Escherichia coli and persistent diarrhea
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Chapter 5

Lack of association between IL-8 inducing capacity of diarrheagenic *Escherichia coli* and diarrhea in returned travelers
Abstract

Although an increasing number of virulence factors associated with diarrheagenic *Escherichia coli* is described, the etiology of diarrhea, particularly when associated with enteroaggregative *E. coli* (EAggEC) and diffuse adherent *E. coli* (DAEC), remains largely unknown. The capacity of EPEC, EAggEC, and DAEC strains to induce IL-8 secretion by HT-29 cells and the association of this capacity with acute and persistent diarrhea was investigated in this study.

Overnight cultures of EPEC, EAggEC, DAEC and non-adherent strains, isolated during a prospective case-control study among returned travelers, were added to HT-29 cells and IL-8 release in the culture supernatant was determined using ELISA. No significant differences in IL-8 release inducing capacity between the various categories of *E. coli* from patients and controls were observed. Both adherent and non-adherent *E. coli* strains were capable of inducing IL-8 release. In addition, no association between IL-8 release inducing capacity and acute or persistent diarrhea was observed.

Subsets of *E. coli* strains appeared to be capable to induce IL-8 release by HT-29 cells but these strains were not associated with diarrhea. Therefore it is concluded that such *E. coli* strains form part of the commensal flora and are important for the physiological balance between pro-inflammatory- and downregulating cytokines in the intestines.
Lack of association between IL-8 inducing capacity of diarrheagenic 
*Escherichia coli* and diarrhea in returned travelers

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Introduction

Diarrheagenic *Escherichia coli* are divided in distinct categories, according to their pathogenic potential, epidemiology and the type of diarrhea they cause. Six categories of diarrheagenic *E. coli* are now recognized: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), entero-invasive *E. coli* (EIEC), aggregative-adherent *E. coli* (EAggEC) and diffuse-adherent *E. coli* (DAEC) (1).

EPEC, EAggEC and DAEC can be distinguished on the basis of their mannose-resistant adherence pattern to Hep-2 cells. EPEC show a localized pattern of adherence, in which the bacteria form microcolonies on the epithelial cell surface. EAggEC adhere to the cell surface and the glass slide in a so called "stacked-brick" pattern. DAEC adhere in a diffuse pattern to the entire epithelial cell surface.

EPEC has been recognized as a cause of acute diarrhea (1). A considerable number of EPEC virulence factors has been characterized in recent years (1). EPEC produces fimbriae and a wide array of proteins, which together allow the bacteria to adhere intimately to epithelial cells and to cause attaching-effacing lesions. In addition, EPEC has been shown to stimulate NFκB in intestinal epithelial cells (2). Stimulation of NF-κB results in IL-8 production and secretion by the cells, which may be associated with intestinal inflammation (2).

Although several studies showed an association of EAggEC with persistent diarrhea (3), the pathogenicity of this *E. coli* has not been fully elucidated. A number of potential
virulence factors, such as fimbriae, and production of toxins, have been characterized, but volunteer studies showed variability in the ability of various EA ggEC strains to cause diarrhea (4). In a recent study among children with persistent diarrhea in Brazil, infection with EA ggEC was associated with persistent diarrhea and with increased levels of IL-8 in stools (5). In vitro experiments with 2 prototype EA ggEC strains in CaCo-2 cells, showed a significant induction of IL-8 production by both strains and their culture supernatants, but not by strains from other categories of E. coli or by a Shigella strain (5).

It remains controversial whether DAEC can cause diarrhea. Several epidemiological studies have been performed and yielded conflicting results (1). In addition, the role of DAEC as a diarrheagenic E. coli could not be clarified in volunteer studies (6). Beside adhesins, no other virulence factors of DAEC are known.

In a prospective case-control study on the role of diarrheagenic E. coli in the etiology of acute and persistent diarrhea in travelers returned from tropical areas, we found that only ETEC was significantly associated with (acute) diarrhea. EA ggEC and DAEC were present in stool samples from a large number of patients as well as controls. Therefore, we investigated whether the ability of EPEC, EA ggEC, and DAEC strains to induce IL-8 secretion by intestinal epithelial cells, is associated with acute or persistent diarrhea. We postulated that, in case of such an association, subsets of EA ggEC and DAEC which are capable of inducing IL-8 production, would occur more frequently in stool samples from patients with diarrhea than in stool samples from controls.

Materials and Methods

Study population

Patients were included who presented with diarrhea at the out-patient department of Tropical Medicine at the Academic Medical Centre, Amsterdam, and the Leopold Institute of Tropical Medicine, Antwerp. Cases were defined as patients who developed diarrhea during a stay in tropical areas or within the first two weeks after their return. Diarrhea was defined as at least three loose stools in 24 hours, or any number of watery stools, or 1 or 2 loose stools in 24 hours accompanied by at least one of the following symptoms: nausea, vomiting, abdominal cramps, fever > 38°C. Diarrhea was considered persistent if it lasted for 14 days or more.
Individuals who presented at the out-patient departments for other reasons than diarrhea and who did not experience diarrhea during the previous 2 months, were included as controls. The first consecutive patient who presented after a patient with diarrhea was included as a control.

Examination of stool samples

Stool samples from patients and controls were collected on the day of presentation at the out-patient department, immediately transported to the laboratory, stored at 4°C and processed within 24 hours. All stool samples were subjected to extensive bacteriological and parasitological examination, as described elsewhere (manuscript in preparation). In addition, stool samples were assessed for the presence of ETEC using PCR as described elsewhere (7)(chapter 4).

For detection of EPEC, EAggEC and DAEC, stool samples were inoculated onto CLED agar plates and, after incubation for 18 hours at 37°C, a “sweep” of the complete bacterial growth on the agar was collected using a sterile cotton swab, and stored in glycerol-pepton at -70°C, as described previously (7). Sweeps were inoculated onto CLED agar plates and a second sweep was collected, which was hybridized with polynucleotide probes for detection of attaching-effacing E. coli, EAggEC and DAEC (8-10). From all sweeps which yielded weak to strong signals after hybridization, the stored sweeps were again inoculated on CLED agar plates and 3 to 5 E. coli-like colonies were isolated and identified biochemically as E. coli. These colonies were tested in a 6-hours HEp-2 adherence assay for phenotypical confirmation of their adherence pattern (11). Strains were considered EPEC if they were positive after hybridization with the eae probe and showed localized adherence in the Hep-2 test. Colonies which yielded an aggregative or diffuse adherence pattern in the Hep-2 test, in addition to positive hybridization with the EaggEC or the daaC probe, were considered EAggEC or DAEC respectively. Isolates which showed an adherence pattern compatible with both EAggEC and DAEC were categorized separately and designated DAAA (Diffuse adherent/Aggregative adherent). EAggEC, DAEC, EPEC, DAAA, and a random selection of non-adherent strains obtained from weakly hybridizing sweeps, from both cases and controls, were tested on HT-29 cells for their IL-8 inducing capacity. From each patient or control, one representative strain from each category of E. coli detected, was chosen.
**Bacterial preparations**

For cytokine release experiments, overnight static cultures of the bacteria in Luria broth (LB) containing 1% mannose, were centrifuged (3000g), the supernatant aspirated and the pelleted bacteria washed twice in phosphate buffered saline (PBS), pH 7.4. After the last centrifugation, the bacteria were suspended in Dulbecco’s Modified Eagle’s Medium (DMEM) with HEPES (Flow Laboratories, USA) to a final concentration of $1 \times 10^8$/ml, using a spectrophotometer at A540. One-hundred microliter of each suspension was added to duplicate wells containing HT-29 cells (see below).

Initial experiments were performed with strain 5a. This strain was isolated from a patient with ulcerative colitis, hybridized with the EAggEC-probe, and showed aggregative adherence to Hep-2 cells. In preliminary experiments this strain appeared capable of inducing IL-8 secretion in HT-29 cells. Culture filtrates of strain 5a were prepared by filtrating the aspirated overnight culture supernatant through a 0.22 um filter (Millipore, S.A. Molsheim, France). One-hundred microliter of the filtrate was added to duplicate wells containing HT-29 cells.

**HT-29 cl19A cell culture and IL-8 release**

HT-29/19A cells, kindly provided by S.J. van Deventer (Academic Medical Centre, Amsterdam), were cultured in 25 cm² tissue cultured flasks in DMEM buffered with 44mM NaHCO₃ (Flow Laboratories) at pH 7.2, to which 10% foetal calf serum (FCS) was added. After growth at 37°C in 5% CO₂ for 7 days, the cells were trypsinized and cultured in 24-wells tissue culture plates (Falcon, NY, USA) until confluency. Each well thus contained approximately $3.0 \times 10^4$ cells per ml. Prior to each experiment, the medium in each well was replaced with 1 ml DMEM, buffered with HEPES at pH 7.2, containing 10% FCS.

For induction of IL-8 release, $1 \times 10^8$ bacteria, or 100 µl of bacterial filtrate of strain 5a, was added to the wells in duplicate and the HT-29 cells were incubated for 3 hours. After 3 hours, the tissue culture medium was removed, cells were washed once with PBS and fresh medium, containing 100 µg/ml gentamicine, was added. The cells were incubated for another 2-3 (strain 5a and clinical isolates) or 21 hours (strain 5a) and the medium was removed, duplicates were pooled and stored at -20°C until testing of IL-8 concentration.

During the initial series of experiments with strain 5a, A23187 (4-bromo-calcium ionophore) (200 nM)/phorbol myristic acid (PMA) (Sigma Chemical Company, St. Louis, USA) (100 ng/ml) was included as a standard and TNFα (10 µg/ml) as a positive control (12).
Constitutive release of IL-8 was measured after addition of gentamycin only. During each experiment with clinical isolates, TNFα (10 μg/ml) was included as a positive control.

**Measurement of IL-8 concentration**

The concentration of IL-8 in the cell culture medium was determined by ELISA (PeliKine compact™, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). All samples were tested in duplicate. To compensate for variability between experiments, levels of IL-8 were expressed as a ratio of the concentration of IL-8 which was induced by A23187/PMA or by TNFα. This ratio was defined as the mean concentration of IL-8 induced by each isolate, divided by the mean concentration of IL-8 induced by A3187/PMA (strain 5a) or TNFα (clinical isolates) during the same experiment and expressed as a percentage.

**Statistical analysis**

Isolation rates of diarrheagenic *E. coli* from patients and controls were compared using χ²-test or Fisher’s exact test. IL-8 levels were compared using non-parametric tests (Wilcoxon-rank sum test).

**Results**

**Isolation of diarrheagenic *E. coli* from stool samples**

Stool samples from 171 patients with diarrhea and 109 controls were examined for the presence of EAggEC, DAEC and EPEC (table). EAggEC was isolated from 18 cases and 7 controls. DAEC was found in 22 cases and 15 controls while EPEC was found in 3 cases and 6 controls. In 2 cases and 3 controls DAAA was identified. The isolation rates for each category of *E. coli* did not differ significantly between patients and controls. Concomitant infection with common bacterial enteropathogens (*Campylobacter jejuni*, *Shigella* sp., *Salmonella* sp., and/or ETEC) occurred in 13 patients with diarrhea and 3 controls. Concomitant infection with cyclospora occurred in 1 patient.
<table>
<thead>
<tr>
<th></th>
<th>Patients (N=171)</th>
<th>Controls (N=109)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>enteropathogenic E. coli</td>
<td>3 (1.8)</td>
<td>6 (5.5)</td>
</tr>
<tr>
<td>entero-aggregative E. coli</td>
<td>18 (10.5)</td>
<td>7 (6.4)</td>
</tr>
<tr>
<td>diffuse adherent E. coli</td>
<td>22 (12.9)</td>
<td>15 (13.8)</td>
</tr>
<tr>
<td>diffuse/aggregative adherent E. coli</td>
<td>2 (1.2)</td>
<td>3 (2.8)</td>
</tr>
</tbody>
</table>

**IL-8 induction by EAggEC strain 5a and TNFα**

HT-29 cells were incubated with A23187/PMA, strain 5a, TNFα or culture medium alone. After 3 hours incubation, the cells were washed once and the medium was replaced with medium containing 100 μg/ml gentamycin. The cells were allowed to incubate for another 2-3 hours or for a total of 24 hours. The median concentration of IL-8 induced by A23187/PMA was 95 pg/ml (range 50-300 pg/ml) after 5-6 hours of incubation and 310 pg/ml (range 220-900 pg/ml) after 24 hours of incubation. Strain 5a and TNFα induced significantly higher levels of IL-8 secretion than the control with only medium containing gentamycin, after both 5-6 and 24 hours of incubation (p=0.05, Wilcoxon rank sum test). The median level of IL-8 induced by strain 5a after 5-6 hours incubation was 220% of the levels induced by A23187/PMA. This level was 330% for TNFα and 100% for solely medium. Levels of IL-8 induced after 24 hours of incubation were 150% of levels induced by A23187/PMA for strain 5a, 668% for TNFα and 67% for medium only. Supernatant of strain 5a was also capable of inducing IL-8 production, at similar levels as the bacteria. The 5-6 hour assay was chosen for subsequent testing of clinical isolates from patients with and without diarrhea.

**IL-8 induction by clinical E. coli isolates**

Induction of IL-8 release by HT-29 cells was studied for 7 EPEC strains, 24 EAggEC strains, 29 DAEC strains, 5 DAAA strains and 18 non-adherent strains, which were isolated from 53 patients with diarrhea and 29 controls. The median level of IL-8 induce by TNFα in these experiments was 650 pg/ml (range 270-1150 pg/ml). The strains from each E. coli category from patients and controls induced similar levels of IL-8, which were expressed as a
percentage of the ratio induced by TNFα during the same experiment (figure 1). In particular, EAggEC strains did not induce higher levels of IL-8 than the other categories of *E. coli*, including non-adherent *E. coli* (figure 1). The levels of IL-8 induced by the 53 *E. coli* isolates obtained from patients with diarrhea were lower than the levels induced by the 29 isolates obtained from controls, but differences were not significant (figure 2). Finally, no significant difference in IL-8 release inducing capacity, was observed between the 22 isolates obtained from patients with acute and the 31 isolates obtained from patients with persistent diarrhea (figure 3).
Figure 1 (opposite page). **IL-8 induced by different categories of diarrheagenic E. coli isolated from patients with diarrhea and controls**

DAEC: Diffuse adherent *E. coli*  
EAggEC: Enteroaggregative *E. coli*  
EPEC: Enteropathogenic *E. coli*  
DAAA: Diffuse/Aggregative adherent *E. coli*  
NA: Non-adherent *E. coli*

The level of IL-8 induced by each isolate is expressed as a percentage of the concentration of IL-8 induced by TNFα during the same experiment.

--- indicates median

● = *E. coli* isolate from case

○ = *E. coli* isolate from control
Figure 1

IL-8/IL-8

TNF (%)

[90]

[80]

[70]

[60]

[50]

[40]

[30]

[20]

[10]

[0]

BSS

DAEC

EAggEC

EPEC

DAAA

NA

N=28

N=24

N=7

N=5

N=18

N=1
Figure 2 (opposite page). **IL-8 induced by adherent and non-adherent* E. coli isolated from patients and controls**

The level of IL-8 induced by each isolate is expressed as a percentage of the concentration of IL-8 induced by TNFα during the same experiment.

• = median

○ = isolate from case

● = isolate from control

Figure 3 (page 102). **IL-8 induced by adherent and non-adherent* E. coli isolated from patients with acute and persistent diarrhea**

The level of IL-8 induced by each isolate is expressed as a percentage of the concentration of IL-8 induced by TNFα during the same experiment.

• = median
Figure 2

\[ \text{[IL-8]/[IL-8]} \text{ TNF (\%)} \]

N=53  N=29

cases  controls

cases N=53  controls N=29

Figure 2 demonstrates the distribution of IL-8/TNF levels in cases and controls. The data are represented as a scatter plot with the percentage of IL-8/TNF levels on the y-axis and case/control status on the x-axis. The plot shows a higher percentage of IL-8/TNF levels in cases compared to controls, indicating a potential association between the two variables. The sample sizes for cases (N=53) and controls (N=29) are indicated, along with the mean values for each group.
Figure 3

The level of TNF produced by each subject is expressed as a percentage of the concentration of [IL-8]/[IL-8]TNF during the acute episode.

[IL-8]/[IL-8]TNF (%)

- N=22 acute
- N=31 persistent
Discussion

We evaluated whether the presence of *E. coli* strains capable of inducing IL-8 release, is associated with acute or persistent diarrhea. This ability may be limited to strains with the capacity to adhere to epithelial cells. Therefore, we also compared isolates with different adherence patterns to HEp-2 cells (which is the gold standard to determine adherence of *E. coli* strains) (1), with non-adherent strains. We did not observe an association between the presence of IL-8 releasing *E. coli* and diarrhea. Furthermore, we found that, in each category of adherent *E. coli*, strains exist which induce IL-8 release in HT-29 cells, and strains which do not. Neither EPEC nor EAggEC induced significantly higher levels of IL-8 than DAEC, DAAA or non-adherent strains. Moreover, within each category of *E. coli* strains, including the non-adherent ones, a considerable variation in levels of IL-8 induced by single isolates, was observed.

Studies on the ability of non-invasive diarrheagenic *E. coli* to induce IL-8 release are limited to a selected number of strains only (2,5). EPEC strain E2348/69 is able to induce transepithelial migration of polymorphonuclear leukocytes (PMN's) across T84 intestinal epithelial cell monolayers, through activation of NF-κB, which leads to activation of production and secretion of IL-8 (2). Inflammatory cell infiltration has been observed in clinical biopsy samples and in animal models, after infection with EPEC (1). EAggEC strains 17-2 and 042 have been shown to induce IL-8 production in Caco-2 intestinal epithelial cells (5). EAggEC can cause acute watery as well as persistent diarrhea but the pathophysiology of EAggEC diarrhea is unknown. While the EAggEC-associated acute diarrhea may be ascribed to the production of enterotoxins (3), persistent diarrhea has been shown to be associated with intestinal inflammation, as indirectly demonstrated by elevated levels of fecal lactoferrin, IL-8 and IL-1β (5).

We used a type of intestinal epithelial cells to test IL-8 release, which differs from the cell lines used by other investigators for studying IL-8 release by diarrheagenic *E. coli*. Differences in epithelial cell surface receptors between T84, Caco-2 and HT-29 cells may be responsible for differences in levels of IL-8 induced by isolates from each category of diarrheagenic *E. coli*. HT-29 cells were shown to be more responsive to LPS than T84 and CaCo-2 cells (13), and produced IL-8 in response to infection with non-invasive *E. coli* O157 and *E. coli* DH5α, in contrast to T84 and CaCo-2 cells (14). Such differences would also

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explain the discrepancy between the results obtained with EPEC strain E2348/69, which induced low IL-8 release in one study using Caco-2 cells (5), but was shown to induce high levels of IL-8, in comparison with non-pathogenic strains, in another study using T84 cells (2). However, the current study is the first in which a large number of clinical E. coli isolates are compared and we can conclude from our experiments that in HT-29 cells no differences in IL-8 inducing capacity can be observed between adherent and non-adherent E. coli strains from patients with diarrhea and controls.

It remains unclear to what extent adherence is required for induction of IL-8 release by diarrheagenic E. coli. The non-adherent strains tested, induced IL-8 secretion to a similar level as the adherent strains. In addition, culture supernatant of EAggEC strain 5a was capable of inducing IL-8 release, as also reported for EAggEC strains 17-2 and 042 (5). Studies with mutant EPEC strains indicated that secreted proteins, rather than intimate adherence, are essential for NF-κB activation in T84 cells, resulting in production and secretion of IL-8 (2). Although we did not test the adherence of all the clinical isolates to HT-29 cells, but only to HEp-2 cells which may show different properties with respect to adherence of E. coli strains, large differences in adherence pattern to HT-29 cells and to HEp-2 cells are unlikely to occur. We tested the adherence of EAggEC strain 5a and DAEC strain 72540 to the HT-29 cells and they respectively showed clear aggregative and diffuse adherence. Secretion of proteins may therefore appear to be more important for stimulation of IL-8 production and secretion than adherence capacity.

We did not measure invasiveness of the bacteria. In vitro experiments with invasive micro-organisms, in particular Salmonella and Shigella species, indicate that intestinal epithelial cells produce pro-inflammatory cytokines in response to cellular invasion by the bacteria (14). Production of pro-inflammatory cytokines leads to neutrophil transmigration which would explain the intestinal inflammation, observed during infection with these invasive pathogens (15,16). It may be argued, in particular for isolates which did not hybridize with any of the DNA-probes used, that invasive strains were present among IL-8 release inducing strains. However, in contrast with the earlier reports, recent studies indicate that invasion is not required for NF-κB activation, but that microbial adherence to the epithelial cells by invasive microorganisms, together with the secretion of virulence associated proteins, may be sufficient for IL-8 production by the epithelial cells (15-18).

We did not observe any association between the presence of E. coli strains capable of IL-8 induction and persistent diarrhea. In various states of chronic intestinal inflammation, for
example during relapses of inflammatory bowel disease, increased levels of IL-8 can be
observed (19). High levels of IL-8 in the stools of such patients with persistent diarrhea may be
the result of stimulation of intestinal epithelial cells by a specific pathogen but could also be
the consequence of changes in the intestinal barrier function, for example as observed during
malnutrition (20). Although we did not observe an association with diarrhea, the presence of
IL-8 inducing *E. coli* among cases and controls is interesting. When we consider these strains
as part of the normal intestinal flora, their presence provides arguments for the hypothesis that
in the normal physiological state, a balance exists between pro-inflammatory and
downregulating cytokine synthesis by host intestinal cells, induced by bacteria (21).
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Reference List


