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Research paper

Simultaneous inactivation of *espB* and *tir* abrogates the strong, but non-protective, inflammatory response induced by EPEC

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) belong to the attaching and effacing (A/E) family of bacterial pathogens that represent a worldwide health concern. These non-invasive bacteria attach to intestinal enterocytes through a type III secretion system (T3SS), leading to intestinal inflammation and severe diarrhea. To dissect the signals leading to the induction of the inflammatory response and to understand its role in the pathogenesis of infection, we used the rabbit model, which represents a close model of human infections. Rabbits were orally inoculated with either the wild type O103:K-:H2 E22 EPEC strain or with the E22Δ*tir*/*espB* strain, which bears mutations in two genes involved in the injectisome structure and function. To monitor the development of the inflammatory response, we developed a quantitative real-time RT-PCR (qPCR) assay specific for a panel of rabbit genes. Using combined immunohistochemistry and qPCR, we show here that the inflammatory response triggered by wild type EPEC occurs very early, preceding the bacterial colonization of the epithelium. However, this early response is unable to prevent bacterial attachment on enterocytes. Moreover, our results show that expression of a complete bacterial injectisome is required for the development of inflammation. Finally, infection by the virulent strain, but not by the doubly mutated strain, rapidly induces the development of a specific immune response in the mesenteric lymph nodes, which is not associated with protection. Our findings suggest that the induction of a strong inflammatory response by T3SS dependent components represents a selective advantage for T3SS+ bacteria, thereby facilitating their colonization.

1. Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a well-established cause of severe diarrhea in young children mainly in developing countries (Camguilhem and Milon, 1989; Donnenberg and Kaper, 1992; Nataro and Kaper, 1998). Numerous EPEC strains are also pathogenic for several animal species, in which they induce diarrhea and mortality (China et al., 1998; Pearson et al., 1989; Peeters et al., 1984; Robins-Browne et al., 1994; Zhu et al., 1994). Among them, rabbits are particularly sensitive to EPEC infections (Blanco et al., 1996; Camguilhem and Milon, 1989). Altogether, this pathovar represents a worldwide public health challenge.

Since EPEC strains isolated from humans do not cause diarrhea in animals, there is no good animal model to study human infections. However, virulence mechanisms and resulting pathology observed during natural infections

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; A/E, attaching/effacing; T3SS, type III secretion system; qPCR, quantitative reverse transcription PCR; MLN, mesenteric lymph node; d.p.i., day post-infection.

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of rabbits with O103:K-:H2 EPEC strains are very similar to that of human EPEC infection (De Rycke et al., 1997; Levine, 1987; McDaniel et al., 1995; Robins-Browne et al., 1994). Thus, rabbit infections represent a valuable animal model for the study of human EPEC infections (Milon et al., 1999; Nataro and Kaper, 1998).

EPEC infection is associated with a typical "attaching and effacing" (A/E) lesion of enterocytes, characterized by the loss of the enterocyte microvilli and intimate adherence of the bacteria (Finlay et al., 1992; Knutton et al., 1989). The virulence factors and the mechanisms leading to the A/E lesion formation are well described. The first step of interaction between bacteria and intestinal epithelial cells is mediated by specific adhesins (Krejany et al., 2000; Milon et al., 1990; Pillien et al., 1996). Following this loose adhesion, EPEC binds closely to the cells via another adhesin, intimin (Jerse and Kaper, 1991). Intimin mediates a close contact between the bacteria and the target cell upon interaction with its translocated receptor Tir (for Translocated Intimin Receptor) (Deibel et al., 1998; Kenny et al., 1997). This intimate attachment is associated with the A/E lesion of enterocytes (Finlay et al., 1992; Knutton et al., 1989). A chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) contains all the genes necessary for A/E lesion formation (McDaniel et al., 1995) and encodes proteins with a wide range of functions, including a type III secretion system (T3SS), intimin (Jerse et al., 1990), Esp proteins (Donnenberg et al., 1997; Elliott et al., 1998) and Tir. The bacterial proteins translocated into cell cytoplasm interfere with normal cell metabolism (Savkovic et al., 1997; Taylor et al., 1999) and are required for the formation of the A/E lesion (Abe et al., 1998; Frankel et al., 1998; Rosenshine et al., 1996).

Although it is well known that the inflammatory response is one of the main pathogenic features induced by EPEC infections (Heczko et al., 2000; Moon et al., 1983; Tzipori et al., 1989), the innate or specific immune responses to EPEC are still poorly described. The relationship between the mucosal immune response and pathogenic mechanisms forms a delicate balance that determines the outcome of infection. For example, it has been shown in mice that intestinal IL-6 production is protective against *Yersinia enterocolitica* and *Citrobacter rodentium* infections (Dube et al., 2004; Dann et al., 2008) while during Salmonellosis, the production of IL-1β, another pro-inflammatory cytokine, favors the dissemination of bacteria by macrophages from the gut to systemic tissues (Guiney, 2005).

It is known that EPEC infection induces a strong infiltration of neutrophils in the lamina propria, associated with their diapedesis into the intestinal lumen (Moon et al., 1983; Tzipori et al., 1989). These results have been linked with *in vitro* studies showing IL-8 production (a major chemokine responsible for the recruitment of neutrophils) by epithelial cells after EPEC infection (Klapproth et al., 1995, 1996; Savkovic et al., 1996; Schuller et al., 2009). Recently, an *in vivo* study showed that a large panel of pro-inflammatory cytokines is produced by enterocytes after a rabbit EPEC infection (Ramirez et al., 2005). However, no study has simultaneously analyzed the nature, the kinetics and the role of the inflammatory response induced locally and at the systemic level following an EPEC infection.

In this study, we have used the rabbit model of colibacillosis to determine the profile of the inflammatory response, associated with the subsets of leucocytes recruited into the mucosa at different time points after the infection. We further characterized the nature of the specific immune response induced in mesenteric lymph nodes (MLN). In addition, to determine the concomitant role of two bacterial effector proteins associated with the injectisome, EspB and Tir, in the inflammatory response, rabbits were orally inoculated either with the virulent E22 O103:K-:H2 EPEC strain or with an attenuated strain E22Δ*tir*/*espB*, mutated in both *espB* and *tir* virulence genes. This attenuated strain exhibits a very promising potential to be used as a vaccine strain (Boullier et al., 2003a). Our studies were facilitated by the development of a real-time PCR (qPCR) assay allowing quantifying transcripts associated with the innate and the specific immune responses in rabbit species (Boullier et al., 2009).

2. Materials and methods

2.1. Bacterial strains

An enteropathogenic *E. coli* belonging to O103:H2:K- serotype (strain E22) was used (Camguilhem and Milon, 1989). E22Δ*tir*/*espB* is a vaccine strain obtained in the laboratory (Boullier et al., 2003a). BM21 is a prototropic laboratory strain (Boullier et al., 2003a). REPEC strains and BM21 were cultured overnight (O.N.) at 37 °C under shaking in Luria–Bertani (LB) broth with kanamycin, 50 μg ml^{-1} and chloramphenicol, 25 μg ml^{-1} for E22Δ*tir*/*espB* and with nalidixic acid, 50 μg ml^{-1} for BM21.

2.2. Experimental infections and tissue sampling

Thirty two-day-old New Zealand white weaned rabbits were used for experimental infections. Rabbits were housed in cages of three animals and fed daily with antibiotic-free commercial feed supplemented with a coccidiostatic agent (Robenidine). Water was ad libitum. Rabbits were divided in 3 groups. Two groups of 9 rabbits were respectively inoculated *per os* with E22Δ*tir*/*espB* and BM21 and a group of 12 rabbits was inoculated *per os* with E22 (2×10^4 CFU each, corresponding to 1 LD_{50} for the w-t strain E22). Clinical examination was done daily. At different time points post-infection (day 1 for E22 group only, day 2, day 4 and day 7), 3 rabbits of each group were sacrificed by intravenous injection of ketamine and sodium-barbital in accordance with the European council directive 86/609. Tissues from the distal ileum (10 cm from ileo-caecal junction) and the mesenteric lymph nodes (MLN) were removed immediately after euthanasia. Samples were split in two. One half was immediately frozen in 1 ml of Trizol (Invitrogen, CA) and kept at −80 °C until use. The other half was fixed in 10% formaldehyde and embedded in paraffin.