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 LD₅₀). 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 LD₅₀) 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

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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-IROMP 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°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 (LPS) assay was carried out [30] to detect the presence of LPS in purified IROMPs preparation.