Outer Membrane Protein Antigens in an Enzyme-Linked Immunosorbent Assay for *Salmonella* Enteric Fever and Meningococcal Meningitis

J. E. SIPPEL,[†] H. K. MAMAY, E. WEISS,* S. W. JOSEPH, AND W. J. BEASLEY

Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014

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Outer membrane protein preparations were obtained from strains of *Salmonella* and *Neisseria meningitidis*. Solubilized cell envelope (CE) fractions from *S. typhi* and *Salmonella* groups A, B, C, and E had very similar electrophoretic mobilities on polyacrylamide gel, and common antigens were demonstrated by immunodiffusion. CE appeared to be a more satisfactory antigen than the more purified preparation (T/TEI) in the enzyme-linked immunosorbent assay (ELISA) with sera from typhoid and paratyphoid patients. With either antigen, however, the presence of antibodies was demonstrated in acute- and convalescent-phase sera. In the case of *N. meningitidis* infections, the crude (STA) and the more purified antigens (T/TEI) were equally satisfactory, and a rise in antibody titer could easily be demonstrated with paired acute- and convalescent-phase sera. The ELISA appears to be a simple but highly sensitive test for the detection of antibodies by using outer membrane protein antigens.

The outer membranes of many gram-negative bacteria contain a major protein with a molecular weight of approximately 40,000 (16-18). *Shigella* and *Escherichia coli* appear to have major outer membrane proteins (OMP) of identical molecular weight when compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). Also, OMP from *Pseudomonas aeruginosa* strains appear to be similar to each other (14), and an extensive study of *Neisseria meningitidis* group A strains demonstrated that all have identically migrating OMP (19). *Neisseria gonorrhoeae* and *N. meningitidis* group B strains, on the other hand, can be separated into types on the basis of the SDS-PAGE patterns produced by their OMP (4, 6, 11). These protein type antigens are immunologically specific as determined by immunodiffusion, and their antigenic diversity has epidemiological significance (5, 11).

Because the surface structures of *Salmonella* (H, O, and Vi antigens) show a high degree of diversity, we were prompted to study their OMP by SDS-PAGE and by the enzyme-linked immunosorbent assay (ELISA) to determine whether a more homogeneous component could be identified. The ELISA has been shown to be effective with different kinds of antigens (1, 3, 8, 10, 13, 15, 20) and proved particularly useful for our purpose. Meningococcal OMP was used in

the ELISA as an example of a heterologous antigen.

MATERIALS AND METHODS

Organisms. Clinical *Salmonella* isolates were collected in Indonesia by S.W.J. and in Egypt by J.E.S. The *E. coli* and *Shigella boydii* strains were from the departmental collection. *Klebsiella pneumoniae* was a U.S. hospital isolate. These enteric strains were stored in tightly sealed Trypticase soy agar stab cultures at room temperature. The *N. meningitidis* group A cultures were those used by Sippel and Quan (19), and the group B strain was a type 2 used by Hill and Weiss (9). Meningococci were either stored at -70°C or lyophilized in Greaves medium (5% bovine serum albumin [BSA] and 5% monosodium glutamate). All strains were grown according to the methods of Hill and Weiss (9).

Antigens. Cell envelope (CE) material from *N. meningitidis* and *S. typhi* was obtained according to the modified Schnaitman techniques of Hill and Weiss (9), using discontinuous sucrose gradients to separate CE from cytoplasmic membrane. CE was further purified by extraction of lipopolysaccharide (LPS) with Triton X-100 and Triton X-100 plus ethylenediaminetetraacetic acid (EDTA) (9). This Triton-EDTA insoluble material will be referred to as T/TEI. Meningococcal serotype antigens (STA), which are probably analogous to the outer membrane of CE, were obtained by the lithium chloride extraction procedure of Frasch and Gotschlich (6) and by the following modification of this procedure. An overnight broth culture was harvested by centrifugation and treated with 0.5 M LiCl for 2 h at 45°C with shaking. The material was then centrifuged at 30,000 × g for 15 min, and the

[†] Present address: U.S. NAVACTS U.K., Box 31, F.P.O. New York 09510.

supernatant was again centrifuged at this speed for 40 min. Instead of pelleting the insoluble OMP by ultracentrifugation, the supernatant was treated either (i) with 2 volumes of cold 95% ethanol or (ii) with 2 volumes of cold water. These preparations were kept at 4°C overnight and centrifuged at $30,000 \times g$ for 15 min. The precipitated protein was suspended at approximately 1 mg/ml in water and tested for OMP by immunodiffusion. Purified *S. typhi* LPS was obtained commercially (Difco Laboratories, Detroit, Mich.)

Miscellaneous procedures. OMP preparations were examined by the SDS-PAGE procedures described previously (6). Protein was determined by the method of Lowry et al. (12) with Dade Lab-trol (American Hospital Supply Corp., Miami, Fla.) as the standard. Immunodiffusion tests were performed in agarose gels, using rabbit sera against whole cells and the above-described antigens. Anti-meningococcal sera were prepared in rabbits by a series of injections of formalin-killed and live cells as previously described (19). Anti-*S. typhi* sera were prepared in New Zealand white rabbits by four 1-ml intravenous injections of 10^9 Formalin-killed cells at weekly intervals and bleeding 2 weeks after the last dose.

Enzyme-linked antiglobulin conjugates. Alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was conjugated to the immunoglobulin G fraction of goat antiserum against rabbit or human immunoglobulin (Microbiological Associates, Bethesda, Md.) as described by Halle et al. (8). The rabbit and human conjugates were diluted 1:500 in 0.36 M sodium borate-buffered saline, pH 7.85, containing 0.5% BSA fraction V (Matheson, Coleman, and Bell, Norwood, Ohio), 0.5% Tween 20 (Sigma), and 0.01% NaN_3 (BBS-AT) immediately before use. These were the highest dilutions of the conjugates to produce maximal activity with immune sera.

ELISA. The method originally described by Engvall and Perlmann (2) and adapted by Halle et al. (8) was modified for use with disposable flexible polyvinyl chloride microtitration plates (Cooke Laboratory Products, Alexandria, Va.). One-tenth milliliter of the insoluble OMP antigen, suspended in 0.1 M sodium carbonate buffer, pH 9.6, was added to each well of the microtitration plates, and the plates were incubated for 3 h at 37°C. The contents of the wells were then discarded, and the plates were washed three times by adding approximately 0.2 ml of BBS-AT to each well and allowing the fluid to remain in the wells for about 1 min each time. Diluted serum (0.1 ml) was then added to the wells; the wells were allowed to incubate for 1 h at room temperature and then discarded. The plates were again washed three times with BBS-AT buffer, after which time 0.1 ml of conjugate was added to each well, which was allowed to incubate for 1 h at room temperature. The conjugate was then discarded, the plates were washed a final three times, and 0.1 ml of phosphatase substrate (Sigma 104), which had been freshly prepared at a concentration of 1 mg/ml in 0.05 M carbonate buffer, pH 9.8, was then added. The plates were allowed to incubate for 30 to 60 min, after which time the contents of each well were transferred to microcuvettes containing 0.1 ml of 1 N NaOH to stop the reaction. The volume in each tube was brought to 1.0 ml with distilled water, and

the optical density (OD) was read at 400 nm on a Beckman model 25 spectrophotometer, using distilled water as the blank.

RESULTS

The lithium chloride extraction procedure is much simpler than the Schnaitman technique for obtaining antigens from *Neisseria*, since several strains can be processed simultaneously (19). The lithium chloride procedure was therefore tested on several strains of *S. typhi*, and the extracted proteins were examined by SDS-PAGE (Fig. 1e). All strains had a major protein band migrating at approximately the same rate as the BSA marker; only faint 40,000-dalton bands were detected. Since this was not typical of OMP from gram-negative organisms, the more laborious Schnaitman technique was used to extract a crude OMP, containing CE, and purified OMP (T/TEI). As in the experiments by Hill and Weiss (9) with meningococci, the combination of Triton X-100 and EDTA treatments greatly reduced the LPS of *S. typhi* OMP, as demonstrated by immunodiffusion (Fig. 2) and mouse toxicity. Mice given an intraperitoneal injection of CE (protein content, 10 µg) had a pyrogenic response as demonstrated by the rough appearance of their fur for as long as 24 h. Mice given an equivalent dose of T/TEI, on the other hand, always maintained a normal appearance.

When *Salmonella* CE were examined by SDS-PAGE, the high-molecular-weight band obtained by lithium chloride extraction was absent, and more typical double bands were observed (Fig. 1a-c) that were similar but not identical to the double bands seen in *E. coli* (Fig. 1d). CE obtained by the Schnaitman procedure from seven *S. typhi*, two *S. paratyphi* A, and one each of *Salmonella* groups B, C, and E all produced very similar double bands. CE from all *Salmonella* strains tested by immunodiffusion reacted with rabbit antiserum prepared against whole cells of *S. typhi* (Fig. 3). The intensity of the reaction differed depending upon the *Salmonella* group and was particularly strong with group B. At least one common antigen was present, as shown in Fig. 3a and in other tests using other combinations of adjacent CE preparations. Only a faint reaction was detected with CE from *E. coli*, *K. pneumoniae* (Fig. 3b), and *Shigella*.

Because of the similarity in electrophoretic patterns and antigenic properties among *Salmonella* CE preparations, the CE of *S. typhi* was used as coating antigen in the ELISA. The T/TEI fraction of *S. typhi* and the STA and T/TEI antigens of *N. meningitidis* group B were

a b c d e

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+

FIG. 2. Immunodiffusion reaction of antiserum prepared against whole cells of *S. typhi* (center well) with CE, T/TEI, and LPS antigens as indicated.

also tested. Titrations of these antigens with serial dilutions of homologous hyperimmune rabbit sera are illustrated in Fig. 4. The antibody titer was defined as the serum dilutions, calculated by interpolation, that produced an OD reading of 0.1. The OD readings of normal sera were <0.1 and were approximately the same with all dilutions. The potency of the antigens was judged by the titers of the sera obtained with various concentrations of antigen (Fig. 4A) and by the OD readings produced with serum diluted 1:200 (Fig. 4B). It appeared by both methods that $10 \mu\text{g}$ of any of the antigen preparations per ml was the smallest concentration to react optimally with the homologous serum. There were, however, marked differences in some of the antigen-antibody reactions. The OD readings of the meningococcal antigens were higher than those of the typhoid antigens, even with due consideration to differences in titer. The two meningococcal antigens (STA and T/TEI) did not differ significantly in reactivity in the region of optimal antigen concentration, but the CE antigen of *S. typhi* appeared to be more satisfactory than T/TEI. The relatively low OD readings of the typhoid antigens, between 0.2 and 0.3, did not introduce a technical difficulty because they were obtained after a 1:10 dilution of fluids in the microtiter plates. The reactions in the microtiter plates themselves were quite visible.

The specificity of the reactions is illustrated in Fig. 5. *S. typhi* antigen did not react with antiserum against *N. meningitidis* group A and vice versa. There was, however, moderate cross-

FIG. 3. Immunodiffusion reaction with antiserum prepared against whole cells of *S. typhi* (center wells). (a) Tests with *Salmonella* CE antigens; (b) tests with control CE preparations. A, B, D, C, and E designate the *Salmonella* group (D = *S. typhi*). Ec, *E. coli*; Kp, *K. pneumoniae*.

reactivity between group A and B *N. meningitidis*, despite the fact that the more purified T/TEI antigens were used and the proteins belonged to different serotypes (6, 19).

The above-described antigens were tested against a few acute- and convalescent-phase sera from proven human cases of typhoid fever and

FIG. 1. SDS-PAGE patterns of bacterial proteins. CE obtained by the Schnaitman procedure: (a) *Salmonella* group C; (b) *S. paratyphi* group A; (c) *S. typhi*; (d) *E. coli* and BSA. Proteins released by lithium chloride: (e) representative *S. typhi* strain.

FIG. 4. Determination of optimal antigen dilution by ELISA with rabbit hyperimmune sera prepared with whole cells of the homologous strain. The final dilution of the reaction mixtures was 1:10 before OD determinations. (A) Measured by antibody titer (dilution eliciting 0.1 OD); (B) measured by OD with serum diluted 1:200.

group A meningococcal meningitis. The reactions of paired sera from three typhoid and one paratyphoid fever cases from Indonesia against T/TEI and CE *S. typhi* antigens are shown in Table 1. The sera from three tuberculous (TB) meningitis patients from Egypt served as controls, although the histories of previous exposure to enteric infections are not known. The CE antigen (Table 1) proved to be much more satisfactory than the T/TEI antigen (not shown) in eliciting higher OD readings and more clearly differentiating sera from enteric fever patients

FIG. 5. Specificity of the reaction in the ELISA. Antisera: N, normal; Nm A, Nm B, *N. meningitidis* groups A and B, respectively; St, *S. typhi*. Indented bars, serum antibody titer < 1:200.

from those of TB meningitis patients. Except for convalescent-phase serum from patient 3, which had a higher titer than the rest, there appeared to be a linear relationship between OD reading and serum dilution. By the criterion used previously of defining the titer as the serum dilution giving 0.1 OD reading, all sera had titers in excess of 1:1,600. The titers obtained with convalescent-phase sera were not higher than those of acute-phase sera, possibly because the acute-phase specimens were taken on the 4th to 8th day of fever. Some Widal-negative sera (from blood culture-positive patients) produced high ELISA activity, whereas none of the specimens with high Widal titers had low ELISA activity.

A similar study was conducted with paired sera from cases of group A meningococcal meningitis patients in Egypt. As with the typhoid fever patients, the relationship between OD reading and serum dilution was approximately linear, but in contrast with the paired typhoid sera there was a pronounced difference in titer between acute- and convalescent-phase sera. When the OD readings of sera diluted 1:200 are shown in relation to day of admission (Fig. 6), in at least four cases the convalescent-phase serum titers were considerably higher than those of the acute-phase sera.

Since the *N. meningitidis* STA antigen proved to be very valuable for these studies, an attempt was made to isolate it by ethanol precipitation, which does not require the use of a high-speed centrifuge. The antigen prepared by this simplified procedure is indistinguishable by immunodiffusion from the conventional STA. When used in the ELISA with rabbit hyperim-

TABLE 1. Titration of human sera from cases of typhoid and paratyphoid fevers

Patient					Widal test				ELISA (OD reading)			
No.	Culture isolated	Fever days before admission	Previous vaccination	Serum	H	O	A	B	1/200 ^a	1/400	1/800	1/1,600
1	<i>S. typhi</i>	8	No	Acute	320 ^b	<20	<20	40	0.348	0.324	0.238	0.157
2	<i>S. typhi</i>	6	Yes	Convalescent ^c	320	<20	<20	<20	0.462	0.340	0.224	0.147
				Acute	20	<20	<20	<20	0.412	0.290	0.214	0.133
3	<i>S. paratyphi A</i>	4	Yes	Convalescent	<20	<20	<20	<20	0.355	0.252	0.198	0.123
				Acute					0.470	0.333	0.264	0.175
4	<i>S. typhi</i>	8	Yes	Convalescent	<20	<20	<20	<20	0.498	0.487	0.395	0.195
				Acute	160	<20	<20	<20	0.407	0.305	0.224	0.143
5-7	(Tuberculous meningitis)			Convalescent	40	<20	<20	<20	0.390	0.313	0.207	0.129
									0.161	0.149	0.079	0.062

^a Serum dilution with CE antigen.^b Inverse of serum dilution.^c Obtained about 2 weeks after acute-phase serum.

FIG. 6. ELISA of human sera from cases of meningococcal meningitis group A and TB meningitis controls. All sera were diluted 1:200, and the reactions with STA were compared on the basis of absorbance.

mune sera, the results were identical to those shown in Fig. 4.

DISCUSSION

The inability of LiCl to extract typical OMP in satisfactory amounts from *Salmonella* indicates that there are significant structural differences in the CE of gram-negative bacteria. This was also suggested by the failures of the Schnaitman method to produce cell wall material from

Vibrio parahaemolyticus (J. E. Sippel, unpublished finding) and the observation of Hill and Weiss (9) that the structure of meningococcal T/TEI differs from that of *E. coli* as evidenced by electron microscopy. Furthermore, Frasch et al. (7) report that the protein (and LPS) composition of meningococcal outer membranes can be altered by changes in cultural conditions. Since the structure of CE seems to differ in different genera and the components are quantitatively dependent on growth conditions, the method used for obtaining OMP is critical.

OMP from 12 *Salmonella* strains encompassing five serogroups were very similar by SDS-PAGE. Schnaitman reported (18), and we confirmed, that *E. coli* and *Shigella* have identical OMP on SDS-PAGE. Although differences were noted by immunodiffusion among *Salmonella* CE antigens, there were strong cross-reactions. Evidently, these antigens are more uniform in enteric bacteria than in pathogenic *Neisseria*, which except for group A meningococci can be separated into types by SDS-PAGE of OMP antigens.

While the STA and T/TEI of *N. meningitidis* reacted comparably in the ELISA, there was a pronounced difference between CE and T/TEI antigens of *S. typhi*. The difference between the T/TEI of the two microorganisms may reflect the greater complexity of the *Salmonella* surface antigens. Possibly, some of the antigenic components of *S. typhi* were lost during T/TEI extraction or modified in such a manner that the coating process for the ELISA was impaired.

The TB meningitis patients whose sera were used for controls were from an area that is endemic for both typhoid fever and meningococcal disease. Previous studies have shown that TB meningitis patients are rarely meningococcal

carriers (J. Sippel and N. Girgis, submitted for publication), and, since they were hospitalized, they probably had no recent experience with typhoid. It is quite possible, however, that they were not true negative controls and had low titers against typhoid antigens. The effect of asymptomatic typhoid and meningococcal carriage on ELISA activity must be further investigated. Although the slopes of the ELISA titration reactions vary with the antigen used, they do appear to be comparable when human sera are used with a single antigen. Serum titers therefore correlate with the OD values obtained with 1:200 dilutions. Only when serum titers are very high are the low-dilution OD readings misleading. Single serum dilutions may therefore be sufficient for identifying high-titer sera, making ELISA an ideal method for screening large numbers of specimens. This method was recently used to good advantage for determining antibodies against *N. meningitidis* (3).

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