Salmonella typhi iron-regulated outer-membrane proteins cause oedema and hyperalgesia during inflammation induced in a rat model

Typhoid fever remains a serious health problem worldwide and is a cause of concern especially in developing countries. Although a number of different vaccines are currently available against typhoid, each one has certain drawbacks, which has led to the development of a new generation of typhoid vaccines. In addition to Vi-polysaccharide vaccines (alone or conjugated to protein), 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 (Isibasi et al., 1988; Singh et al., 1999a). It is now known that in vivo expression of virulence determinants differs from expression under in vitro conditions (Smith, 1998). Therefore, there is a renewed interest in understanding the behaviour of pathogens in different host environments such as acidic pH, elevated temperature, different osmotic strengths and the presence of cationic peptides as well as the availability of ions and nutrients.

Like other enteric pathogens, Salmonella typhi requires iron for growth, proliferation and a variety of enzymic reactions. The human body, however, has an iron-withholding defence system, preventing the organism from acquiring essential iron for survival inside the host. 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 environmental stress. S. typhi has been reported to express iron-uptake systems, characterized by secreted siderophores, along with certain proteins on the cell’s surface referred to as iron-regulated outer-membrane proteins (IROMPs).

Here, in order to assess the effect of iron availability, iron-deficient and iron-sufficient conditions were created in vitro by adding 200 μM dipyridyl and 200 μM ferrous sulphate, respectively, to nutrient broth. The iron content of the media was estimated using ferrozine by the method of Carter (1971). S. typhi (Ty2) was grown in the iron-deficient and iron-sufficient media and OMPs were extracted (Chander et al., 2004). The expression of three distinct bands in the outer-membrane profile of S. typhi, indicating enhanced expression of IROMPs when under iron-deficient conditions compared with the expression under normal or iron-sufficient conditions. The expression of these proteins seems to result from a modification of the existing proteins under iron-deficient conditions rather than being from de novo synthesis. The expression of three distinct bands in the molecular mass range from 66 to 97 kDa is in agreement with an earlier study (Fernandez-Beros et al., 1989) of IROMPs from the same strain (S. typhi Ty2). However, it differs from the profile shown recently by Chibber & Bhardwaj (2004) using the same strain. The dipyridyl used in that study may not have been efficient in causing the chelation of iron present in the medium. Thus, the quantification of iron-chelation becomes essential.

In the present study, the inflammatory potential of these proteins was assessed. The inflammatory reaction is readily produced in rats in the form of paw oedema with the help of inflammagens such as carrageenan, bradykinin, histamine, 5-hydroxytryptamine, mustard, egg white and micro-organisms and their products. To assess the inflammatory potential of IROMPs, six rats were injected with 0·1 ml of normal saline containing 7·5 μg (after standardization) of protein in the right paw and the same volume of normal saline in the left paw to compare the oedema readings. Carrageenan (1 % w/v), a sulphated polysaccharide obtained from seaweed (Rhodophyceae), was used as a positive control. It produces inflammation and oedema by causing the release of histamine, 5-hydroxytryptamine, bradykinin and prostaglandins. The footpad oedema was measured using a plethysmometer rather than crudely measuring the footpad swelling with Vernier calipers. With this instrument, the oedema could be measured accurately by dipping the paw in mercury and directly reading the displacement from the scale attached to the mercury column.

Fig. 1 shows the outer-membrane profile of S. typhi, indicating enhanced expression of IROMPs when under iron-deficient conditions compared with the expression under normal or iron-sufficient conditions. The expression of these proteins seems to result from a modification of the existing proteins under iron-deficient conditions rather than being from de novo synthesis. The expression of three distinct bands in the molecular mass range from 66 to 97 kDa is in agreement with an earlier study (Fernandez-Beros et al., 1989) of IROMPs from the same strain (S. typhi Ty2). However, it differs from the profile shown recently by Chibber & Bhardwaj (2004) using the same strain. The dipyridyl used in that study may not have been efficient in causing the chelation of iron present in the medium. Thus, the quantification of iron-chelation becomes essential.

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the paw oedema induced after 3 h by OMPs extracted under different conditions. The results agree with those of Galdiero et al. (1990) using porins of *Salmonella typhimurium*. This study thus indicates the inflammatory potential of non-porin proteins expressed under iron stress.

Assessment of thermal hyperalgesia during inflammation was assessed using the paw immersion (warm water) test. The rats’ paws were immersed separately in a warm water bath (47 ± 0.5 °C) until paw withdrawal (flicking response) or signs of struggle were observed (cut off 10 s). The withdrawal response was measured for 5 h at 1 h intervals. Fig. 4 shows that the time required for the withdrawal of the paw injected with IROMPs was significantly shorter than the control paw, indicating hyperalgesia. Hyperalgesia can increase diacylglycerol levels and activate protein kinase C, which has been implicated in changes in pain perception.

The uncontrolled release of cytotoxic substances and proinflammatory mediators including cytokines (TNF and interleukins) (Klimpel et al., 1995) due to hyperinflammation may damage the host tissues as well. Therefore, under such conditions, it is necessary to manage the detrimental effects of these inflammatory mediators, which may be crucial in modifying the clinical course of the disease. In our earlier studies we observed the role of *S. typhimurium* surface components in modulating the inflammation mediated through alterations in the levels of oxidants and anti-oxidants following immunization (Choudhary et al., 2005).

While evaluating any antigen for its vaccine potential, it is desirable to determine the minimum concentration of the antigen that gives a protective effect. It has been shown by Chibber & Bhardwaj (2004) that 50 μg of IROMPs and 2.5 μg of VI-IROMPs conjugate of *S. typhi* gives protection in a mouse peritonitis model. The use of the same concentrations has been reported for the OMPs and VI-OMP conjugate expressed under normal conditions.

*Fig. 3.* Inflammatory potential of IROMPs measured by plethysmometer scale. All values are mean ± SD of six animals. *Inflammation caused by iron-deficient OMPs and carrageenan vs normal OMPs and iron-sufficient OMPs (P < 0.05).*

*Fig. 4.* Paw-withdrawal (flicking) response in rats injected with 7.5 μg of IROMPs and controls. All values are mean ± SD of six animals. *Control vs IROMPs (P < 0.05). White bars, injected with normal saline (control); black bars, injected with IROMPs.*

(Tabaric et al., 1994; Singh et al., 1999b). When the organism is under any type of stress, it is reported to behave differently as it expresses different virulence determinants. Hence, the standardization of the dose becomes an important factor, particularly when vaccination is considered as a cost-effective means for preventing any infection. In our study on IROMPs, 7.5 μg of purified antigen (eluted from the gel and purified by HPLC) has been found to afford 90% protection against 480 × LD₅₀ of *S. typhi* Ty2 in 5% hog mucin using the same mouse peritonitis model, in contrast to 50 μg of purified antigen used in the recent report (Chibber & Bhardwaj, 2004). We have also seen that IROMPs are able to induce humoral as well as cellular immune responses at the systemic and mucosal level (unpublished data). Therefore, the use of IROMPs in future as a potential vaccine candidate is worth exploring.

The experimental protocol described in the study had ethical approval from the Institutional Animal Ethic Committee, Panjab University, Chandigarh.

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