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Role of *Salmonella* surface components in immunomodulation of inflammatory mediators

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Abstract

Salmonella enterica serovar Typhimurium and its surface components were assessed for their inflammatory potential by footpad oedema test using plethysmometer. Inflammation was found to be the highest when outer membrane proteins (OMPs) were used as inflammagen followed by lipid associated protein-lipopolysaccharide complex (LAP-LPS) and lipopolysaccharides (LPS). Inflammation produced by OMPs was found to be comparable to that by carrageenan (a known positive inflammagen). However, injection of L-histidine (an antioxidant) prior to administration of carrageenan or *Salmonella enterica* serovar Typhimurium inhibited the inflammation, which indicated the involvement of oxidants during inflammatory response. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and nitric oxide (NO) production by peritoneal macrophages from infected mice exhibited a significant increase as compared to those of the immunized mice. In contrast, glutathione production was found to be the maximum in the macrophages taken from OMPs-immunized mice followed by LAP-LPS and LPS alone. The biochemical studies correlated well with histopathological studies of intestinal tissue of animals from various groups. Based upon these parameters, inflammation seems to be modulated by OMPs and LAP-LPS, which may be because of the protein moieties present in the components. Hence, immunization with protein moieties having L-histidine or L-histidine-like structures may suggest an alternative to the potential therapeutic values of anti-inflammatory drugs. Thus the results of this study form the basis for evaluating these antigens (either alone or in combination with polysaccharides) for preventive intervention rather than therapeutic. (Mol Cell Biochem **270**: 167–175, 2005)

Key words: *Salmonella*, OMPs, LAP-LPS, L-histidine, inflammation, free radicals

Introduction

Inflammation is a basic host defense process, which occurs in response to noxious stimuli and to local injury. Amongst these, *Salmonella enterica* serovar Typhimurium is a leading causative agent of gastroenteritis in humans. In Salmonellosis, the ensuing inflammatory response of the intestinal mucosa has long been associated with *Salmonella* virulence [1]. Infiltrating inflammatory cells participate and ensue the destruction of the invading microbe by release of nonenzymatic mediators such as oxygen and nitrogen metabolites as well as enzymatic mediators such as elastase, cathepsin and collagenase [2]. Reactive oxygen species (ROS) such as superoxides (O_2^-) and hydroxyl radicals ($^\circ$OH) propagate through a series of chain reactions and attack the polyunsaturated fatty acids present in the membrane. These radicals can also cause damage to DNA and proteins [3]. The other inflammatory mediator, nitric oxide (NO) is a gaseous free radical produced in biological systems. This regulates a diverse array of physiological functions and acts as an inter and extracellular

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messenger in most mammalian organs. Many types of cells produce NO during enzymatic conversion of L-arginine to L-citrulline by NO synthase (NOS). NO has been demonstrated to have a beneficial effect in host defense mechanisms against various pathogenic bacteria and protozoa [4–6]. The uncontrolled release of these cytotoxic substances [7] and proinflammatory mediators including cytokines (tumor necrosis factor and interleukins) [8, 9] by the migrating cells may damage the host tissues as well. Therefore, under such conditions, it is necessary to manage the hyper inflammation to useful level, to change the clinical manifestation of the disease.

The development of tissue injury depends upon the balance between the generation of toxic radicals and the tissue antioxidant status [10]. As a defense mechanism, all types of cells are endowed with enzymatic and nonenzymatic antioxidants, which fight against the toxic free radical species. One potential approach is the use of L-histidine or *N*-acetyl-L-cysteine, a precursor of glutathione, which maintain a favorable redox environment [11] and are effective in scavenging hydroxyl radicals. Several authors have reported an inverse correlation between the levels of GSH and lipid peroxides (LPOs). It seems that GSH apart from being a substrate for some antioxidant enzymes could be an effective free radical scavenger directly [12]. The alternative possible approach is to modulate the release of these mediators through immunoprophylaxis. It is known that LPS, Vi antigens and outer membrane proteins have an inflammatory potential, which results in the release of cytokines [13, 14]. These have also been reported to interact with the host immune cells efficiently [15–18]. Therefore, in the present study, the potential of *Salmonella* surface components in modulating the inflammatory response following immunization has been discussed.

Materials and methods

Chemicals

All the chemicals used in the present study were of analytical grade and were procured from standard firms.

Bacterial strain

Salmonella enterica serovar Typhimurium (1402/84) was procured from Central Research Institute, Kasauli (HP), India. The strain was checked biochemically and serologically prior to storage and use. It was maintained in 10% glycerol broth and also stored as lyophilized ampules.

Animal model

Balb/C mice each weighing 15–25 g (8–10 weeks old) and Wistar rats weighing 150–200 g were obtained from Central Animal House, Panjab University, Chandigarh, India. The animals were fed on commercially available diet and were given water ad libitum. Care and use of animals was followed in accordance with guidelines of the institutional ethical committee.

Extraction of outer membrane proteins (OMPs)

To study the expression of stress-inducted proteins, OMPs were prepared by the method as described earlier [19]. Briefly, bacterial growth was harvested at 10000 rpm for 20 min. The pellet obtained was suspended in 20 mM Tris–HCl buffer (pH 7.6) containing 2 mM $MgCl_2$. Cells were washed twice and suspended in the same buffer containing 2 mM phenyl methyl sulphonyl fluoride (PMSF, Sigma, USA). Bacterial cells were disrupted by sonication using ultratip lab-sonic system (10 cycles of 30 s with 1 min interval in between) and undisrupted material was removed by low speed centrifugation (3000 $\times$ g, 4 °C, 20 min). Supernatant was then ultracentrifuged (Beckam Coulter, Optima™ XL-100 K Ultracentrifuge, USA) at 100,000 $\times$ g for 60 min at 4 °C and the pellet was suspended in 1% sodium lauryl sarcosyl (ICN, USA) in 20 mM Tris–HCl buffer (pH 7.6). After incubation for 2 h at 37 °C, detergent insoluble OMP fraction was collected by ultracentrifugation at the same speed. The recovered outer membrane (OM) fraction was suspended in 20 mM Tris buffer containing 2 mM PMSF and stored at −20 °C.

Extraction of lipid associated protein-lipopolysaccharide complex (LAP-LPS) and lipopolysaccharides (LPS)

For LAP-LPS preparation, conventional phenol extraction procedure of Westphal and Jann [20] as modified by Morrison and Leive [21] was used. For making LPS preparation, pronase was added to LAP-LPS preparation. The LPS content in both the preparations was measured by determining the 2-keto-3-deoxyoctonate (KDO) levels.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein content of various preparations (OMPs, LPS and LAP-LPS) was estimated by the method of Lowry *et al*. [22]. SDS-PAGE [23] was carried out to see the expression of protein and lipid profiles.

Inflammatory potential of Salmonella enterica serovar Typhimurium and its surface components

The inflammatory potential of *Salmonella enterica* serovar Typhimurium and its surface components were studied using the acute paw oedema test in rats [24]. Whole cell suspension (5×10^2 organisms/ml), OMPs (50 μg/ml), LAP (50 μg/ml), LAP-LPS (50 μg/ml), L-histidine (150, 250 and 350 mg/ml), Carrageenan (positive control, 1%, w/v) and L-histidine (negative control, 250 mg/ml) were used to study the inflammatory potential. In brief, the hind paws of test animals were marked above the tibiotarsal junction to ensure that every time paw was dipped to the same level in the mercury column of Plethysmometer. Rats were injected with 0.1 ml of the antigen preparation described above in the right paw and an equivalent amount of normal saline in the left paw. The mercury displacement occurring as a result of dipping of the paw was directly read from scale attached to the mercury column. The amount of mercury displaced in cm (on plethysmometer scale) on dipping rat paw was recorded in each case for 5 h at an interval of 1 h.

Treatment protocol and experimental design

Fifty mice were divided into five groups (10 mice in each group) as follows. Group A (control group): Mice injected with 0.5 ml saline intraperitoneally. Group B (infected group): Mice injected with 0.5 ml of whole cell suspension (2×10^6 cells/ml) intraperitoneally. Group C (OMPs immunized-infected): Mice immunized intraperitoneally with 50 μg OMPs. Group D (LAP-LPS immunized-infected): Mice immunized intraperitoneally with 50 μg of LAP-LPS. Group E (LPS immunized-infected): Mice in three subgroups (10 mice in each group) were immunized against LPS (5–50 μg/mouse).

Mice in C, D and E groups were given booster doses of serovar Typhimurium on the 14th day and challenge dose of serovar Typhimurium on the 21st day. The challenge dose was calculated by the method of Reed and Muench [25]. Mice in each group were sacrificed on third and seventh day of challenge dose to assess the anti-inflammatory role of each preparation.

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity

NADPH oxidase activity was measured following the method of Bhatnagar *et al*. [26]. In short, the peritoneal macrophage preparation from each group was interacted with different antigen(s) for 1 h at 37 °C under 5% CO_2 atmosphere. After centrifugation the cell deposits were resuspended in 1 ml of Kreb's ringer phosphate buffer (KRP buffer, pH 7.4) in separate tubes. These were again centrifuged at 2000 rpm and the pellets obtained were suspended in 1 ml ice cold potassium phosphate buffer (pH 5.5). Cell preparations, after three cycles of freezing and thawing, were sonicated (three cycles each of 5 s at 4 mA) and then centrifuged. The clear supernatant obtained on centrifugation (30 min at 2000 rpm) was carefully collected in test tubes. Its protein concentration was adjusted to 70 μg/ml. For NADPH oxidase assay, 100 μl of sample was mixed with 100 μl of NADPH (0.1 mM in KRP buffer, pH 7.4) and the volume was made to 1 ml with potassium phosphate buffer in quartz cuvettes equilibrated at 25 °C. Decrease in absorbance was recorded at 340 nm for 3 min using double beam spectrophotometer. Amount of NADPH oxidized was calculated and results were expressed as *n*-moles of NADPH oxidized/min/mg protein.

Nitric oxide estimation

Nitric oxide in the cell free supernatant was quantitated following the method of Green *et al*. [27]. In brief, 100 μl of supernatant was mixed in equal proportion with Griess reagent. The mixture was kept at room temperature for 10 min prior to measuring its optical density at 550 nm. Nitric oxide was quantitated with $NaNO_2$ (Sigma) as a standard.

Total glutathione levels

Glutathione content was estimated by the method of Tietz *et al*. [28]. In brief, 1 ml of peritoneal macrophage suspension and 110 μl TCA were mixed in a test tube. The mixture was centrifuged at 4 °C for 10 min at 10,000 $\times$ *g*. Supernatant was separated carefully and used for total glutathione estimation. Supernatant diluted with buffer (1:10 times) was mixed with 700 μl of NADPH, 100 μl of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and 80 μl of buffer, was kept at room temperature for equilibration and then 10 μl of glutathione reductase was added (just before the readings) and immediately the changes in OD were recorded for 90 s. Concentration of glutathione was calculated as below.

$$\text{GSH} = \frac{\text{change in OD (Test)}}{\text{change in OD (standard)}} \times \text{concentration of standard}$$

Histopathological studies

Mice at random from each group were sacrificed through cervical dislocation and their intestines were removed aseptically. It was cut into small pieces and fixed in 10% buffered formalin. Preserved samples were processed and stained using hematoxylin and eosin.

Statistical analysis

The results were statistically evaluated using Student's unpaired *t*-test and nonparametric statistics using Kruskal–Wallis comparison of one way analysis of variance (ANOVA).

Results

Analysis of surface components

The SDS-PAGE analysis of OMPs showed several bands in the range of 14–67 kDa molecular weight (lane B, Fig. 1). SDS-PAGE of LPS did not show any protein bands (lane D, Fig. 1) but couple of protein bands were seen in LAP-LPS preparation (lane C, Fig. 1). When the gels were stained with silver, the LPS portion of the LAP-LPS complex demonstrated an electrophoretic mobility pattern similar to that of the protein free LPS. Both antigens demonstrated ladder-like structures of *Salmonella* LPS (lanes A and B, Fig. 2). However, the LPS contamination in OMPs preparation was not observable in the gel stained with silver (lane C, Fig. 2). Quantitative analysis also showed that LPS was devoid of protein and contained 0.13 mg/ml LPS only where as LAP-LPS contained both LPS (0.16 mg/ml) and protein (0.125 mg/ml).

Inflammatory potential

All the antigenic preparations including LPS, LAP-LPS, OMPs and whole bacterial cell suspensions were found to be effective as inducers of inflammation as assessed by rat paw oedema test (Fig. 3A, representative photographs showing inflamed paws). The inflammation was visible within 1 h of infection, this became optimal by 4 h. The magnitude of inflammatory oedema induced by these antigenic preparations was maximum in OMPs followed by LAP-LPS and LPS. Inflammation induced by OMPs (0.43 ± 0.018 cm) preparation was comparable to that caused by carrageenan (0.48 ± 0.015 cm), a positive control (Fig. 3B). Administration of L-histidine (up to 15 mg/0.1 ml) to rats prior to injecting carrageenan or whole cells reduced the inflammatory response by 0.3 cm. Rats injected with higher doses of L-histidine did not show any inflammation.

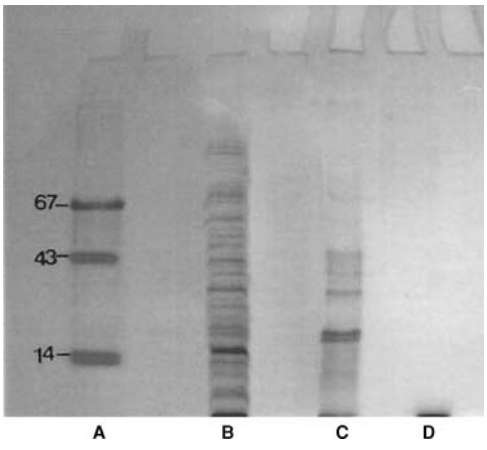

Fig. 1. SDS-polyacrylamide gel stained with Coomaasie blue showing the electrophoretic pattern of proteins in different preparations extracted from *Salmonella enterica* serovar Typhimurium. Lane A: Molecular size markers: albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (14 kDa); lane B: OMPs; lane C: LAP-LPS; lane D: LPS.

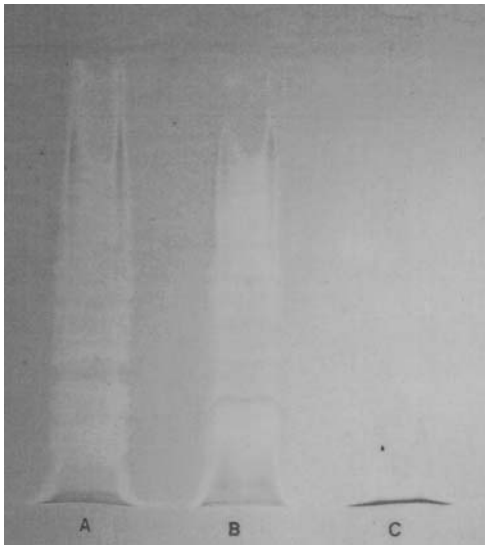

Fig. 2. SDS-polyacrylamide gel stained with silver showing the electrophoretic pattern of different preparations extracted from *Salmonella enterica* serovar Typhimurium. Lane A: LPS; lane B: LAP-LPS; lane C: OMPs.

Inflammatory response following immunization

LD_{50} for serovar Typhimurium administered intraperitoneally was found to be 3.2×10^3 organisms/mouse. The immunoprotection of mice immunized in separate groups against: OMPs (50 μg/mouse), LAP-LPS (50 μg/mouse) or protein free LPS (5 μg/mouse) was 100% against $100 \times LD_{50}$ challenge dose of serovar Typhimurium. However, higher doses of LPS resulted in decrease in the survival in the test group.

Besides differences in magnitude of inflammatory response to different antigens, significant differences ($p < 0.001$) were also observed in NADPH oxidase activity of peritoneal macrophages derived from infected and control groups at third and seventh day of post-infection. NADPH oxidase

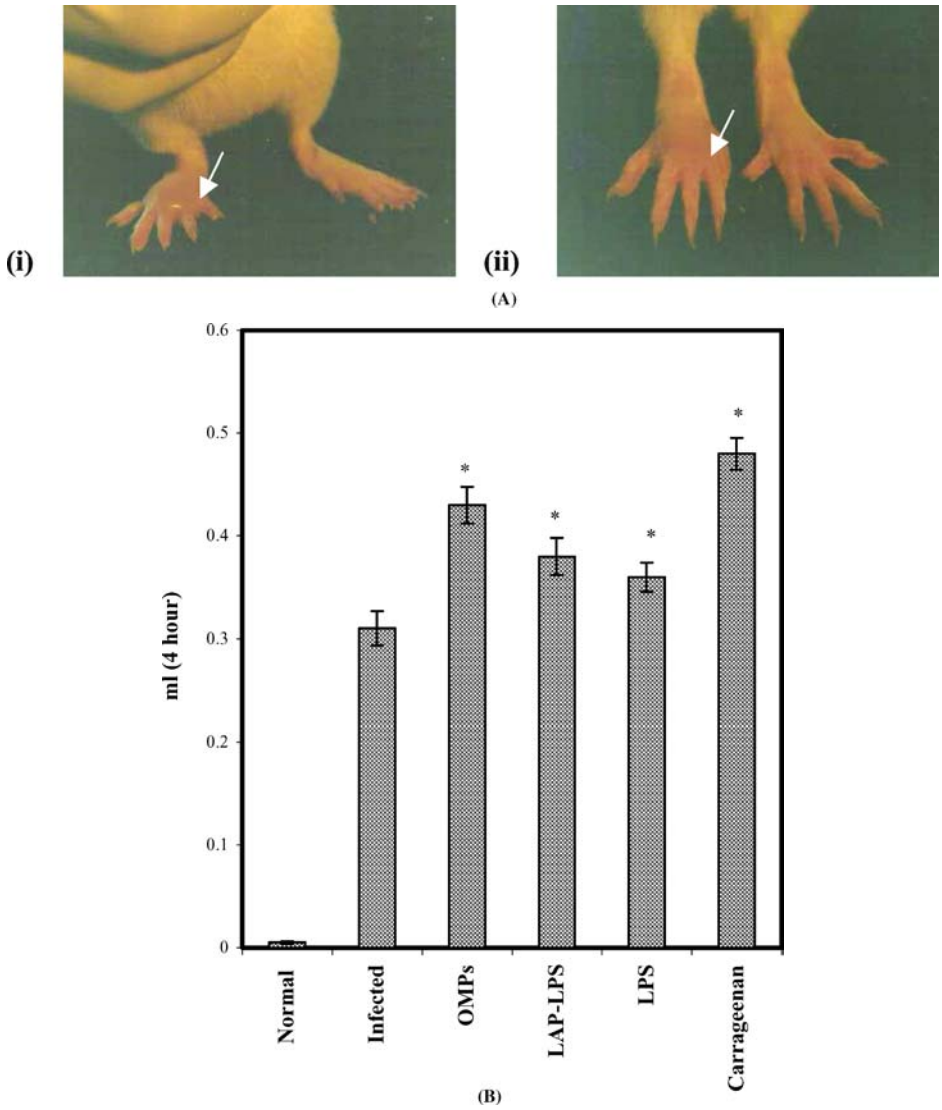

Fig. 3. (A) Representative photographs of rat paw showing oedema. (i) Right paw injected with carrageenan (positive control), (ii) right paw injected with OMPs. Left paws injected with PBS served as negative control. (B) Inflammatory potential of *Salmonella* and its surface components measured by plethysmometer scale. All values are mean ± S.D. of six animals. *Inflammation caused by OMPs, LAP-LPS, LPS and carrageenan vs. *Salmonella* ($p < 0.005$) as compared by Student's unpaired *t*-test.

activity was the highest in the LPS-immunized group and the least in mice group immunized with OMPs (Fig. 4). Significant increase in NO production was observed by peritoneal macrophages ($p < 0.001$) in infected group in contrast to the control animals (Fig. 5). Increase in NO production in immunized mice groups was observed to be highest in LPS followed by LAP-LPS and OMPs. Contrary to NADPH oxidase and NO production, a decrease in glutathione levels ($p < 0.005$) was observed in immunized and infected mice groups as compared to control group on third day. Glutathione levels were lower in LPS immunized group ($p < 0.001$) as compared to LAP-LPS and OMPs immunized-infected group (Fig. 6).

Histopathological studies

Histopathological studies showed a significant disorganization with detached mucosal cells, distorted epithelial cells and mononuclear infiltration with oedema as is evident in the intestine of infected mice group. Comparing the histopathological alterations in various immunized-infected groups: the disorganization and detachment of mucosal tissue was highest in LPS immunized-infected group where in distorted villi were seen. The changes were mild in LAP-LPS immunized-infected group. Microscopic examination of intestinal tissue from OMPs immunized-infected mice did not show many changes. These findings were comparable

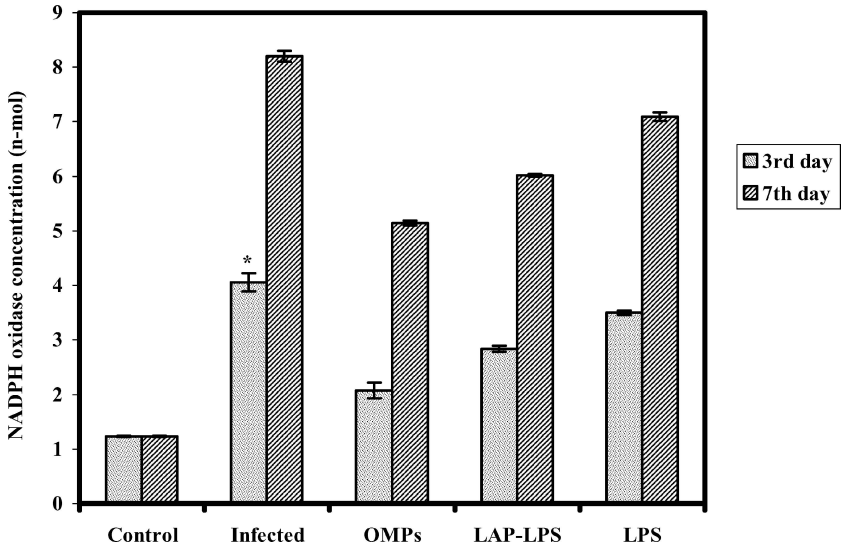

Fig. 4. Concentration of NADPH oxidase in murine peritoneal macrophages isolated from control, infected, OMPs immunized-infected, LAP-LPS immunized-infected and LPS immunized-infected mice. Values are expressed as mean of three experiments carried out in duplicates ±S.D. *NADPH oxidase concentration, infected vs. control group ($p < 0.001$).

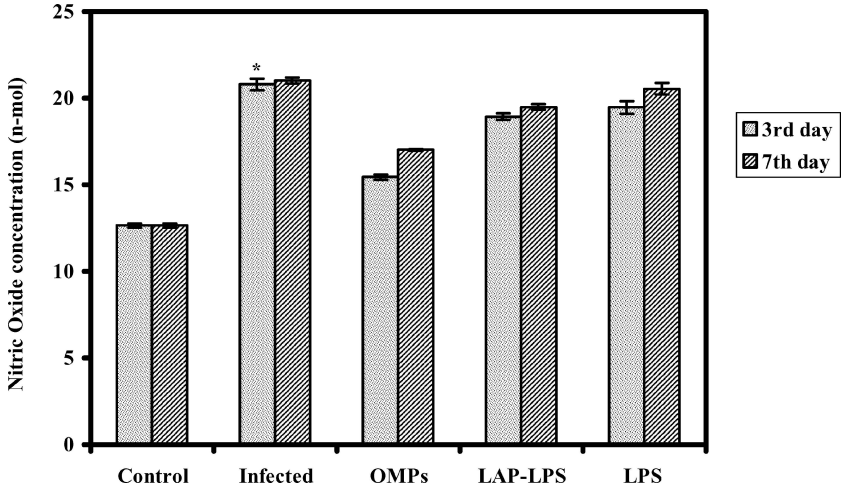

Fig. 5. Levels of nitric oxide in murine peritoneal macrophages isolated from control, infected, OMPs immunized-infected, LAP-LPS immunized-infected and LPS immunized-infected mice (values are mean ± S.D.). *NO concentration, infected vs. control group ($p < 0.001$).

with control group where no special treatment was given (Fig. 7).

Discussion

Most often the changes in LPS profile of pathogen alter the bacterial virulence as well [29]. Since, the LPS can activate the compliment, even the minutest differences in carbohydrate structure results in differential rate of complement activation and ensuing inflammatory response. LPS sterically binds to serum and plasma proteins and forms LAP-LPS complex [30]. Such complexes are biologically less active with respect to induction of fever, neutropenia, thrombocytopenia and complement activation [14]. Curiously, some of the pathogens exploit this strategy evading the host defense too, by modifying their LPS to escape the host surveillance [31]. Knowing the biological activities of LPS as a known inducer of inflammation, its ability to form complexes with host proteins prompted us to investigate the inflammatory potential of LPS alone and complexed to proteins as LAP-LPS in addition to OMPs.

Measurement of paw oedema is a convenient method for evaluating the degree of inflammation and effectiveness of

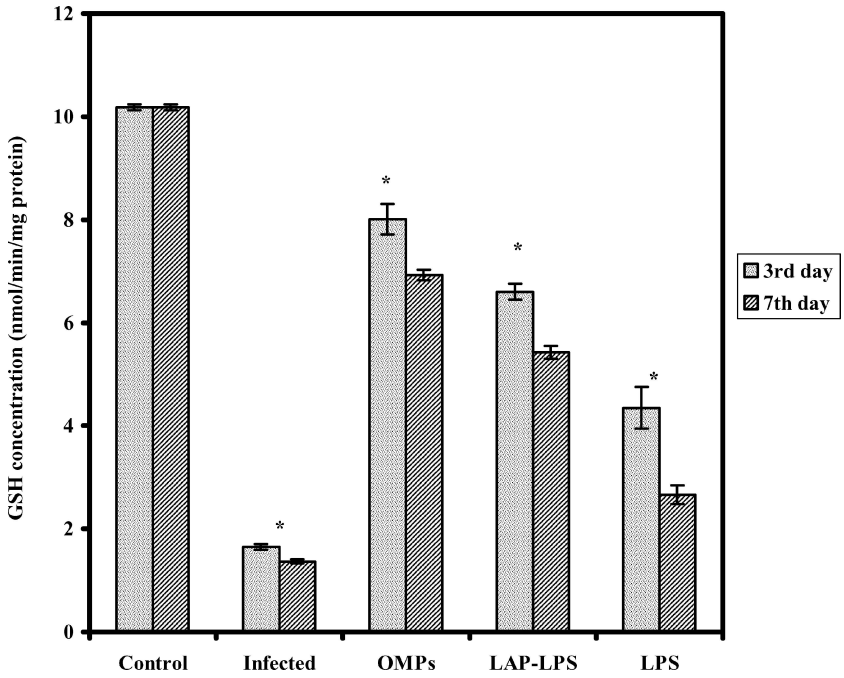

Fig. 6. Concentration of total glutathione in murine peritoneal macrophages isolated from control, infected, OMPs immunized-infected, LAP-LPS immunized-infected and LPS immunized-infected mice. Values are expressed as mean of three experiments carried out in duplicates ±S.D. *Glutathione concentration, infected vs. control group ($p < 0.005$).

Fig. 7. Micrographs showing histopathological changes in the intestines from (A) normal, (B) infected, (C) OMPs immunized-infected mice (magnification 40×, H and E).

therapeutic drugs. Surprisingly, contrary to the current reports on LPS as a known inflammatory agent [32], the inflammation induced by LPS alone was lower as compared to the inflammation induced by complexed LPS (LAP-LPS) or OMPs in the present study. The higher inflammatory response observed with LAP-LPS complex apparently indicates the involvement of protein moieties present in LAP-LPS resulting in exacerbated inflammatory response. Similar increase in inflammatory response has been reported with OMPs (porins) wherein oedema induced was comparable to that induced by carrageenan (a known inflammagen) [33].

It is known that serovar Typhimurium induces ruffling in intestinal epithelial cells and probably signals inflammation as well [13]. It could be crucial in modifying the clinical course of the disease. Currently, the detrimental effect of inflammatory mediators is regulated through antioxidants including the use of L-histidine or *N*-acetyl-L-cysteine, the precursors of glutathione (GSH). GSH is known to directly help in maintaining a favorable redox environment [11]. The inflammation induced by serovar Typhimurium was manageable or controllable with L-histidine, which is in agreement with the study carried out by Peterson *et al*. [24].

Apprehending the undesirable effects of anti-inflammatory drugs such as dexamethasone and indomethacin, it was worth exploring the anti-inflammatory potential of bacterial surface antigens as immunogens for preventive intervention rather than as therapeutics. The ROS are produced by a membrane bound enzyme complex, the NADPH oxidase [34, 35] leading to the activation of respiratory burst. Higher NADPH oxidase activity observed *in vitro* in macrophages derived from infected mice group causes extensive damage *in vivo* in various tissues [36–39]. Differences observed in NADPH oxidase levels in animals immunized with surface components (prior to serovar Typhimurium challenge) evidently indicate the role of surface antigens in activation of phagocytic cells. Manipulation of release of ROS and pro-inflammatory mediators (cytokines) that orchestrate the inflammatory response may be useful in neutralizing inflammation-mediated damages in the host.

Peroxinitrite, a potent oxidant formed from NO and superoxide radical is microbicidal for various bacteria including *Salmonella enterica* serovar Typhimurium [40]. It has also been demonstrated that mice deficient in both NADPH phagocyte oxidase (phox) and iNOS were more susceptible to various bacterial infections that were mice deficient in either single enzyme [41]. RNI such as NO and peroxynitrite (ONOO) function as effective chemo attractants [42, 43]. Although NO released against these preparations did not show any significant differences amongst the LPS and LAP-LPS immunized groups, yet the net amount of NO produced was significantly higher ($p < 0.005$) in LPS immunized groups as compared to the OMPs-immunized groups. Partly the differences may be due to the protein concentrations in these preparations.

Glutathione peroxidase is capable of catalyzing the reduction of a wide range of lipid hydroperoxides to their corresponding hydroxy radicals. It requires GSH as substrate, which in turn is regulated by the enzyme glutathione reductase that catalyzes the conversion of oxidized glutathione to GSH. A significant increase in the level of GSH was observed in all the immunized groups. The differences in glutathione released by activated macrophages from the immunized groups and infected group further confirm the subtle differences in interactive activities of these preparations. Decrease in GSH levels has earlier been shown to correlate with intestinal fluid accumulation and morphological damage to tissues [44] following enteric infection. However, earlier work done in our laboratory also indicated an increase in the levels of superoxide dismutase following immunization with OMPs [45] and LAP-LPS [46] complex. It may be possible that both mechanisms could be working synergistically to combat the oxidative stress. Absence of disarray in mice immunized with OMPs may be due to little or controlled inflammatory activities. Histopathalogical changes observed in the intestinal tissues of the infected mice may be related to the differences in NADPH oxidase, NO as well as GSH levels in the immunized groups vis a vis infected mice group.

This study shows that immunization with protein moieties have the anti-inflammatory potential evident by inhibition of oedema and effective inhibition of ROS generation. It seems to inhibit the action of free radicals by increasing the levels of GSH. Immunization with protein moieties may be useful as preventive intervention that is devoid of effects of anti-inflammatory drugs containing L-histidine-like structure. In addition, the associated anti-inflammatory activity of OMPs may further enhance the host immune responses as the proteins intervene in both non-specific and specific immune responses. This response would be long lasting vis a vis LPS alone. Further we plan to study the role of cytokines in this context.

References

1. Giannella RA: Importance of the intestinal inflammatory reaction in *Salmonella* mediated intestinal secretion. Infect Immun 23: 140–145, 1979
2. Henson PM, Johnston RB: Tissue injury in inflammation: Oxidants, proteinases and cationic proteins. J Clin Invest 79: 669–674, 1987
3. Ballmer PE, Reinhart WH, Gey KF: Antioxidant vitamins and disease risk of suboptimal supply. Ther Umsch 51: 467–474, 1994
4. DeGroote MA, Fang FC: Antimicrobial properties of nitric oxide. In F.C. Fang (ed). Niric Oxide and Infection. Kluwer Academic/Plenum, New York, NY, 2000, pp 231–261
5. James SL: Role of nitric oxide in parasite infections. Microbiol Rev 59: 533–547, 1995
6. Nathan C, Shiloh MU: Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc Natl Acad Sci USA 97: 8841–8848, 2000
7. Miesel R, Zuber M: Copper-dependent antioxidase defenses in inflammatory and autoimmune rheumatic diseases. Inflammation 17: 283–294, 1993
8. Stenson WF: Role of eicosanoids as mediators of inflammation in inflammatory bowel disease. Scand J Gastroenterol Suppl 72: 13–18, 1990
9. Klimpel GR, Asuncion M, Haithcoat J, Niesel DW: Cholera toxin and *Salmonella typhimurium* induce different cytokine profiles in the gastrointestinal tract. Infect Immun 63: 1134–1139, 1995
10. Windrow VR, Winyard PG, Morris CJ, Blake DR: Free radicals in inflammation: Second messengers and mediators of tissue destruction. Br Med Bull 49: 506–522, 1993
11. Floreani M, Petrone M, Debetto P, Palatini P: A comparison between different methods for determination of reduced and oxidized glutathione in mammalian tissues. Free Radic Res 26: 449–445, 1997
12. Koul A, Singh A, Sandhir R: Effect of α-tocopherol on the cardiac antioxidant defense system and atherogenic lipids in cigarette smoke-inhaling mice. Inhal Toxicol 15: 513–522, 2003
13. Galdiero F, Antonietta M, Marilena G, Masiello S, Rosa M: Inflammatory effect of *Salmonella typhimurium* porins. Infect Immun 58: 3183–3186, 1990
14. Galdiero F, Cipollario G, Benedetto LN, Galdiero M: Release of cytokines induced by *Salmonella typhimurium* porins. Infect Immun 61: 155–161, 1993

15. Rishi P, Ganguly NK, Sharma BK, Sharma S, Sehgal R: Specific immunoglobulin response in mice immunized with porins and challenged with *Salmonella typhi*. Microbiol Immunol 33: 519–525, 1989
16. Isibasi A, Ortiz V, Vargas M, Paniagua J, Gonzalez C, Morenno J, Kumate J: Protection against *Salmonella typhi* infection in mice after immunization with OMPs isolated from *Salmonella typhi* 9, 12, Vi. Infect Immun 56: 2953–2959, 1988
17. Tabarie B, Sharma BK, Rishi P, Sehgal R, Ganguly NK: Stimulations of macrophages oxygen free radical production and lymphocyte blastogenic response by immunization with porins. Microbiol Immunol 38: 561–565, 1994
18. Singh M, Ganguly NK, Kumar L, Vohra H: Protective efficacy and immunogenicity of Vi-porin conjugate against *Salmonella typhi*. Microbiol Immunol 43: 535–542, 1999
19. Chander H, Majumdar S, Sapru S, Rishi P: Reactivity of typhoid patients sera with stress induced 55 kDA phenotype in *Salmonella enterica* serovar Typhi. Mol Cell Biochem 267: 75–82
20. Westphal O, Jann K: In R.L. Whistler (ed). Methods in Carbohydrate Chemistry. Academic Press, New York, 1965, pp 83–97
21. Morrison DC, Leive L: Fractions of LPS from *E. coli* OIII: B4 prepared by two extraction procedures. J Biol Chem 250: 2919–2930, 1975
22. Lowry DH, Rosenbrough NJ, Farr AL, Randell RJ: Protein measurements with the folin phenol reagent. J Biol Chem 193: 265–275, 1951
23. Laemmli UK: Cleavage of structural properties during the assembly of head of the bacteriophage T4. Nature 227: 68–85, 1970
24. Peterson JW, Boldogh I, Popov VL, Saini SS, Chopra AK: Anti-inflammatory and antisecretory potential of histidine in *Salmonella* challenged mouse small intestine. Lab Invest 78: 523–534, 1998
25. Reed IJ, Muench H: A simple method of estimating fifty percent end points. Am J Hygiene 27: 493–497, 1938
26. Bhatnagar R, Schirmer R, Ernst M, Decker K: Superoxide release by zymosan-stimulated rat kupffer cells in vitro. J Biochem 119: 171–175, 1981
27. Green LC, Wagner DA, Glogowski J, Skipper PI, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and (15 N) nitrate in biological fluids. Ann Biochem 126: 131–136, 1982
28. Tietz F: Enzymatic method for quantitative determination of nanogram amount of total and oxidized glutathione application to mammalian blood cell and other tissues. Anal Biochem 27: 502–522, 1969
29. Valtonen M, Plosila VM, Valtonen VV, Makela PH: Effect of the quality of lipopolysaccharide on mouse virulence of Salmonella enteritidis. Infect Immun 12: 828–832, 1975
30. Munford RS, Hall LC, Lipton JM, Dietschy JM: Biological activity, lipoprotein binding behavior, and in vivo disposition of extracted and native forms of *Salmonella typhimurium* lipopolysaccharides. J Clin Invest 70: 877–888, 1982
31. Liang-Takasaki CJ, Makela H, Leive L: Phagocytosis of bacteria by macrophages: Changing the carbohydrate of lipopolysaccharide alters interaction with complement and macrophages. J Immunol 128: 1229–1235, 1982
32. Ulevitch RJ, Jhonston AR: Modification of biophysical and endotoxic properties of bacterial lipopolysaccharides by serum. J Clin Invest 62: 1313–1324, 1978
33. Monack DM, Roupach B, Hremocykj AE, Falkow S: *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc Natl Acad Sci USA 93: 9833–9838, 1999
34. Morel F, Doussiere J, Vignais P: The superoxide generating oxidase of phagocytic cells, physiological, molecular and pathological aspects. Eur J Biochem 201: 523–546, 1991
35. Dilly AK, Panchatcharam M, Miriyala S, Kalidindi VSNR, Chandrasekar G, Natarajan A, Rengarajulu P: A novel peptide derivative exhibits anti-inflammatory antioxidant activity in adjuvant induced arthritis in rats. Mol Cell Biochem 229: 9–17, 2002
36. Grooth H: Reactive oxygen species in tissue injury. Hep Gastroenterol 41: 328–332, 1994
37. Anderson D: Antioxidant defenses against reactive oxygen species causing genetic and other damage. Mutat Res 350: 103–108, 1996
38. Bauer V, Bauer F: Reactive oxygen species as mediator of tissue protection and injury. Gen Physiol Biophys 18: 7–14, 1999
39. Gupta S, Kumar D, Vohra H, Ganguly NK: Involvement of signal transduction pathway in *Salmonella typhimurium* porin activated gut macrophages. Mol Cell Biochem 194: 235–243, 1999
40. Alam MS, Akaike T, Okamoto S, Kubota T, Yoshitake J, Sawa T, Miyamoto Y, Tamura F, Maeda H: Role of nitric oxide in host defense in murine Salmonellosis as a fur of its antibacterial and antiapoptotic activities. Infect Immun 70: 3130–3142, 2002
41. Shiloh MU, MacMicking JD, Nicholson S, Brause JE, Poter S, Marino M, Fang F, Dinauer M, Nathan C: Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity 10: 29–38, 1999
42. Clancy RM, Abramson SB: Nitric oxide: A novel mediator of inflammation. Proc Soc Exp Biol Med 210: 93–101, 1995
43. Kaplan SS, Lancaster JR, Basford RE, Simmons RL: Effect of nitric oxide on *Staphylococcal* killing and interactive effect with superoxide. Infect Immun 64: 69–76, 1996
44. Greenberg JT, Demple B: A global response induced in *Escherichia coli* by redox cycling agent overlaps with that induced by peroxide stress. J Bacteriol 171: 3933–3939, 1989
45. Kashyap DR, Sood S, Tiwari RP, Rishi P: *Salmonella typhi* OMPs induced immunomodulation in peritoneal macrophages. Ind J Exp Biol 38: 352–362, 2000
46. Rishi P, Batra N, Sood S, Tiwari RP: Modulatory effects of *Salmonella* LAP-LPS on murine macrophages. Ind J Med Micro 20: 187–193, 2002