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Research paper

C57-CD40 ligand deficient mice: A potential model for enterotoxigenic *Escherichia coli* (H10407) colonization

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) are a major cause of diarrheal disease in humans, calves and pigs. In humans, these infections mainly occur in developing countries leading to a high diarrheal morbidity and infant mortality and to travellers' diarrhea. ETEC strains constitute a phenotypically and genetically diverse pathotype with as common characteristics the production of heat-labile (LT) and/or heat-stable enterotoxins (ST) as well as of one or more fimbrial colonization factors. Despite the global importance of these pathogens, a broadly ETEC protective vaccine is not yet available, partially due to the lack of a suitable animal model for human ETEC. Such model would allow to test more ETEC molecules as potential vaccine candidates. The C57-CD40 ligand deficient (C57-*cd40l*$^{-/-}$) mouse has been successfully used to develop infection models of intestinal pathogens, but little is known about its humoral immune response. Therefore, the aims of this study were to characterize the humoral immune response of C57 and C57-*cd40l*$^{-/-}$ mice and to determine the persistence of ETEC H10407 and two of its variants after oral inoculation. The serum IgM, IgG and IgA and faecal IgG and IgA concentrations, of twelve mice per mouse strain (C57 and C57-*cd40l*$^{-/-}$), were determined by ELISA. All serum immunoglobulins and the faecal IgG concentration were significantly lower in C57-*cd40l*$^{-/-}$ than in C57 mice. In contrast the faecal IgA concentration was significantly higher in the C57-*cd40l*$^{-/-}$ mice. This high intestinal IgA concentration might be a compensatory T cell-independent production of IgA production. Both mouse strains were orally inoculated with 5×10^8 ETEC H10407 (LT$^+$, ST-colonization factor antigen I (CFA/I)$^+$) and ETEC in animal faeces was established by culture followed by *st* and *lt* loci identification by PCR until day 14 post infection. Most C57 mice eliminated the strain within 3 days whereas infection remained in C57-*cd40l*$^{-/-}$ mice until day 14. Subsequently both mouse strains were inoculated with ETEC H10407 variants and followed up until day 113. Likewise C57 mice eliminated both ETEC variants within 4 days. All C57-*cd40l*$^{-/-}$ mice had eliminated the LT$^-$ variant at day 31, whereas the ST-CFA/I$^-$ variant remained in mice stools until day 113. These observations suggest that C57-*cd40l*$^{-/-}$ mice are permissive for ETEC H10407 colonization.

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) strains constitute a phenotypically and genetically diverse pathotype that have the production of fimbrial colonization factors and of enterotoxin heat-labile toxin (LT) and/or heat-stable toxin(s) (ST) in common (Fleckenstein et al., 2010). ETEC

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infections are a significant cause of diarrheal disease not only in humans but also in calves and pigs (Nataro and Kaper, 1998). This heterogeneous group of pathogens is species-specific thus strains that produce disease in animals usually do not produce disease in humans and vice versa.

In humans, ETEC infections are a major cause of diarrheal morbidity and infant mortality in developing countries (Qadri et al., 2005), and are also considered the most common cause of travellers' diarrhea (Bouckenooghe et al., 2002). According to the World Health Organization (WHO), ETEC is responsible for 280 million to 400 million episodes of diarrhea and about 170,000–380,000 deaths annually (WHO, 2006, 2009). Moreover, ETEC infections in children from developing countries will lead them to malnutrition and will contribute substantially to their delayed growth; conversely malnourished children appear to be at higher risk of acquiring ETEC infections (Qadri et al., 2007; Petri et al., 2008). Several studies over the last 30 years have revealed that ETEC remains the most frequent agent associated with travellers' diarrhea, resulting in an estimated 10 million cases annually (Bouckenooghe et al., 2002; Jiang et al., 2002; WHO, 2009). Hence, ETEC is a high priority for vaccine development accordingly to WHO (2006).

Among ETEC strains, the acquisition of genes encoding LT and/or ST(s) is the essential and distinctive element of their molecular mechanisms of virulence (Turner et al., 2006). Therefore, the development of a successful pathogenic ETEC clone must involve the production, secretion, and effective deliver of their toxins. A common feature of ST and LT action is the activation of the cystic fibrosis transmembrane regulator (CFTR) chloride channel, which consequences are driving Cl^- and water secretion, as well as inhibition of NaCl absorption, leading to osmotic diarrhea (Sears and Kaper, 1996; Fleckenstein et al., 2010). However, the mechanisms by which these two toxins interact with the host cells are different and only LT induces an antibody response in vivo (Schulz et al., 1990; Chao et al., 1994; Sears and Kaper, 1996; Nataro and Kaper, 1998). The only other ETEC virulence factors that have been well characterized are a heterogeneous group of surface structures that among human isolates are referred to as colonization factors (CFs) (Evans et al., 1975). Therefore, LT toxin and CFs have been considered important targets for vaccine development (Qadri et al., 2005).

However, despite the global importance of this group of pathogens, a broadly protective vaccine is not yet available. A reason could be that much of ETEC pathogenesis remains to be uncovered such as the involvement of other virulence factors in addition to the enterotoxins and CFs. But also, due to the lack of a suitable animal model of a human ETEC isolate, to identify and test ETEC molecules as potential vaccine candidates. Recently, an experimental murine model with the prototype strain ETEC H10407 (O78:H11, LT^+, CFA-I^+ ST^+) has been developed (Allen et al., 2006; Roy et al., 2008). Although this model has been proven to be useful to test the capacity of a set of ETEC mutants in early colonization, unfortunately it employs antibiotics to eliminate local flora and has only been tested for colonization until 72 h. Nevertheless, until now there is not an animal model of ETEC colonization and persistence without previous antibiotic treatment. C57-CD40 ligand deficient (C57-*cd40l*$^{-/-}$) mouse has been successfully used to develop an animal model of *Cryptosporidium parvum* intestinal infection and persistence (Cosyns et al., 1998). Nevertheless, little is known about the humoral immune response of C57-*cd40l*$^{-/-}$ mice (Renshaw et al., 1994). Therefore, the aims of the present study were to characterize the humoral immune response of both C57and C57-*cd40l*$^{-/-}$ mice and to determine the persistence of ETEC H10407 and two ETEC H10407 variants after oral inoculation of C57 and C57-*cd40l*$^{-/-}$ mice.

2. Materials and methods

2.1. Mice strains

Two mice strains were used in this study, C57BL/6 and C57-CD40 ligand knock out, (C57-*cd40l*$^{-/-}$), which was first described by Renshaw et al. (1994). Both mice strains were originally purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Animals were bred and maintained under specific pathogen-free conditions. In all experiments male mice of 6–8 weeks old were used. These were housed in autoclaved microisolator cages with autoclaved bedding and autoclaved drinking water and food, which were given ad libitum. The cages were kept in an environmentally controlled room.

2.2. Faecal and blood sample collection for determination of immunoglobulin concentrations

Faecal pellets and blood samples were collected from mice, prior to inoculation. Serum was collected and aliquoted. Immunoglobulins (Ig) were extracted from faeces using the aprotinin (Sigma–Aldrich) – 1,1,2-trichlorotrifluoroethane (Riedel-deHaen–Sigma–Aldrich) method (Winsor et al., 1988). Serum and faecal extracts aliquots were frozen at −20 °C until tested.

Total IgM, IgG and IgA concentrations were determined in the sera and the faecal extracts by comparing the values of test sample dilution series in ELISA with isotype-specific control standard curves (Cappel 50335, Jackson Immunoresearch 015-000-003, and Cappel 50325 for the IgM, IgG and IgA standard curve, respectively) as previously described (Renshaw et al., 1994). Briefly individual wells of flat bottom ELISA plates (Corning inc. Costar 3590) were coated with 60 μl of capture antibody in a final concentration of 1.0 μg/ml in carbonate–bicarbonate buffer, pH 9.6 (Jackson Immunoresearch 715-005-140, ZYMED 61-6400 and Southern Biotech 1165-01 antibodies were used for capturing IgM, IgG, and IgA, respectively) and incubated overnight at 4 °C. Then plates were washed with 0.1% v/v Tween®20 in phosphate-buffered saline (PBS-T) and subsequently blocked with 1% skimmed milk and 0.1% BSA in PBS-T (blocking solution) for 1 h at 37 °C. Then 60 μl of serum samples (1:500 and 1:1000) and faecal extracts (1:4 and 1:8) both specimens diluted in blocking solution were added to each well for 1 h at 37 °C, followed by washing and incubation with the appropriate horseradish peroxidase conjugated secondary antibodies 1:1000 diluted in blocking solution (IgM Pierce, 31440, IgG Invitrogen, G21040,