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Histological Alterations and Immune Response Induced by Pet Toxin During Colonization with Enteroaggregative *Escherichia coli* (EAEC) in a Mouse Model Infection

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Enteroaggregative *E. coli* (EAEC) is an important aethiological causal agent of diarrhea in people of developed and undeveloped countries. Different *in vitro* and *in vivo* models have been proposed to study the pathogenic and immune mechanisms of EAEC infection. The aim of this study was to analyze whether BALB/c mice could be used as an animal model to study EAEC pathogenesis. Six-week-old BALB/c mice were inoculated with EAEC strain 042 (O44:H18) nalidixic acid resistant, and re-inoculated ten days after. Mice feces were monitored for the presence of the EAEC strain over a period of 20 days. Bacteria were enumerated on MacConkey agar containing 100 μg of nalidixic acid per ml. Results showed that 35% of the animals were colonized for 3 days, 15% for 5 and 10% for more than 7 days. After re-inoculation only 16% of the animals remained colonized for more than 3 days. During the necropsy, the intestinal fluid of some of the infected animals presented mucus and blood. Six of these fluids showed the presence of IgA antibodies against Pet toxin and IgG antibodies raised against the toxin were also detected in the animal serum. Histopathologic evidence confirms the stimulation of mucus hypersecretion, an increased amount of goblet cells and the presence of bacterial aggregates in the apical surfaces of intestinal epithelial cells. Edema was present in the submucosa. These results suggest that BALB/c mice could be used as an animal model for the *in vivo* study of EAEC infection.

Key words: enteroaggregative *Escherichia coli*, Pet toxin, mucosal immunity, animal models

Enteroaggregative *Escherichia coli* (EAEC) is an important cause of persistent diarrhea among children in the developing world (Nataro *et al.*, 1998; Pai *et al.*, 1997; Sang *et al.*, 1997) and as the causative organism in a number of outbreaks in developed parts of the world (Morabito *et al.*, 1998; Smith *et al*, 1997). It has also been identified in patients with AIDS (Mayer and Wanke, 1995; Polotsky *et al.*, 1997; Wanke *et al.*, 1998). The enteric pathogen has been defined by its distinctive aggregative or "stacked-brick" pattern of adherence to cultured human epithelial cells (Nataro *et al.*, 1987).

The pathogenesis of EAEC infection is not completely understood. Several *in vitro* and *in vivo* models have been used and some features have been defined. Formation of a thick mucus gel on the intestinal epithelium mucosa has been observed in gnotobiotic piglets inoculated with EAEC (Tzipori *et al.*, 1992), as well as a bacteria biofilm on the intestinal surface and exfoliation of enterocytes using *in vitro* organ cultures (Hicks *et al.*, 1996). Using rabbit and rat ileal loop models inoculated with EAEC strains, the lesions were characterized by shortening of the villi, hemorrhagic necrosis of the villus tip and a mild inflammatory response with edema and mononuclear infiltration of the submucosa (Vial *et al.*, 1988). Similar histological alterations have been observed in autopsy samples of the ileum from children who died as a consequence of persistent diarrhea associated with EAEC infection (Eslava *et al.*, 1993), as well as in rat jejunal preparation mounted in Ussing chambers and treated with supernatant from EAEC (Navarro-Garcia *et al.*, 1998) suggesting that some of the alterations caused during EAEC infection could be associated with the production of a cytotoxin. Eslava *et al.* (Eslava *et al.*, 1993) also identified two high molecular proteins isolated from a strain of

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EAEC. The gene of one high molecular protein named Pet is located on the 65 MDa EAEC virulence plasmid and was observed to have enterotoxic activity (Eslava *et al.*, 1998; Navarro-Garcia *et al.*, 1998). It was cloned and showed a high homology with type V class autotransporter secreted proteins (Eslava *et al.*, 1998) and its product has shown that it induces cytopathic effects on HEp-2 and $HT29C_1$ culture cells, characterized by the release of the cellular focal contact from glass substratum, rounding and detachment of cells, as well as observation of cytoskeleton contraction and loss of actin stress fibers (Navarro-Garcia *et al.*, 1999).

The aim of this work was to investigate if BALB/c mice could be used as a colonization model by EAEC strains and also, for the study of the immune response and histopathologic changes in the intestinal epithelium of the animals.

Material and Methods

Bacteria

EAEC strain 042 (O44:H18) which has been shown to cause diarrhea in adult volunteers (Nataro *et al.*, 1995), is nalidixic acid resistant (Sainz, 2000), and HB101 strain kanamycin resistant (used as a negative colonization control) were grown in Luria-Bertani broth (LB) at 37°C overnight without shaking prior to infection. Cells were pelleted by centrifugation at 2400× g, washed twice and suspended in phosphate-buffered saline, pH 7.4 (PBS) to give a final viable count of 10^9/ml (infection dose).

Animals

Six-week-old male mice of the BALB/c strain were used in all experiments, and were maintained with sterilized water, food and sawdust before and during the experimentation.

Experimental design

Twenty BALB/c mice (per strain) were inoculated intragastrically (0.2 ml) with each of the two strains by using a 1 ml tuberculin syringe fitted with a 20-gauge needle. Mice were closely observed to ensure that they did not regurgitate or aspirate the inoculum. Five mice were inoculated with 5% (w/v) filter-sterilized sodium bicarbonate (0.2 ml) and used for control. On 10^{th} day, animals were re-inoculated using the same conditions.

Mice feces were monitored for the presence of the test strains over a period of 20 days. For specific enumeration and identification, samples of freshly passed feces were emulsified in sterile PBS and spread on MacConkey plates (Oxoid) containing 100 μg of nalidixic acid, or 50 μg of kanamycin per ml and incubated overnight at 37°C. The identity of colonies resembling *E. coli* was confirmed by agglutination with hyperimmune specific rabbit antisera (SERUNAM). Animals were sacrificed when diarrhea occurred or up to 20 days after infection.

Intestinal fluid and sera collection

Intestinal fluid was obtained from the small intestine of mice by flushing out the intestinal contents with 2 ml of cold PBS. Intestinal fluid was centrifuged at 3000× g and 4°C for 15 min and supernatant was added with EDTA 50 mM (final concentration) and phenylmethylsulfonyl fluoride (PMSF) 1 mM (final conc.) and stored at -70°C until needed. Sera were prepared from clotted blood obtained from an axillar vein and stored at -70°C.

Protein purification

Pet protein was obtained as described by Villaseca *et al.* (Villaseca *et al.*, 2000). Briefly, a culture supernatant of *pet* clone (*E. coli* HB101 pCEFN 1) was precipitated with 75% ammonium sulfate, and further precipitated with 1.15 and 1.75 M potassium phosphate buffer, eluted from Q-Sepharose columns and then from fast-protein liquid chromatography (FPLC) Mono S HR 5/5 columns. The protein concentration was determined by the Bradford method (Bradford, 1976).

Western immunoblots for antibodies detection

Western immunoblots were performed with the purified protein. Samples containing 100 μg of total protein were separated by sodium dodecyl sulphide (SDS)-10% polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1971), and the protein band obtained was transferred to 0.45μm nitrocellulose membranes (Millipore Co., Bedford, Mass. Cat. No. HAHY304F0) as described by Towbin *et al.* (Towbin *et al.*, 1979). The membranes were blocked by incubation for 1 h at room temperature with PBS and 5% low-fat milk and then washed three times with PBS-0.1% Tween 20. Mice intestinal fluids and sera were then added to each membrane using a Mini-Protean II Multiscreen Apparatus (BIO-RAD) and incubated overnight at 4°C. IgA-bound antibodies or IgG-bound antibodies were detected using a 1:1,000 dilution of horseradish peroxidase conjugated goat anti-mouse IgA or goat anti-mouse IgG (Kirkegard & Perry Lab., Gaithersburg, Md.) and developed using chemiluminescent reagent (Du Pont NEN). Positive control lanes in the membranes were incubated overnight with rabbit antisera against Pet enteroaggregative *E coli* toxin as described by Eslava *et al.* (Eslava *et al.*, 1998) diluted 1:100. To visualize the reaction, membranes were incubated with goat-anti-rabbit antibodies conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaitherburg, Md.) and developed.

Tissue preparation for light microscopy

Samples from mice ileum and colons were fixed in 10% neutral buffered formalin (pH 7.3) and embedded in paraffin wax. Serial 7 μm-thick sections were cut onto glass slides and stained with hematoxylin and eosin, silver stain

(modified Warthin-Starry method) and periodic acid leucofuchin (PAS) methods (Staining Procedures used by the Biological Stain Commission, 1960).

Results

Intestinal colonization by E. coli strains

BALB/c strain mice were assessed for their susceptibility to intestinal colonization by HB101 and EAEC strains. Following intragastric inoculation of EAEC, the intestines of the mice became colonized, as evidenced by fecal shedding of the pathogen. None of the animals studied carried kanamycin or nalidixic acid resistant coliform bacteria at the start of the experiment.

Quantitative cultures of mice feces revealed that EAEC strain was excreted and 10% of the animals became colonized for 7 days, 15% for 5 days and 35% for three days. For the purposes of this study, colonization was defined as isolation of the test strains from feces on at least two separate occasions more than 48 h after inoculation. Concentration of bacteria varied from a mean of 10^6 CFU/g on the first day to 10^3 CFU/g on the last.

After re-inoculation, animals feces' were monitored for ten days and results showed that 11.1% of the animals were colonized for 4 days, 16.7% for 3 days and 55.6% for 2 days and the mean values for bacterial concentration were the same as for the first inoculation period.

None of the animals inoculated with the negative control strain (HB101) were colonized; no bacteria was recovered 24 h after inoculation.

Two of the twenty infected mice developed diarrhea and lost weight during the infection period and none during the re-inoculation period.

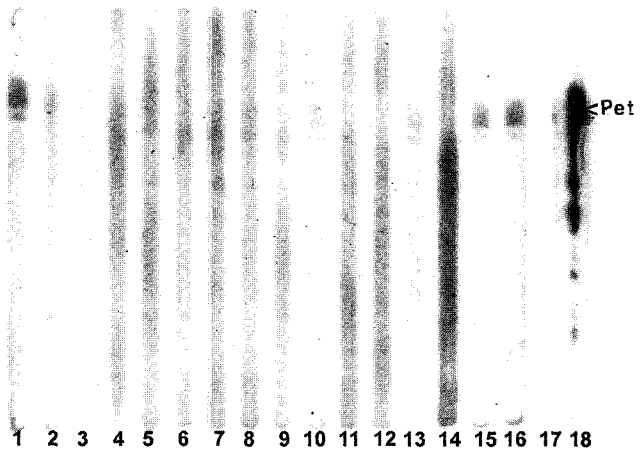

Fig. 1. Western blot analysis of intestinal fluids obtained from mice colonized with EAEC strain 042. Detection of IgA antibodies raised against Pet toxin in six animals (lanes 1,2,10, 13, 15 and 16). Lanes 5 and 14 correspond to intestinal fluids of non-infected mice (controls). Lane 18 corresponds to positive control.

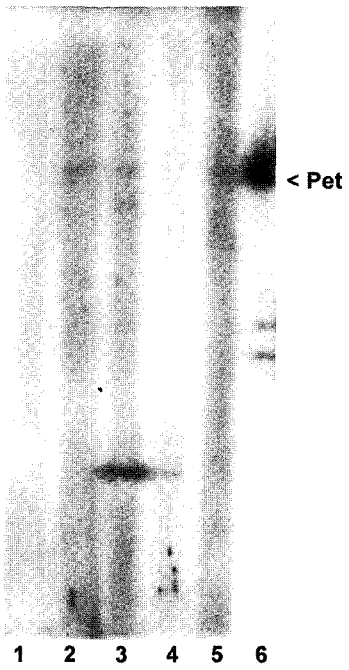

Fig. 2. Western blot analysis of sera obtained from mice colonized with EAEC strain 042. Detection of IgG antibodies raised against Pet toxin. The intensity of the response was variable, a light response was noticed in some mice (line 1) and more detectable in other animals (Lanes 2,3,5). Lane 4 corresponds to sera sample from a non-infected mouse (control). Lane 6 corresponds to the positive control using Pet toxin and rabbit specific antibodies.

Antibody response against Pet toxin

Intestinal fluids and sera of the animals were analyzed for the presence of IgA and IgG antibodies against Pet toxin. Western blot analysis using intestinal fluids of 14 re-infected mice showed that six animals raised IgA antibodies against Pet toxin secreted by this EAEC strain (Fig. 1).

Sera analysis revealed that IgG antibodies were raised against Pet toxin (Fig. 2) in the animals challenged with EAEC strain. There was a correlation in the production of intestinal IgA and serum IgG antibodies in three of the studied animals.

Histological alterations induced by EAEC strain 042 on intestinal samples

Ileum mucosal samples obtained from the gut of negative control mice, and mice inoculated with *E. coli* HB101 and with 042 strains were examined. The staining methods used to characterize the histopathologic changes in the gut samples showed normal morphology of intestinal mucosa in ileum samples of the negative control mice (Fig.3 A, B, C) and mice inoculated with *E. coli* HB101 strain (Fig. 4 A, B, C).

Consistent with previous reports, EAEC strains enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm. Ileum samples of

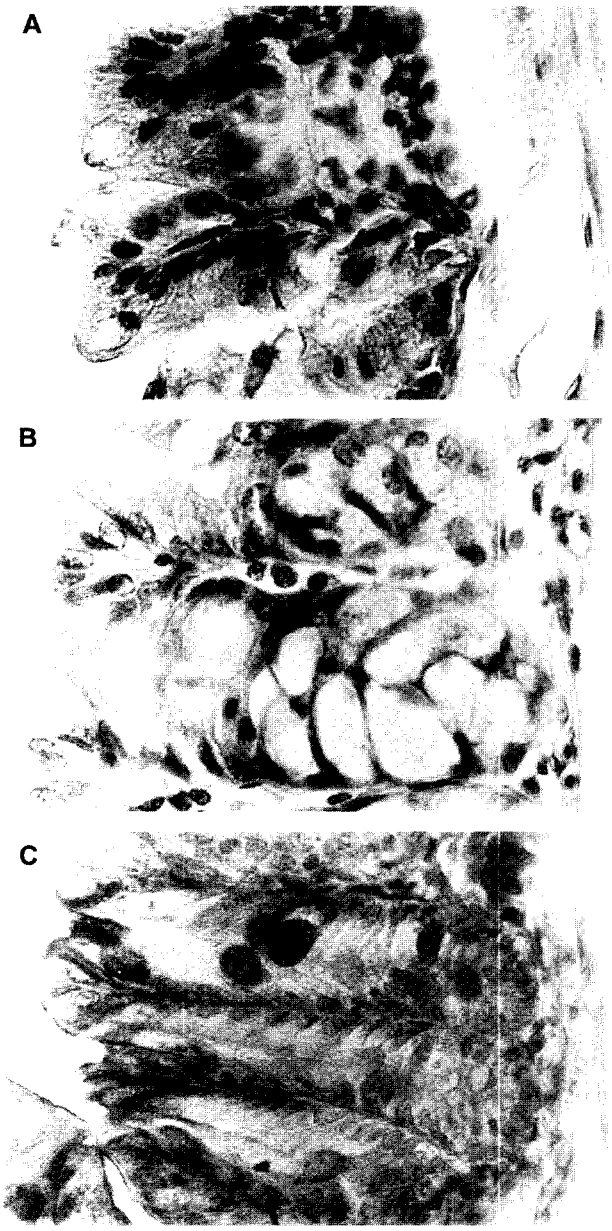

Fig. 3. Transverse cut of ileum from negative control mouse. (A): The epithelial and goblet cells morphology appear normal, the apical surface with increased staining density corresponds to the glycocalyx, nuclei are defined by condensed chromatin, the lamina propria shows a regular proportion of cellular nuclei in the apical glandular region as well as in the transverse cut in relation to the submucosa, separated by the muscularis mucosae. (Hematoxylin and eosin stain. Magnification, x650). (B): No bacterial aggregates using the Warthin-Starry stain procedure were observed. (Magnification x1300). (C): Mac Manus (modified PAS) stain was used to identify mucopolisacharides located in the glandular portion of the apical surface of enterocytes, as well as inside the goblet cells. (Magnification x1300).

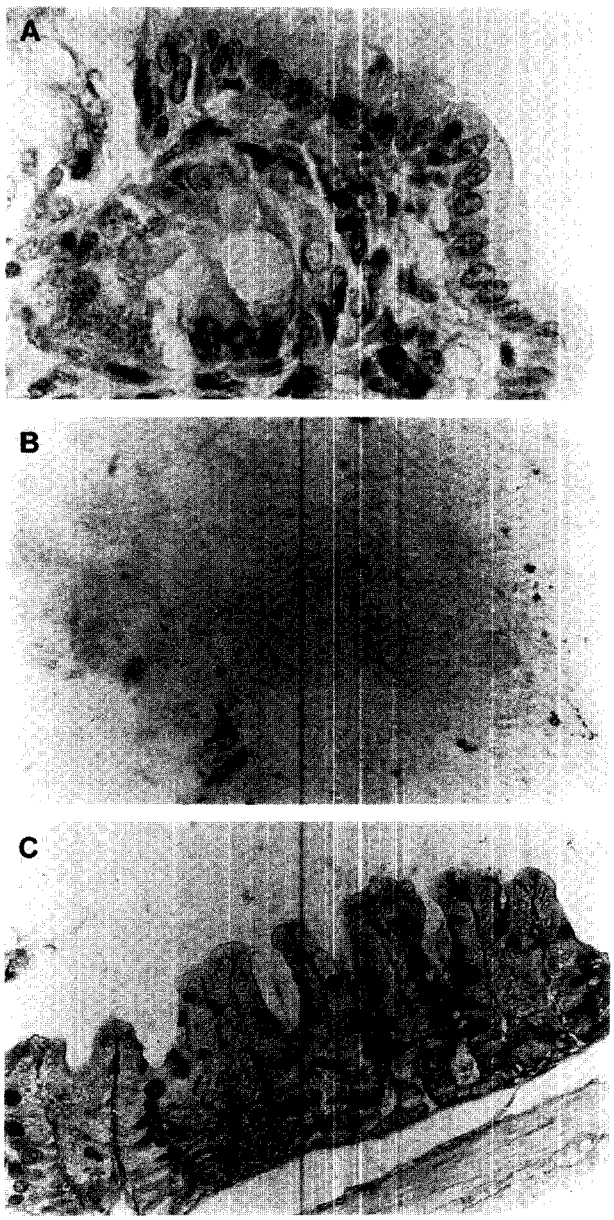

Fig. 4. Ileum transverse cut of mouse inoculated with *E. coli* HB101 strain. (A): light changes in staining density are observed in the glycocalyx at the apical surface and the glandular region without affecting the epithelial continuity and the nuclei, the lamina propria appears like in Fig. 3A (Hematoxylin and eosin stain. Magnification, x1300). (B): a few bacterial aggregates are located on the apical surface of enterocytes (Warthin-Starry stain procedure. Magnification x1300). (C): polysaccharides appear stained within the goblet cells and epithelial cells of some mucosal glands but no significant changes are observed in the cell number or amount of mucus secretion (Mac Manus stain. Magnification x650).

mice inoculated with EAEC 042 showed histological alterations characterized by a mild inflammatory response with edema and mononuclear infiltration in the lamina propria, derangement of the enterocytes and stunting or shortening of the villi. It was also observed that goblet cells appeared increased in number in relation to the epithelial cells (Fig. 5A). Bacterial aggregates were observed on the apical membrane near the intestinal lumen (Fig. 5B). An increase in the amount of goblet cells and mucus

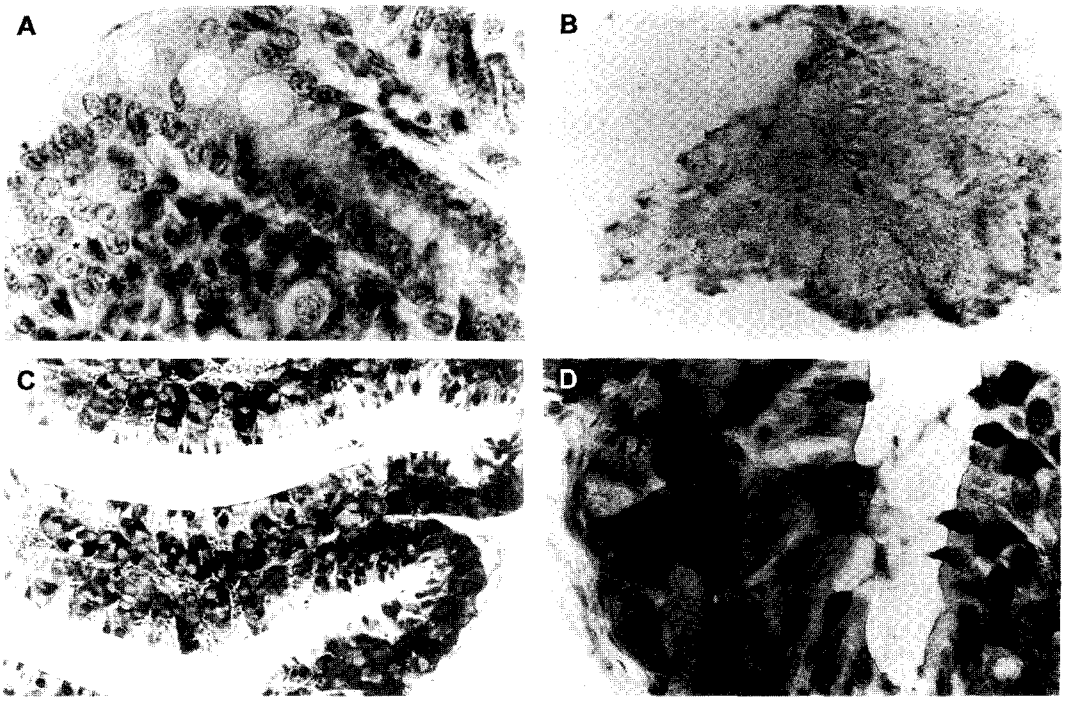

Fig. 5. Histological section of ileum of a mouse inoculated with EAEC 042. (A): Intestinal alterations observed were: (i) a mild inflammatory response with edema and mononuclear infiltration (*) in the lamina propria, (ii) derangement of the enterocytes and stunting or shortening of the villi. It was also observed that goblet cells appeared increased in number in relation to the epithelial cells (Hematoxylin and eosin stain. Magnification, x1300). (B): bacterial aggregates are observed on the apical membrane near the intestinal lumen (Warthin-Starry stain. Magnification x1300). (C): an increase in the amount of goblet cells and mucus secretion is observed, lamina propria also exhibits augmented cellularity. (Mac Manus stain. Magnification x650). (D): High magnification (x1300) of the ileal mucosa of a mouse inoculated with EAEC strain 042. Note the different stages in the secretion of mucus gel adhering to the intestinal mucosa. Pitting of goblet cells suggests stimulation of mucus hypersecretion.

secretion was observed, lamina propria also exhibited augmented cellularity (Fig. 5C). Different stages in the secretion of mucus gel adhering to the intestinal mucosa are also shown and pitting of goblet cells suggests stimulation of mucus hypersecretion (Fig. 5D). Colon samples also showed an augmented cellularity in the intestinal glands with hypertrophy and hypersecretion of mucus (data not shown).

Discussion

Enteroaggregative *E. coli* is considered an emergent pathogen that causes diarrheal disease in different countries of the world. Human voluntary studies and animal models, have been used to investigate the pathogenic and immune mechanisms induced by EAEC strains. Nevertheless, these models partially helped in understanding its pathogenic properties.

Previous experiments have employed intact mice inoculated with enteropathogenic (EPEC) *E. coli* (Robins-Browne *et al.*, 1994) and showed the colonization of the intestinal tract with these bacteria. Conlan and Perry (Conlan and Perry, 1998) using an *E. coli* O157:H7 strain, re-inoculated three different mice strains. These authors showed that BALB/c mice strain developed a certain resistance to re-infection when treated with these bacteria. We also found certain resistance to re-colonization as the period of bacterial shedding became shorter when compared with the initial inoculation time. The reason is uncertain, but the fact that some animals developed IgA and IgG antibodies against a secreted toxin (Pet), could be related to such a finding.

The ability of EAEC to bind to intestinal mucus has been demonstrated *in vitro* (Wanke *et al.*, 1990), and volunteers fed EAEC, developed diarrhea which is predominantly mucoid (Nataro *et al.*, 1995). The role of excess mucus production in EAEC pathogenesis is unclear; however, the formation of a heavy biofilm may be related to the diarrheagenicity of the organism and, perhaps, to its ability to cause persistent colonization and diarrhea (Nataro and Kaper, 1998).

In our study we also observed that there is an augmented production of mucus. Previous work done in our laboratory showed that Pet toxin is directly involved in fodrin disruption (Villaseca *et al.*, 2000), and we can speculate that this augmented secretion of mucus could be related with the disruption of the goblet cells and the release of mucus induced by the action of the toxin.

The production of an inflammatory response (Steiner *et*

al., 1998) and edema with the characteristic presence of mononuclear infiltration of the submucosa in animal models has also been reported (Vial *et al.*, 1988). This same process was observed in this study. Chemotaxis activity studies developed in our laboratory, showed that Pet toxin is directly involved in this process (manuscript in preparation) and we consider that this inflammatory response is directly associated with this toxin secreted by EAEC 042 strain, yet we could not find any inflammatory response in tissue samples of mice challenged with *E. coli* HB101 strain (negative control).

We considered this animal model useful to understand the colonization mechanisms of EAEC and the development of the disease whose symptoms and distribution of bacteria in the body mimicked the human form of the infection. It allows the study of the histological alterations that take place in the intestine during the evolution of the disease. It's a reproducible model also useful to evaluate the local (intestinal) and systemic (sera) immunological responses against Pet toxin, considered to be a virulence factor for enteroaggregative *E. coli*, as well as for the assay of some immunogenic products for specific protection.

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