Protection mediated by antibodies to iron-regulated outer-membrane proteins of *S. typhi* in a mouse peritonitis model

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Abstract

Iron limitation induces the expression of iron-regulated outer-membrane proteins, which are not expressed under iron sufficient growth conditions. In the present study, these proteins were purified in order to evaluate their protective potential in the experimental model. Anti IROMPs antiserum was raised in rabbits. In mice, passively transferred anti-IROMPs antibodies provided 60% protection against the serovar Typhi challenge dose ($9.6\,LD_{50}$). The hyperimmune serum containing anti-IROMPs antibodies were also found to be bactericidal in the presence of complement whereas no bacterial killing was observed with pre-immunized serum. Bactericidal titre of anti-IROMPs serum was fond to be 2000 as more than 50% killing was observed with serum diluted to 1:2000. The role of IROMPs was assessed in actively-immunized mice followed by challenge with serovar Typhi. These proteins provided protection in 90% mice against challenge ($480\,LD_{50}$) with the pathogen. The levels of isotypes of antibodies (IgG, IgM & IgA) in the sera and secretory antibodies (sIgA) in the gut fluid of immunized mice correlated with the protection. This study, thus indicates that anti IROMPs antibodies may play an important role in providing protection at systemic as well as at mucosal level. (Mol Cell Biochem **273**: 69–78, 2005)

Key words: antibodies, IROMPs, protection, *Salmonella*

Introduction

Typhoid fever remains a serious health problem of children as well as adults worldwide and is a cause of concern especially in the developing countries. Though, a number of different vaccines are currently available against typhoid, each one of these has certain drawbacks, which has led to the development of a new generation of typhoid vaccines. The new immunogens include several attenuated *S. typhi* strains for use as oral vaccines [1–3]. Various subunit vaccines have also been evaluated which include Vi-polysaccharide and lipopolysaccharide alone or conjugated to proteins [4–6]. Attention has also been focused on the role of outer membrane proteins (OMPs), particularly porins of Gram-negative bacteria in the induction of specific immunity [7–9]. It is now known that *in vivo* expression of virulence determinants is different from those expressed under *in vitro* situations. Therefore, it is renewed interest in understanding the behaviour of pathogens in different environments of the host like low pH, elevated temperature, changes in osmotic strength, presence

of cationic peptides as well as the availability of ions and nutrients.

Like other enteric pathogens, *S. typhi* requires iron for growth, proliferation and variety of enzymatic reactions [10]. The human body however, has different iron withholding defense system, preventing the organism from acquiring essential iron for survival inside the host [11–13]. The regular interaction of the host and the pathogen causes the latter to express certain regulons for sensing the host environment and develop survival strategies against the host environmental stress, including the survival under hypoferric conditions. Under iron stressed conditions *Salmonella enterica* serovar Typhimurium has been observed to induce increased expression of *hilA* (hyper invasive loci) gene in addition to other genes [14].

In response to this selective pressure, bacteria have been observed to evolve efficient iron uptake systems, which include iron uptake through surface receptor proteins [15–17]. Iron limitation has been shown to induce synthesis of a separate set of OMPs, designated as iron-regulated outer membrane proteins (IROMPs) in some pathogens [18–20]. These IROMPs have been reported to be amongst the determinants of virulence [21–23].

In an earlier report from our laboratory, it has been shown that iron limitation causes *S. typhi* to induce the expression of three OMPs (IROMPs) *in vitro*, which were not expressed under iron replete conditions [24]. These IROMPs have been reported to be reactive with the sera of typhoid patients suggesting their *in vivo* expression [25]. In the present study, potential of anti-IROMPs antibodies to provide protection against *S. typhi* infection in mouse peritonitis model has been discussed.

Material and methods

Bacterial strain

Salmonella enterica serovar Typhi, was procured from Central Research Institute (CRI) Kasauli, India. The lyophilised strains were revived in nutrient broth, preserved in glycerol media and stored at $-70\,^{\circ}$C. The strain was checked biochemically as well as serologically.

Growth conditions

For the isolation of iron-regulated outer membrane proteins (IROMPs), cells were grown on nutrient broth under iron replete as well as iron deplete conditions i.e. in the presence of ferrous sulphate (200 μM) and 2,2′-dipyridyl (200 μM) respectively at 37 °C. The iron content of the media in presence and absence of dipyridyl was estimated using ferrozine [26] as described by us earlier [24].

Animals

Female inbred Balb/c mice, 4–6 weeks old (16–22 g), used in the present study were procured from the Central Animal House, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. Animals were kept in well aerated rooms and were fed on standard pellet diet and water. Mice were acclimatised in the laboratory conditions for one week before using for the experiments. Four young male New Zealand rabbits (1–2 kg), procured from Central Animal House, PGIMER, Chandigarh, were used for raising antisera against IROMPs. Animals were handled and disposed according to the guidelines of the institutional ethical committee.

Preparation of outer membrane proteins

Outer membrane proteins were prepared from cells harvested after growing the organism in nutrient broth [24]. Briefly, cells were grown in iron replete and iron deplete conditions at 37 °C overnight on nutrient agar. Cells were harvested and suspended in 10 mM Tris buffer (pH 8.0) after two washings in the same buffer. Cells were disrupted by ultrasonication (12 cycles of 30 s each with one-minute interval in between) (Sonicator, Ultrasonic Processor XL, Misonix, USA) and undisrupted material removed by centrifugation at 800 × *g* for 20 min. Supernatant was centrifuged at 100000 × *g* (Beckam Coulter, Optima™ XL-100K Ultracentrifuge, USA) for 60 min; pellet was suspended in Tris buffer containing 1% sodium sarkosyl (ICN, USA) and incubated at 37 °C for 40 min with gentle shaking. The detergent insoluble outer membrane fraction was collected by centrifugation at 100000 × *g* and suspended in Tris buffer containing 2 mM phenylmethanesulfonylflouride (PMSF, Sigma USA) [27].

Purification of IROMPs

The OMPs enriched fraction was solubilised in sample buffer (containing SDS and β-mercaptoethanol) and loaded onto 10% acrylamide gel. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli [28] to separate outer-membrane proteins. The gel, containing IROMPs was sliced and the proteins were eluted by the chemical elution method given by Hager and Burgess [29]. Eluted proteins were further purified by loading onto HPLC column (Protein Pack-125) and the fractions containing IROMPs were collected carefully and were again subjected to HPLC. Limulus amoebocyte lysate (LAL) assay was carried out [30] to detect the presence of LPS in purified IROMPs preparation.

Antiserum

Antisera raised in rabbits against purified IROMPs as described by Bolin and Jenson [31] were pooled. Immunodiffusion studies were carried out to see the production of antibodies and antibody titre was determined by ELISA as per the standard procedure.

Protection with anti-IROMPs serum
(passive protection studies)

The protective potential of anti IROMP antibodies was determined by minor modification in the method described by Muthukumar and Muthukkaruppan [32]. Two groups of 10 mice each were taken. In the control group, all mice received 0.2 ml normal saline intravenously. In the second group of mice, 0.2 ml anti-IROMPs serum raised in rabbit was administered intravenously. Three hours after the serum transfer, the mice were challenged with 9.6 LD_{50} of *S. typhi* Ty2 (after standardizing the dose). Lethal dose was determined by the method of Reed and Muench [33]. The number of survivors was recorded till 21 days after the challenge.

Bactericidal assay of anti-IROMP antibodies

The bactericidal activity of anti-IROMPs serum raised in rabbits was assayed by the method given by Chen *et al.* [34]. The complement source for the experiment was the fresh human serum from healthy individuals. Serial dilutions of the heat inactivated anti-IROMP serum were prepared. The bactericidal assay was performed by mixing 100 μl of bacterial suspension (approximately 2×10^6 organisms) in PBS (containing 1 mM $CaCl_2$ and 0.2 mM Mg Cl_2) with 50 μl of heat-inactivated serum and incubating for 30 min at 4 °C. About 30 μl of the complement containing serum was then added (1:10 diluted) to give a final concentration of 20% and mixture was incubated overnight at 37 °C in 96 well plates. Following morning, the samples from wells were plated on nutrient agar plates and allowed to incubate overnight at 37 °C. The controls for this assay consisted: (i) bacterial suspension + immune serum + heat inactivated complement, (ii) bacterial suspension + preimmune serum + fresh complement., (iii) bacterial suspension + preimmune serum + heat inactivated complement.

Colony forming units (CFU) was calculated after incubation of nutrient agar plates and %killing was calculated as follows:

$$\frac{\text{CFU from control} - \text{CFU from sample}}{\text{CFU from control}} \times 100$$

Protection studies with IROMPs (active protection studies)

This study was performed as described by Isibasi *et al.* [8]. The inbred Balb/c mice were taken in six groups of ten mice each. Five groups of animals were actively immunized with five different concentrations of IROMPs, 1, 2.5, 5, 7.5, and 10 μg to find out the optimum concentration of IROMPs. Animals were injected intraperitoneally with 0.5 ml of sample in normal saline on day 0 and 14. Control group received only normal saline. After seven days of last injection of immunization, all the mice were challenged intraperitoneally with 480 LD_{50}. Deaths and survivals were recorded till 20 days post infection.

Immunization of mice

Additional protection studies were performed after finding the optimum dose (7.5 μg). Four groups of inbred Balb/c mice each consisting of 20 mice were immunized intraperitoneally as detailed below:

Group-1 (control): Mice in this group were given 0.5 ml of normal saline. *Group-2 (infected)*: Mice in this group were given 0.5 ml of *S. typhi* (1×10^4 organisms) in presence of mucin. *Group-3 (IROMP immunized)*: Mice were immunized in 0.5 ml with 7.5 μg of purified IROMP in 0.5 ml saline at day 0 and 14. *Group 4 (IROMP-immunized-**challenged**)*: Mice were immunized as in group 3. Then the mice were challenged intraperitoneally with 480 LD_{50} (Challenge dose) of *S. typhi* Ty2 in 5% hog mucin seven days after the second dose of immunization. Mice in each group were sacrificed at different time intervals after immunization/challenge to assess the antibody response.

Collection of serum and gut fluid

The blood samples were collected from jugular veins of different groups of mice. They were kept at 37 °C for 2 h and then at 4 °C for 2 h. Then the samples were centrifuged for 10 min at 2000 rpm, the supernatants were collected as sera and stored at −20 °C. For the collection of gut fluid, the small intestines from different groups of mice were removed and washed with 0.5 ml of chilled PBS. The washed out fluids were centrifuged at 4000 rpm for 20 min at 4 °C, the supernatants were collected and stored at −20 °C.

Measurement of antibody response

Anti-IROMP IgG, IgA and IgM antibodies were determined using ELISA technique described by Forrest [35]. In each case, antigen (IROMP, 1 μg) was coated on to the wells of microtitre plates in carbonate − bicarbonate coating buffer

(pH 9.6, 100 μl/well) overnight at 4 °C. The plates were washed with 0.15 M PBS (pH 7.2–7.4) containing 0.05% Tween 20 (PBS-T) and the non-specific sites were blocked by adding 2% BSA (150 μl/well) and incubated at 37 °C for 2 h (or overnight at 4 °C). After washing with PBS-T twice and then with PBS, 100 μl of two fold serially diluted serum was added in wells and incubated at 37 °C for 2 h. The plates were washed extensively with PBS-T and then with PBS and 100 μl of HRP-conjugated IgG, IgA or IgM diluted to 1:1000 with 0.01 M PBS was added respectively. The plates were then incubated at 37 °C for 45–60 min. The plates were washed 4–5 times and 100 μl of orthophenylene-diamine (OPD) [5 mg/10 ml of 0.15 M citrate buffer (pH 5.0) and 5 μl H_2O_2] was added and the plates were kept at room temperature in dark for 30 min. The reaction was stopped by the addition of 100 μl of 6N H_2SO_4. The intensity of colour developed was read at 492 nm in an ELISA reader. Secretory IgA (sIgA) was determined in the gut fluid of mice in the same way.

Statistical analysis

For statistical significance, the data was analysed using unpaired student's t-test and two way analysis of variance (ANOVA) with multiple comparison. Data was considered significant at $p < 0.05$.

Results

Iron deficient conditions induces the expression of three high molecular weight proteins in *S. typhi* which are not expressed under iron replete conditions as reported by us earlier. These were denoted as IROMPs with high molecular masses ranging from 68 to 85 kDa approximately [24].

Purification of IROMPs

The IROMPs, eluted by chemical elution method from the polyacrylamide gels were used for the purification by HPLC Protein Pack-125 column, and the fraction containing IROMPs showed a single peak (Fig. 1). Purified preparation of IROMPs was found to contain only 0.02% of LPS contamination.

Passive protection with anti-IROMPs serum

Passive transfer of anti-IROMPs serum given to mice (intravenously) afforded 60% protection against 9.6 LD_{50} of *S. typhi*, whereas there was no protection in the control group (where normal serum was given). Protected mice in this group could survive till 19th day post challenge.
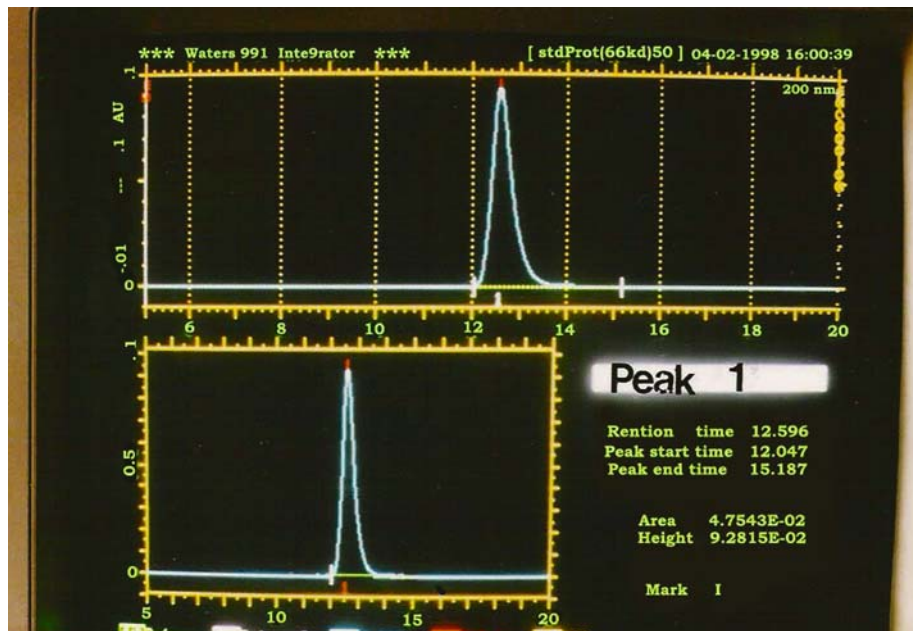

Fig. 1. HPLC profile of IROMPs preparation. Proteins were eluted chemically from the gel and loaded on to HPLC column. Fraction containing IROMPs collected carefully and was again subjected to HPLC.
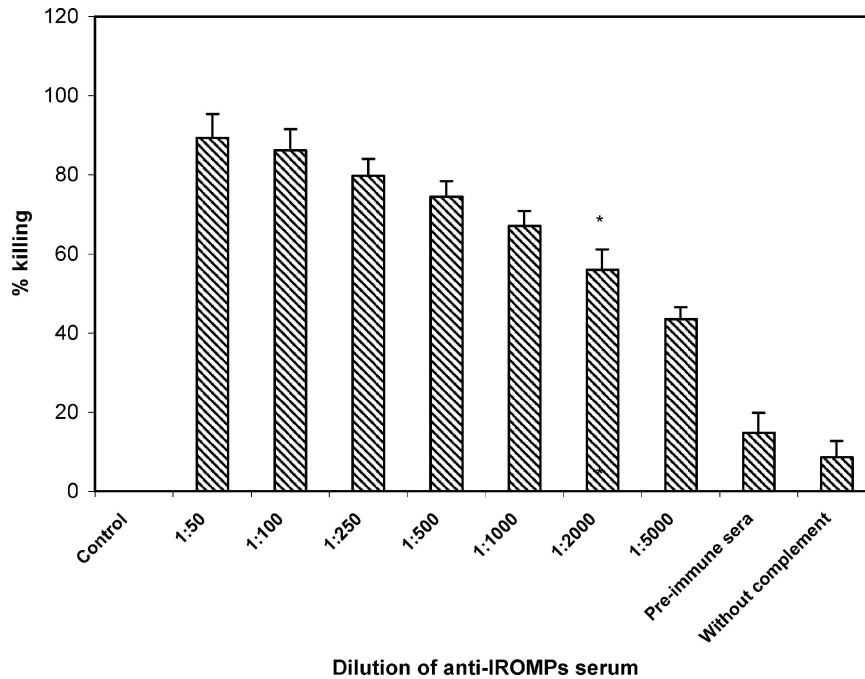
Fig. 2. Bactericidal activity of anti-IROMPs serum raised in rabbits. Serial dilutions of anti-IROMPs serum were incubated with bacterial suspension in the presence of complement, *indicates the dilution causing more than 50% killing.

Bactericidal assay

After incubating *S. typhi* cells with heat inactivated anti-IROMPs antibodies in the presence of fresh human serum (which served as the complement source), it was observed that the organisms were killed. Bacterial killing was abolished in the absence of human serum indicating the complement dependent bactericidal activity. There was also no bacterial killing when *S. typhi* was incubated with pre-immune sera from rabbits, indicating the bactericidal activity of hyper immune sera against IROMPs. Bactericidal titre of anti-IROMPs serum was found to be 2000 as more than 50% killing was observed with serum diluted to 1:2000 (Fig. 2).

Protection with IROMPs

Protective dose of IROMPs was assessed by immunizing mice with different concentrations of purified proteins. 7.5 μg of IROMPs provided maximum (90%) protection against the challenge dose (480 LD_{50}), which decreased with the decrease in the concentration of protein. Hence for the subsequent studies, 7.5 μg of IROMPs was used as the immunization dose in mice (Fig. 3).

Isotypes of anti-IROMPs antibodies in serum

There was significant increase ($p < 0.001$) in the level of IgM antibodies in the infected and IROMPs-immunized groups

on 3rd and 7th day as compared with the control group. On both the days, the level of IgM antibodies in the immunized-infected group, decreased significantly ($p < 0.001$) compared to immunized as well as infected group (Fig. 4A). However, the level was still significantly higher than in the control group. From the results it was clear that on day 15 there was significant decrease ($p < 0.01$) in IgM levels in all the groups as compared with the levels on 3rd and 7th day. The infected group showed increase ($p < 0.01$) in IgM levels on day 15 also as compared to the control group.

In the immunized and immunized-challenged groups, the levels of IgG significantly increased ($p < 0.001$) as compared with control as well as infected group throughout the study period. However, the infected group showed a significant increase ($p < 0.01$) compared to the level in control group only on 3rd day. But on 7th and 15th day post infection, the IgG levels came down almost to the same level as in the control group (Fig. 4B).

In the infected, immunized and immunized challenged groups, IgA levels in serum were significantly increased ($p < 0.001$) as compared to control group on all the days (days 3, 7 and 15). immunized-challenged group showed significant increase ($p < 0.01$, $p < 0.001$) in the level as compared with infected as well as immunized groups on days 3 and 7, and the level was found to be maximum on 7th day. Although it decreased significantly on day 15, still remained higher in contrast to other groups. However, the levels in the
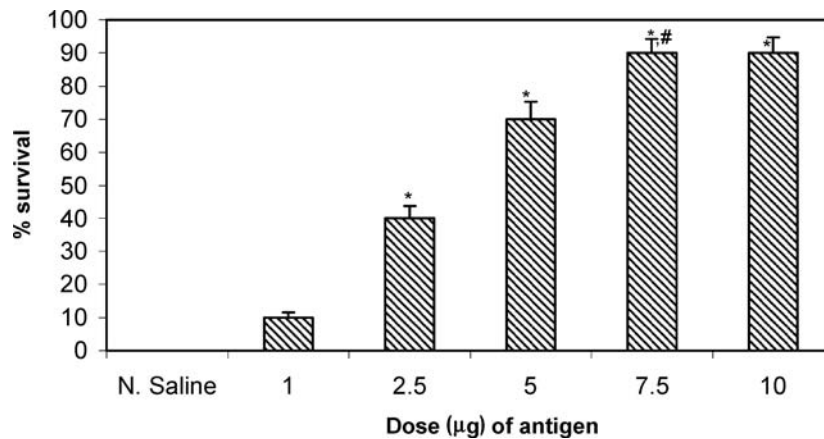
Fig. 3. Protection provided by 7.5 μg of IROMPs immunization against 480 LD$_{50}$ of *S. typhi* Ty2 in 5% hog mucin. $^*p < 0.05$ as compared to the control group, $^\#p > 0.05$ as compared to the protection provided at 10 μg dose.

infected and immunized groups were almost same on the 15th day (Fig. 4C).

Secretory IgA response in gut fluid

Figure 5 shows that anti-IROMPs sIgA levels in gut fluid of infected, immunized as well as immunized-challenged groups were significantly high ($p < 0.001$) as compared with control group on 3rd, 7th and 15th day. There was significant increase ($p < 0.001$) in sIgA levels in immunized and immunized-challenged groups compared with infected group on 3rd and 7th day. However, no such increase was observed on day 15 in these groups when compared with the infected group. Secretory IgA also followed the same trend as that of circulating IgA.

Discussion

The classical role for specific antitoxic and bactericidal antibodies in preventing many microbial infections present a satisfying explanation for the humoral response almost invariably seen in a convalescing host. The humoral response to *Salmonella enterica* serovar Typhi is important for protective immunity against typhoid fever, as indicated by the protection obtained with killed cell vaccines and component vaccines (OMPs, Vi-antigen) in animals and human beings [36, 37]. Earlier studies have shown that porins of *S. typhi* induce both cellular and humoral immune response through induction of T-cell response [7–9]. However, there are not many reports available about these antigens in reference to host environment. Since the expression of virulence determinants change in the diverse array of the host environment, the present study was undertaken with the aim of obtaining

a clearer picture of the antibody response against the environmentally regulated proteins. Therefore, this study investigated the modulation of humoral response after manipulating at least one of the conditions encountered by the pathogen in the host i.e. by creating iron limited conditions *in vitro*.

In order to carry out the protection studies, the IROMPs were purified by HPLC system employing protein Pack-125 column after elution from the gel. This method employing the direct application of eluate on HPLC column is faster and efficient in comparison to the conventional gel filtration chromatography [38] used for purifying iron regulated proteins from *Yersinia*.

Generally, the efficacy of humoral immunity is established by demonstrating protection after passive transfer of specific antibodies and/or correlating protection against infection with the presence of antibodies in the serum of infected individuals. The passively transferred OMPs and egg yolk immunoglobulins have been reported to have protective effect against experimental *Pseudomonas aeruginosa* and enterotoxigenic *Escherichia coli* infection [39, 40] respectively. It is indicated from the existing literature that circulating immune mechanism can influence the course of infection caused by intracellular pathogens [41]. It has also been reported that antibodies can protect against the effect of LPS and can activate complement-mediated lysis [42, 43]. Antibodies raised against *Salmonella*, *Cryptosporidium* and *Chlamydia* species have been reported to afford protection by interfering with the entry and invasion of normal cells [44, 45]. In the present study, 60% protection obtained after passively transferred anti-IROMPs antibodies emphasizes the role of humoral immunity against *S. typhi* infection which are in conformity with earlier observations where anti-IROMP antibodies significantly reduced *E. coli* septicemia and severity of gross lesions in turkeys [31]. In the present study, following passive immunization with anti-IROMPs antibodies
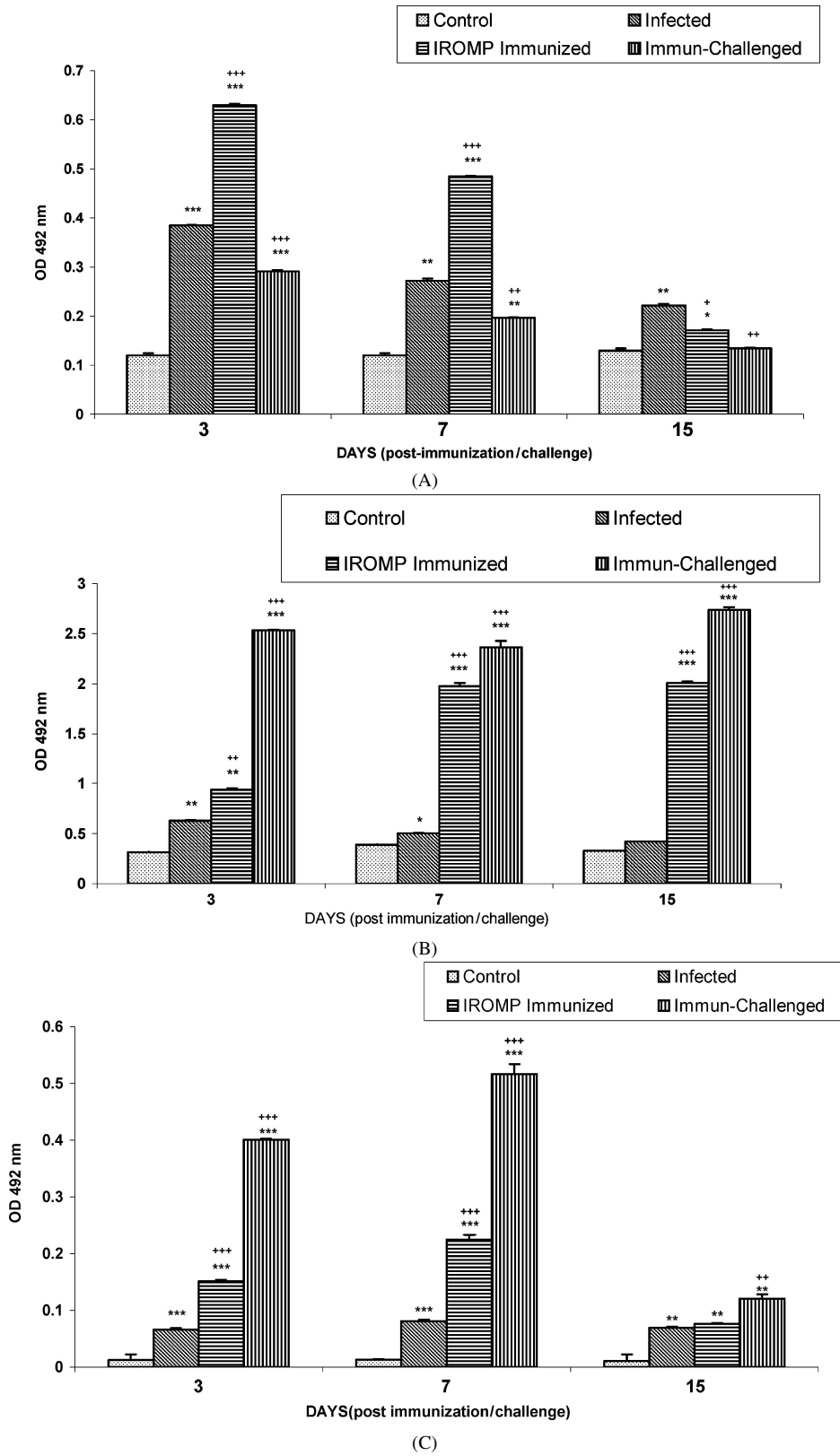
Fig. 4. Anti IROMPs antibody response in serum. A: IgM, B: IgG, C: IgA Values are mean ± S.E.M. $^{*} p < 0.005$; $^{**} p < 0.01$, $^{***} p < 0.001$ as compared to the control group $^{+} p < 0.005$; $^{++} p < 0.01$, $^{+++} p < 0.001$ as compared to the infected group.
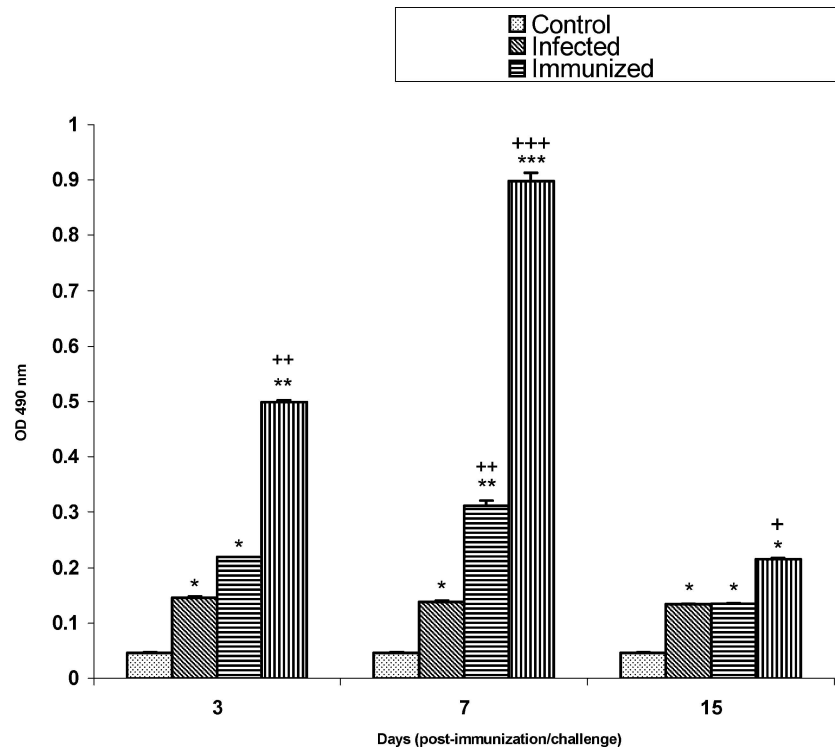
Fig. 5. Secretory IgA response in the intestinal fluid. Results are expressed as mean ± S.E. Values are mean ± S.E.M. $^* p < 0.005$; $^{**} p < 0.01$, $^{***} p < 0.001$ as compared to the control group $^+ p < 0.005$; $^{++} p < 0.01$, $^{+++} p < 0.001$ as compared the to infected group.

protected mice survived for 19 days. In these mice, antibodies may have bound to bacteria and acted as opsonins, either directly or as result of complement activation [46]. Alternatively, anti IROMPs antibodies may also interfere with the iron uptake ability of *Salmonella* thereby inhibiting bacterial growth.

Due to certain inherent problems, use of antibodies in human subjects is not an acceptable approach. Pure preparation of gamma- globulin is needed which can only be obtained from volunteers recovered from the disease. Besides this, shelf life of gamma globulin, purification of globulin prior to use making the therapy costlier and multiple doses of globulin leading to serum sickness limit their use [36]. These reasons prompted us to study the protective potential of active immunization with IROMPs. IROMPs conferred 90% protection against *S. typhi* challenge in experimental mouse peritonitis model. These results are in line with the earlier observations where it has been shown that immunization with IROMPs protects against infection [31]. Little amount of LPS present in the preparation does not account for the observed magnitude of protection [9] but may give the adjuvant effect.

IgG anti IROMPs antibodies were significantly higher in the immunized group in which 90% protection was observed as revealed by laboratory potency test in mice. The IgG level persisted upto 15 days. IgA specific antibodies were also higher in the immunized mice up to seven days only and then the level declined. However, IgM specific antibodies did not follow the same trend. This anti-IROMPs antibodies mediated protection against challenge in mice may be operating by controlling the spread of the organisms during the stage of bacteremia. Bacterial infection of the gastrointestinal tract normally produces a secretory response (sIgA), which prevents the invasion of epithelial cells. In earlier studies, it has been observed that porins alone or conjugated to Vi induces sIgA response in the intestine. In the present study, higher level of sIgA after IROMPs immunization were obtained up to seven days. This indicates, in addition to porins, the non-porin proteins like IROMPs might be playing an important role in inducing the mucosal response under the iron deficient conditions prevailing in the host. This study thus suggests the role of mucosal immunity at the initial stage followed by systemic immunity at the later stage following infection. It is clear from the study that anti IROMP antibodies are important and related to protection against salmonellosis though cellular factors may play a major role. Passive protection (60%) observed by anti-IROMPs antibodies against $9.6\,LD_{50}$ in contrast to active protection (90%) provided by IROMPs against $480\,LD_{50}$ suggest the role of cell-mediated immune response. Studies are under way to evaluate the role of CD^{4+} and CD^{8+} cells alongwith the lymphokine production in inducing the

systemic and mucosal immunity. Better understanding of immune response against intracellular pathogens in reference to the ever changing environment of the host may be important for fundamental immunology, vaccine design and development of immunotherapy.

Acknowledgments

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