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Immunomodulatory Properties of Porins of Some Members of the Family *Enterobacteriaceae*

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The outer membrane protein (OMP) preparation of *Salmonella typhi* was observed to have several immunomodulatory properties. Treatment of mice with an intraperitoneal injection of the OMP preparation enhanced both cellular and humoral responses of the mice to an unrelated antigen, a killed vaccine of *Mycobacterium vaccae*; both the delayed-type hypersensitivity (DTH) response and the antibody titers were enhanced. The predominant isotype of the antibody shifted from immunoglobulin G1 (IgG1) to IgG2a upon treatment with OMP. Treatment of mice with the OMP preparation improved the efficiency of in vitro antigen presentation by the peritoneal cells and also induced the cells to secrete interleukin-1. Treatment with the lipopolysaccharide (LPS) preparation of *S. typhi* had the opposite effect; i.e., the DTH response to *M. vaccae* was suppressed. Treatment with OMP neutralized the suppressive effects of LPS. The OMP preparation also had an enhancing effect on the innate immune mechanisms of the mice. Intraperitoneal injection of the OMP preparation enhanced the microbicidal activity of the peritoneal cells, and production of nitric oxide intermediates was stimulated. Injection of the OMP preparation into footpads of naive nonimmune mice induced a sustained hypersensitivity response that peaked at 24 h. Purified porins of the OMP preparation could induce both immunomodulation and hypersensitivity. Porins prepared from five different *Salmonella* strains and a strain of normal fecal *Escherichia coli* also exhibited immunomodulatory and hypersensitivity-inducing activities.

Several bacterial products such as lipopolysaccharides (LPS), muramyl dipeptide (MDP), and pertussis toxin are known to have immunomodulatory properties (4, 8, 18). Recently, preparations containing outer membrane proteins (OMP) of *Salmonella typhimurium* have been shown to induce tissue inflammation in nonimmune animals (13) by stimulation of secretion of cytokines by macrophages and natural killer cells (14). It is therefore logical to expect the OMP of *Salmonella* to have immunomodulatory properties also.

It is now recognized that the biological properties of adjuvants depend on their ability to activate selectively either T-helper 1 (Th1) or T-helper 2 (Th2) cells (26), either by their action on antigen-presenting cells (APCs) such as macrophages or by targeting the antigen to the appropriate APC (1, 39). For example, the two commonly used adjuvants in animal experiments, alum and Freund's complete adjuvant (FCA), favor Th2 and Th1 responses, respectively (1, 39). By use of an appropriate adjuvant, it is now possible to achieve the desired type of immune response against immunogens and vaccines. The biological response modifiers are also of interest as immunostimulants for therapeutic use. For example, the mycobacterial MDP and its derivatives have undergone clinical trials (17). Therefore, the immunomodulatory properties of OMP of *Salmonella* would be of immense interest. In this report, a detailed investigation of the immunomodulatory properties of the OMP of *Salmonella typhi* and a few other enterobacteria is presented.

MATERIALS AND METHODS

Organisms. *Salmonella typhi* Ty2, *S. enteritidis*, *S. typhimurium*, *S. paratyphi* A, and *S. paratyphi* C were obtained from the National Collection of Type Cultures, London, England. *Escherichia coli* was isolated from the fecal sample of a normal healthy volunteer. *Mycobacterium vaccae* was kindly supplied by J. L. Stanford, Middlesex Hospital, London, England, and cultivated in the medium of Doub and Youmans (9) for antigen production.

Mice. BALB/c, C3H/HeJ, and Swiss white mice, 8 to 10 weeks old, obtained from the National Institutes of Health, Bethesda, Md., and maintained at the Cancer Research Institute, Bombay, India, were used. Prior to use, all mice were tested for delayed-type hypersensitivity (DTH) response to sonicates of *S. typhi* and *M. vaccae*. Ten percent of the batches of mice used were also tested for serum antibodies against the 65-kDa antigen of *M. vaccae* (referred to as anti-65-kDa antibody).

Immunization. Suspensions in phosphate-buffered saline (PBS) of an overnight growth of *S. typhi* Ty2 and 10-day-old culture of *M. vaccae*, killed by γ irradiation in a ^{60}Co source (1 Mrad), were used for immunization. Mycobacterial cell density was adjusted as described earlier (35). Mice were immunized intradermally (i.d.) in the flanks with 10^8 cells.

OMP preparation of *S. typhi*. The OMP preparation was made by the method of Nakae and Ishii (28). Washed cells of *S. typhi* Ty2 (6 to 7 g [wet weight]) in 50 mM Tris-HCl (pH 6.8) were sonicated for 4 min to prepare the cell envelopes. The sonicate was centrifuged at 750 × *g* for 10 min. The supernatant was collected and spun again at 30,000 × *g* for 30 min to pellet the envelopes. The pellet was washed twice in Tris-HCl, washed twice in 2% sodium dodecyl sulfate (SDS) in distilled water, and dialyzed against distilled water. The envelopes, suspended in a minimum volume of 5 mM Tris-HCl (pH 7.4), were digested with lysozyme (100 μg/ml; Sigma) at 37°C for 2 h. Papain (Sigma) was added to the lysozyme-digested envelope preparation in the proportion of 1.0 mg of enzyme per 15 mg of protein, 0.1 M L-cysteine hydrochloride was added in the proportion of 0.5 ml per 30 ml of digest, and the reaction was carried out at 37°C for 2 h; then 0.13 M iodoacetamide (0.2 ml per 10 ml of mixture) was added to stop the reaction, and the mixture was chilled on ice. The mixture was then centrifuged at 100,000 × *g* for 1 h to remove debris and reduce the LPS content, the supernatant was collected and dialyzed against PBS, and protein was estimated by the method of Lowry et al. (25).

Preparation of the porin fraction of OMP of *S. typhi*. The OMP porin fraction was prepared as described by Nikaido (29). Cell envelopes prepared as before were suspended in 2 ml of NaCl buffer (50 mM Tris-HCl [pH 7.7], 0.4 M NaCl, 1% SDS, 5 mM EDTA, 0.05% 2-mercaptoethanol, 3 mM NaN_3), and extraction was done at 37°C for 2 h. The suspension was then centrifuged at 100,000 × *g* for 1 h at 20°C, the supernatant was collected and dialyzed against PBS, and protein was estimated.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (24). Ten-microliter samples containing 5 μg of protein in the sample buffer (1.25 M Tris-HCl [pH 6.8] containing 2% SDS and 5% 2-mercaptoethanol) were loaded on a two-layered polyacrylamide minigel 8.5 by 8.5 by 0.075 cm thick. The upper stacking gel layer was 1.5 cm. Electrophoresis was carried out at a 25-mA constant current for 1 h; the gel was fixed and stained with silver as described by Blum et al. (3).

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Preparation of sonicates and 65-kDa antigen of *M. vaccae*. An overnight growth of *S. typhi* Ty2 and a 10-day-old growth of *M. vaccae* were irradiated, washed, and suspended in PBS as before and were disrupted in a Sonifier (Branson model B-30, 20 kHz, 50% duty cycle) for 45 min. The sonicates were centrifuged at 100,000 × *g* for 1 h to remove the cell debris and to reduce the LPS content of the salmonella sonicate. The supernatants were filtered through 0.22-μm-pore-size membrane filter (Millipore), and their protein content was assayed. The 65-kDa antigen was purified from the sonicate of *M. vaccae* by preparative agarose isoelectrofocusing, using ampholines (pH range of 3.5 to 5.0) as described earlier (22).

Preparation of LPS from *S. typhi*. LPS was extracted from a log-phase culture of *S. typhi* Ty2 by the phenol-water extraction method of Westphal as described elsewhere (23). LPS content was assayed by the thiobarbituric acid method (31).

Staphylococcal enterotoxin B (SEB) and concanavalin A (ConA) were purchased from Sigma. Phytohemagglutinin M (PHA) was purchased from Gibco.

CNBr treatment. OMP and LPS preparations were treated with cyanogen bromide (CNBr) by the method of Cahnmann et al. (5). OMP and LPS preparations (5.0 mg/ml) in 0.0175 M phosphate buffer (pH 6.3) were mixed with an equal volume of CNBr solution, 1.5 mg/ml, in 0.6 M HCl. The reaction mixture was incubated at room temperature for 4 h with continuous shaking. The digest was dialyzed for 4 h against 0.1 M phosphate buffer (pH 7.8) and then overnight at 4°C against PBS.

Hypersensitivity test. Hypersensitivity tests were done with naive and immunized mice by the technique of Grey and Jennings (15). A 0.05-ml volume of the test material (sonicates, OMP, or LPS preparations containing the required amount of protein or LPS) was injected into a hind footpad. The other footpad received the buffer used for dissolving the protein or the LPS. Measurements of footpad thickness were taken before and 24 and 48 h after injection. Differences in the values of footpad thickness measured after injection and before injection were calculated and designated a footpad swelling (FPS). The difference between the FPS value for sonicate, OMP, or LPS and the FPS value for the corresponding buffer simultaneously tested in the same animal constitutes the measure of hypersensitivity induced by the particular agent and was designated footpad enlargement (FPE).

DTH. DTH, which is due to activation of $CD4^+$ T effector cells by the antigen injected into the footpad of an immunized animal and is distinctly different from hypersensitivity induced in naive animals, was assayed by the technique of Grey and Jennings (15). Forty-eight-hour hypersensitivity to the sonicate or the 65-kDa antigen of *M. vaccae* was tested simultaneously in normal, nonimmune naive mice and also the mice immunized with *M. vaccae* by the i.d. route. FPE values for normal and immunized animals were determined. The mean FPE value of normal mice subtracted from the FPE value for the same antigen in the immunized mice was used as a measure of DTH and was designated corrected footpad enlargement (CPE). The CPE values were analyzed by Student's *t* test by comparison with zero.

Lymphoproliferation assay. The assay was carried out by the standard microculture technique (30), using 2×10^5 cells in 0.2 ml of RPMI 1640 with 10% fetal calf serum (FCS) and 20 μg of sonicate of *M. vaccae* per well. Proliferation was assayed by incorporation of label after a 16-h pulse of [^{3}H]thymidine, given at 120 h.

Separation of T cells. T cells were separated on nylon wool columns by the method of Julius et al. (21).

Peritoneal cells (PC). Mice were given an intraperitoneal (i.p.) injection of 5.0 ml of RPMI 1640 and sacrificed, the abdomen was massaged gently, within 5 min a laparotomy was performed, and peritoneal contents were aspirated with a sterile Pasteur pipette. Aspirates of several mice were pooled and suspended in the culture medium, and viable cell number was assayed by trypan blue exclusion.

Anti-65-kDa antibody assay. Antibody levels against the 65-kDa antigen of *M. vaccae* and their isotypes in the sera of primed mice were assayed by indirect sandwich enzyme-linked immunosorbent assay (ELISA). Plates were coated overnight at 4°C with rabbit anti-65-kDa antibody (10 μg/0.1 ml). Washed wells were incubated with 100 μl of lysozyme papain-digested sonicate of *M. vaccae* as a source of 65-kDa antigen. The washed wells were then incubated with 100 μl of different dilutions of test mouse sera, normal mouse sera, and different dilutions of a standard high-titer antiserum of mice immunized with purified 65-kDa antigen in incomplete Freund's adjuvant. The bound mouse antibody was detected by horseradish peroxidase (HRP) conjugate of anti-mouse immunoglobulin G (IgG; heavy-chain specific) antibody (optimum dilution, 1:10,000; Sigma). TMB (Sigma) and H_2O_2 were used as the substrate, the reaction was stopped with 2.0 M H_2SO_4, and plates were read at 450 nm in an ELISA plate reader (Titertek Multiscan; Flow Laboratories). Results were computed and expressed as absorbance units (AU) per milliliter, using the following formula: AU/ml = (A_{450} of test serum/A_{450} of standard anti-65-kDa serum) × dilution of test serum × 10.

For assay of the IgG2a/IgG1 ratio of the serum anti-65-kDa antibody, we used the assay as described above except that instead of anti-mouse IgG-HRP conjugate, conjugates of anti-mouse IgG1 and IgG2a specificities were employed. The relative amounts of the two isotypes were expressed as a ratio, AU/ml (IgG2a)/Au/ml (IgG1).

Rabbit and mouse anti-65-kDa antibodies. The antibodies were raised by repeated immunizations with 4.3-pI antigen of *M. vaccae* in Freund's incomplete adjuvant at 10-day intervals. Rabbit immunization was repeated until a precipitin titer (by agar gel diffusion) of 1:16 was reached. For generation of murine anti-65-kDa antibody, Swiss white mice were given four immunizations. For confirmation of antibody specificity, SDS-PAGE of *M. vaccae* sonicate was done, followed by a Western blotting with the rabbit antibody, employing goat anti-rabbit IgG-HRP conjugate (Sigma). A single band of 65 kDa and a faint band of higher-molecular-mass aggregates were seen, indicating that the rabbit antibody preparation was specific for the 65-kDa antigen.

IL-1 assay. Soluble interleukin-1 (IL-1) in the supernatants of PC and spleen cell cultures was assayed by the mouse thymocyte costimulation assay as described by Grey et al. (16). Briefly, C3H/HeJ thymocytes were cultured in microtiter plates (1.5×10^6 cells/well) in culture medium containing 1% PHA and 100 μl of the culture supernatants being tested for IL-1 content. The cultures were incubated at 37°C in 5% CO_2 and pulsed 48 h later with ^{3}H-labeled thymidine for 16 h, and incorporation of the label was assayed.

As a source of standard IL-1, supernatant of spleen cell cultures stimulated with ConA was used. Normal spleen cells of C3H/HeJ mice were cultured at 10^6 cells/ml in medium containing 5.0 μg of ConA per ml for 36 h, and the supernatant was harvested.

Assay of microbicidal activity of peritoneal macrophages. The assay used was a modification (27) of the method originally described by Blandan et al. (2). Briefly, 1.0 ml of an overnight culture of *S. typhi* Ty2 in nutrient broth adjusted to give 2×10^5 to 4×10^5 cells/ml was mixed with 9.0 ml of PBS containing 20% heat-inactivated rabbit antiserum against *S. typhi* somatic or O antigen (agglutination titer, 1,024) and incubated at 37°C for 1 h for opsonization. An aliquot was set aside at 4°C for determination of the exact viable count. One-half milliliter of the opsonized suspension containing 1×10^4 to 2×10^4 *S. typhi* cells was injected i.p. into each of a batch of Swiss white mice. After exactly 5 min, which is the time taken for phagocytosis of the salmonellae by the peritoneal macrophages, the mice were sacrificed. Two milliliters of PBS containing 10% newborn calf serum and 20 IU of heparin was injected i.p., the abdomen was massaged gently, and the PC were aspirated. PC from a number of mice were pooled and kept on ice. An aliquot was set aside for viable count. The PC suspension was spun at 110 × *g* for 5 min at 4°C; the supernatant was also set aside at 4°C for enumeration of unphagocytosed salmonellae. The PC pellet was washed twice and suspended in PBS containing 10% newborn calf serum at 6×10^6 cells/ml. One-milliliter aliquots were incubated at 37°C for 5 to 35 min. After the incubation period, the aliquots were removed and chilled immediately to 4°C. To the different aliquots, 0.1 ml of Saponin (Sigma) was added to a final concentration of 2.5% to lyse the PC. The lysates were diluted and plated on MacConkey's agar to determine the number of viable salmonellae in the original suspension, the PC suspension immediately on harvest, the supernatant of the PC suspension (unphagocytosed salmonellae), and the PC aliquots incubated for various durations. The percentage of salmonellae killed at each time point was calculated by taking the number of organisms injected as 100%.

IFN-γ. Tau interferon (IFN-γ)-containing culture supernatants were prepared in the laboratory. C3H/HeJ and BALB/c mice were immunized with *M. vaccae* by the i.d. route. One week later, the mice were sacrificed and the spleens were harvested and cultured with *M. vaccae* sonicate (20 μg/ml) as the antigen for 48 h. The cultures were then harvested and spun, the supernatants were collected and pooled, and IFN-γ was assayed by ELISA, using a commercial kit (Genzyme). On average, the culture supernatants so prepared contained 1,000 IU of IFN-γ per ml.

Nitrite estimation. Nitrite estimation as an index of nitric oxide (NO) synthesis was done as described by Isobe and Nakashima (20).

RESULTS

Effect of treatment with OMP on the immunogenicity of an unrelated antigen. Swiss white mice (15 per group) were each given an i.p. injection of 250 μg or 1.0 mg of the OMP preparation of *S. typhi* in a 0.5-ml volume; 72 h later, the mice were immunized with a vaccine containing 10^8 killed *M. vaccae* cells by the i.d. route; 72 h later, the OMP injection was repeated. Seven days after the immunization, DTH responses of the mice to the sonicate and the immunodominant 65-kDa antigen of *M. vaccae* were tested. The mice were then bled from the retro-orbital plexus; blood samples of five identically treated and immunized mice were pooled to obtain three pools per group. Serum from each pool was separated, and antibody titers against the 65-kDa antigen of *M. vaccae* and the IgG2a/IgG1 ratio of the antibody were assayed by ELISA.

The OMP-treated mice showed enhancement of both DTH and anti-65-kDa responses, in comparison with the responses of untreated mice. These experiments were repeated twice with essentially similar results (Table 1). The IgG1 fraction of the anti-65-kDa antibody was greater than the IgG2a fraction in the sera of untreated mice given an i.d. immunization with

TABLE 1. DTH and antibody responses to 65-kDa antigen of *M. vaccae*: effect of treatment with OMP and porins of *S. typhi* on T-helper responses of mice against *M. vaccae*

Treatment (dose)	Mean DTH CPE (mm) ± SE		Mean anti-65-kDa antibody titer (AU/ml) ± SE	AU/ml (IgG2a)/ AU/ml (IgG1)
	Sonicate	65-kDa antigen		
None	0.27 ± 0.03	0.25 ± 0.02	162 ± 47	0.8
OMP				
500 μg	0.40 ± 0.02	0.39 ± 0.02	1,691 ± 335	1.4
2.0 mg	0.42 ± 0.06	ND[a]	ND	ND
Porin (500 μg)	0.39 ± 0.01	0.41 ± 0.03	1,310 ± 42	ND
Porin, CNBr treated (500 μg)	0.25 ± 0.02	0.27 ± 0.02	176 ± 24	ND
PE-OMP[b] (500 μg)	0.35 ± 0.01	ND	ND	

[a] ND, not done.
[b] PE-OMP, OMP prepared from cell envelopes after salt extraction of porins.

M. vaccae. In contrast, in the sera of OMP-treated mice, the IgG2a fraction was greater than the IgG1 fraction. The IgG2a/IgG1 ratio of the antibody of the OMP-treated mice was almost twice that observed in the sera of untreated mice (Table 1).

Comparison of effects of OMP and LPS of *S. typhi* on immunogenicity of *M. vaccae*. OMP preparations can be contaminated with LPS; as a result, one may argue that the observed effects are due to contaminating LPS. To examine this possibility, Swiss white mice (10 per group) were given an i.p. injection of OMP (500 μg/mouse), LPS (10 to 20 μg/mouse), or a mixture of OMP (500 μg) and LPS (10 μg) in two equally divided doses before and after immunization with *M. vaccae* by the i.d. route as before, and the DTH responses of the animals to the sonicate of *M. vaccae* were tested 7 days later. OMP treatment enhanced the DTH response to *M. vaccae* by increasing the mean 48-h CPE from 0.29 ± 0.03 to 0.40 ± 0.04 mm. In contrast, the LPS treatment suppressed it. The mean 48-h CPE was reduced to 0.18 ± 0.04 mm. Interestingly, the mean 48-h CPE of mice treated with both LPS and OMP was of the same order as that of the untreated mice, i.e., 0.27 ± 0.03 mm, indicating that the OMP abrogated the suppression caused by LPS.

In view of the OMP-induced enhancement in the response of mice to immunization with *M. vaccae*, we decided to investigate the effect of OMP treatment on antigen presentation and IL-1 production by APCs. As a source of APCs, we used PC because they were previously shown to be not efficient in antigen presentation (34).

Antigen presentation by PC of mice treated with OMP of *S. typhi*. Nonimmune naive BALB/c mice were each given 500 μg of the OMP preparation by the i.p. route; 72 h later, the PC were harvested and were given 800-rad irradiation from a ^{60}Co source. These cells were then used as APCs in a lymphoproliferation assay. As a source of standard efficient APCs, naive nonimmune irradiated spleen cells were used. A separate set of BALB/c mice were given an i.d. immunization with 10^8 killed *M. vaccae* and sacrificed 1 week later, spleens were harvested, and single-cell suspensions were made and passed over a nylon wool column to obtain T cells. Then 10^5 i.d.-primed T cells were mixed with an equal number of irradiated spleen cells or PC (normal and OMP treated) to serve as APCs and were cultured with *M. vaccae* sonicate as the antigen. A standard 6-day lymphoproliferative assay was performed.

Normal spleen cells as APCs induced an excellent antigen-specific proliferative response. Normal, naive, untreated PC did not induce a proliferative response. However, OMP-treated PC induced a proliferative response as efficient as that induced by the spleen cells (Table 2). Based on these results, it was concluded that OMP treatment improves the antigen-presenting efficiency of the PC.

Effect of in vitro stimulation with OMP of *S. typhi* on IL-1 secretion by PC. PC were harvested from normal naive C3H/HeJ mice. This strain was selected for two reasons: it does not respond to LPS (36), and it is the appropriate strain to use in a thymocyte costimulation assay for IL-1. The PC were cultured in RPMI 1640 containing 10% FCS at a cell density of 10^6/ml (2×10^5/well) in microtiter plates. The cells were stimulated with 5 or 25 μg of OMP per ml incorporated in the culture medium. The cultures were incubated for 36 h at 37°C in 5% CO_2 in humidified air. Supernatants were harvested and assayed for IL-1 by the thymocyte costimulation assay. As a positive control, supernatant of cells treated with ConA (see Materials and Methods) was used. Normal PC did not secrete IL-1, and OMP induced its secretion by the PC (Table 3).

Hypersensitivity (inflammatory) response of nonimmune naive mice to OMP of *S. typhi*. OMP (50 μg in 0.05 ml) was injected in the hind footpads of nonimmune naive Swiss white mice (10), and FPE was measured. The mean 24- and 48-h FPE values (± standard error) were 1.20 ± 0.03 and 0.39 ± 0.02 mm, respectively. The mice were then sacrificed, and their

TABLE 2. Comparison of antigen presentation by naive and OMP-treated PC

APCs	Proliferative response of primed T cells (mean cpm ± SE)	Stimulation index
Spleen cells	10,453 ± 776	6.5
Naive PC	578 ± 23	1.2
OMP-treated PC	18,129 ± 410	9.5

TABLE 3. OMP induces IL-1 synthesis by PC[a]

Stimulus	Proliferative response of thymocytes (mean cpm ± SE)	SI
PHA only	1,981 ± 408	3.8
PHA + ConA sup	7,375 ± 71	14.7
PHA + PC sup A	2,040 ± 328	4.1
PHA + PC sup B	5,790 ± 325	11.5
PHA + PC sup C	6,304 ± 637	12.6

[a] ConA sup, supernatant of spleen cells cultured with ConA; PC sup A, supernatant of PC cultured without OMP; PC sup B, supernatant of PC cultured with OMP (5 μg/ml); PC sup C, supernatant of PC cultured with OMP (25 μg/ml); SI, stimulation index.

TABLE 4. Hypersensitivity reaction of naive mice to OMP and porins of *S. typhi*

Amt/footpad	Mean FPE (mm) ± SE at 48 h	
	Untreated	CNBr treated
OMP		
60 μg	0.33 ± 0.03	0.20 ± 0.02
15 μg	0.24 ± 0.02	0.13 ± 0.01
(7.5 μg)	0.23 ± 0.02	0.09 ± 0.01
3.75 μg	0.23 ± 0.02	0.08 ± 0.01
Porin		
7.5 μg	0.41 ± 0.02	0.15 ± 0.01
3.75 μg	0.35 ± 0.02	0.07 ± 0.01
PE-OMP,[a] 3.75 μg	0.31 ± 0.02	ND[b]
LPS		
5 μg	0.83 ± 0.03	0.75 ± 0.01
1 μg	0.53 ± 0.01	0.49 ± 0.01
100 ng	0.16 ± 0.01	0.15 ± 0.03
50 ng	0.11 ± 0.01	0.09 ± 0.01

[a] PE-OMP, OMP prepared from cell envelopes after salt extraction of porins.
[b] ND, not done.

footpads were biopsied. Histological examination of the footpads revealed acute inflammation with infiltration by a pleomorphic infiltrate rich in macrophages and lymphocytes; a significant neutrophilic component was also seen in the exudate. The experiment was repeated twice more with similar results.

It may be argued that the hypersensitivity reaction of the naive mice to the OMP preparation was caused by the contaminating LPS and not the proteins. Different amounts of LPS (50 ng to 5 μg) were injected into the hind footpads of naive Swiss white mice (five/dose), and 48-h FPE was assayed as before. LPS also induced footpad swelling in the naive mice (Table 4). It was therefore considered essential to rule out the possibility that the footpad swelling induced by the OMP preparation was due to contaminating LPS.

CNBr fragments proteins and has no effect on LPS. Hence, the effect of CNBr treatment of LPS and OMP preparations on their ability to induce footpad swelling was investigated. LPS and OMP preparations were treated with CNBr (see Materials and Methods), and the resulting fragmentation of peptides was confirmed by SDS-PAGE, revealing the absence of 35-kDa and larger bands seen in the untreated OMP preparation (Fig. 1). Different amounts of treated and untreated LPS and OMP were injected into the hind footpads of nonimmune naive Swiss white mice (five per group), and the 48-h FPE was measured as

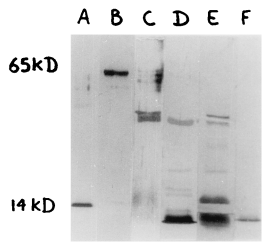

FIG. 1. SDS-PAGE of OMP and porin preparations. Lanes: A, lysozyme; B, bovine serum albumin; C, porin preparation of *S. typhi*; D, OMP preparation of *S. typhi* envelopes from which porins were extracted earlier by Nikaido's procedure (29); E, crude OMP preparation of *S. typhi*; F, CNBr-treated porin preparation of *S. typhi.*

before. It was observed that the FPE induced by the CNBr-treated OMP was considerably less than that induced by untreated OMP when 15 or 60 μg was injected. At lower doses, practically no FPE was observed. This dose-related effect could be due to incomplete degradation of the OMP by CNBr treatment. CNBr treatment had no effect on LPS-induced FPE (Table 4). It was therefore concluded that the proteins in the OMP preparation of *S. typhi* were responsible for the hypersensitivity induced in naive mice.

OMP of *S. typhi* do not behave as T- or B-cell mitogens or superantigens. Spleen cells of naive nonimmune BALB/c mice were cultured with different amounts (500 ng to 50 μg per ml) of an OMP preparation. As a positive control, separate sets of spleen cells were cultured with the superantigen SEB (1.0 μg/ml), ConA (5 μg/ml), and LPS (5 μg/ml). The cultures were pulsed with ^{3}H-labeled thymidine at 48 or 120 h for a period of 16 h and harvested, and incorporation of label was assayed. The SEB, ConA, and LPS induced an excellent proliferative response, whereas the OMP did not (data not shown).

Effect of treatment with OMP on microbicidal activity of PC. Normal Swiss white mice were given an i.p. injection of an OMP preparation (500 μg per mouse); 72 h later, the mice were challenged with 2×10^4 to 4×10^4 live *S. typhi* Ty2 cells by the i.p. route. After exactly 5 min, the time allowed for phagocytosis by the peritoneal macrophages, the mice were sacrificed, PC were harvested and incubated for various durations, and surviving salmonellae were assayed at each time point.

PC of normal nontreated mice killed only 17.1% of the challenge salmonellae in 5 min, and the killing did not increase significantly upon further incubation up to 35 min. The OMP-treated PC killed 52.5% of challenge salmonellae in 5 min, and as for naive PC, no further increase in killing was observed upon incubation up to 35 min.

OMP treatment enhances NO production by the peritoneal macrophages. PC were harvested from normal, naive Swiss white mice and cultured in microtiter plates at 5×10^6 cells/ml (10^6 cells/well) in RPMI 1640 containing 10% FCS. The PC were stimulated with different amounts of OMP and LPS preparations incorporated in the culture medium. As a positive control, the PC were stimulated with IFN-γ (250 IU/ml) incorporated in the medium. To assess maximum nitrite production by 10^6 PC, a separate set of Swiss white mice were given by the i.p. route six doses of indomethacin (50 μg per mouse) at 12-h intervals; PCs were harvested 12 h after the last dose and cultured with and without IFN-γ. All cultures were incubated at 37°C for 20 h, supernatants were harvested, and nitrite levels were assayed as an index of NO production.

Even normal, unstimulated PC produced nitrites. Stimulation with OMP, LPS, and IFN-γ increased nitrite production. No synergism was observed between OMP and LPS or OMP and IFN-γ in stimulation of nitrite production (Table 5). Effects of OMP and IFN-γ on NO production by PC of C3H/He mice were also studied, and the results were essentially similar (data not shown). This step was essential because the animals used were not germ free; the only way one could be certain that natural exposure to LPS did not play a significant role in the responses of PC to OMP and IFN-γ was to use a strain that is nonresponsive to LPS.

Characterization of the protein or peptide in the OMP preparation having immunomodulatory and hypersensitivity-inducing properties. (i) Analysis of SDS-PAGE profiles. SDS-PAGE of the crude OMP preparation revealed 35-kDa bands of porins and several low-molecular-mass bands of up to 7.5 kDa. The gels also revealed faint bands of higher molecular

TABLE 5. OMP stimulates NO production by the peritoneal cells

Treatment (concn)	Mice used as PC source	Mean nitrite concn (μmol/10^6 cells) ± SE
None	Swiss white	17.0 ± 1.3
	Swiss white, indomethacin treated	37.0 ± 3.3
OMP (5 μg/ml)	Swiss white	38.5 ± 0.6
LPS (100 ng/ml)	Swiss white	34.5 ± 0.3
LPS (1 μg/ml)	Swiss white	37.0 ± 0.3
OMP (5 μg/ml) + LPS (100 ng/ml)	Swiss white	37.0 ± 0.6
IFN-γ (250 IU/ml)	Swiss white	39.5 ± 0.6
OMP (5 μg/ml) + IFN-γ (250 IU/ml)	Swiss white	41.5 ± 0.3
IFN-γ (250 IU/ml)	Swiss white, indomethacin treated	94.0 ± 5.1

TABLE 6. Porins of *Enterobacteriacae* have immunomodulatory properties[a]

Source of porins injected before immunization with *M. vaccae*	Mean DTH CPE (mm) ± SE	Mean anti-65-kDa antibody titer (AU/ml) ± SE
None	0.23 ± 0.01	165 ± 8
S. enteritidis	0.35 ± 0.01	ND
S. typhimurium	0.36 ± 0.02	1,246 ± 99
S. paratyphi A	0.33 ± 0.01	555 ± 37
S. paratyphi C	0.33 ± 0.02	ND
E. coli	0.30 ± 0.02	548 ± 17

[a] DTH and antibody responses against the 65-kDa antigen of *M. vaccae* of mice treated with porins of different *Salmonella* strains and *E. coli* before i.d. immunization with the organism. ND, not done.

mass, formed from the aggregates of porins and other proteins (Fig. 1).

Since porins constituted the major component of OMP, we investigated whether the immunomodulatory and hypersensitivity-inducing properties of the OMP were due to porins. Porins were extracted from the cell envelopes; the residual envelopes after salt extraction were treated with lysozyme papain to obtain porin-depleted OMP. SDS-PAGE of the porin preparation revealed the characteristic 35-kDa bands. Lower-molecular-mass peptides were absent. Faint bands of higher-molecular-mass aggregates were also seen. However, SDS-PAGE of the OMP, prepared from the envelopes after porin extraction, revealed practically all of the bands present in the original crude OMP preparation, including those of porins, indicating that the NaCl extraction procedure did not remove porins completely (Fig. 1).

(ii) Immunomodulatory and hypersensitivity-inducing properties reside in porins. Three groups of Swiss white mice (five per group) were injected i.p. (500 μg/mouse) with a crude OMP preparation, a porin preparation, or a preparation of OMP from cell envelopes after porin extraction, and modulatory effects on the responses of the mice to an i.d. immunization with *M. vaccae* were studied as before. All the three preparations showed the immunomodulatory property. The DTH and antibody titers against the 65-kDa antigen of *M. vaccae* were enhanced when crude OMP or the porin preparations were used (Table 1). With the porin-depleted preparation of OMP, only the DTH response was tested; it was enhanced. Since the porin preparation lacking low-molecular-weight peptides showed the immunomodulatory activity, it was felt that the property resided in the porin fraction of the OMP (Table 1).

To confirm that the porins had the immunomodulatory activity, a porin preparation was treated with CNBr to fragment the proteins. SDS-PAGE of such a preparation clearly demonstrated lack of the 35-kDa band (Fig. 1). This preparation, when tested in Swiss white mice (500 μg/mouse), failed to enhance the DTH and antibody responses against *M. vaccae*, confirming that the immunomodulatory activity resided in porins (Table 1). As pointed out earlier, the salt extraction procedure for porins was not exhaustive; as a result, the preparation of OMP, from the residual cell envelopes after salt extraction of porins, revealed porin bands on SDS-PAGE and also the immunomodulatory property.

Experiments were also undertaken with crude OMP, porin fraction, porin-depleted OMP, and CNBr-treated porin preparations to investigate if porins were also capable of inducing hypersensitivity in naive mice. As before, 0.05 ml of each of the four preparations, containing 3.75 to 7.5 μg of protein, was injected into the hind footpads of Swiss white mice, and the 48-h FPE was measured. All preparations except the CNBr-treated porin preparation induced FPE, indicating that the hypersensitivity-inducing activity also resided in the porin preparation (Table 4).

Porins of different *Salmonella* strains and even *E. coli* have immunomodulatory activity. Five groups of Swiss white mice (five per group) were injected i.p. with 500 μg of a porin preparation made from *S. enteritidis*, *S. typhimurium*, *S. paratyphi A*, *S. paratyphi C*, or nonpathogenic normal fecal *E. coli*, and the modulatory effects of the various porins on the responses of the mice to immunization with *M. vaccae* were studied as before. Treatment with porins of all the salmonellae and even *E. coli* resulted in enhancement of DTH and antibody responses against *M. vaccae* (Table 6). Porins of all the salmonellae and normal fecal *E. coli* also induced 48-h FPE ranging from 0.26 to 0.32 mm in naive, nonimmune mice. Thus, both immunomodulatory- and hypersensitivity-inducing activities were shown by the porins of not only pathogenic salmonellae but also *E. coli* from normal gut.

DISCUSSION

Data presented in this report reveal several immunomodulatory properties of OMP of *S. typhi*: (i) enhancement of humoral and cellular immune responses to an unrelated antigen; (ii) improvement in the efficiency of antigen presentation by even nonprofessional APCs such as peritoneal macrophages; (iii) induction of IL-1 secretion by the PC; (iv) induction of a hypersensitivity response (tissue inflammation) in nonimmune mice; and (v) enhancement of microbicidal activity of the peritoneal macrophages, which could be due to stimulation of production of reactive oxygen and reactive NO intermediates (ROI and RNOI). Evidence for the latter was also presented.

The data also lead to some interesting observations. The OMP treatment enhanced both DTH and antibody responses against the 65-kDa antigen of *M. vaccae*. The antibody isotype favored by OMP treatment was IgG2a. Based on these data, it appears the OMP treatment favors a Th1 type of response. This is similar to the effect of OMP of *S. typhimurium*, favoring Th1 responses (38), by inducing IFN-γ secretion, possibly by natural killer cells. The improvement in the efficiency of antigen presentation by the PC could also be due to a similar effect. The effect of IFN-γ as a modulator of the humoral response is quite complex. It changes the isotype of the antibody formed to IgG2a (10). However, its effect on the antibody titer is depen-

dent on its concentration, high concentrations being inhibitory. Low concentrations do not have the inhibitory effect and could even induce enhancement of the IgG2a titers (7). The Th1 cells also secrete IL-2, a cytokine known for helping B-cells to generate IgG2a antibodies (7). Under the influence of an in vivo Th1 response, particularly a primary one, high titers of IgG2a antibodies have been reported (7).

The observed OMP-induced neutralization of the suppressive effect of LPS on the T-cell response to an antigen is very significant. T-cell suppression induced by LPS could be due to several causes, such as stimulation of macrophages to secrete prostaglandins (33) and ROI and RNOI synthesis (20). Prostaglandins are known to inhibit T-cell responses (32) and secretion of cytokines such as IFN-γ (6) and IL-2 (19), thus changing the T-cell response pattern to a low-grade Th2 type of response (19). RNOI have been shown to inhibit antigen-induced T-cell proliferation (20) and also favor a Th2 response (37). Incidentally, OMP treatment of mice was found to improve the efficiency of antigen presentation by the PC, despite a higher level of NO secretion. We have not studied the effect of OMP on prostaglandin secretion by the macrophages. LPS is a known B-cell mitogen and can induce B cells to secrete IL-10 (12), which favors a Th2 response (11). Again, secretion of IFN-γ under the influence of OMP can antagonize this effect. Thus, OMP seems to neutralize the T-cell-suppressive effect of LPS by multiple mechanisms.

OMP of *S. typhi* also appear to have major effects on the innate immune mechanisms. Induction of a hypersensitivity response in nonimmune naive mice could be due to IL-1 synthesis and/or induction of tumor necrosis factor alpha secretion by macrophages as reported earlier (14). Enhancement in the microbicidal activity of the peritoneal macrophages by stimulation of RNOI and possibly ROI is another significant effect of OMP on the innate immune mechanism. There was no synergism between OMP and either LPS or IFN-γ in RNOI synthesis. It may be argued that the amounts of OMP, LPS, or IFN-γ that we used for stimulation of PC were so high that maximum NO production occurred under the influence of a single agent. As a result, there was no scope for further enhancement when two agents were added to the PC cultures. However, under the influence of indomethacin and IFN-γ, nitrite levels generated were twice the levels seen with IFN-γ, porins, or LPS alone (Table 5).

A crude OMP preparation contains several components, namely, porins (35 kDa) and low-molecular-weight peptides such as lipoproteins and OMP A. Both the immunomodulatory and hypersensitivity-inducing activities could be ascribed to the porins.

Porins of all five *Salmonella* species and a strain of *E. coli* isolated from normal human feces revealed both the immunomodulatory and hypersensitivity-inducing properties, implying that the porins of members of the family *Enterobacteriacae* have these activities.

It is of interest that the porins favored a Th1 type of response (important in immunity against facultative intracellular bacteria) while increasing the antibody titer. In contrast the currently permitted adjuvant for human use, alum, induces a Th2 type of response (1). The *Bordetella pertussis* suspension often used along with alum does not significantly alter the adjuvant effect of the latter. However, the pertussis toxin when administered along with antigen has been shown to enhance the DTH response also (39). Another adjuvant, the mycobacterial MDP, induces a Th2-like response when administered in saline along with antigen and a Th1-like response with a high antibody titer when administered as a water-in-oil emulsion (1, 39). FCA also increases the antibody titer while inducing a Th1-like response (1). In this respect, the adjuvant effect of porins is similar to that of MDP (water-in-oil emulsion) and FCA. The special advantage of porins is that the unique immunomodulatory effects (Th1-like response with high antibody titers) are induced when porins are administered in saline. This finding raises the possibility that the porins of *Enterobacteriacae* may prove to be useful as adjuvants or as immunostimulants.

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