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Basic Science

Immunological Evaluation of Formulated Drugs against Typhoid

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Key words: Typhoid; Nitric oxide (NO); Delayed type of hypersensitivity (DTH); CMI.

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Abstract

OBJECTIVES: Typhoid fever an important causes of illness and death, particularly among children and adolescents in south-central and Southeast Asia, where enteric fever is associated with poor sanitation and unsafe food and water. Cell-mediated immunity (CMI) plays an important role for the survival of the host in experimental salmonellosis. Nitric oxide (NO) is the one of the product of macrophages activated by cytokines, microbial compounds or both, is derived from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS or NOS2) which acts as antimicrobial molecule.

AIM: The aim was to examine the induction of DTH reaction in the animals treated with L-Arginine, ciprofloxacin and their combination followed by immunization with *S. typhimurium* cell lysate as assessed by the footpad swelling test.

RESULTS: The results of the present study showed that the induction of DTH reaction in the animals treated with L-Arginine, ciprofloxacin and their combination followed by immunization with *S. typhimurium* cell lysate using an antigen revealed that the treatment with combination increased foot pad swelling significantly as compared to saline treated control animals at 48 hour which was followed by a decrease of the swelling at 72 hour.

CONCLUSIONS: Animal treated with L-arginine, ciprofloxacin and their combination showed increased cell mediated immune responses as evident by DTH response whereas groups (B+S) shows decreases CMI responses.

Introduction

In the recent year NO has been recognized as an important molecules in the immune system having its involvement in pathogenesis and control of infectious disease. Formation of NO with the help of iNOS, has also been reported in a wide variety of cells, including vascular endothelial cells, neuronal cells, PMN cells, bronchial epithelial cells, hepatocytes, and activated macrophages [1-6], which is induced by lipopolysaccharide and various proinflammatory cytokines such as IFN-γ and TNF-α and produces an excessive amount of NO [7].

Cytokines are the key regulators of host response to intracellular pathogens [8], various bacterial products from *Salmonella* are potent inducers of cytokines expression by immune cells [9-11]. NO produced excessively, in particular by activated macrophages, has been shown to function as a cytotoxic or cytostatic molecule and inhibits the growth of a diverse array of infectious agents [12, 13].

Salmonella encounters a diversity of environments throughout the course of systemic infection. In Peyer`s patches Salmonella rapidly internalized and get partially phagocytized and neutralized. Some cells escape the mucosal immunity and the T and B lymphocyte activation is provoked. *S. typhimurium* is an intracellular facultative bacterium; therefore cell-mediated immune responses are very important for removal of invading organism. Studies in both humans and mouse model of typhoid fever suggest the importance of these responses. The immune response to *S. typhi* infection is complex and involves cellular, secretory and serum components. In the mouse model, cellular components are involved in resistance to salmonella infection [14, 15]. Cell-mediated immunity is of major importance for the survival of the host in experimental salmonellosis [16].

However, humoral antibodies also contribute to protection, especially in the initial phase of the infection. The beneficial qualities of the cell-mediated immune effector mechanism involving cytokines are well known [17]. The activated T-cells produce lymphokines that activate tissue macrophages, resulting in enhanced host protection. Akeda *et al.* [18] reported a synergic contribution of macrophages and antibodies in protection against *Salmonella typhimurium.*

We have examined the roles of CMI as assessed by the footpad swelling test.

Materials and Methods

Animals

Swiss albino mice (25-30 g) and Wistar rat of both 6-8 weeks old were obtained from the central animal house of Hamdard University, New Delhi, India. The animals were kept in Polypropylenecages in an air-conditioned room at 22°/25°C and maintained on a standard laboratory feed (Amrut Laboratory, rat and mice feed, Navmaharashtra Chakan Oil Mills Ltd, Pune) and water *ad libitum*. Animals were allowed to acclimatize for one week before the experiments under controlled light/dark cycle (14/10 h). The studies were conducted according to ethical guidelines of the "Committee for the purpose of control and supervision of Experiments on Animals (CPCSEA)" on the use of animals for scientific research.

Bacteria

Standard strain of *S. typhi* and *S. typhimurium* (wild), were obtained from National *Salmonella* Phage Typing Centre, Lady Harding Medical College, New Delhi, India, and was used for these studies. The bacterial strains were characterized further at the microbiology Department of Microbiology Majeedia Hospital Hamdard University to confirm their identity.

Briefly, *S. typhimurium* was grown at 37°C as stationary overnight cultures in nutrient broth. The innoculum was diluted in PBS (Phosphate buffer saline) and injected into peritoneum of mice. Dilution and pour plating onto agar plate was done to check the number of viable bacteria in each innoculum.

Dose and Dosage

Animals were divided into eight groups. Each group comprised of six animals.

Table 1: The study comprised of following treatment schedules.

Groups	Treatments
Group1	Negative control (Normal Saline)
Group2	Positive control (*S. typhimurium)* (0.6xLD50)+Saline
Group3	Ciprofloxacin (400mg per kg b. wt)
Group4	*S. typhimurium* (0.6xLD50)+Ciprofloxacin (400mg per kg b. wt)
Group5	Arginine (1000mg per kg b. wt)
Group6	*S. typhimurium* (0.6xLD50)+Arginine (1000mg per kg b. wt)
Group7	*S.typhimurium* (0.6xLD50)+Arginine (500mg per kg b. wt)+Ciprofloxacin (200mg per kg b. wt)
Group8	*S. typhimurium*(0.6xLD50)+Arginine (250mg per kg b. wt)+Ciprofloxacin (200 mg per kg b. wt)

Effects of above drugs on infected mice by *S. typhimurium* were analyzed. Mice were divided into eight groups having six mice in each group. Post-treatment of drugs were done at above dose orally to the experimental animals, first group was considered as control that receive only saline, second group considered as positive control which was challenged with sub lethal dose of *S. typhimurium* along with saline.

Third group was only received only full dose of ciprofloxacin. Fourth group was challenged with sub lethal dose of *S. typhimurium* and then mice were treated with standard drug ciprofloxacin. Fifth group was received full dose of Arginine only. In sixth group after infection with *S. typhimurium* animals were treated with full dose of Arginine. In seventh and eight group animals were challenged with *S. typhimurium* and then half and one fourth dose of Arginine was administered along with half dose of Ciprofloxacin respectively.

Preparation of sonicated antigen

The sonicated antigen was made as described by Tiwari and Kamat, (1986) [19]. Briefly, *S. typhimurium* was grown at 37°C as stationary overnight cultures on nutrient agar were suspended in phosphate buffered saline (PBS), pH 7.2. Bacteria were washed in PBS and disrupted by sonication (Ultrasonic Processor, Heat system Ultrasonic, Inc, USA). The resultant material was centrifuged at 10,000 rpm for 1hour. The supernatant was lyophilized and the protein content of the lyophilized material was estimated.

Delayed type of hypersensitivity (DTH)

DTH studies were carried out by standard footpad swelling method as described by Collins and Mackanees, (1968) [20]. The mice were treated with sublethal dose of S.*typhimurium* (Wild) intraperitoneally for the purpose of sensitization of animals and then treated with different concentration of drugs. After 7 days, about 50 µg of sonicated *S. typhimurium* cell lysate were injected into the right footpad. An equal amount of saline was injected into left footpad. The footpad swelling was measured at different time intervals (3, 6, 12, 24, 48 and 72 hrs) after injection by digital Vernier caliper (Guanglu, China). The values obtained for the swelling induced by saline in the left footpad were subtracted from the values obtained for the swelling induced by sonicated *S. typhimurium*. Finally the effect of drugs on DTH was measured.

Statistical analysis

All data are expressed as means ± standard errors of the means (SEM). The statistical difference was determined by the two-tailed unpaired *t* test. A *P* of <0.05 was considered statistically significant.

Results

As *S. typhimurium* is an intracellular facultative bacterium, the CMI response is very important for the clearance of invading microorganisms. Studies in both human and mouse model of typhoid fever suggest the importance of these responses. Nitric oxide plays a central role in eradication of *Salmonella.* Therefore, assessment of CMI in animals treated with NO donor compounds should be able to give an evaluation of the role of these compounds in protection against *S. typhimurium* challenge.

Delayed Type Hypersensitivity (DTH)

Delayed type hypersensitivity is considered as an *in vivo* manifestation of cell-mediated immune response, and development of a positive DTH reaction is correlated with protective cell-mediated immune response. L-arginine and Ciprofloxacin were screened for their ability to induce DTH reactivity in mouse sensitized with sublethal dose (0.6 x LD50) of *S. typhimurium* by using *S. typhimurium* cell lysate as an antigen. The results of these studies are shown in Figure1. Treatment with L-arginine, Ciprofloxacin and their combination increased foot pad swelling significantly ($p<0.01$) as compared to saline treated control animals at 48 hour followed by a decrease in swelling at 72 hour. These results correlate with the fact that nitric oxide donor and ciprofloxacin is involved in the induction of DTH response as a mechanism to protect the animals. Treatment with L-arginine significantly enhanced the DTH reaction at 48 hour but the effect was less than the ciprofloxacin

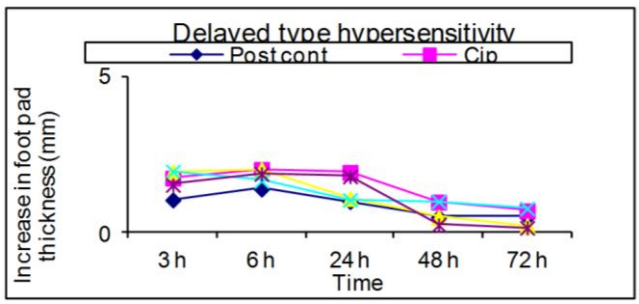

Figure 1: Effect of L-arginine, ciprofloxacin and their combination on DTH response: Mice were immunized with 0.6xLD_{50} of S. typhimurium and then animals were treated with above drugs. Control received saline only. On 8th day after PI, the bacterial sonicate of 50µl volume correspond 50µg of protein was injected into the right hind footpad in all the experimental groups. The left hind footpad received an equal volume of saline, which was served as a control. (pos cont=positive control, Cip= ciprofloxacin, Arg= arginine).

Discussion

Delayed type hypersensitivity

A frequent consequence of infectious diseases is the development of delayed-type hypersensitivity (DTH) to one or more specific microbial antigens [21]. Similar to other facultative intracellular parasitic infections *Salmonella* infections have also been reported to be mediated by Cell Mediated immunity mechanism. NO is known to exert its effect at multiple cellular and molecular levels that include direct effects on different immune cell types (eg, Tcells, neutrophils, or mast cells) either at the site of inflammation or in the lymph node [22]. NO has also been reported to regulate expression of different cytokines and cell surface markers that are essential for the cell-mediated immune response [23]. The results of the present study showed that the induction of DTH reaction in the animals treated with L-Arg, ciprofloxacin and their combination followed by immunization with *S. typhimurium* cell lysate using an antigen revealed that the treatment with combination increased foot pad swelling significantly as compared to saline treated control animals at 48 hour which was followed by a decrease of the swelling at 72 hour. These results correlate with the fact that nitric oxide is involved in the induction of DTH response as a mechanism to enhance cell mediated immune response resulting in protection of the animals.

In conclusion, animal treated with L-arginine, ciprofloxacin and their combination showed increased cell mediated immune responses as evident by DTH response whereas groups (B+S) shows decreases CMI responses.

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