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Longevity of duodenal and peripheral T-cell and humoral responses to live-attenuated *Salmonella* Typhi strain Ty21a

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ABSTRACT

Background: We have previously demonstrated that polyfunctional Ty21a-responsive CD4$^+$ and CD8$^+$ T cells are generated at the duodenal mucosa 18 days following vaccination with live-attenuated *S.* Typhi (Ty21a). The longevity of cellular responses has been assessed in peripheral blood, but persistence of duodenal responses is unknown.
Methods: We vaccinated eight healthy adults with Ty21a. Peripheral blood and duodenal samples were acquired after a median of 1.5 years (ranging from 1.1 to 3.7 years) following vaccination. Cellular responses were assessed in peripheral blood and at the duodenal mucosa by flow cytometry. Levels of IgG and IgA were also assessed in peripheral blood by enzyme-linked immunosorbent assay.
Results: No T-cell responses were observed at the duodenal mucosa, but CD4$^+$ T-cell responses to Ty21a and FliC were observed in peripheral blood. Peripheral anti-lipopolysaccharide IgG and IgA responses were also observed. Early immunoglobulin responses were not associated with the persistence of long-term cellular immune responses.
Conclusions: Early T-cell responses which we have previously observed at the duodenal mucosa 18 days following oral vaccination with Ty21a could not be detected at a median of 1.5 years. Peripheral responses were observed at this time. Immunoglobulin responses observed shortly after vaccination were not associated with cellular immune responses at 1.5 years, suggesting that the persistence of cellular immunity is not associated with the strength of the initial humoral response to vaccination.

1. Introduction

Salmonella enterica serovar Typhi (*S.* Typhi) is a facultative intracellular pathogen and the causative agent of typhoid fever. This bacterium, which is restricted to its human host, is spread via the faecal-oral route, and causes systemic illness following invasion via the mucosal surface of the small intestine [1]. A live-attenuated oral vaccine, designated Ty21a, was developed in the 1970s [2]. Vaccination with three doses of Ty21a is moderately protective, with a calculated cumulative efficacy of 48% between two and half and three years following vaccination [3]. It is estimated that 58% of all cases of disease in endemic regions occur in children under 5 years [4]. Although Ty21a has not been routinely administered in children, it has been demonstrated that when administered in liquid suspension, Ty21a is immunogenic in children aged between 2 and 6 years [5,6].

Ty21a is able to induce humoral and cellular immune responses, both of which have been implicated in protection against disease [7]. The peripheral humoral response to Ty21a has not previously been assessed beyond 42 days [8]; however, one novel live-attenuated oral vaccine candidate, CVD 909, has demonstrated the capacity to generate memory B cells which persist for at least one year [9]. Peripheral cellular responses targeting soluble *S.* Typhi flagella (FliC) as well as infected host cells have been assessed following vaccination with Ty21a, and data indicate that T cells responding to these antigens can persist for at least two years post-vaccination [10–12].

Recently, controlled human infection has demonstrated that polyfunctional CD8$^+$ T cells are associated with protection against

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disease when volunteers are challenged with approximately 10^3 CFU [13], but are associated with an increased susceptibility to disease when volunteers are challenged with approximately 10^4 CFU [14]. It has been suggested that higher dose inoculum generates stronger inflammatory responses than the lower dose inoculum and that exposure to this inflammatory environment may favour systemic dissemination [14]. Thus, polyfunctional $CD8^+$ T cells do appear to play a dominant role in protection against typhoid fever in humans. We have previously demonstrated that vaccination with Ty21a generates robust, polyfunctional $CD4^+$ and $CD8^+$ T-cell responses at the duodenal mucosa and in peripheral blood at day 18 [7]; however, whether early duodenal responses persist in the long-term has yet to be determined.

An increased understanding of the longevity of immune responses both at the intestinal mucosa and in peripheral blood may allow us to identify both early and late functional correlates of vaccine-mediated protection, which are currently unknown. Here, we have assessed humoral immunity in peripheral blood and cellular immunity at the duodenal mucosa and in peripheral blood approximately 1.5 years following oral vaccination with Ty21a, and compared responses with those observed in a control group. These data provide a unique insight into the longevity of human mucosal and peripheral immune defence.

2. Materials and methods

2.1. Ethical approval, recruitment, and study protocol

All volunteers provided written informed consent. This study was approved by the United Kingdom National Research Ethics Service (13/NW/0282). Eighteen healthy adult volunteers were enrolled into the study. Ten volunteers (5 males and 5 females; median age 24 years) were recruited to an unvaccinated control group. Eight volunteers (3 males and 5 females; median age 23.5 years) who had previously been vaccinated with live-attenuated *S.* Typhi (Ty21a; Vivotif®) as part of a previous study (10/H1005/20) were recalled (Table 1). During the previous study, volunteers were vaccinated with live-attenuated *S.* Typhi (Ty21a; Vivotif®), according to the manufacturer's instructions – a single oral capsule was taken on days 0, 2 and 4, approximately 1 h before a meal with a cold or lukewarm drink. Since we wished to assess the longevity of responses generated from the original vaccination, recalled volunteers were not revaccinated. Full details of the previous study (10/H1005/20) are presented in the Supplementary Materials and Methods.

2.2. Mucosal mononuclear cell (MMC) isolation

Mucosal samples were acquired approximately 1.5 years following vaccination. O_2 was administered nasally, and saturation was monitored throughout endoscopic biopsy. Sedation was offered to all volunteers; those who requested sedation were given up to 5 mg of midazolam intravenously. By use of large-capacity forceps (Boston Scientific), 12–15 single-bite mucosal biopsy specimens were acquired during flexible video-endoscopy from the duodenal mucosa at parts D2-D3 (n = 16) MMCs were isolated from biopsy specimens, using a modified version of a previously described method [15]. Full details are presented in the Supplementary Materials and Methods.

2.3. Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood samples were collected in lithium heparin Vacutainers (BD Biosciences) (n = 17) 6 days prior to mucosal sampling. PBMCs were isolated using Histopaque-1077™ (Sigma-Aldrich), according to the manufacturer's instructions. Full details are presented in the Supplementary Information Materials and Methods.

2.4. Antigenic stimulation and incubation

PBMCs (1×10^6 cells/well) and MMCs (approximately 1×10^6 cells/well) were seeded in complete medium in 96 well v-bottom plates. Cells in each well were stimulated with either 5×10^6 colony forming units (CFU) heat-killed *Salmonella* Typhi Ty21a (Vivotif; suspended in Dulbecco's PBS, quantified using the Miles and Misra technique, and killed by incubation at 95 °C for 30 min) or 10 ng FliC protein flagella. One positive control well was stimulated with 100 ng staphylococcal enterotoxin B (SEB; Sigma-Aldrich). One negative control well was left untreated to adjust for non-antigen-specific background cytokine production. Cells were then incubated at 37 °C in 5% CO_2. After 2 h, 1 μL brefeldin A (BD GolgiPlug; BD Biosciences) and 1 μL monensin (BD GolgiStop; BD Biosciences) was added to each well, and the plate incubated for a further 16 h at 37 °C in 5% CO_2.

2.5. Flow cytometric analyses

Following incubation, PBMCs and MMCs were washed, stained for viability and surface phenotype and, following fixation and permeabilisation, stained for intracellular cytokine production. Details of the antibodies that were used are presented in the Supplementary Materials and Methods. Cells were washed, resuspended and stored in the absence of light at 4 °C until data were acquired using a LSR II flow cytometer (BD Biosciences). Compensation beads (BD Biosciences) were used to create compensation matrices and sequential cell isolation used to identify populations of interest (Fig. 2). Full details are presented in the Supplementary Materials and Methods.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Each well in flat-bottomed 96-well microtitre plates (Nunc) was coated with 100 μL carbonate-bicarbonate buffer containing either 50 ng *S.* Typhi lipopolysaccharide (LPS; Sigma-Aldrich) and incubated at 4 °C overnight. Plates were washed 3 times with PBS-Tween. Plates were blocked with 1.0% bovine serum albumin and incubated for 2 h at room temperature. A standard was created using serum obtained from a convalescent patient with a diagnosis of typhoid. Volunteer samples were diluted 4 times across an optimised range for optimum comparison against the standard. Plates were washed, samples were added in duplicate and incubated at 4 °C overnight. For detection of immunoglobulin G (IgG), plates were washed and incubated with 1:4000 anti-human IgG-alkaline phosphatase (Sigma-Aldrich) for 2 h. For detection of immunoglobulin A (IgA), plates were washed and incubated with 1:4000 anti-human-IgA (AbD Serotec) for two hours; plates were washed again and then incubated with 1:2000 streptavidin to alkaline phosphotase (AbD Serotec) for 1 h. For detection of both IgG and IgA, plates were washed and incubated with 100 μL *p*-nitrophenyl phosphate (Sigma-Aldrich). Optical density was measured at 405 nm using a FLUOstar Omega ELISA plate reader (BMG Labtech).

2.7. Statistical analyses

Comparisons were made using paired and unpaired *t* tests based on 1000 bootstrapped samples, as indicated. Statistical analyses were performed using SPSS v22 (IBM). Differences were considered significantly different if bootstrapped confidence intervals did not cross zero.