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Heat killed multi-serotype *Shigella* immunogens induced humoral immunity and protection against heterologous challenge in rabbit model

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ABSTRACT

Recently we have shown the homologous protective efficacy of heat killed multi-serotype *Shigella* (HKMS) immunogens in a guinea pig colitis model. In our present study, we have advanced our research by immunizing rabbits with a reduced number of oral doses and evaluating the host's adaptive immune responses. The duration of immunogenicity and subsequently protective efficacy was determined against wild type heterologous *Shigella* strains in a rabbit luminal model. After three successive oral immunizations with HKMS immunogens, serum and lymphocyte supernatant antibody titer against the heterologous shigellae were reciprocally increased and remained at an elevated level up to 180 days. Serogroup and serotype specific O-antigen of lipopolysaccharide and immunogenic proteins of heterologous challenge strains were detected by immunoblot assay. Up-regulation of IL-12p35, IFN-γ and IL-10 mRNA expression was detected in immunized rabbit peripheral blood mononuclear cells (PBMC) after stimulation with HKMS in vitro. HKMS-specific plasma cell response was confirmed by production of a relatively higher level of HKMS-specific IgG in immunized PBMC supernatant compared to control group. Furthermore, the immunized groups of rabbits exhibited complete protection against wild type heterologous shigellae challenge. Thus HKMS immunogens induced humoral and Th1-mediated adaptive immunity and provided complete protection in a rabbit model. These immunogens could be a broad spectrum non-living vaccine candidate for human use in the near future.

1. Introduction

Shigellosis is bacillary dysentery caused by shigellae. It is a major cause of infant morbidity and mortality, with about 165 million cases each year, predominantly in children under the age of 5 years. This leads to as many as 1.1 million deaths annually worldwide (Kweon, 2008; Kotloff et al., 1999; Levine et al., 2007; Pasetti et al., 2011). *Shigella* is an antigenically diverse pathogen with four serogroups namely *Shigella dysenteriae, Shigella flexneri, Shigella boydii and Shigella sonnei*, including 50 serotypes and subserotypes. Prevalent serogroups that cause shigellosis differ over time and geographical region (Pal, 1984; Dutta et al., 1989, 2002; Nair et al., 2010; Ghosh et al., 2011). Shigellosis is also a great clinical problem in other developing countries besides India, as well as developed countries, and shigellosis is at the top of the global priority prevention list of WHO (Lindberg et al., 1991). It is a highly contagious infection, capable of inducing human disease with only 100 microorganisms (Dupont et al., 1989).

Management of shigellosis includes improvement of sanitation, rehydration therapy (if dehydration occurs) and most essentially, antibiotic therapy. Shigellosis is starting to become to an untreatable disease due to the global emergence of multidrug resistance (MDR) (Hoge et al., 1998; Alanis, 2005). Improvement of sanitation is a major challenge in many Asian and African countries due to lack of resources. Therefore, developing anti-*Shigella* vaccines is currently the only foreseeable way of preventing shigellosis. Scientists, academicians, and public health experts are working to develop suitable therapies against shigellosis. Although several vaccine candidates have undergone or are currently undergoing clinical trials in several phases (Phalipon and Sansonetti, 2003; Girard et al., 2006;

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Levine, 2006), no licensed vaccine is available (Levine et al., 2007), except in China (Kweon, 2008). This is due largely to the diversity of *Shigella* spp, and a lack in knowledge of the nature of protective antigens and antigen delivery systems (Lindberg et al., 1991).

Lipopolysaccharide LPS is a major bacterial surface antigen, both in terms of the adaptive immune response and the innate immune response, and also a major virulence factor for *Shigella*. The great variability of the LPS terminal O polysaccharide chain results in the distinct *Shigella* serotypes (Strockbine and Maurelli, 2005). In fact, the protection provided by natural infection or vaccination is considered to be from O-serotype-specific antigen. The O-antigen of *S. flexneri* 2a, 3a and 6 cross reacts with another ten *S. flexneri* serotypes (Noriega et al., 1999; Formal et al., 1991). According to the global condition of shigellosis, a *Shigella* vaccine must protect against 16 serotypes and sub-serotypes, namely *S. dysenteriae* 1, *S. sonnei*, and all 14 classical *S. flexneri* types and sub-serotypes. Additionally, *S. boydii* infection is predominant in the Indian subcontinent. With these points in mind, we have formulated a heat-killed hexavalent prototype vaccine from a combination of six *Shigella* strains: *S. dysenteriae* 1 (NT4907Δstx), *S. flexneri* 2a (B294), *S. flexneri* 3a (C519), *S. flexneri* 6 (C347), *S. sonnei* (IDH00968) and *S. boydii* 4 (BCH612). Our recent laboratory study showed that these newly formulated hexavalent immunogens successfully induce a homologous protective response in guinea-pig rectocolitis model (Barman et al., 2013).

In this present study, we evaluated different immunological parameters, specially T cell response and antigen specific plasma cell response in immunized rabbits. We also studied protection against heterologous predominant circulating strains in a rabbit luminal model after three doses of oral immunization.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Invasive strains of *S. dysenteriae* 1 (NT4907, Δ*stx*), *S. flexneri* 2a (B294), *S. flexneri* 3a (C519), *S. flexneri*6 (C347), *S. sonnei* phase I (IDH00968) and *S. boydii* 4 (BCH612) were taken for HKMS immunogen preparation. *S. dysenteriae* 1 (HK811), *S. flexneri* 2a (2457T), *S. flexneri* 3a (NK3758), *S. flexneri* 6 (NK4025), *S. sonnei* (NK3918) or *S. boydii* type 2 (NK4023) were taken for challenge study. These strains were obtained from the National Institute of Cholera and Enteric Diseases (NICED) culture collection division. Invasiveness of these strains was confirmed phenotypically by Sereny test, invasion assay in a Caco2 cell line (Mitra et al., 2013) and in a guinea pig colitis model (Barman et al., 2011). The genotypic characterizations of all strains were observed through PCR based methods with the primers for virulence genes (Mitra et al., 2012). All strains were kept in 15% glycerol in brain heart infusion broth (Difco, USA) at −80 °C. Prior to experimentation, each strain was grown in tryptic soy broth (TSB; Difco, USA) at 37 °C under shaking conditions (100 rpm) or on plates in tryptic soy agar (TSA; Difco, NJ, USA)

2.2. Animals

New Zealand white rabbits of either sex weighing 2.0–2.5 kg were used for oral immunization by HKMS immunogen. All rabbits were collected from the animal resource department, National Institute of Cholera and Enteric Diseases, Kolkata. They were individually caged and maintained at 24 °C with 65% humidity and fed sterile food and water.

2.3. Ethics statement

All the animal experiments were conducted following the standard operating procedure as outlined by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India. The animal experimental protocol was approved by the Institutional Animal Ethical Committee of National Institute of Cholera and Enteric Diseases with Registration No. 68/1999/CPCSEA dated 11-03-1999 (Approval No.: Apro/77/24/11/2010, Ref. No. NICED/CPCSEA (AW) 215/2009–2015).

2.4. Preparation of HKMS immunogen

Overnight culture on TSA of either *Shigella* strains, *S. dysenteriae* 1 (NT4907Δstx), *S. flexneri* 2a (B294), *S. flexneri* 3a (C519), *S. flexneri* 6 (C347), *S. sonnei* (IDH00968) or *S. boydii* 4 (BCH612) were inoculated in 5 ml TSB and cultured overnight at 37 °C. Overnight broth cultures were spread separately on TSA and incubated at 37 °C overnight. The lawn generated by each bacterium was scraped off and suspended in phosphate buffered saline (PBS, pH 7.4) and centrifuged at 10,000 g for 10 min. The resulting pellet was washed twice and resuspended in PBS to a concentration of 10^6 cfu/ml by measuring OD at 600 nm. Each of the suspensions was heated at 70 °C for 1 hour in normal pressure, washed twice by centrifugation and resuspended in PBS. Non-viability of heat treated suspensions was checked by spreading 100 μl of the suspension on TSA plates. Equal volumes of the six suspensions were mixed thoroughly. The HKMS immunogen thus prepared was aliquoted into small vials. Vials were stored at −80 °C until used for oral immunization.

2.5. Oral immunization with HKMS immunogen

Eighteen New Zealand white rabbits were immunized with 1 ml (10^6 cfu) of HKMS immunogen and eighteen rabbits were given 1 ml of PBS on days 0, 14 and 28, according to the method of Sack et al., 1988. Briefly, rabbits were fasted for 36 h, but water was given *ad libitum*. 30 min before the oral immunization, each rabbit was anesthetized by intramuscular injection of a mixture of Ketamine (35 mg kg^{-1} body weight, Neon Laboratories Pvt. Ltd., India) and Xylazine (5 mg kg^{-1} body weight, Astra Zeneca Pharma India Ltd., India). Intravenous ranitidine (50 mg kg^{-1} body weight, Ranbaxy, India) was administered after 5 min, followed by two boluses of sodium bicarbonate (15 ml of a 5% solution, SRL, India) at 15 min intervals, introduced directly into the stomach through a feeding tube (Ramsons Sci. and Surg. Ind. Pvt. Ltd., India). The second bolus was immediately followed by oral administration of HKMS immunogen (1 ml, 10^6 cfu) for the immunized group of rabbits and the same volume of PBS for the non-immunized group. The rabbits were returned to the cage and given limited amounts of sterile food and water. Blood samples were collected on 0th, 7th, 14th, 21th, 28th, 35th, 56th, 63rd, 77th, 120th and 180th day of 1st oral immunization for the measurement of serum immunoglobulin and PBMC were isolated on 0th, 14th, 28th, 35th, 63rd, 120th and 180th day of 1st immunization from HKMS-immunized and non-immunized rabbits.

2.6. ELISA for serum immunoglobulin

Antibody titer in HKMS-immunized and non-immunized serum was measured using enzyme-linked immunosorbent assay (ELISA), as described previously by Mitra et al. 2013 against each heterologous challenge strains. Briefly, each well of a disposable polystyrene microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 μl of each of the challenge strain (10^9 live cells/ml) and incubated for 18 h at 4 °C. Control wells were coated with 100 μl of PBS (pH