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The effect of intestinal colonization of germ-free pigs with *Escherichia coli* on calprotectin levels in plasma, intestinal and bronchoalveolar lavages

Igor Šplíchal[a,*], Magne K. Fagerhol[b], Ilja Trebichavský[a], Alla Šplíchalová[a], Jürgen Schulze[c]

[a]*Division of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Doly 183, 54922 Nový Hrádek, Czech Republic*
[b]*Department of Immunology and Transfusion Medicine, Ullevaal University Hospital, Oslo, Norway*
[c]*Ardeypharm GmbH, Herdecke, Germany*

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Abstract

Calprotectin levels were measured by ELISA in plasma, terminal small bowel lavage and bronchoalveolar lavage from 8-day-old germ-free piglets or gnotobiotic piglets 24 h after colonization with one of the following *Escherichia coli* strains: non-pathogenic O86, probiotic Nissle 1917 or enteropathogenic O55. The concentration of calprotectin in plasma was about 30 ng/ml only in germ-free piglets and piglets associated with non-pathogenic *E. coli*. Piglets infected with O55 showed a significant increase of plasma calprotectin and the highest mean level of calprotectin in the bronchoalveolar lavage, which was coincident with septicaemia. However, in the lumen of the small intestine, *E. coli* Nissle 1917 alone elicited a significant increase of the calprotectin level which was confirmed by immunofluorescence and APAAP immunohistochemistry on cryostat sections through the small bowel. The relevance of this finding to the therapeutic effect of *E. coli* Nissle 1917 in inflammatory bowel disease is discussed.

Keywords: Calprotectin; Probiotics; Inflammatory bowel disease

Introduction

At birth the gut is sterile and provides a fertile soil for bacteria acquired from the environment. Within 3 h after birth, a small microbial population occurs in the gut. In most animals, the initial colonizing microorganisms are *Escherichia coli*, streptococci, clostridia and lactobacilli (Savage, 1977). *E. coli* colonizing the intestine belong to microbes that provide antigens and thus stimulate the gut-associated lymphatic tissues and the immune system in general. Several *E. coli* strains have been tested as probiotics. Among them, *E. coli* O83:K24:H31 (Colinfant Newborn vaccinal strain,

Abbreviations: APAAP, alkaline phosphatase–anti-alkaline phosphatase; Bal, bronchoalveolar lavage; CFU, colony forming unit; DPBS, Dulbecco's phosphate buffered saline; DS, diluting solution; EcN, *Escherichia coli* strain Nissle 1917; Eco55, *Escherichia coli* strain O55; EcO86, *Escherichia coli* strain O86; EPEC, enteropathogenic *Escherichia coli*; GF, germ-free; mAb, monoclonal antibody; PBSS, phosphate buffered saline with saponin; RT, room temperature; WS, washing solution

*Corresponding author. Tel.: +420 491 478 216; fax: +420 491 478 264.
E-mail address: splichal@biomed.cas.cz (I. Šplíchal).

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Dyntec, Terezín, Czech Rep.) is used for preventive colonization of infants (Lodinova-Zadnikova et al., 1998), and the *E. coli* strain Nissle 1917 (O6:K5:H1; Mutaflor®, Ardeypharm GmbH, Herdecke, Germany) is successfully used for the treatment of various diseases of the digestive tract, especially for maintenance therapy in patients with ulcerative colitis in the state of remission (Bruckschen and Horosiewicz, 1994; Floch, 2003; Fric and Zavoral, 2003; Kruis et al., 1997; Kruis et al., 2004; Kuzela et al., 2001; Malchow, 1997; Mollenbrink and Bruckschen, 1994; Rembacken et al., 1999; Tromm et al., 2004).

Bacterial lipopolysaccharide stimulates mechanisms of natural immunity. It also induces the release of calprotectin (Johne et al., 1997; Kido et al., 2003), a calcium- and zinc-binding protein belonging to the S100 family. Calprotectin is produced predominantly by neutrophils, with an estimated cytosolic concentration of 5–15 mg/ml, constituting about 60% of the cytosolic protein. It is a heterotrimer consisting of one light (8 kDa) and two heavy (14 kDa) chains. The names MRP-8/MRP-14 (migration-inhibitory factor-related proteins), cystic fibrosis antigen, L1 antigen, calgranulin A/B, and S100A8/S100A9 have all been used for calprotectin (Striz and Trebichavsky, 2004). The calprotectin concentration in plasma and other biological fluids or excretions is markedly increased during bacterial infections and inflammation in the relevant organs. High levels in feces have become a useful non-invasive marker of inflammatory bowel disease and intestinal tumours (Bjarnason and Sherwood, 2001; Fagerhol, 2000; Poullis et al., 2003; Roseth, 2003; Roseth et al., 1992). It exerts antimicrobial effects primarily through competition for zinc, and by preventing bacteria from binding to mucosal epithelial cells (Nisapakultorn et al., 2001). Presumably, it can protect against bacterial infections of the gut.

Here, we report for the first time measuring of calprotectin levels in germ-free state and describe changes in calprotectin levels after infection with *E. coli* strains of different virulence.

Materials and methods

Animals

Colostrum-deprived germ-free piglets were obtained by hysterectomy of gilts under halothane anesthesia on the 112th day of gestation. Thirty-six piglets were reared in sterile positive-pressure fibreglass isolators and fed an autoclave-sterilized milk diet with mineral and vitamin supplements (Mandel, 2004). They were checked for absence of bacterial contamination by culturing rectal swabs aerobically and anaerobically and by staining methods.

Bacteria for colonization

E. coli O86 (EcO86) is a normal intestinal non-pathogenic wild-type strain in the pig. It does not produce enterotoxins or cytotoxins and is neither enteroinvasive nor uropathogenic. *E. coli* O86 showed cross-reactivity with human red blood cell antigens and was originally used to study the formation of hemagglutinins (Tlaskalova-Hogenova et al., 1970). The immune response of gnotobiotic piglets against this strain has been recently described (Cukrowska et al., 2001).

E. coli Nissle 1917 (EcN; serotype O6:K5:H1) is a probiotic strain and the active substance in the pharmaceutic preparation Mutaflor® which shows an antagonistic effect against various enteropathogenic bacteria in vitro (Sonnenborn and Greinwald, 1991) and in vivo (Schulze et al., 1992). This strain produces neither toxins nor mannose-resistant hemagglutinating "adhesins". It secretes, however, six different iron-binding substances (siderophores), two microcins (Patzer et al., 2003) and expresses common fimbrial adhesins: F1A (type 1), F1C and curli fimbriae (Blum et al., 1995; Blum-Oehler et al., 2003). All these factors contribute to the probiotic properties of this microbe. Nevertheless, it is serum-sensitive and considered non-virulent. A single nucleotide exchange in the *wzy* gene is responsible for the serum sensitivity (Grozdanov et al., 2002).

E. coli O55 (EcO55) is an enteropathogenic strain (EPEC) causing diarrhea both in infants and in young piglets. EPEC produce specific attaching-and-effacing (A/E) lesions on gut enterocytes characterized by intimate bacterial adhesion, reorganization of host cytoskeletal proteins into pedestal-like structures beneath the adherent bacteria, and destruction of the brush border microvilli. In germ-free pigs, O55 causes septicemia leading to death within a few days. Strains belonging to the O55 serogroup show tissue tropism and restricted adhesion to follicle-associated epithelium of Peyer's patches, involving A/E activity, with no apparent adhesion to the duodenum or colon (Fitzhenry et al., 2002).

Colonization of germ-free piglets

Germ-free pigs, 7-day-old, were inoculated in breeding isolators via a stomach tube with 5 ml of milk diet containing 10^8 CFU bacteria, freshly cultivated for 24 h on sloped meat-peptone agar (blood agar base No. 2, Immuna, Šarišské Michal'any, Slovakia). The control group of uninfected germ-free piglets received the same