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Mechanism of Protective Immunity Induced by Porin-Lipopolysaccharide against Murine Salmonellosis

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Investigations were undertaken to characterize the protective immunity induced by porin-lipopolysaccharide (LPS) against *Salmonella typhimurium* infection in mice. Mice immunized with porin-LPS showed higher levels of antiporin immunoglobulin G than mice which received porin alone. Further, T cells from porin-LPS-immunized mice showed an augmented proliferative response to porin in vitro compared with the response of T cells from porin-injected animals. The passive transfer of anti-LPS antibodies conferred significant protection (17%), while antiporin serum failed to protect mice against lethal challenge, indicating the protective ability of anti-LPS antibodies. However, the transfer of serum obtained from porin-LPS-immunized mice resulted in better protection (30%) than did anti-LPS or antiporin antibodies alone. In contrast to LPS, monophosphoryl lipid A completely failed to induce protection against lethal infection. However, comparable to the effect of LPS, injection of porin with monophosphoryl lipid A enhanced antibody response and the protective ability of porin (81.25%). The transfer of T cells from porin-LPS-immunized mice provided higher levels of protection (47%) against lethal challenge than did T cells from porin-immunized mice (23%). The combination of T cells and serum from porin-immunized mice transferred 36% protection. However, a combination of T cells and serum from porin-LPS-immunized mice conferred the highest level of protection (92%), which was reflected by the number of survivors (100%) in the porin-LPS-immunized group. These results demonstrate that besides the protective effect of anti-LPS antibodies, the ability of LPS to augment humoral and cell-mediated immune responses to porin confers effective protection against *Salmonella* infection.

Several studies have stressed the significance of proteins as protective antigens in murine salmonellosis (3, 17, 20, 31, 46). Cell surface proteins and protease-sensitive culture supernatant factor from *Salmonella typhimurium* were found to protect mice from lethal challenge (20, 31). However, this supernatant factor was protective only if O antigens were present in the supernatant (32). In another study, Plant et al. (33) have reported that protein-lipopolysaccharide (LPS) complex isolated from *S. typhimurium* was able to induce protective immunity in mice. Though LPS-free proteins extracted from rough mutants of *S. typhimurium*, *Salmonella minnesota*, and *Salmonella dublin* conferred protection against lethal salmonellosis, the protective ability of purified proteins was lower than that of the corresponding crude extracts, suggesting that LPS might play a significant role in inducing protective immunity (39). Rabbit anti-O antibodies passively administered to mice significantly enhanced the clearance of *S. typhimurium* challenge (11). Similarly, sera raised in rabbits against polysaccharide-protein conjugates having specificity to O antigens conferred protection upon transfer (40). The protective ability of antibodies of the immunoglobulin M (IgM) isotype with specificity to the O-antigenic determinant has been reported elsewhere (38).

In addition to its ability to induce protective antibodies, LPS has been shown to be a powerful adjuvant capable of enhancing the immune response to many antigens (8). LPS isolated from various gram-negative bacteria stimulated a 20-fold-greater antibody response to ovalbumin in rabbits than did the administration of antigen alone (9). In addition to the in vivo effects, addition of LPS during the first 12 to 24 h of in vitro cultures of spleen cells in the presence of sheep erythrocytes (SRBC) remarkably enhanced the anti-SRBC response (28). Chiller and Weigle (6) have reported that LPS can convert a tolerogenic regimen of deaggregated human gamma globulin into an immune stimulus. Further, the ability of LPS to induce a characteristic switch in antibody responses from IgM to IgG has also been reported previously (47). Considerable evidence indicates that LPS is capable of potentiating a variety of manifestations of T-cell-mediated immune responses (8). LPS was found to enhance the delayed-type hypersensitivity response to SRBC (19). Further, by using athymic nude mice, the requirement of T cells for the adjuvant action of LPS to enhance the antibody response to T-dependent antigens has been demonstrated elsewhere (25).

Although LPS is a strong immunopotentiating agent for various protein antigens, its use as immunological adjuvant is precluded by the fact that LPSs are extremely toxic and pyrogenic for most animal species (34). However, monophosphoryl lipid A (MPL), the nontoxic form of lipid A, has been shown to retain the biological activities of LPS, such as B-cell mitogenicity, adjuvancity, activation of macrophages, and induction of interferon synthesis (10). The effect of MPL in enhancing antibody response to SRBC (10) and ovalbumin (35) was found to be comparable to the adjuvant effect of toxic lipid A. Further, MPL has been shown to be a good adjuvant in restoring age-dependent losses in immune responses and in augmenting the antibody response to SRBC in LPS-hyporesponsive C3H/HeJ mice (42).

There have been only limited attempts to understand the role of outer membrane proteins in inducing protective immunity against salmonellosis. Crude porin and crude outer membrane proteins prepared from the cell wall of *S. typh-*

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imurium protected mice against lethal challenge (18, 44). Passive transfer of antiserum raised against purified porin did not protect mice, whereas antiserum obtained from mice immunized with LPS-reconstituted porin was protective (17). Similarly, LPS–lipid A-associated protein complex but not LPS alone has been shown to protect mice against lethal *Salmonella* infection (14). Porin isolated from *S. typhimurium* when injected along with LPS induced higher level of protection than did porin or LPS alone (46). Though the significance of LPS in the protective immunity against *S. typhimurium* is evident from these studies (17, 46), it is not clear whether it is due mainly to anti-LPS antibodies or also to the augmentation of immune responses to porin by LPS. Therefore, the present study was aimed to characterize the enhanced protective immunity induced by porin-LPS and to test the efficacy of porin-MPL in the protection against *S. typhimurium* infection in mice.

MATERIALS AND METHODS

Mice. BALB/c mice of both sexes aged 6 to 10 weeks from our colony were used. They were fed with commercial feed (Hindustan Lever, Bombay, India) and water ad libitum.

Bacterial strain. A virulent strain of *S. typhimurium* C5 was obtained from C. E. Hormaeche, University of Cambridge, Cambridge, United Kingdom. This strain was used for isolation of porin as well as a challenge bacterium, and the 50% lethal dose (LD_{50}) of C5 in BALB/c mice was 10^5 cells as reported and discussed earlier (43). Briefly, the organisms used for challenge were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.), and for porin preparation, the bacteria were grown in nutrient broth containing peptone (1%), beef extract (1%), and NaCl (0.5%).

Reagents. Smooth LPS of *S. typhimurium* was obtained from Sigma Chemicals (St. Louis, Mo.). MPL purchased from Ribi Immunochemicals (Hamilton, Ohio) was dissolved in 0.2% triethylamine. Working dilutions from the stock was made in saline before use. Freund complete adjuvant (FCA) was purchased from Difco Laboratories.

Preparation of porin. Porin from C5 was prepared by following the method of Tokunaga et al. (41) with minor modifications. Briefly, bacterial cells suspended in 0.05 M Tris-HCl (pH 7.2) were sonicated (Labline Ultratip; Labsonic System, Melrose Park, Ill.) and centrifuged at 80,000 × *g* for 30 min at 4°C. The cell wall pellet was suspended in 0.05 M Tris-HCl containing 2% (wt/vol) sodium dodecyl sulfate (SDS) and incubated overnight at 37°C. After centrifugation at 80,000 × *g* for 30 min at 20°C, the pellet obtained was treated with Tris–0.005 M EDTA and incubated for 2 h at 37°C. After further centrifugation as indicated in the previous step, porin was extracted in Tris-EDTA containing 0.2% SDS and 0.4 M NaCl. Porin was purified from this preparation by gel filtration in a Sepharose 6B column. Porin digested with an equal volume of sample buffer by heating at 100°C for 2 min was subjected to SDS-polyacrylamide (10%) gel electrophoresis followed by Coomassie blue (45) or silver (21a) staining and showed a single band at the position of 36,000 without any detectable polypeptide or LPS contamination.

Preparation of porin-LPS. Porin dialyzed against a buffer containing 5 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 0.025% 2-mercaptoethanol at 25°C for 48 h was mixed with LPS in a ratio of 2:1 (wt/wt) and incubated at 37°C for 1 h. Appropriate amounts were removed and used for immunization. Under similar conditions LPS was shown to form a complex with porin (51). The same preparation of porin was used for immunization along with MPL or porin alone.

Immunization and determination of protection. Mice were immunized subcutaneously (s.c.) with optimal doses of porin (100 μg), LPS (50 μg), MPL (50 μg), or porin (100 μg) plus either LPS or MPL (50 μg) either once or twice at 15-day intervals. Different doses of antigen or combinations of porin and LPS or MPL were tested for their ability to induce protection against lethal challenge. The dose which conferred maximum protection was selected as the optimal dose as reported earlier for porin and LPS (46). Control mice received similar injections of either Tris buffer containing 0.2% SDS or 0.05% triethylamine in saline. Ten days after the last injection, animals were challenged intravenously (i.v.) with 10 LD_{50}s of virulent *S. typhimurium* C5 in 0.2-ml volumes. The daily mortality was observed for up to 21 days postchallenge, and the percentage of survivors in each group was calculated at the end of this period.

Enumeration of bacteria in the spleen. The number of viable bacteria in the spleens of both control and experimental mice was determined as previously described (22). The spleens were aseptically removed from mice at various time points and homogenized separately in 5-ml portions of sterile phosphate-buffered saline (PBS) with Teflon homogenizer. The cell suspension was serially diluted in PBS, and aliquots were plated in duplicate on MacConkey agar plates. The colonies were counted 24 h after incubation at 37°C. The calculation was made by assuming that each CFU represented a single bacterium.

Collection of sera. Control and immunized mice were bled retroorbitally with Pasteur pipettes. The sera were separated from the clot by centrifugation, inactivated at 57°C for 30 min, and stored at −20°C until use.

Determination of antibody levels. Antiporin or anti-LPS antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described by Voller et al. (48) with a few modifications. Briefly, 96-well flat-bottom polystyrene plates (Immunoplate I; Nunc, Roskilde, Denmark) were coated with optimal concentrations of porin (0.5 μg per well) or LPS (1.3 μg per well) dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Horseradish peroxidase-conjugated goat anti-mouse total or heavy-chain-specific immunoglobulins (Cappel, West Chester, Pa.) were used as probes to detect specific binding of mouse immunoglobulins. The substrate solution consisted of 0.04% *O*-phenylene diamine (Sigma) and 0.012% hydrogen peroxide in phosphate citrate buffer (pH 5). The log dilution of antibodies corresponding to an A_{490} of 0.15 was considered to be the titer. In some experiments antibody titers were expressed as ELISA titer units and were calculated as follows: total antibody titer/1,000 = number of units.

Purification of T cells. The enriched T cells were purified as described in detail elsewhere (12). Briefly, a single-cell suspension was prepared from inguinal lymph nodes or spleens in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were then washed three times and passed through a nylon wool column to remove adherent cells, and the T cells were eluted from the column with warm medium. The purity of T cells isolated was usually greater than 90%, as determined by complement-mediated lymphocytotoxicity (37) using anti-Thy 1.2 antibodies and guinea pig complement (Pel-Freez, Rogers, Ark.). For instance, the responses of isolated T cells from porin-LPS-immunized mice in the tests of purity with concanavalin A and LPS were 27,069 ± 2,672 and 3,624 ± 234 cpm, respectively. The

response to these mitogens was similar in cell preparations from other groups of mice.

Passive transfer of immune serum and cells. Control or immune serum (0.1 or 0.2 ml) was administered i.v. to naive animals 3 h prior to lethal challenge with *S. typhimurium* C5. The enriched splenic T cells (3×10^7) collected from control and immunized mice were transferred i.v. to naive mice. Mice were challenged i.v. with a lethal dose of C5 organisms 24 h later.

Proliferation assay. The enriched T cells isolated from control and immunized mice were cultured in RPMI 1640 medium containing 10% fetal calf serum by using 96-well flat-bottom microtiter plates. Cells were cultured for 5 days at a density of 2×10^5 per well and in a final volume of 0.2 ml at 37°C with 5% CO_2. DNA synthesis was determined by addition of 0.5 μCi of [^{3}H]thymidine (specific activity, 6.6 Ci/mol; Bhabha Atomic Research Center, Bombay, India) during the last 16 h of the culture period. Cultures were harvested onto glass fiber filters with a PHD cell harvester, and the radioactive counts were determined in a liquid scintillation counter (LKB, Bromma, Sweden).

Statistics. The data were expressed as arithmetic means ± standard errors (SE). Two-tailed Student's *t* test was used to assess the significance of the data.

RESULTS

Effect of LPS on antiporin IgM and IgG responses. In order to characterize the protective immunity induced by porin-LPS, the qualitative and quantitative differences in the antiporin antibody responses were analyzed when porin was injected alone or with LPS. As shown in Fig. 1A, the antiporin IgM response was not enhanced by LPS. However, the magnitude of antiporin IgG response in porin-LPS-immunized mice was severalfold higher than that in the porin-immunized group, and the difference was highly significant ($P < 0.0005$), starting from the first week on (Fig. 1B). The peak antiporin IgG titer observed in the fifth week was about sevenfold higher than that in porin-immunized mice. The peak titer was maintained approximately at the same level for at least 8 weeks. These results indicate that LPS when injected along with porin induced a profound enhancement of the antiporin IgG response.

Passive transfer of protective immunity by immune serum and T cells. In addition to the augmentation of the antiporin IgG response, immunization of porin along with LPS induced a strong delayed-type hypersensitivity response to porin compared with the response in mice which received porin alone (data not shown). These results indicated that LPS could enhance the cell-mediated immune response to porin as well. Hence, it has become critical to determine the relative contributions of humoral and cellular factors in the protective immunity induced by porin-LPS. Therefore, passive-transfer studies were carried out with immune serum and T cells. As shown in Table 1, the transfer of serum obtained from porin-immunized mice failed to protect recipients against lethal challenge whereas equal volumes of anti-LPS and anti-porin-LPS sera protected 20 and 33% of the recipients, respectively. The transfer of enriched T cells obtained from porin-LPS-immunized mice conferred a higher level of protection (47%) than did T cells from porin-immunized mice (23%). On the other hand, T cells from LPS-administered mice completely failed to mediate protection. The highest level of protection (92%) was observed when animals received a combination of T cells and serum obtained from porin-LPS-immunized mice. On the other hand, a similar combination of T cells and serum collected from porin-immunized mice could transfer only 36% protection. The transfer of T cells collected from LPS-immunized mice plus anti-LPS serum resulted in 25% protection against lethal challenge, suggesting that T cells from LPS-immunized mice did not play a critical role in protection. These results indicate the superiority of immune serum and T cells obtained from porin-LPS-immunized mice in mediating protection against *Salmonella* infection.

Analysis of protective ability of anti-porin-LPS serum. As indicated in Table 1, the transfer of serum obtained from porin-immunized mice completely failed to give protection while serum collected from porin-LPS-immunized mice conferred 33% protection. Therefore, an attempt was made to understand whether the increased level of protection achieved by anti-porin-LPS serum was mediated by anti-LPS or antiporin antibodies. Serum obtained from mice immunized with porin-LPS showed 9 U of anti-LPS and 140.6 U of antiporin titers (Table 2). This serum upon transfer protected 30% of the recipient mice, while anti-LPS serum (titer = 7.1 U) showed 17% protection. Both normal and antiporin sera (titer = 15 U) failed to confer protection. The transfer of antiporin serum obtained from mice immunized with porin emulsified in FCA also failed to confer protection, though it contained a high titer of antiporin antibodies (75.5 U). Notably, even the transfer of 0.1 ml of anti-porin-LPS sera gave 25% protection. These results showed that anti-LPS antibodies could protect mice but that the antibodies to pure porin could not. However, antibodies raised against porin-LPS were more protective, which could be due to an additive effect of anti-LPS antibodies plus the subclass of antiporin IgG generated as a result of immunization with porin-LPS. After secondary immunization, for the titers of antibodies shown in Table 2, the proportion of antiporin IgM versus IgG titers in antiporin serum was 1:2.6 and in anti-porin-LPS serum was 1:24. The proportions of anti-LPS IgM versus IgG in anti-LPS and anti-porin-LPS sera were found to be 1:1.3 and 1:2.3, respectively (data not shown). Though 2.6-fold more antiporin IgG antibodies were detected after secondary immunization with porin alone, the proportion was dramatically lower than the level seen in porin-LPS-immunized mice (24-fold). Further, in addition to anti-LPS antibodies, the high antiporin IgG titer and the possible variations in the subclass of IgG generated in porin-LPS-immunized mice might have caused the enhanced level of protection.

Effect of LPS on proliferation of T cells responding to porin. To characterize whether injection of porin along with LPS influences the T-cell response to porin, the proliferative response of T cells in vitro was tested. Interestingly, the proliferative response of T cells from porin-LPS-immunized mice was found to be twofold higher than the response of T cells obtained from porin-immunized animals (Fig. 2). The optimal dose of porin for in vitro stimulation was found to be 40 μg/ml. However, the augmented proliferative response of T cells was seen at other doses of porin tested. T cells from control and LPS-immunized mice did not respond to porin in vitro. The magnitude of the T-cell response in porin-LPS-immunized mice was well above the sum of the responses of T cells from mice which received porin and LPS separately. A similar augmented proliferation was seen in response to porin in vitro in T cells obtained after secondary immunization with porin-LPS. Further, T cells obtained from LPS-immunized mice did not augment the proliferation of T cells from porin-injected mice (data not shown). Therefore, it can be suggested that the enhanced T-cell response resulting

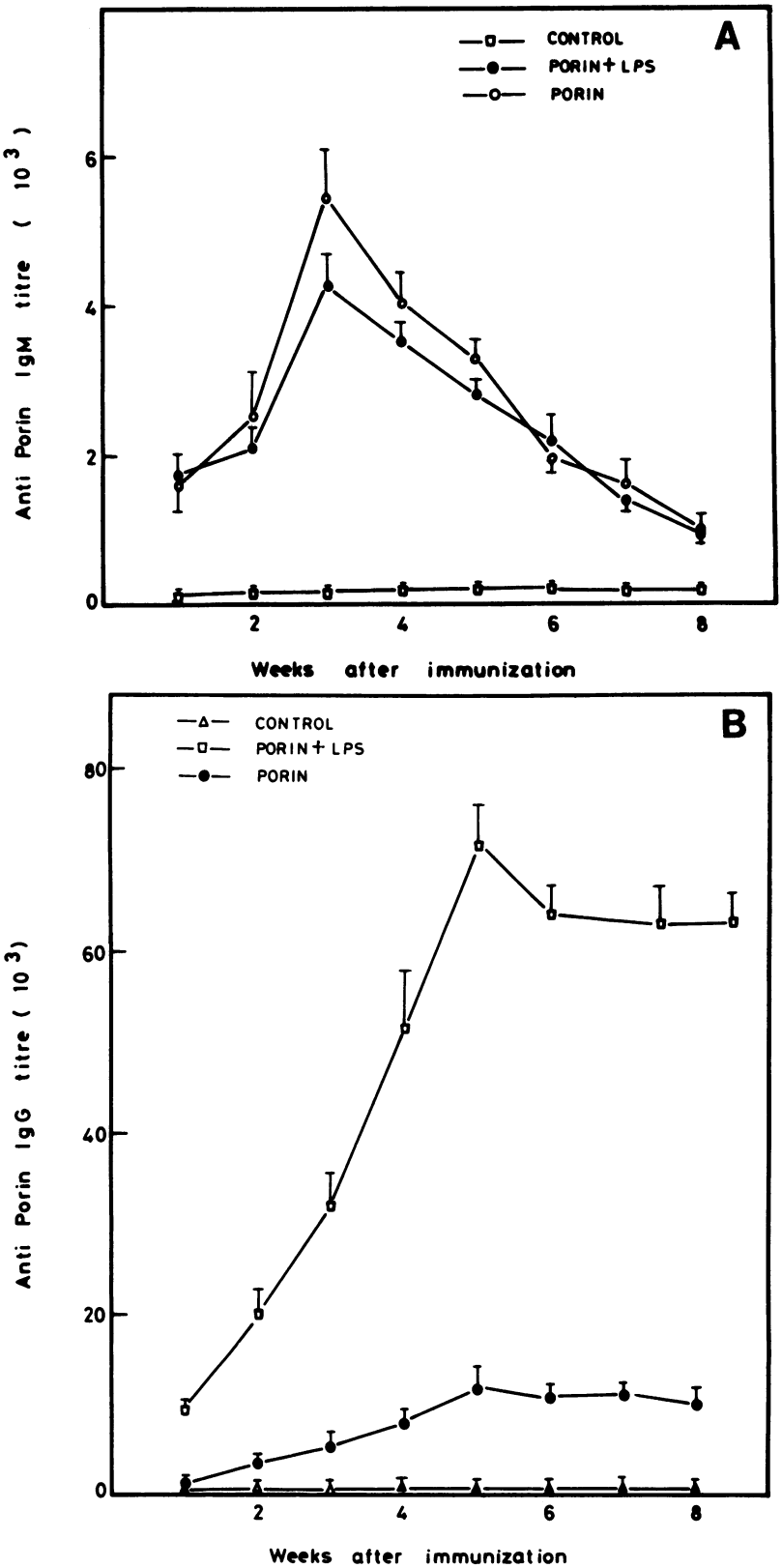

FIG. 1. Effect of LPS on antiporin IgM and IgG responses. Mice were immunized s.c. with porin (100 μg) or porin (100 μg) plus LPS (50 μg). The control mice received 0.2 ml of Tris buffer containing 0.2% SDS. Sera were titrated for antiporin IgM (A) and IgG (B) by ELISA at the indicated time points as described in Materials and Methods. Each point represents mean titer ± SE for six mice.

TABLE 1. Passive transfer of protection by immune serum and T cells[a]

Transfer material	No. of survivors/total no. of mice (%) receiving material from donor mice immunized with:			
	Tris (control)	Porin	LPS	Porin + LPS
Serum	0/10 (0)	0/12 (0)	3/15 (20)	4/12 (33)
T cells	0/11 (0)	3/13 (23)	0/10 (0)	7/15 (47)
T cells + serum[b]	0/10 (0)	4/11 (36)	3/12 (25)	11/12 (92)

[a] Donor mice were immunized with either porin (100 μg), LPS (50 μg), or porin (100 μg) plus LPS (50 μg) s.c. twice at 15-day intervals. Control mice received two similar injections of Tris buffer containing 0.2% SDS. Ten days after the last injection, spleens were removed and T-cell-enriched fractions were separated as described in Materials and Methods. Sera from control and immunized mice were also collected and pooled. Each recipient mouse received 3×10^7 T cells, 0.2 ml of pooled serum, or a combination of serum and cells i.v. The mice were challenged i.v. with 10 LD_{50}s of *S. typhimurium* C5 either 3 h after serum transfer or 24 h after T-cell transfer. The number of survivors 21 days after challenge is indicated.

[b] In the group of mice which received the combination of T cells and serum, the T cells were given 21 h before serum transfer.

from the administration of porin-LPS occurred during the priming phase and that LPS can augment the proliferation of T cells which are responding to porin.

Effect of MPL on antibody response to porin. It was of interest to understand whether MPL, a detoxified form of LPS, could enhance the antibody response to porin. As shown in Table 3, MPL markedly enhanced the antiporin antibody response. Fifteen days postimmunization, the antiporin titer in the porin-MPL-immunized group was significantly higher than the level seen in porin-immunized mice. The adjuvant effect of MPL on the antiporin antibody response was clearly evident after secondary immunization and was about fivefold higher than that in the porin-immunized group. The maximum increase in the antiporin response was seen in porin-LPS-immunized animals and was about ninefold higher than that of the porin-immunized group. These results indicated that MPL significantly enhanced the antibody response to porin. However, the adjuvant effect of LPS in inducing the antiporin antibody response was more remarkable than that of MPL.

TABLE 2. Passive transfer of protective immunity by immune serum

Serum treatment[a]	ELISA titer (U)[b]		No. of survivors[c]/ total no. of mice	% Survivors
	Anti-LPS	Antiporin		
Normal mouse	0.8	0.5	0/9	0
Anti-LPS	7.1	1.0	2/12	17
Antiporin	0.9	15.0	0/9	0
Antiporin (FCA)	1.1	75.5	0/10	0
Anti-porin-LPS	9.0	140.6	3/10	30
Anti-porin-LPS (0.1 ml/mouse)	9.0	140.6	2/8	25

[a] Groups of donor mice were immunized s.c. twice with LPS (50 μg), porin (100 μg), porin (100 μg) emulsified in FCA, or porin (100 μg) plus LPS (50 μg) at 15-day intervals. Control mice were given an equal volume of Tris buffer containing 0.2% SDS. Serum from each group of mice was collected and pooled 10 days after the last immunization.

[b] Estimated as described in Materials and Methods (1 U = antibody titer of 1,000). Except for the indicated group, groups of 8 to 12 mice were injected with pooled serum (0.2 ml per mouse) i.v.

[c] Three hours after serum transfer the mice were challenged i.v. with 10 LD_{50}s of *S. typhimurium* C5. The number of survivors at 21 days after challenge is indicated.

Effect of MPL on the protective ability of porin. Since MPL augmented the antibody response to porin, whether a similar effect in the level of protection was possible was tested. Groups of mice were given two injections of MPL, LPS, porin, porin-MPL, and porin-LPS at 15-day intervals. Ten days after the last injection, mice were challenged lethally with *Salmonella* cells and protection was assessed. In contrast to mice which received LPS (35.7% survived), none of the mice immunized with MPL survived the lethal challenge, demonstrating that MPL was not a protective antigen (Table 4). While all control animals died, immunization with porin resulted in 25% protection. On the other hand, administration of porin along with MPL significantly increased the survival rate to 81.25%, while porin-LPS induced a strong protective immunity, as evident by 100% survivors. These results indicate that MPL is capable of enhancing the protective immunity induced by porin but porin-LPS is a better candidate to induce complete protection.

In order to assess protective immunity more precisely, the in vivo growth pattern of challenge organisms was taken as another criterion. Mice injected with a combination of porin and MPL effectively controlled bacterial multiplication (Fig. 3). However, the group of animals immunized with porin-LPS was more efficient in restricting the bacterial growth than any other group. Immunization with MPL alone completely failed to restrict the growth of challenge bacteria. In control and MPL-immunized mice, the challenge bacteria multiplied actively and resulted in death prior to 10 days. While 50 to 65% of the mice which received either porin or LPS also died in the first 10 days, the survivors in these groups began to control the growth of challenge organisms slowly. In contrast, mice immunized with porin-MPL or porin-LPS showed bacterial growth until day 6, and subsequently an effective restriction in the bacterial load was observed. These results demonstrate the development of an effective antibacterial mechanism in porin-MPL-immunized mice as well as in porin-LPS-immunized mice.

DISCUSSION

We have previously reported that porin in association with LPS evoked a higher level of protective immunity in mice against salmonellosis than either of these antigens alone (46). In the present study, an attempt was made to characterize the protective immunity induced by porin-LPS. The results shown in Fig. 1B indicate that porin in combination with LPS induced a profound antiporin IgG response compared with the response in mice which received porin alone. However, no such enhancement in the antiporin IgM response was observed (Fig. 1A). Upon transfer, the serum collected from porin-LPS-immunized mice protected a higher percentage of mice than did anti-LPS or high-titer antiporin sera obtained with FCA (Table 2). It is possible that the elevation in the level of antiporin IgG plays an important role in mediating protective immunity in the porin-LPS-immunized group. However, this contention seems unlikely because a similar increase in the antibody response to porin by FCA did not cause an elevated level of protection as determined by passive-transfer studies (Table 2). These results were consistent with the earlier findings of Udhayakumar and Muthukkaruppan (46), in which mice immunized with porin emulsified in FCA did not have an enhanced level of protection. Therefore, the mere increase

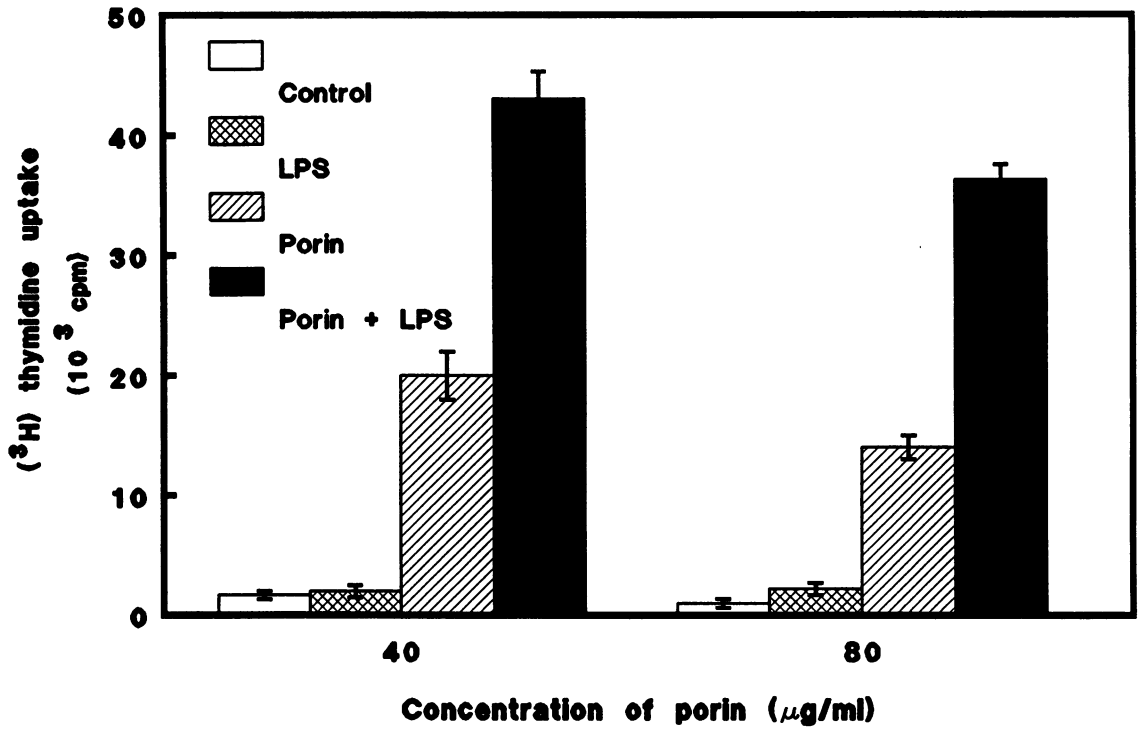

FIG. 2. Effect of LPS on proliferation of T cells responding to porin. Mice were immunized s.c. with porin (100 μg), LPS (50 μg), or porin (100 μg) plus LPS (50 μg). Control mice were given an equal volume of Tris buffer containing 0.2% SDS. Inguinal lymph node T cells from various groups of mice were obtained 15 days postimmunization. T cells (2×10^5 per well) were cultured in RPMI 1640 medium containing 10% fetal calf serum with the indicated concentrations of porin for 5 days and pulsed with [^{3}H]thymidine during the last 16 h of culture. The mean counts per minute ± SE of triplicate cultures from a representative experiment is shown. For example, in the tests of purity, the concanavalin A- and LPS-induced mitogenic responses of isolated T cells from porin-immunized mice were 33,675 ± 2,012 and 3,491 ± 176 cpm, respectively. Similar mitogenic responses were seen in T cells isolated from other groups of mice.

in antiporin titer cannot be attributed to the enhanced protection observed in porin-LPS-immunized mice.

Administration of protein antigens along with FCA induced mostly antibodies of the IgG1 subtype. On the other hand, immunization of the same antigen along with LPS preferentially evoked antibodies of the IgG3, IgG2b, and IgG2a subtypes (16). Among murine antibodies, the IgG2a subtype is most effective in activating the complement and antibody-dependent cellular cytotoxicity mechanisms and mediating better protection against parasitic infections (49, 50). Similarly, the protection against *S. typhimurium* infection by IgG3 monoclonal antibodies was demonstrated by Colwell et al. (7). Recent results from our laboratory indicated a qualitative difference between antiporin antibodies generated as a result of immunization of porin along with LPS and FCA (23a). This qualitative difference in the antiporin antibody response might also be a reason for the

TABLE 3. Effect of MPL on antibody response to porin

Immunization[a]	Antiporin titer[b] on day:	
	15	25
Tris buffer	713 ± 121	965 ± 96
0.05% triethylamine	1,127 ± 206	894 ± 108
MPL (50 μg)	1,435 ± 140	1,229 ± 189
LPS (50 μg)	1,630 ± 201	2,315 ± 120
Porin (100 μg)	5,427 ± 1,176	15,201 ± 2,650
Porin (100 μg) + MPL (50 μg)	16,428 ± 2,015	81,005 ± 17,216
Porin (100 μg) + LPS (50 μg)	32,624 ± 3,456	140,267 ± 15,268

[a] Groups of mice (six mice per group) were immunized s.c. twice with the indicated dose of antigens at 15-day intervals. Control mice received similar injections of either Tris buffer containing 0.2% SDS or 0.05% triethylamine in saline.

[b] Estimated by ELISA as described in Materials and Methods with sera collected 15 days after the first injection or 10 days after the last injection. The results represent mean titers ± SE for six mice. For porin versus porin plus MPL, $P < 0.0005$ (day 15) and $P < 0.0025$ (day 25).

TABLE 4. Effect of MPL on the protective ability of porin

Immunization[a]	No. of survivors[b]/ total no. of mice	% Survivors
Tris buffer	0/9	0
0.05% triethylamine	0/8	0
MPL (50 μg)	0/14	0
LPS (50 μg)	5/14	35.7
Porin (100 μg)	3/12	25.0
Porin (100 μg) + MPL (50 μg)	13/16	81.25
Porin (100 μg) + LPS (50 μg)	16/16	100.0

[a] Groups of mice were immunized with indicated doses of antigen s.c. twice at 15-day intervals. Ten days after the last injection mice were challenged i.v. with 10 LD_{50}s of *S. typhimurium* C5.

[b] At 21 days after challenge.

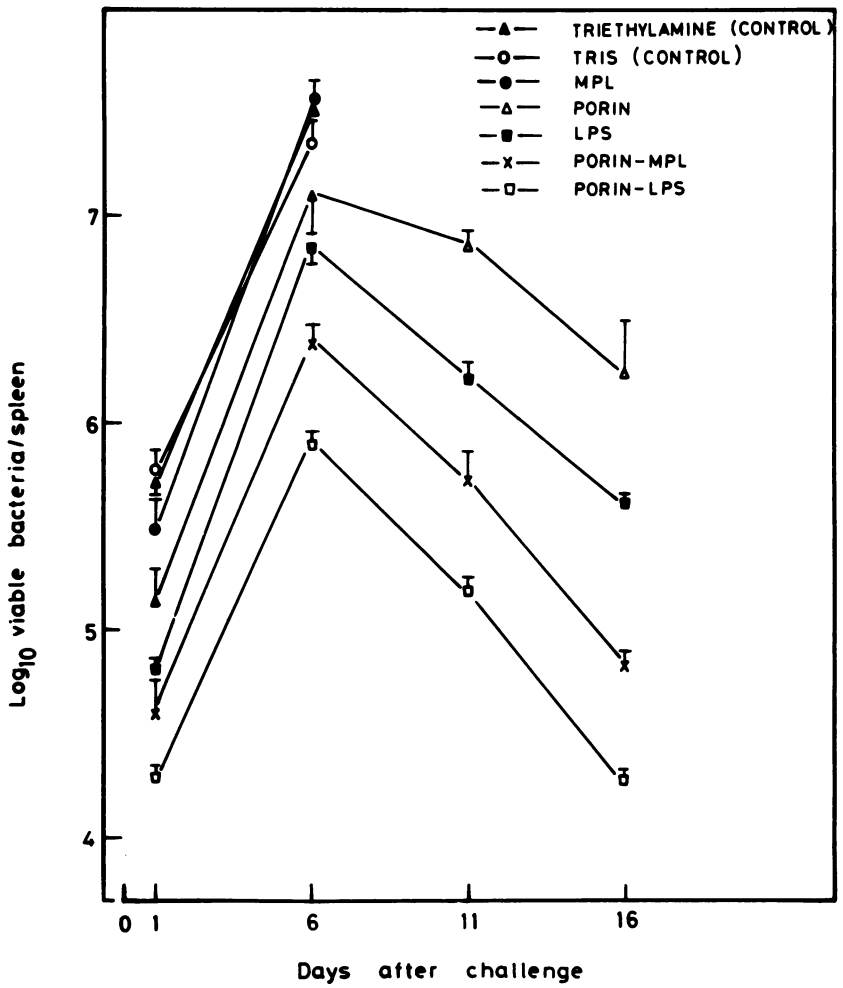

FIG. 3. Effect of MPL on the protective ability of porin as determined by the growth of challenge bacteria. Mice were immunized s.c. twice with porin (100 μg), LPS (50 μg), or porin (100 μg) plus LPS (50 μg) at 15-day intervals. Control mice were administered either Tris buffer containing 0.2% SDS or 0.05% triethylamine in saline. Ten days after the last injection, animals were challenged with 10 LD_{50}s of *S. typhimurium* C5. At different time intervals, four animals in each group were sacrificed and the number of viable bacteria in the spleen was determined. Each point represents the mean number of viable bacteria present in four mice, and the vertical bar denotes SE.

differential protective ability of serum obtained from mice immunized with porin-LPS and porin emulsified in FCA.

The importance of anti-LPS antibodies in immunity to murine salmonellosis has been indicated by several workers (7, 15, 38, 40). This importance was also evident from the present findings that passive transfer of anti-LPS serum provided better protection than did antiporin serum to the lethal challenge (Tables 1 and 2). However, anti-LPS antibodies did not seem to be the only factor in the enhanced level of protection induced by porin-LPS complex for two reasons: first, there was no significant enhancement in the anti-LPS titer above the level observed in LPS-immunized mice, and second, the passive transfer of anti-porin-LPS serum provided better protection than did anti-LPS serum alone (Table 2). Earlier findings of Kuusi et al. (17) also supported this possibility. They have shown that the protective ability of sera raised against porin-LPS complex remains unaltered even after absorption of anti-LPS antibodies. Further, addition of MPL significantly improved the protective ability of porin against lethal challenge (Table 4 and Fig. 3). The increased level of protection induced by a combination of porin and MPL may be analogous to the situation in which porin injected along with Rb_2 (rough) LPS protected mice against lethal challenge (46). Further, Rb_2 LPS was found to be as good as smooth LPS in improving the protective ability of porin (46). However, unlike porin-LPS, immunization with porin-MPL did not result in complete protection (Table 4), suggesting that anti-LPS antibodies play a significant role in protection against *Salmonella* infection. Therefore, it can be suggested that the increased level of protection observed in porin-LPS-immunized mice might be due to the combined effect of anti-LPS antibodies and the subclass of antiporin IgG generated as a result of immunization of porin along with LPS.

Previously, LPS been shown to potentiate a variety of manifestations of cell-mediated immune responses (19, 24, 30). However, the mechanism by which LPS immunopotentiates protein antigens and improves their protective ability is not yet well understood. Immunization of mice with 100 μg of porin induced a moderate delayed-type hypersensitivity response. However, the same dose of porin when injected along with LPS resulted in a strong delayed-type hypersensitivity response (data not shown). These results imply that LPS has the ability to influence the cellular

functions either directly or indirectly. In order to test this possibility, the T cells obtained from porin- or porin-LPS-immunized mice were tested for their ability to respond to porin in vitro. T cells from porin-LPS-immunized mice showed a level of proliferation which was more than twofold higher than the response of cells obtained from porin-immunized animals (Fig. 2). Further, the magnitude of the T-cell response in mice injected with porin-LPS was well above the sum of the responses of T cells from mice immunized with porin and LPS separately. Therefore, the enhanced T-cell response resulting from porin-LPS immunization can be considered a synergistic response. Similarly, the SRBC-specific T-cell proliferative response was found to be elevated when SRBC were injected along with lipid A (21). It is possible that LPS helps in enhancing the clonal expansion of T cells responding to porin. Therefore, it is interesting to correlate the earlier observations that LPS exerts its adjuvant effect mainly through antigen-reactive T-helper cells (1, 2, 26). LPS-mediated enhancement of antigen-specific proliferation was observed in several T-helper cell lines (4). The passive transfer of T cells from porin-LPS-immunized mice conferred a level of protection to the lethal challenge (47%) higher than that in the group which received the same number of T cells from porin-immunized animals (23%) (Table 1). These results indicate that T cells from porin-LPS-immunized mice are effective in mediating protection against *Salmonella* infection and that this may be due to the increased level of proliferation of antigen-specific T cells when reexposed to porin. Further, the fact that adoptive transfer of T cells from LPS-immunized mice failed to transfer any protection, while the cells from porin-immunized mice conferred a low level of protection (23%), also suggested the significance of porin-specific T cells in immunity against *Salmonella* infection.

The passive transfer of a combination of T cells and serum obtained from porin-LPS-immunized mice conferred 92% protection, and immunization of mice with porin-LPS resulted in complete protection against lethal challenge (Table 4). In contrast, a similar combination of T cells and serum from porin-immunized mice transferred only 36% protection (Table 1), and correspondingly only a low level of protection (25%) was seen in mice which received porin alone (Table 4). Thus, the high level of protection seen in porin-LPS-immunized animals appears to be due to the augmentation of both humoral and T-cell-mediated immune responses to porin. Several adoptive transfer studies indicated that the protection against facultative intracellular bacteria depends on T-cell immunity (5, 13, 27). The support mediated by specific T cells may be through macrophage activation and/or target cell lysis. In the present study, the adoptive transfer of a combination of immune serum and T cells into syngeneic mice considerably increased the level of protection against lethal challenge over that produced by either of these alone. Thus, defense mechanisms such as antibody-dependent bacteriolysis and phagocytosis might play a role, together with activation of macrophages for killing ingested salmonellae by soluble mediators such as gamma interferon secreted by T cells.

In the cell membrane, porin exists in the form of a complex with LPS (29). The tightly bound nature of porin and LPS even after heat denaturation in the presence of SDS has been described by Rocque et al. (36). Further, the porin-LPS complex in *Escherichia coli* was found to be necessary for phage T4 receptor activity (23). These findings suggest the importance of porin-LPS interaction in performing their biological functions. It is possible that similar interaction with LPS enhances the immune potential of porin. In conclusion, besides generating protective anti-LPS antibodies, the ability of LPS to augment both humoral and cell-mediated immune responses to porin plays an important role in providing protection against *Salmonella* infection in mice.

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