Protective immunity induced by porin against *Salmonella* infection in mice

V. Udhayakumar* & Vr. Muthukkaruppan

Department of Immunology, School of Biological Sciences
Madurai Kamaraj University, Madurai

Porin, a major outer membrane protein was purified from *Salmonella typhimurium* and its immune potential was studied in mice. Active immunization with porin induced about 45 per cent protection to an intravenous challenge with $10LD_{50}$ of *S. typhimurium*. Further, in porin immunized mice significant level of anti-porin antibodies and DTH reaction were detected. Attempts were also made to improve the immune potential of porin. Freund's complete adjuvant when mixed with immunogenic doses of porin enhanced the anti-porin antibody titre. However, it could not improve the protective ability of porin. On the other hand, porin when injected along with lipopolysaccharide (LPS) induced a higher level (55% survival with $50LD_{50}$) of protection than porin or LPS alone. This finding was also substantiated by the significantly reduced *in vivo* growth of challenge organisms in mice immunized with porin plus LPS. These results indicate that porin is a protective antigen and LPS significantly enhances the protective ability of porin.

Importance of cell wall proteins as protective antigens in mouse salmonellosis has been stressed[1-3]. Porin, a major outer membrane protein of *Salmonella typhimurium* has been shown to be a suitable antigen for eliciting delayed type hypersensitivity (DTH) reaction[4]. The protective ability of purified porin is not fully understood. Passive transfer of rabbit antibodies to crude preparation of porin but not to purified porin was able to protect mice against a subsequent lethal challenge with *S. typhimurium*[3]. Further, the sera raised against porin-lipopolysaccharide (LPS) complex remained protective even after the absorption of anti-LPS antibodies by passing through an affinity column. However, in these studies no attempt has been made to understand the effectiveness of porin in inducing cell mediated immunity (CMI), since the development of CMI has been shown to be important for mediating protection in murine salmonellosis. Hence, we have studied the immunogenic potential of porin in terms of its ability to induce cell mediated and humoral immune responses as well as protection. The present study demonstrates that porin is a protective antigen and its immune potential is significantly enhanced by LPS.

Present address : *Rm 205, Sander-Brown Center on Aging, University of Kentucky, Lexington, KY 40536-0230

Material & Methods

Both male and female Balb/c mice, 6 to 8 wk old were used.

A virulent strain of *S. typhimurium* C5 and a rough (Rb2 chemotype) mutant strain (SH9013) of *S. typhimurium* derived from LT2 were used[5]. The LD_{50} of C5 strain in Balb/c mice is 1×10^5, as reported and discussed earlier[6].

Purification of porin : Porin from strain C5 was purified by the method of Tokunaga *et al*[7]. We have described the purity of the porin preparation elsewhere[4].

Smooth LPS (S-LPS) of *S. typhimurium* was obtained from Sigma Chemicals Co. (St. Louis, USA). LPS from the rough Rb2 (Rb2-LPS) strain of SH9013 *S. typhimurium* was prepared by the trichloroacetic acid (TCA) method[8]. Purity of this preparation has been described elsewhere[5].

Mice were immunized with various doses of porin and 50 μg LPS subcutaneously twice at 15 days interval and then challenged with 10 (1×10^6) or 50 (5×10^6) LD_{50} of C5 intravenously 10 days after the last injection.

Determination of antibody levels to porin : Sera were prepared from control and immunized mice, decomplemented and stored at −20°C until used. Anti-porin antibodies were detected by enzyme linked immunosorbent assay (ELISA)[9] with a few modifications. Briefly, flat bottom 96 well microtitre plates (Nunc-immunoplate I, Denmark) were coated with optimal concentration of porin (0.5 μg in 100 μl) or LPS (1.6 μg in 100 μl) dissolved in 0.1 *M* carbonate-bicarbonate buffer (pH 9.6). Horseradish peroxidase conjugated sheep anti-mouse immunoglobulin (HRP-SMI Serotech, England)

was used as a probe to detect specific binding of mouse immunoglobulins. The substrate solution consisted of 0.04 per cent O-phenylene diamine dihydrochloride (Sigma Chemicals, St. Lousis, USA) and 0.012 per cent hydrogen peroxide in phosphate citrate buffer (pH 5.0). The antibody titre was expressed as the $\log^2$ of the dilution corresponding to an absorbance of 0.15 at 490 nm.

Delayed type hypersensitivity : Delayed type hypersensitivity (DTH) test was performed in the foot-pad of mouse as described elsewhere[6]. The DTH was expressed as absolute increase in foot-pad thickness at 24 h post elicitation, the time at which maximum reaction was observed.

Control and immunized groups challenged with lethal doses of virulent bacteria were observed for 14 days. Daily death count was recorded and the percentage of survival in each group was calculated.

Number of viable bacteria in liver and spleen of both control and experimental groups were determined as described elsewhere[5,6,10].

The data were expressed as mean±SE. Two-tailed Student's 't' test was used to assess the significance of the data.

Results

Protective immunity induced by porin : The survial data (Table I) indicate that porin was capable of conferring protection to a challenge dose of 10 LD_{50} but failed to give protection when the challenge dose was increased to 50 LD_{50}. A minimum of 50 μg porin was necessary to achieve this level of protection and increasing the immunizing dose to 200 μg did not result in enhanced protection (Table I).

Table I. Protective immunity induced by porin

Immunization*	Challenge**	Survivors†/ total number of mice	Per cent survivors
Tris buffer containing 0.2% SDS	$10LD_{50}$	0/7	0
	$50LD_{50}$	0/7	0
Porin 25 μg	$10LD_{50}$	0/8	0
	$50LD_{50}$	0/7	0
Porin 50 μg	$10LD_{50}$	5/14	35.7
	$50LD_{50}$	0/9	0
Porin 100 μg	$10LD_{50}$	5/12	41.6
	$50LD_{50}$	0/7	0
Porin 200 μg	$10LD_{50}$	4/9	44.4
	$50LD_{50}$	0/7	0
FCA alone	$10LD_{50}$	0/8	0
Porin, 100 μg+FCA	$10LD_{50}$	4/9	44.4
Porin, 200 μg+FCA	$10LD_{50}$	4/10	40

*Groups of mice received two injections subcutaneously of indicated doses of antigen either with or without FCA at 15 days interval; **S. typhimurium C5 was injected iv 10 days after the last immunization dose.; † Number of survivors at the end of 14 days after challenge

Effect of FCA/LPS on HI response to porin :
Serum antibody levels to porin in immune sera were determined by ELISA and the results are shown in Fig. 1. In the control group, the $\log^2$ anti-porin titer was 2 whereas in immunized group it was raised to 5. When porin was injected in FCA an increase in the antibody titer was noticed. Interestingly, a significant increase ($P <$ 0.001) in anti-porin titre was seen in mice immunized with porin plus LPS when compared to mice immunized with porin alone (Fig. 1). However, no such enhancement could be seen in anti-LPS antibody titre

in porin plus LPS immunized group (Table II) when compared with mice immunized with LPS alone.

Effect of LPS on the protective ability of porin : Since LPS has been shown to augment antibody response to porin, it was aimed to see whether a similar effect could be possible in the level of protection. To highlight the efficacy of porin-LPS complex in inducing protection, challenging dose was increased to 50 LD_{50}. As evident in Fig. 2, none of the control and porin immunized mice survived higher lethal (50 LD_{50}) challenge. In S-LPS immunized group 22 per cent of the mice survived and it increased to 55 per cent when porin was
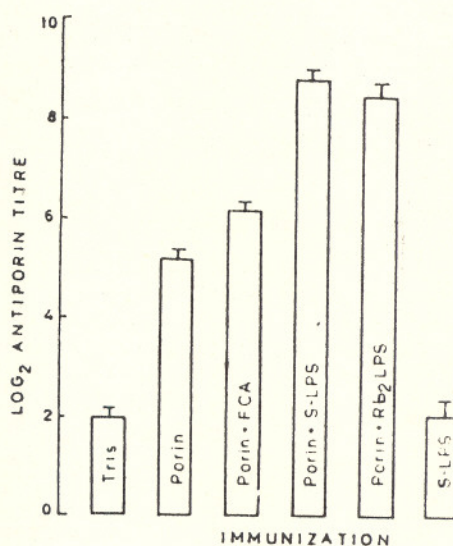

Fig. 1. Anti-porin activity as determined by ELISA, with initial dilution of 1 : 250, in sera collected 10 days after the last injection in groups of mice which received two injections (15 days interval) of either porin (100 μg) or porin mixed with FCA or 50 μg S-LPS of 50 μg Rb2 LPS. Control group received Tris buffer similarly and another group of mice was injected with 50 μg S-LPS alone. Values are mean (of at least 5 sera) ± SE.

Table II. Anti-LPS antibody response

Immunization*	Anti-LPS titre (ELISA)**		P value
	15th day	25th day	
Tris-buffer with 0.2% SDS	345±76	525±210	>0.05
Porin 100 μg	NT	1200±230	
Smooth LPS 50 μg	275±75	5825±1684	>0.2
Porin 100 μg + Smooth LPS 50 μg	570±106	8750±3347	
Rb2 LPS 50 μg	457±119	5600±979	>0.2
Porin 100 μg + Rb2 LPS 50 μg	375±63	4900±1268	

*Groups of mice were immunized twice with indicated doses of antigen at 15 days interval.   **Serum dilution corresponding to OD 0.15 was taken as titre (mean±SE);   NT, not tested
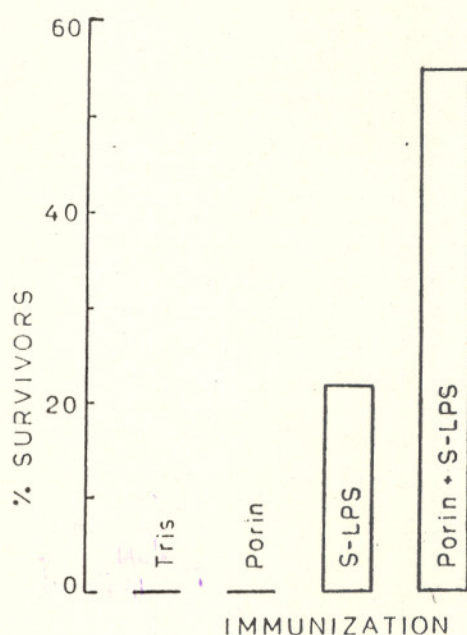

Fig. 2. Per cent survivors 14 days after iv challenge with 50 LD_{50} of *S. typhimurium*. Groups of mice (10-20) injected twice at 15 days interval with 100 μg porin or 50 μg S-LPS or 100 μg porin mixed with 50 μg S-LPS.

injected along with S-LPS (Fig. 2). The finding suggests that S-LPS could enhance the protective ability of porin.

This finding also led us to explore further whether this enhanced protection by the addition of LPS was due to cumulative effect of immune response evoked by O-chain of LPS and porin, or due to any other changes induced by LPS. To distinguish between these possibilities, LPS from Rb2 (which lacks O-specific chain) was used for further studies. In these experiments, *in vivo* growth pattern of challenge organisms was taken as criterion to assess the protective immunity more precisely. As shown in Fig. 3, Rb2-LPS was as good as that of smooth LPS in enhancing the protective activity of porin, as evidenced by the efficient suppression of growth of challenge organism. In control and Rb2-LPS immunized group the challenge organism multiplied actively and reached near lethal proportion by day 6 and subsequently they killed all the recipients before day 11. In
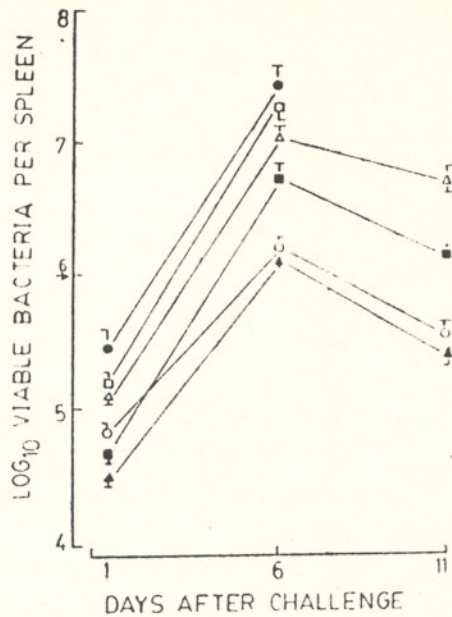
Fig. 3. Effect of LPS on the protective ability of porin. Growth of challenge bacteria in the spleen of control mice and mice immunized sc with two doses of various antigens at an interval of 15 days. Ten days after last injection they were challenged with $10LD_{50}$ of S. *typhimurium* C5. The arrow in Figure indicates challenge dose. Each point represents the mean number of viable organism present in three mice and vertical bars denote SE. ●——● Tris buffer containing 0.2% SDS □——□ Rb2 LPS 50 μg △———△ Porin 100 μg ■——■ Smooth LPS 50 μg ▲———▲ Porin+smooth LPS O——O Porin+Rb2 LPS. On day 6, porin + Rb2 LPS or porin+smooth LPS vs other groups, $P < 0.01$.

contrast, in all other immunized groups, an initial growth of bacteria was noticed till day 6 and subsequently the level started to decline. Thus, it was clear that both smooth and Rb2-LPS have a marked influence on the protective immunity induced by porin.

Effect of LPS on DTH response to porin : An attempt was also made to find out the effect of LPS on DTH response to porin (Fig. 4). Porin immunized and porin plus LPS immunized groups showed a positive DTH reaction ($P < 0.001$ when compared to control) but there was no significant difference in the level of response among these groups ($P > 0.2$). This study indicates that LPS does not have an enhancing effect on DTH response generated by porin, as seen in the case of antibody response and protection.

Discussion

In the present study, it has been shown that active immunization with appropriate doses of porin could induce significant level of protection to a lower lethal challenge of $10LD_{50}$. Further, porin is a good eliciting antigen for DTH[4] as well as capable of inducing cell mediated immunity.
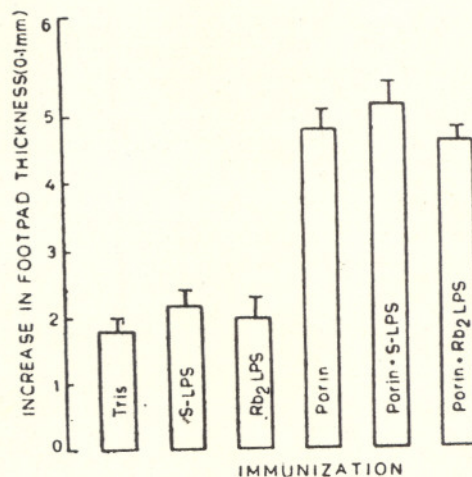

Fig. 4. Delayed type hypersensitivity response to porin in groups of mice (5-11) immunized twice with 50 μg LPS, or 100 μg porin or 100 μg porin mixed with 50 μg LPS. Control group received Tris-buffer similarly. Ten days after the last injection 5 μg porin was injected into foot-pad (mean± SE).

However, it was unable to induce protection to a relatively higher lethal challenge (50 LD_{50}) even by increasing the dose of immunization. These results agree with the previous report[3] that antibodies raised against highly purified porin failed to protect mice in passive transfer experiments. The reason for the low protective ability of purified porin is not clearly understood. Porin when injected as porin plus LPS complex induced a high level of protection to otherwise higher lethal challenge. This observation suggests that (i) anti-LPS antibodies could have contributed for protection; (ii) LPS could have acted as an adjuvant; and (iii) LPS could have complexed with porin so as to change the configuration of porin to a more immunogenic form.

Among these possibilities, the involvement of anti-LPS antibodies in inducing high level of protection is unlikely, because the anti-LPS antibody titre was not increased in porin-LPS complex immunized group compared to LPS group. Further, the protective ability of the sera raised against porin-LPS complex remained unaltered even after absorption of anti-LPS antibodies[3]. Thus, anti-LPS antibodies do not seem to contribute for the enhanced protection.

Interestingly, a highly significant increase in anti-porin titre has been observed in porin-LPS complex immunized group when compared with the group immunized with porin alone. It may be argued that this increased anti-porin antibody titre could have contributed for the observed higher level of protection in these groups. However, this is unlikely because similar increase in the antibody titre to porin by FCA did not result in enhanced level of protection. Therefore, the question whether LPS could qualitatively alter the antibody response

to porin needs to be explored, in addition to the fact that LPS can act as an adjuvant to a variety of antigens, thus enhancing the antibody response[11,12].

Although Rb 2-LPS failed to induce protection by itself, it was equally efficient as smooth-LPS in enhancing the protective ability and antibody levels induced by porin. It may therefore be suggested that immune enhancement by LPS is independent of O-specific chain and thus the role of O-specific antibodies as the major protective factor has also been excluded.

It is known[13] that in native membrane, the porins exist in the form of complex with LPS. It has been shown that association of LPS with porin was necessary to maintain the biological activity of porin. A similar interaction with LPS may be necessary to enhance the immune potential of porin, and thus the protection. However, further studies are required to understand the mechanism of LPS mediated immuno-potentiation of porin against salmonellosis.

Acknowledgment

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Reprint requests : Dr Vr. Muthukkaruppan, Professor of Immunology, School of Biological Sciences Madurai Kamaraj University, Madurai 625021