic solution of the test sugar. The swelling rate, i.e., dA^{-1}/dt, was calculated as reported by de Gier et al. (8). In a control experiment, the MOMP fraction which had been denatured by heating in 1% SDS at 100°C for 10 min and precipitated with 50% acetone to remove SDS was used for the swelling assay.

Murine antibodies against MOMP of *S. dysenteriae* type 1. BALB/c mice, originally obtained from Jackson Laboratories (Bar Harbor, Maine), were reared and maintained in the animal facility at the National Institute of Cholera and Enteric Diseases, Calcutta, India. The mice were injected intraperitoneally with 30 μg of purified MOMP emulsified with Freund complete adjuvant (Gibco). This was followed by two booster doses at an interval of 10 days. Antisera were collected by puncturing of the supraorbital plexus.

ELISA. Ninety-six-well micro-enzyme-linked immunosorbent assay (ELISA) plates (Gibco) were coated with either 100 μl of purified MOMP (10 μg per well in 50 mM Tris-HCl, pH 8.1) or 100 μl of cells of *S. dysenteriae* type 1 (10^8 cells per ml). After overnight incubation at 4°C, the wells were blocked with 250 μl of 2% BSA. The plates were incubated for 2 h at room temperature (RT). After blocking, 100 μl of murine anti-MOMP antibody previously absorbed with LPS of *S. dysenteriae* type 1 was added to the wells at different dilutions, and the plates were incubated for 1 h at RT. After incubation, the wells were washed with PBS (pH 7.2) containing 0.05% (vol/vol) Tween 20. One hundred microliters of peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG; Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) at a 1:2,000 dilution was then added, and the plates were incubated for 45 min at RT. The wells were washed, and 100 μl of 0.1% *O*-phenylenediamine (Sigma) in 0.1 M citrate buffer (pH 4.5) containing 0.03% (vol/vol) H_2O_2 was added. After 15 min of incubation, the reaction was stopped by the addition of 2 N H_2SO_4, and the 96-well plates were measured at 492 nm in a Titertek Multiskan ELISA reader (Flow Lab, Helsinki, Finland).

Immunoelectron microscopy. For surface localization of MOMP components, bacteria were pelleted by centrifugation and suspended in PBS, pH 7.2. Five microliters of bacterial suspension was deposited on a pioloform-coated nickel grid. The excess bacterial suspension was blotted out and treated successively at RT with 1% BSA for 15 min and anti-MOMP antibody for 30 min. The grid was then washed with PBS and incubated with colloidal gold (particle size, 5 nm) conjugated with protein A (Sigma) for 30 min at RT. The grid was then washed thoroughly with PBS, stained briefly with 2% phosphotungstic acid (pH 7.0), and examined with a Philips 420T transmission electron microscope.

Elution of *S. dysenteriae* type 1 MOMP-specific IgG from nitrocellulose membrane and Western blotting (immunoblotting). Murine IgG was eluted from nitrocellulose-bound MOMP of *S. dysenteriae* type 1 with a buffer containing 500 mM NaCl, 0.5% Tween 20, 100 μg of immunoglobulin-free BSA (Sigma) per ml, and 50 mM glycine, pH 2.3 (30). The membranes were washed twice with the buffer for 10 min, and the eluted IgG was neutralized by supersaturated $NaHPO_4$. The IgG was used to probe MOMP of *S. flexneri* type 2a, *S. boydii* type 11, and *S. sonnei* in a cross-reactivity study.

Electrophoresed MOMP of *Shigella* spp. was transferred to a nitrocellulose membrane (0.45 μm) at 90 V for 60 min in a Bio-Rad minitransblot chamber (44). After the transfer, membranes were blocked with 3% gelatin in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) for 2 h. Then the membranes were incubated for 1 h at RT with anti-MOMP IgG of *S. dysenteriae* type 1 obtained as described above. Antigen-antibody complexes were detected with alkaline phosphatase-conjugated goat $F(ab')_2$ anti-mouse IgG (Jackson Immunoresearch) at a 1:2,000 dilution for 45 min at RT. The

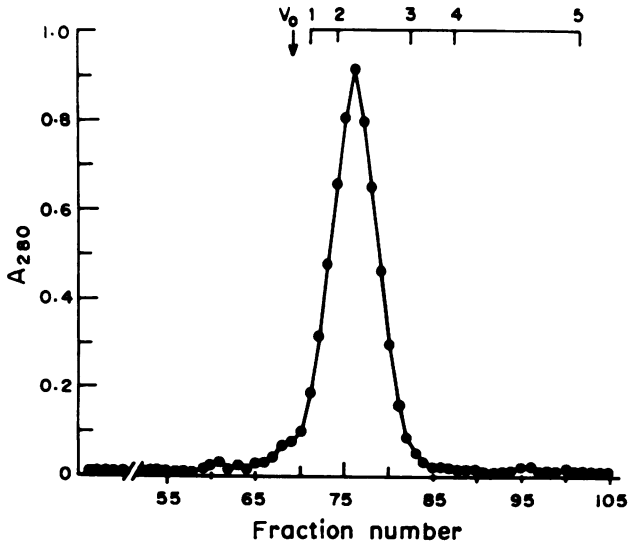

FIG. 1. Profile of MOMP of *S. dysenteriae* type 1 eluted through a Sephacryl S-200 HR column. Fractions, 1 ml, were collected at a flow rate of 9.5 ml/h. The following Pharmacia gel filtration protein standards (molecular weight) were used: 1, catalase (232,000); 2, aldolase (158,000); 3, BSA (67,000); 4, ovalbumin (43,000); 5, chymotrypsinogen A (25,000). V_0, void volume.

nitrocellulose membranes were washed thoroughly in Tris-buffered saline–Tween 20 and developed with 0.3 mg of *p*-nitroblue tetrazolium chloride per ml and 0.15 mg of toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate per ml in carbonate buffer (pH 9.8) containing 0.1 M $NaHCO_3$ and 1.0 mM $MgCl_2$ to visualize the bands.

RESULTS

Purification of MOMP of *S. dysenteriae* type 1. MOMP of *S. dysenteriae* type 1 eluted through a Sephacryl S-200 HR gel filtration column as a single peak and had an apparent molecular weight of 130,000 in the presence of SDS (Fig. 1). Neutral sugar estimation showed that purified MOMP contained less than 1% LPS. The protein ran on SDS-PAGE as a single oligomer band with an apparent molecular weight of 78,000 when it was not heated prior to electrophoresis and with an apparent molecular weight of 38,000 when it was heated in SDS to 100°C for 5 min prior to electrophoresis (Fig. 2).

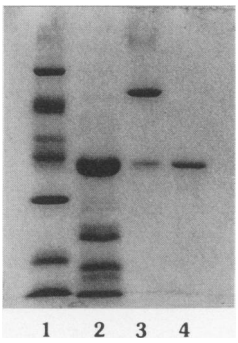

FIG. 2. SDS-PAGE of OMP and MOMP of *S. dysenteriae* type 1. Lane 1, Pharmacia molecular weight standards (from the top): phosphorylase *b* (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400); lane 2, 18 μg of OMP fraction; lane 3, 5 μg of MOMP in SDS at 37°C; lane 4, 5 μg of MOMP in SDS heated at 100°C for 5 min prior to electrophoresis.

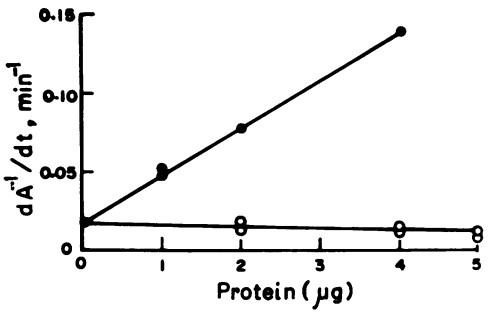

FIG. 3. Swelling of the liposome membrane reconstituted with increasing amounts of MOMP of *S. dysenteriae* type 1. The rate of A_{400} change induced by a 30 mM solution of glucose in 5 mM Tris-HCl (pH 7.4) with MOMP (●) or heat-denatured MOMP (○) was determined.

Assay for pore-forming activities of MOMP. The pore-forming activity of MOMP of *S. dysenteriae* type 1 was examined to determine the ability of MOMP to form hydrophilic diffusion channels by incorporation of various sugars into artificial liposome membranes. Liposomes containing purified MOMP of *S. dysenteriae* type 1 exhibited a marked increase in the initial rate of swelling because of the penetration of glucose into liposomes, while liposomes containing heat-denatured MOMP were barely active (Fig. 3). The swelling rates of MOMP-containing liposomes after the addition of mono- and oligosaccharides, such as arabinose, glucose, sucrose, raffinose, and stachyose, were then compared (Fig. 4). It was found that arabinose and glucose with relative molecular weights (M_r) of 150 and 180 permeated the MOMP-containing liposome membranes, while sucrose (M_r = 342) penetrated only slowly and raffinose (M_r = 504) and stachyose (M_r = 666.7) permeated poorly.

SDS-PAGE profile of purified MOMP preparations of *S. flexneri*, *S. boydii*, and *S. sonnei*. MOMPs were prepared from *S. flexneri* type 2a, *S. boydii* type 11, and *S. sonnei* following the procedure for purification of *S. dysenteriae* type 1 MOMP described in Materials and Methods. The SDS-PAGE profiles

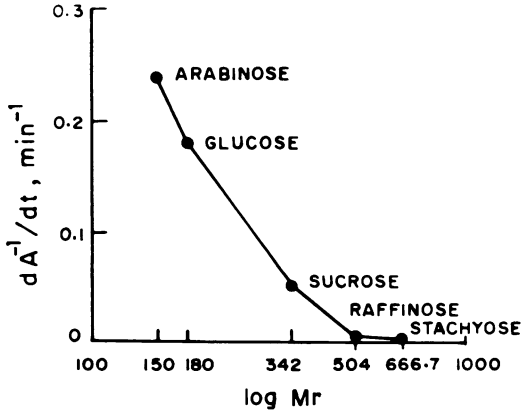

FIG. 4. Swelling of the liposome membrane reconstituted with MOMP of *S. dysenteriae* type 1 by mono- and oligosaccharides. The rate of A_{400} change induced by a 30 mM solution of various sugars in 5 mM Tris-HCl (pH 7.4) was determined.

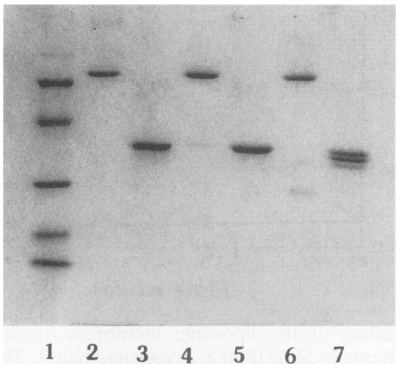

FIG. 5. SDS-PAGE of MOMP preparations (6 μg of protein per lane) of *S. flexneri* type 2a, *S. boydii* type 11, and *S. sonnei* obtained by gel filtration through a Sephacryl S-200 HR column. Lane 1, Pharmacia molecular weight standards (see legend to Fig. 2 for details); lane 2, *S. flexneri* type 2a MOMP in SDS at 37°C; lane 3, *S. flexneri* type 2a MOMP in SDS heated at 100°C for 5 min prior to electrophoresis; lane 4, *S. boydii* type 11 MOMP in SDS at 37°C; lane 5, *S. boydii* type 11 MOMP in SDS heated at 100°C for 5 min; lane 6, *S. sonnei* MOMP in SDS at 37°C; lane 7, *S. sonnei* MOMP in SDS heated at 100°C for 5 min.

of purified MOMPs of all of the *Shigella* spp. showed a single oligomer band with an apparent molecular weight of 78,000 (Fig. 5), as found for the homogeneous MOMP of *S. dysenteriae* type 1 when it was not heated prior to electrophoresis (Fig. 2). However, when it was heated to 100°C for 5 min prior to electrophoresis and the profile was compared with that of *S. dysenteriae* type 1, the protein showed a single band corresponding to a molecular weight of 38,000 for *S. boydii* and two bands of molecular weights of 38,000 and 37,000 for *S. flexneri* and *S. sonnei* (Fig. 5). In fact, *S. sonnei* MOMP, when electrophoresed after heating to 100°C, showed another band of 35,000. Therefore, although the purified MOMPs of all four *Shigella* spp. showed a single oligomer band of 78,000 when not heated before electrophoresis, species-specific variable sizes of MOMPs were found to be generated by heating to 100°C for 5 min.

Antigenicity and antigenic relatedness of MOMP of *S. dysenteriae* type 1. Murine antibodies raised against the purified MOMP of *S. dysenteriae* type 1 recognized both whole-cell and purified MOMPs of *S. dysenteriae* type 1 as the antigen in an ELISA (Fig. 6), suggesting that the strongly antigenic MOMP possessed surface components. Further, colloidal gold-protein A reacted with anti-MOMP antibody-coated bacteria (Fig. 7), indicating surface localization of the components.

IgG was eluted from nitrocellulose membrane-bound MOMP of 38,000 molecular weight of *S. dysenteriae* type 1 and was used to probe MOMPs of *S. flexneri*, *S. boydii*, and *S. sonnei* ranging in molecular weight from 38,000 to 35,000 (Fig. 8). Extensive structural homology among the MOMPs of *Shigella* spp. was observed, as described for detection of OmpD and -C within members of the family *Enterobacteriaceae*, including *S. boydii*, using monoclonal antibodies (40).

DISCUSSION

Since MOMP of *S. dysenteriae* type 1 shares several properties with porins, notably, SDS insolubility, we attempted to purify the protein by a method similar to those described for other bacterial porins. Purification of the MOMP to homogeneity was achieved by size exclusion chromatography after selective extraction with detergents. The purified preparation contained <1% LPS. During purification of the SDS-resistant oligomeric form, the peptidoglycan-porin complex was dissociated by performing the extraction with SDS at RT in the presence of 0.4 M NaCl. Moreover, in order to avoid spontaneous aggregation of the native form, the chromatography was performed in the presence of SDS. The MOMP of *S. dysenteriae* type 1 was purified by procedures similar to those used for bacterial porins, and when assessed for its ability to form transmembrane aqueous channels, the MOMP was found to possess pore-forming ability.

The outer membranes of gram-negative bacteria are immunologically important structures because of their accessibility to host defense mechanisms. In this study, we have found that antibody to MOMP was present in sera of mice immunized with purified MOMP. Recognition of *S. dysenteriae* type 1 in a

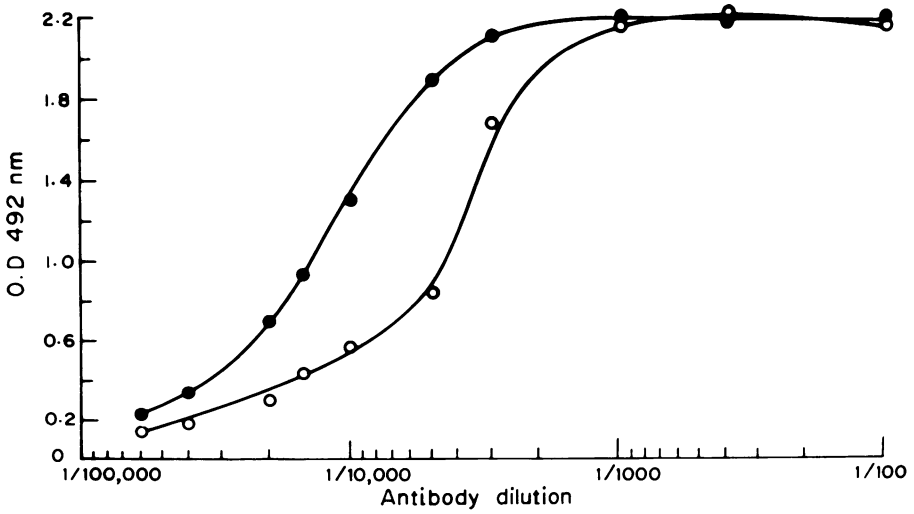

FIG. 6. ELISA for detection of purified MOMP (●) and whole cells (○) of *S. dysenteriae* type 1. Various dilutions of anti-MOMP antibody were used. O.D., optical density.

FIG. 7. Immunolocalization of the antibody to MOMP of *S. dysenteriae* type 1. The electron micrograph shows protein A-colloidal gold (5 nm) labels on the *S. dysenteriae* type 1 surface. Magnification, ×79,380.

whole-cell ELISA by murine antibody raised against MOMP suggested that *S. dysenteriae* type 1 MOMP is surface exposed, as is known for porins of other bacteria (22, 45). The surface exposure of MOMP was also confirmed by immunoelectron microscopy. Besides being strongly immunogenic and exposed to the surface, the MOMP of *S. dysenteriae* type 1 was found to be antigenically related to those of other *Shigella* spp. The antigenic relatedness of MOMP would make it useful as a potential immunogen-crossing barrier of species specificities, as was found for the mouse antibody response to *S. dysenteriae* type 1.

In summary, our study indicates that the 38,000-molecular-weight MOMP purified from *S. dysenteriae* type 1 is a common antigen among *Shigella* spp. and could be a significant macromolecule to study, particularly when a vaccine is being sought to counteract emerging drug-resistant strains of *Shigella* spp.

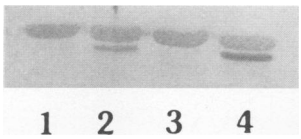

FIG. 8. Western blot analysis of MOMPs of *Shigella* spp. IgG with specificity for *S. dysenteriae* type 1 MOMP was eluted from a nitrocellulose membrane and incubated at RT with blotted MOMPs of *S. dysenteriae* type 1 (lane 1), *S. flexneri* type 2a (lane 2), *S. boydii* type 11 (lane 3), and *S. sonnei* (lane 4).

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