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Immunoblot Detection of Class-Specific Humoral Immune Response to Outer Membrane Proteins Isolated from *Salmonella typhi* in Humans with Typhoid Fever

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The studies reported here were undertaken to assess the ability of the outer membrane proteins (OMPs) of *Salmonella typhi* to induce a humoral immune response in humans with typhoid fever. OMPs were isolated with the nonionic detergent Triton X-100 and were found to be contaminated with approximately 4% lipopolysaccharide. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns showed protein bands with molecular size ranges from 17 to 70 kilodaltons; the major groups of proteins were those that correspond to the porins and OmpA of gram-negative bacteria. Rabbit antiserum to OMPs or to *S. typhi* recognized OMPs after absorption with lipopolysaccharide. Sera from patients with typhoid fever contained immunoglobulin M antibodies which reacted with a protein of 28 kilodaltons and immunoglobulin G antibodies which reacted mainly with the porins, as determined by immunoblotting. These results indicate that the porins are the major immunogenic OMPs from *S. typhi* and that the immune response induced in the infection could be related to the protective status.

Typhoid fever is an unsolved health problem in the world. There are approximately 12 million cases per year, and in developing countries the incidence is on the order of 540 new cases per 100,000 inhabitants (6). Eradication of the disease in these countries depends mainly on improvement of sanitary and nutritional conditions, but in the meantime, a good vaccination program could help in the control of the disease. The main problem for the development of an effective vaccine is that up to now little is known about the microbial factors that determine pathogenicity and those that elicit a protective immune response in humans with typhoid infection.

Available vaccines containing either acetone or heat-killed *Salmonella typhi* are of limited value, not only because they confer short-lived protection but also because they produce unacceptable side effects, due mainly to the presence of endotoxin, which prevent their use in children (8, 18). Germanier and Furer have developed an oral vaccine from a strain of *S. typhi* (Ty21a) which is deficient in the enzyme UDPgalactose epimerase (10). Although this vaccine lacks the ill effects of the previous ones, it is expensive and has shown a variable efficacy in the trials that have been done in Egypt (97%) and Chile (67%) (9, 32). At the moment there are also several vaccines prepared from Vi antigen still in the phase of experimentation in humans (1).

Up to now, the antigens of *S. typhi* that participate directly in inducing protection are not known. In humans, the humoral immune response against some superficial antigens has been studied. In the sera of typhoid fever patients, there are antibodies against O, H, and Vi antigens (13, 28). Nevertheless, high titers of these antibodies have no relationship to the state of protection or to the evolution of the disease. That is why the search for specific antigens involved in protection is still in progress.

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Due to the localization of the outer membrane proteins (OMPs) on the surface of gram-negative bacteria (25, 27), OMPs have been recently considered as important antigens in the induction of a specific, protective immune response. OMPs from *S. typhi* have molecular sizes in the range from 17 to 80 kilodaltons (kDa) (14). When they are isolated by the method of Schnaitman (29), a contamination with lipopolysaccharide (LPS) of 4% is obtained. We have demonstrated in a mouse model that these OMPs induce protection against a challenge with *S. typhi* (15); the same protective effect was obtained when mice were passively injected with rabbit hyperimmune anti-OMP sera; when the rabbit antisera were previously absorbed with OMPs this protective effect disappeared, but when they were absorbed with LPS there was no significant loss of protection. These results indicate that the OMPs are responsible for the protection observed in this animal model.

Considering the results mentioned above, it is important to find out whether OMPs from *S. typhi* are also immunogenic in humans. For this reason we analyzed the immune response to OMPs in patients with typhoid fever. In this work, we demonstrate that the humoral immune response of such patients is directed mainly to porins in the convalescent phase of the disease.

MATERIALS AND METHODS

Bacterial strains. The virulent strain *S. typhi* 9,12,Vi:d (hereafter referred to as M01) was isolated from a patient with typhoid fever and has been maintained in culture in our laboratory since 1979. *S. typhi* O-901, Ty2, and Ty21a and *Salmonella typhimurium* 1,4,5,12 were kindly donated by the Instituto Nacional de Higiene, Mexico City, Mexico.

Isolation of outer membrane proteins. OMPs were obtained as described by Schnaitman (29). Briefly, *S. typhi* M01 was grown in minimum salts medium (medium A) containing 0.5% yeast extract and 1.25% glucose and incubated in a

shaker at 37°C for 8 h to mid-exponential growth to an optical density of 0.36 at 660 nm (previously determined by a growth curve). Cells were harvested and suspended in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. Bacteria were treated with an OmniMixer (Ivan Sorvall, Inc., Norwalk, Conn.) to remove flagella and then were disrupted by sonication, using an Ultratip Labsonic system (Lab-Line, Melrose Park, Ill.), and centrifuged at 7,000 × *g* for 10 min to remove intact cells. The supernatant fluid was centrifuged at 200,000 × *g* for 45 min at 4°C to obtain the cell envelope. The cytoplasmic membrane was removed with 0.01 M HEPES containing 2% Triton X-100, followed by centrifugation at 200,000 × *g* for 45 min at 4°C. to achieve solubilization of the OMPs, the Triton X-100-insoluble fraction (outer membrane and peptidoglycan) was suspended in 0.05 M Tris containing 5 mM EDTA and 2% Triton X-100, pH 7.4, and was allowed to stand 10 min at 37°C. Finally, the suspension was centrifuged as described above but at 37°C, and the OMPs were recovered in the supernatant fraction. Protein concentration was determined by the method of Lowry et al. (24). LPS contamination of the protein samples was 4%, as determined indirectly by measuring the concentration of 2-keto-3-deoxyoctulosonic acid (20); as a standard, 2-keto-3-deoxyoctulosonic acid obtained from Sigma Chemical Co., St. Louis, Mo., was employed.

Isolation of LPS. *S. typhi* O-901 LPS was extracted by the phenol-water method (34).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. SDS-PAGE was performed under reducing conditions by using the discontinuous buffer systems of Laemmli (23) with a vertical-slab gel electrophoresis unit (Hoefer Scientific). The separating gel contained 11.2% acrylamide, 2.5% bisacrylamide, and 0.19% SDS on 0.35 M Tris hydrochloride buffer (pH 8.8). The stacking gel contained 5% acrylamide, 0.13% bisacrylamide, and 0.1% SDS in 0.12 M Tris hydrochloride buffer (pH 6.8). The running buffer was 0.025 M Tris hydrochloride–0.192 glycine (pH 8.3) with 0.1% SDS. Electrophoresis was performed at 30 mA per plate.

Electrophoretic transfer of OMP from polyacrylamide gels to nitrocellulose paper (NCP) was accomplished in an electroblotting unit (Hoefer Scientific) by means of the transfer buffer described by Towbin et al. (31). The proteins were electrophoresed at 100 mA for 18 h. NCPs were immersed for 1 h at 37°C in blocking buffer (0.15 M NaCl, 0.01 M phosphate buffer [pH 7.2], 0.25% gelatin, 6 mM EDTA). After being washed in 0.15 M NaCl–0.01 M phosphate buffer (pH 7.2)–0.1% Tween 20 (PBS-T), the NCPs were incubated for 3 h at 25°C in a 1:100 dilution of the test serum samples in blocking buffer. After being washed in PBS-T, the NCPs were incubated for 1.5 h at 25°C with optimal concentrations of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or anti-human IgM or IgG (Copper Biomedical, Inc., West Chester, Pa.). After two washes in PBS-T and one in phosphate-buffered saline only, the NCPs were incubated in substrate solution (2 mM 4-chloro-2-naphthol and 0.08% H_2O_2 in phosphate-buffered saline) for 10 min. After a wash in tap water, the NCPs were photographed, air dried, and kept in the dark.

Human sera. Sera from eight persons (ages between 25 and 40) with clinical and bacteriological diagnosis of typhoid fever, hospitalized at the Hospital de Enfermedades Infecciosas, Centro Médico la Raza, IMSS, Mexico City, Mexico, were analyzed for IgM and IgG responses to OMPs in both the acute (1 day after hospital admission) and the convalescent (30 days after release from the hospital) phases. Sera from 10 persons without previous known contact with *S. typhi* were used as controls.

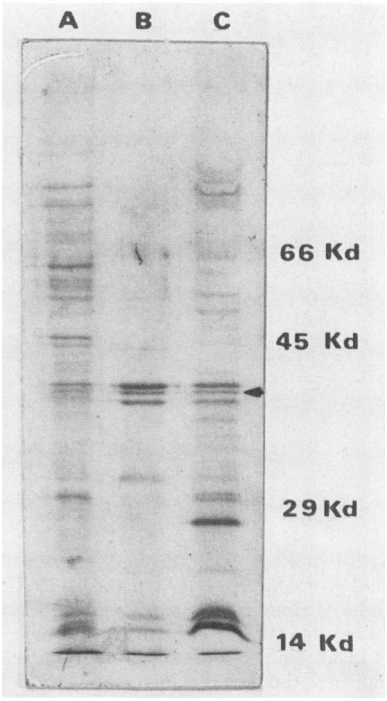

FIG. 1. SDS-PAGE of *S. typhi* M01 OMPs. Lanes: A, soluble fraction in HEPES-Triton X-100; B, insoluble fraction in Tris-EDTA-Triton X-100 (peptidoglycan); C, soluble fraction in Tris-EDTA-Triton X-100 (OMPs). Molecular size markers: bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14.4 kDa).

Anti-OMP antisera. Anti-OMP sera were raised in New Zealand White rabbits (2.5 to 3 kg) intradermally injected with different concentrations of OMPs; the first two injections (1 mg/ml) were given in Freund complete adjuvant on days 0 and 7. After day 15, the rabbits received three further injections of 0.15, 0.5, and 1.0 mg/ml given every other day without Freund complete adjuvant. The rabbits were bled 10 days after the last injection.

RESULTS

OMP preparation. *S. typhi* M01 OMPs isolated as described above were contaminated with 4% LPS. Figure 1 shows the SDS-PAGE electrophoretic pattern. As seen, there are differences between fractions obtained during the process of purification. The fraction soluble in HEPES-Triton X-100 (lane A), which corresponds to the cytoplasmic membrane proteins, shows more than 15 bands, most of them different from those observed in the other two fractions (lanes B and C). The insoluble fraction in Tris-EDTA-Triton X-100 (lane B), which corresponds to peptidoglycan, presents mainly three proteins of molecular sizes around 36 to 41-kDa. The fraction soluble in Tris-EDTA-Triton X-100 (lane C) corresponds to the OMP preparations. It presents some minor proteins with molecular sizes ranging from 17 to 70 kDa and the characteristic group of major proteins of 36 to 41 kDa, as well as the 28- and the 17-kDa bands.

To confirm whether we really had obtained OMPs from *S. typhi* M01, we compared PAGE patterns of similar preparations obtained from other strains of *S. typhi* as well as from *S. typhimurium*, which has been well characterized by other

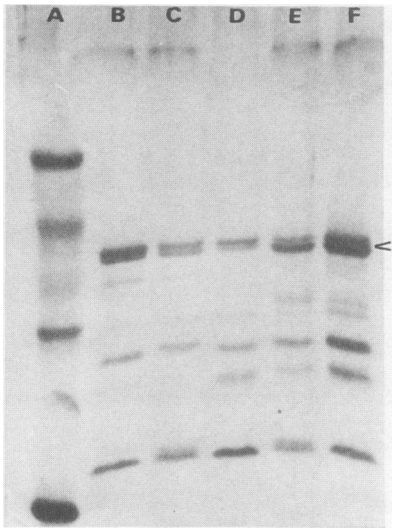

FIG. 2. SDS-PAGE of OMPs from *S. typhi* M01, O-901, Ty2, and Ty21a and from *S. typhimurium* (lanes B, C, D, E, and F, respectively). Molecular size standards: bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and lysozyme (14 kDa) (lane A, from top to bottom, respectively). The arrowhead indicates the porins.

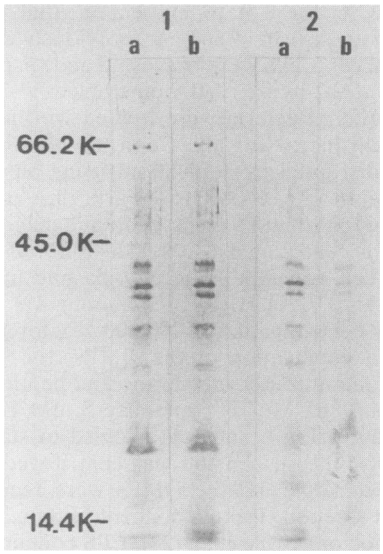

FIG. 3. Immunoblotting of OMPs from *S. typhi* M01 detected by rabbit antibodies and peroxidase-conjugated goat anti-rabbit polyvalent immunoglobulins. Lanes: 1a, rabbit anti-*S. typhi* OMPs; 1b, rabbit anti-*S. typhi* OMPs absorbed with LPS; 2a, rabbit anti-whole *S. typhi*; 2b, rabbit anti-whole *S. typhi* absorbed with LPS. Absorption with LPS was done at equivalence. Nonabsorbed sera were used at a 1:100 dilution, absorbed sera were used at a 1:200 dilution, and the conjugate was used at a 1:1,000 dilution. Molecular weights (in thousands) are given at the left.

authors (19). In Fig. 2, we observe that the *S. typhi* M01 pattern (lane B) is very similar to those of *S. typhi* O-901 (lane C), Ty2 (lane D), and Ty21a (lane E) and *S. typhimurium* (lane F). All of them have the major proteins of 36 to 41 kDa that are called porins.

Immunoblotting with rabbit anti-OMP sera. Sera from rabbits immunized with *S. typhi* OMPs were analyzed by immunoblot to determine the antigenicity of these proteins. The immunoblot analysis is shown (Fig. 3). Anti-OMP rabbit immune sera reacted with greater intensity against the protein bands located between 36 and 41 kDa, which correspond to the so-called porins and OmpA. They also reacted against the other OMPs (Fig. 3, lane 1a). This pattern of recognition was not modified, in comparison with the number of bands seen when the antisera were previously absorbed, at equivalence, with LPS from *S. typhi* (Fig. 3, lane 1b). Rabbits immunized with whole bacteria produced antibodies against OMPs (Fig. 3, lane 2a); when the sera were absorbed with LPS (Fig. 3, lane 2b), no changes were observed. Nevertheless, the intensity of the reaction was much less because the antisera were diluted two times during the absorption procedure. The results demonstrate that the antibodies are directed against OMPs and not against LPS.

Immunoblotting with patient or normal human sera. To determine the antigenicity of the OMPs from *S. typhi* in humans, antibodies against these proteins were detected, by immunoblotting, in sera of patients with typhoid fever, in both the acute and the convalescence phases. The analysis of the primary and secondary responses showed the following results.

(i) IgM class response. Four of eight patients (patients 1, 4, 5, and 8) had antibodies of the IgM class which recognized a protein with a molecular size of 28 kDa during the acute phase; only two of these (patients 5 and 8) showed the same type of response during the convalescence phase (Fig. 4). The 28-kDa protein was the only one recognized by the patient antibodies. None of the 10 healthy controls showed any type of response (data not shown).

(ii) IgG class response. The secondary antibodies present in typhoid fever patients were directed mainly to the major OMPs, that is, to the 17- and 28-kDa proteins and to the porins (Fig. 5). The response to the 17-kDa protein was present in only three of eight patients (patients 1, 2, and 5) in both the acute and the convalescence phases. To the 28-kDa protein, four of eight patients (patients 1, 2, 4, and 5) responded in the same way in the acute and the convalescence phases. During this latter phase, one other patient (patient 3) also showed a positive reaction. Response to the porins was more evident since during the acute phase four of eight patients (patients 1, 2, 4, and 5) gave an intense reaction that was also present during the convalescence phase. In this latter phase, three more patients (patients 3, 6, and 8) had antibodies which recognized the porins. Only 1 of 10 control sera recognized the porins and the 28-kDa protein (Fig. 6, lane a).

DISCUSSION

In the present work, we have demonstrated that OMPs from *S. typhi* M01, a strain isolated from a typhoid fever patient, presents an SDS-PAGE pattern similar to those of other strains of *S. typhi*, such as Ty2 (parenteral vaccine strain), Ty21a (oral vaccine strain), and O-901 (nonflagellated strain). The proteins that are common in all strains are the 17-, 28-, and 36- to 41-kDa proteins. The pattern for *S. typhimurium*, which is very similar to that reported in the literature (19), allowed us to identify the major proteins, including the 36- to 41-kDa proteins which are called porins.

By immunoblot analysis, we showed that the OMPs of *S. typhi* M01 are immunogenic in rabbits when they are administered either in isolated form or as a component of the bacteria. The IgG anti-OMP antibodies recognized the major proteins present in the preparations, including the porins.

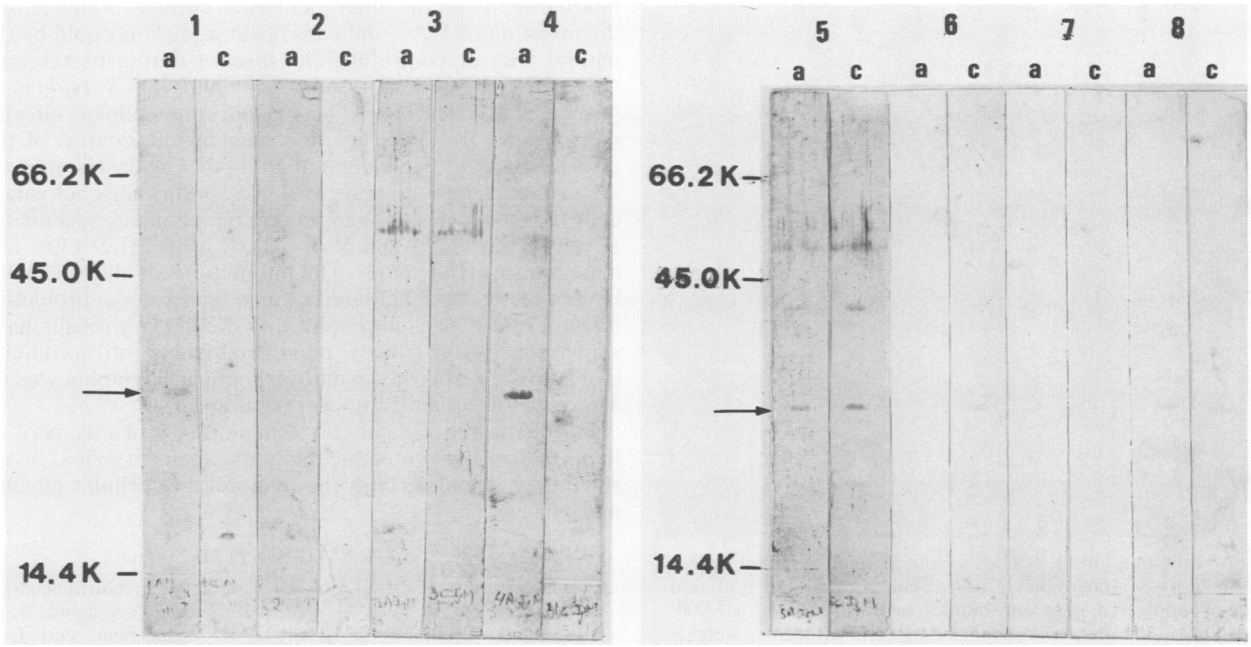

FIG. 4. IgM responses of eight patients with typhoid fever (panels 1 to 8) against OMPs from *S. typhi*, detected by immunoblotting. Sera were diluted 1:50, and peroxidase-conjugated goat anti-human IgM was diluted 1:1,000. Lanes: a, acute phase; c, convalescence phase. Arrows indicate the proteins recognized by sera of patients. Molecular weights (in thousands) are shown.

This reactivity was not essentially modified when anti-LPS antibodies were previously absorbed, which shows the importance of proteins as immunogens.

We have also demonstrated that *S. typhi* M01 OMPs are immunogenic in typhoid fever patients. The immunoblot analysis performed permitted us to identify which proteins are recognized by IgM and IgG antibodies from patients in the acute and the convalescence phases of the disease. We observed that four of eight patients had IgM antibodies against the 28-kDa protein during the acute phase. In the convalescence phase, only two of these patients continued to present this reactivity. We did not observe any reaction to the porins. This is in disagreement with the results obtained by Calderon et al. (4) using enzyme-linked immunosorbent assay. This difference could be related to the nature of the antigen used in both works. In our experiments the OMPs

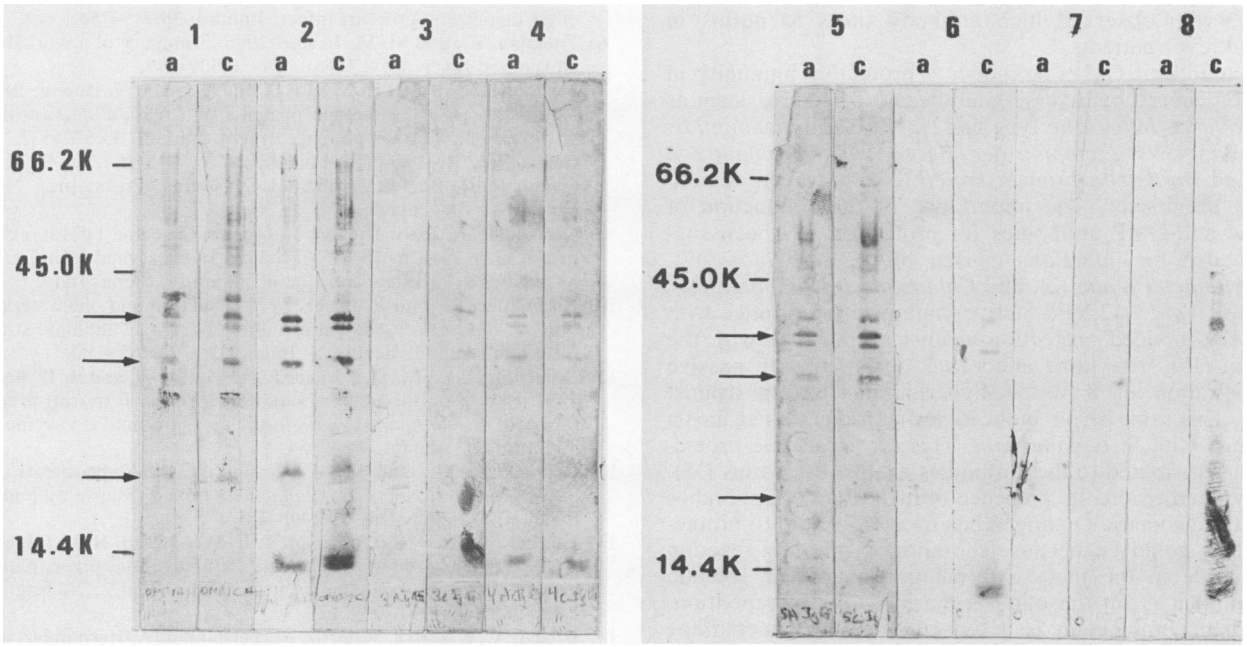

FIG. 5. IgG responses of eight patients with typhoid fever (panels 1 to 8) against OMPs from *S. typhi*, detected by immunoblotting. Sera were diluted 1:50, and peroxidase-conjugated goat anti-human IgG was diluted 1:1,000. Lanes: a, acute phase; c, convalescent phase. Arrows indicate the proteins recognized by sera of patients. Molecular weights (in thousands) are shown.

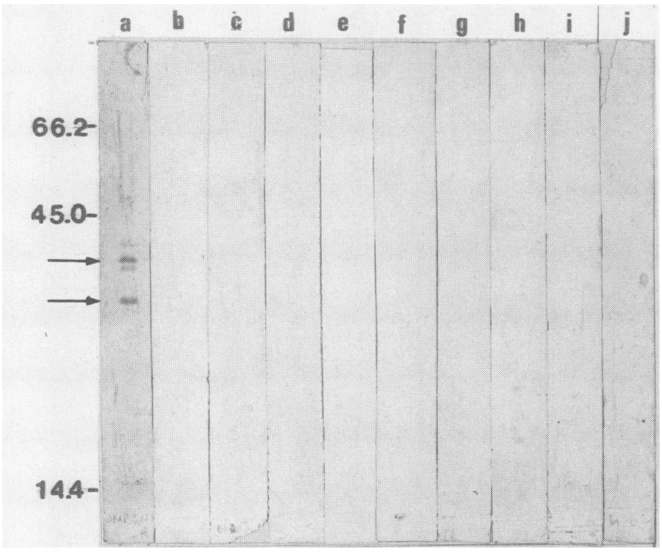

FIG. 6. Immunoblotting of OMPs from *S. typhi* detected in sera from 10 healthy persons (lanes a to j). Sera were diluted 1:50, and peroxidase-conjugated goat anti-human IgG was diluted 1:1,000. Arrows indicate the proteins recognized by sera. Molecular weights (in thousands) are shown.

were denatured, whereas Calderon and co-workers used porins in a native form; therefore, it is possible that IgM anti-porin antibodies react mainly with the proteins in a native form. The IgG response was different from the primary one, since several proteins were recognized in both the acute and the convalescence phases. It is important to point out that the response to porins was the most relevant because an intense reaction was observed in four of eight patients in the acute phase and in seven of eight patients in the convalescence phase. These results indicate that porins are the immunodominant antigens in these OMP preparations, which is in agreement with the results of Calderon et al. (4), who observed high antibody titers to porins in typhoid fever patients.

The ability of OMPs to induce a protective immunity in infection caused by diverse gram-negative bacteria, such as *Haemophilus influenzae* type b (12), *Neisseria meningitidis* group B (33), *Pseudomonas aeruginosa* (11), *S. typhimurium* (22), and *Bordetella bronchiseptica* (26), has been demonstrated previously. The importance of the production of specific anti-OMP antibodies for protection has been suggested also for infections caused by *P. aeruginosa* (7), *Campylobacter jejuni* (2), and *Chlamydia trachomatis* (5).

For *S. typhi* 9,12,Vi:d, active immunization of mice with its OMPs induced protection against a challenge with the bacteria (15). The same effect was observed with passive administration of a rabbit hyperimmune serum against OMPs. This same serum induced partial protection against a challenge with *S. typhimurium*. This cross-reactive protection was attributed to the antibodies against the porins (15).

In typhoid fever, the presence of high titers of antibodies against the somatic O antigen has no relationship to protection. This could be because the antigen-antibody reaction takes place in the most external portion of the somatic antigen, that is, in the oligosaccharide units of repetition, and when complement is activated the C5-C9 membrane attack complex (MAC) cannot be inserted in the membrane to promote bacterial lysis (16, 17). On the other hand, anti-porin antibodies recognize antigenic determinants exposed directly on the outer membrane surface, which allows the insertion of MAC and causes damage. This could be the natural way of controlling the disease during bacteremia. Nevertheless, it is important to remember that *S. typhi* is an intracellular germ; for that reason, the cellular effector mechanisms are also very important in the control of the disease. Because production of antibodies against proteins is a T-cell-dependent immune response, porins must activate T cells (CD4) which will then release lymphokines specific for B cells, (IL4, IL5, and IL6) (21), T cells (IL2) (30), and macrophages (INF-γ) (3). The interactions of all these cells, lymphokines, and antibodies must generate a protective immune status. It is important that preliminary results have shown that porins from *S. typhi* stimulate in vitro-proliferative immune responses in murine immune T lymphocytes (J. Moreno et al., manuscript in preparation).

In conclusion, we can say that porins probably play an important role in protective immunity against typhoid fever, since they stimulate both the humoral and cellular effector mechanisms.

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