High Prevalence of Adherent-Invasive *Escherichia coli* Associated With Ileal Mucosa in Crohn’s Disease

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Background & Aims: Adherent-invasive *Escherichia coli* (AIEC) pathovar has been identified in the intestinal mucosa of patients with Crohn’s disease (CD). AIEC reference strain LF82 is able to adhere to intestinal epithelial cells, to invade epithelial cells via a mechanism involving actin polymerization and microtubules, and to survive and replicate within macrophages. This study was performed to assess the prevalence of AIEC associated with intestinal mucosa of patients with CD, ulcerative colitis (UC), and of controls. Methods: A search for *E. coli* strains was performed with ileal specimens of 63 patients with CD and 16 controls without inflammatory bowel disease (IBD), and with colonic specimens of 27 patients with CD, 8 patients with UC, and 102 controls. The abilities of *E. coli* strains to invade epithelial cells and to survive and replicate within macrophages were assessed using the gentamicin protection assay. Bacterial uptake by epithelial cells was analyzed using cytoskeletal inhibitors. Bacterial adhesion was quantified with Caco-2 and Intestine-407 cells. The presence of known *E. coli* virulence genes was assessed by polymerase chain reaction and DNA hybridization. Results: In ileal specimens, AIEC strains were found in 21.7% of CD chronic lesions vs. in 6.2% of controls. In neoterminal ileal specimens, AIEC strains were found in 36.4% of CD early lesions (P = 0.034 vs. controls) and 22.2% of healthy mucosa of CD patients. In colonic specimens, AIEC strains were found in 3.7% of CD patients, 0% of UC patients, and 1.9% of controls. Conclusions: AIEC strains are associated specifically with ileal mucosa in CD.

The pathogenesis of Crohn’s disease (CD) is complex and consists of 3 interacting elements: genetic susceptibility factors, priming by the enteric microflora, and immune-mediated tissue injury.1–4 Experimental and observational data suggest that intestinal inflammation arises from abnormal immune response to bacterial flora in the intestines of individuals who are genetically susceptible.2 Characteristic pathologic elements of CD have long suggested a microbial cause, including aphthous ulcers of the mucosa, mural abscesses, suppurative fistulas, and macrophage and epithelioid cell granulomas. These occur in well-recognized infectious diseases of the intestines of humans and animals.5 The role of luminal bacteria in the pathogenesis of CD is supported by observations that patients with CD clinically improve when luminal bacterial concentrations are decreased.6–11 The importance of the luminal flora also is suggested by studies of postsurgical endoscopic relapse, which occurs at a rate of 73% within 1 year. Diverting ileostomy prevents postoperative recurrence, and, if the anastomosis is isolated from the fecal flux by an upstream stoma, no lesion occurs. In contrast, relapse occurs rapidly when fecal flow through the anastomosis is reconstituted.12 In that situation, there is a significant increase in the mucosal-associated bacterial counts in the neoterminal ileum and this colonization may be related to postoperative relapse.13 There is still controversy regarding whether the virulence traits of bacteria in CD are expressed broadly or just in a small subset of bacteria.14 Individual bacterial species within the indigenous flora vary in their capacity to cause intestinal inflammation. In experimental models, some bacteria such as *Bacteroides vulgatus* can cause colitis alone when monoassociated in the HLA-B27 transgenic rat model.15 Others, including *Lactobacillus* and *Bifidobacterium* species, have no proinflammatory capacity and have been used as probiotics.16,17 In patients with inflammatory bowel disease (IBD), systematic approaches to this issue have been hampered by the limited knowledge of the intestinal flora. *Escherichia coli* strains are

**Abbreviations used in this paper:** AIEC, adherent-invasive *Escherichia coli*; LB, Luria–Bertani.

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0016-5085/04/$30.00
The hypothesis of the involvement of invasive bacteria in CD. Such ulcers occur in shigellosis, salmonellosis, and yersinial enterocolitis, in which invasiveness is an essential virulence factor of the bacteria involved. The identification of mutations in the NOD2/CARD15 encoding gene in patients with CD showed a link between innate immune response to invasive bacteria and the development of CD. Indeed, NOD2 is a cytosolic receptor responding to the presence of bacterial components such as peptidoglycan through muramyl dipeptide detection. The hypothesis of the involvement of invasive bacteria in CD was strengthened by the recent findings that intestinal epithelial cells expressing a NOD2/CARD15 variant were unable to constrain the replication of intracellular bacteria. Moreover, in vitro interaction between adherent E. coli and human intestinal epithelial cells leads to overexpression of the major histocompatibility complex class I–related molecule MICA. Intestinal biopsy specimens from CD patients overexpressed MICA, confirming a possible role of pathogenic E. coli in CD.

We previously characterized the invasive ability of E. coli strain LF82, which was isolated from damaged ileal mucosa of a patient with CD, and showed that it was a true invasive strain. It efficiently invades cultured epithelial cells, its uptake is dependent on actin microfilaments and microtubules, and it survives intracellularly and replicates in the host cell cytoplasm after lysis of the endocytic vacuole. However, strain LF82 has none of the invasive determinants of the invasive E. coli known to be involved in acute gastrointestinal infections, that is, the ipaC plasmid gene encoding the invasin of Shigella flexneri and enteroinvasive E. coli, the aae gene encoding the intimin of enteropathogenic E. coli, the afaD gene involved in invasion of diffusely adhering E. coli, or the tia gene encoding a 25-kilodalton outer-membrane protein involved in enterotoxigenic E. coli invasiveness. In vitro analysis of the interactions of invasive E. coli strains isolated from CD patients with macrophages indicated that these strains were able to survive and replicate within the phagocytic cells without inducing cell death. We also showed that infected macrophages secreted large amounts of tumor necrosis factor α. We thus defined a new pathogenic group of E. coli, designated AIEC for adherent-invasive E. coli.

The aim of the present study was to assess the prevalence of AIEC associated with the intestinal mucosa of patients with CD, ulcerative colitis (UC), and controls. We thus investigated for the presence of invasive E. coli and determined whether the invasive strains belonged to the AIEC pathovar by analyzing the invasive process, searching for known genetic invasive determinants, quantifying the bacterial adhesion to differentiated Caco-2 and undifferentiated Intestine-407 intestinal epithelial cells, and by studying the survival and replication of the internalized bacteria within J774-A1 macrophages.

Patients and Methods

Patients and Controls

E. coli strains included in this study were isolated from ileal specimens of patients with CD or of controls, and from colonic specimens of patients with CD, UC, or of controls. All patients and controls gave their informed consent, and approval from the local ethics committee was obtained.

Ileal specimens (CD). A total of 63 mucosal specimens from patients with CD were studied.

Resection specimens (chronic lesions). Twenty-three patients with CD (11 women, 12 men; mean age, 30 yr; range, 19–42 yr) who had undergone ileocolonectomy for the first time were included. Fourteen patients had ileal involvement, and 9 had ileocolonic CD. All patients received cefoxitin (2 g intravenously) at the time of incision. Specimens were taken from the mucosa of resected ileum with an endoscopic forceps.

Neoterminal ileal specimens. Specimens were obtained from the neoterminal ileum (10 cm from anastomosis) at endoscopy in 40 patients who had undergone ileocolonectomy with end-to-end ileocolonic anastomosis, except in 2 patients having a definite ileostomy. Endoscopic findings were scored 11–14 according to the criteria of Rutgeert et al. Endoscopic biopsy specimens were taken from early recurrent ileal lesions (referred to as early lesions) (scored ≥ 12) in 22 patients (12
women, 10 men; mean age, 33 yr; range, 19–55 yr), 8 of whom underwent biopsy examinations 3 months after surgery, and 14 of whom underwent biopsy examinations at 1 year. Similarly, endoscopic biopsy specimens were taken from healthy ileal mucosa (referred to as healthy mucosa) of 18 patients with CD (10 women, 8 men; mean age, 28 yr; range, 18–43 yr) who had no endoscopic recurrence (Rutgeerts score ≤ 1) at 3 months (n = 12) or 1 year (n = 6) after surgery. None of the patients had received antibiotics within 4 weeks of sampling.

Ileal specimens (controls). Sixteen patients (12 women, 4 men; mean age, 65 yr; range, 45–89 yr) with right colonic cancer who had undergone right hemicolectomy were included. Specimens were obtained at surgery in 11 patients. All patients received cefoxitin (2 g intravenously) at the time of incision. Specimens were taken from the mucosa of resected ileum with an endoscopic forceps. In 5 patients specimens were obtained postoperatively from the neoterminal ileum during surveillance endoscopy.

Colonic specimens (CD). Specimens were obtained from the left colon using endoscopic forceps in 27 patients with CD (mean age, 33 yr; range, 19–74 yr). Seven patients had undergone surgical resection with end-to-end anastomosis (6 right ileocolic resection, 1 small bowel resection). Specimens were taken from colonic lesions in 15 patients and from normal mucosa in 12 patients. None of the patients had received antibiotics within 4 weeks of sampling.

Colonic specimens (UC). Specimens were obtained from the left colon using endoscopic forceps in 8 patients with UC (mean age, 48 yr; range, 28–70 yr). Specimens were taken from colonic lesions in 5 patients and from normal mucosa in 3 patients. None of the patients had received antibiotics within 4 weeks of sampling.

Colonic specimens (controls). Specimens were obtained from normal mucosa of the left colon using endoscopic forceps in 9 patients with irritable bowel syndrome and from macroscopic lesions of colitis in 93 patients with acute self-limited colitis (mean age, 38 yr; range, 18–94 yr). None of the patients developed IBD at follow-up evaluation.

Microbiologic Methods

The intestinal specimens were collected into sterile vials containing either phosphate-buffered saline (PBS) or cysteinated one-quarter strength Ringer’s solution. One biopsy specimen weighing approximately 5–10 mg was tested for each patient or control. The specimens were crushed and 10-fold dilutions were plated on blood agar medium. Suspicious E. coli colonies were identified by the API system (bioMérieux sa, Marcy l’Etoile, France). Up to 3 colonies for each patient or control were characterized further. All E. coli strains were stored in Mueller-Hinton broth (Institut Pasteur Production, Marne-la-Coquette, France) with glycerol (15% vol/vol) at −80°C, and grown in Luria–Bertani (LB) broth without shaking or on Mueller–Hinton agar plates overnight at 37°C.

The minimal bactericidal concentration (MBC; concentration that reduced the bacterial count by 99.9%) of gentamicin for all strains included in this study was determined because this drug is used to distinguish between extracellular adherent bacteria and intracellular bacteria during the invasion assays. E. coli strains resistant to gentamicin were excluded from the study.

Reference Bacterial Strains

Enteropathogenic E. coli H10407,46 and AFA A3037 E. coli strains, which belong to different pathogenic groups that are responsible for gastrointestinal or urinary tract infections, were used as sources of the aae, tia, and afaD genes. Shigella flexneri strain SC30138 was used as a source of the ipaC gene. E. coli strain K-12 C600 was used as a noninvasive control.

Cell Lines and Cell Culture

The following epithelial cell models were used: the Caco-2 cell line established from a human colonic adenocarcinoma that exhibits structural and functional differentiation patterns in postconfluence culture characteristic of mature enterocytes of the small intestine, the Intestine-407 cell line derived from human embryonic jejunum and ileum as a model of undifferentiated intestinal epithelial cells, and the HEP-2 cell line derived from a human laryngeal carcinoma. All cell lines were purchased from Flow Laboratories (Flow Laboratories Inc., Mc Lean, VA). The murine J774-A1 macrophage-like cell line (American Type Culture Collection no. TIB67; ATCC, Manassas, VA) was used as a macrophage model. Cell culture was performed as described previously.

Invasion Assays

Intestine-407 and HEP-2 cells were seeded in 24-well tissue culture plates (Polylabo, Strasbourg, France) at a density of 4.10³ cells/well and incubated for 20 hours. The cell monolayers were washed twice with PBS (pH 7.2). Bacterial invasion of epithelial cells was measured using the gentamicin protection assay.39 Each monolayer was infected in 1 mL of the cell culture medium lacking antibiotics with a multiplicity of infection that reduced the bacterial count by 99.9% (gentamicin, Sigma, St. Louis, MO) was added for 1 hour to kill extracellular bacteria before lysis of the monolayers with 1% Triton X-100 (Sigma) in deionized water. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 minutes. The samples were diluted and plated onto Mueller–Hinton agar plates to determine the number of colony-forming units. All results of E. coli invasive ability with Intestine-407 and HEP-2 cell lines were expressed as the percentage of intracellular bacteria compared with the initial inoculum, taken as 100%. All of the assays were performed at least 3 times in separate experiments.
Table 1. Oligonucleotide Primers to Generate PCR Amplification Products Used as Nucleic Probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Polymerase chain reaction product size (bp)</th>
<th>Probe</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae-I</td>
<td>GGTACTGAACGCGATACGC</td>
<td>831</td>
<td>eae</td>
<td>M58154</td>
</tr>
<tr>
<td>eae-II</td>
<td>CGACATCGTAAACACGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipaC-I</td>
<td>ATCATTGTCGCTTTAGTGC</td>
<td>862</td>
<td>ipaC</td>
<td>J04117</td>
</tr>
<tr>
<td>ipaC-II</td>
<td>GCAATCTGACTTGGCTGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tia-F</td>
<td>ACCAGCCGCTACGTAGG</td>
<td>382</td>
<td>tia</td>
<td>U20318</td>
</tr>
<tr>
<td>tia-R</td>
<td>GCCAGATTATCCAGAGGG</td>
<td></td>
<td></td>
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<tr>
<td>Afa-70</td>
<td>TGGCTCTTTTCTACGCTTGGCCACACACAG</td>
<td>500</td>
<td>afaD</td>
<td>X76688</td>
</tr>
<tr>
<td>Afa-92</td>
<td>GGGATTTTCTGACGCGTACGCTG</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Adhesion Assays

Adhesion to differentiated Caco-2 and undifferentiated Intestine-407 intestinal cells was measured as reported previously20 with minor modifications. Briefly, Caco-2 cells were seeded at a density of 2.10^5 cells/well and used at postconfluence after 15 days of culture. Intestine-407 cells seeded at 4.10^4 cells/well were incubated for 20 hours. The cells were washed with PBS before the adhesion test and a suspension of 10^8 bacteria/mL in the cell line culture medium was added to the tissue culture and incubated for 3 hours at 37°C. After 3 washes with PBS, the cells were lysed with 1% Triton X-100 and the number of colony-forming units was determined by plating. For each experiment the mean number of Caco-2 cells after 15 days of culture or of Intestine-407 after 20 hours of culture was determined. Adhesion assays were performed in triplicate, and the mean number of bacteria per cell was determined.

Effect of Eukaryotic Cytoskeletal Inhibitors

HEp-2 cells were preincubated for 30 minutes before the invasion assay in cell culture medium lacking antibiotics with 1 μg/mL of cytochalasin D or 0.5 μg/mL of colchicine (Sigma). The inhibitors were present throughout a 3-hour bacterial infection period. The inhibitory effect of each inhibitor on bacterial uptake was evaluated against a control assay without inhibitor, which was defined as 100% of bacterial uptake. All of the assays were performed at least 3 times in separate experiments.

Bacterial Survival and Replication Within Macrophages

Bacterial uptake, survival, and replication were measured by the gentamicin protection assay. J774-A1 macrophages were seeded in 24-well tissue culture plates (Polylabo) at a density of 2.10^5 cells per well and were grown for 24 hours. Before infection, the cell monolayers were washed twice with PBS and the medium was replaced with 1 mL of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum. Each monolayer was infected with a multiplicity of infection of 10 bacteria per macrophage cell. After a 2-hour incubation period at 37°C with 5% CO2, infected macrophages were washed twice with PBS, and fresh cell culture medium containing 100 μg/mL of gentamicin was added to kill extracellular bacteria. After incubation for an additional hour, the medium was removed and fresh medium containing 20 μg/mL of gentamicin was added for 1 or 24 hours. Cells were washed once with PBS, and 0.5 mL of 1% Triton X-100 (Sigma) in deionized water was placed in each well for 5 minutes to lyse the eukaryotic cells. Samples were removed, diluted, and plated onto Mueller–Hinton agar plates to determine the number of colony-forming units recovered from the lysed monolayers. The number of bacteria surviving the gentamicin kill assay was determined after 1 and 24 hours of gentamicin treatment. Survival and replication at 24 hours postinfection was expressed as the mean percentage of the number of bacteria recovered after 1 hour postinfection, defined as 100%. All assays were performed at least 3 times in separate experiments and in duplicate.

Transmission Electron Microscopy

Cross-sections of infected cultured cells were prepared as follows. After infection, cells were fixed with 3% glutaraldehyde in 0.2 mol/L cacodylate buffer at 4°C for 2 hours and postfixed in 1% OsO4 in cacodylate buffer at 4°C for 1 hour. After dehydration in a graded series of ethanol, the cultures were embedded in a 2-mm thick Epon coating (Resolution Performance Product, Houston, TX) in the tissue culture well and polymerized for 3 days at 60°C. Suitable areas were oriented parallel to the cell layer surface on Epon blocks with an Epon mixture. Ultracections were contrasted with uranyl acetate and lead citrate.

Hybridization Experiments

DNA fragments used as nucleic probes were generated by polymerase chain reaction. Oligonucleotides used for amplification of ipaC-specific, eae-specific, tia-specific, and afaD-specific sequences were synthesized on the basis of published nucleotide sequences (Table 1). Polymerase chain reaction protocols were performed as described previously.30 Polymerase chain reaction fragments obtained from each specific amplification of DNA from reference E. coli strains were used as nucleic probes for colony blot hybridization experiments. Polymerase chain reaction fragments were purified from the agarose gel by using QIAEX II gel extraction kit (Qiagen S.A., Courtaboeuf, France) and radiolabeled with (α-32P) deoxyadenosine triphosphate (5000 Ci/mmol; Amersham International,
Amersham, UK) using a random-primed DNA labeling kit (Boehringer Mannheim, Meyher, France) according to the manufacturer’s specifications. Colony blot hybridizations were performed with rapid hybridization buffer (Amersham) overnight at 65°C as described previously.30

Definition of AIEC

Determination of E. coli strains as belonging to the AIEC pathovar was performed using the following criteria: (1) the ability of the bacteria to invade Intestine-407 and HEp-2 epithelial cells with an invasion index equal or superior to 0.1% of the original inoculum, (2) the involvement of host cell actin polymerization and microtubule recruitment in bacterial uptake, (3) the absence of known invasive determinants, (4) the ability to adhere to differentiated Caco-2 and/or undifferentiated Intestine-407 intestinal epithelial cells with an adhesion index equal or superior to 1 bacteria per cell, and (5) the ability to survive and to replicate within J774-A1 macrophages.40

Statistical Analysis

The data were analyzed by the χ² test unless the variables needed a 2-tailed Fisher exact test. A P value <0.05 was considered statistically significant.

Results

Search for Invasive E. coli Strains Associated With Intestinal Mucosa of CD and UC Patients and Controls

The invasion level of the noninvasive reference E. coli strain K-12 C600 was 0.0005% ± 0.0005% of the original inoculum (Table 2). Reference strain LF82, included in all of the assays as a positive invasive control, gave a mean invasion level of 1.29% ± 0.55%. E. coli strains were considered invasive when the mean invasion level was superior or equal to 0.1% of the original inoculum. Based on this criteria, invasive E. coli strains isolated from patients with IBD or controls are listed in Table 2. For each patient or control, only the E. coli strain that showed the highest invasive level is listed. The invasion levels ranged from 0.12% to 1.41% and from 0.10% to 3.38% with Intestine-407 cells and HEp-2 cells, respectively. The presence of numerous intracellular bacteria was confirmed by electron microscopy (Figure 1).

In CD patients, invasive E. coli strains were found associated with ileal mucosa in 7 (30.4%) of 23 patients with chronic lesions, associated with the neoterminal ileal mucosa in 8 (36.4%) of 22 patients with early lesions, and in 4 (22.2%) of 18 patients with healthy mucosa (Table 3). In contrast, invasive E. coli was isolated from only 1 (6.2%) of the 16 ileal specimens from controls.

Of the colonic specimens from 27 CD patients included in this study, only 1 (3.7%) was positive for invasive E. coli. A similar low prevalence of invasive E. coli was observed in colonic mucosa of controls (3.9%), and only 1 (12.5%) colonic specimen of the 8 UC patients harbored invasive E. coli.

Characteristics of the Invasive E. coli Strains

Adhesion to intestinal epithelial cells. E. coli strains were considered adherent when the mean adhesion index was equal or superior to 1 bacteria per cell. All the invasive strains had the ability to adhere to differentiated Caco-2 cells and to undifferentiated Intestine-407, with adhesion indices varying from 1 ± 0 to 25 ± 5 bacteria per cell with Caco-2 cells and from 2 ± 1 to 29 ± 8 bacteria per cell with Intestine-407 cells (Table 2). Surprisingly, the most adherent strains did not systematically show the highest invasion levels.

Involvement of actin polymerization and microtubule recruitment in bacterial uptake. Cell monolayers were treated with either cytochalasin D or colchicine to examine the role of actin microfilaments and microtubules in bacterial uptake, respectively. Experiments were performed with HEp-2 cells to compare results with those from previous reports.30 Treatment of HEp-2 cells with either cytochalasin D or colchicine markedly inhibited the entry of all of the invasive strains isolated from ileal or colonic biopsy specimens from patients with CD and UC, giving residual invasion levels ranging from 1.0% to 28.5% (Table 2). In contrast, an actin- and microtubule-independent mechanism was observed for 2 strains (strains LB13 and LB29) that were isolated from colonic specimens of controls.

Search for known invasive determinants. None of the invasive E. coli strains isolated from patients with CD or UC hybridized with intragenic probes corresponding to the ipaC gene encoding the invasin of S. flexneri, the eae gene encoding the intimin of enteropathogenic E. coli, or the tia gene encoding an invasin of enterotoxigenic E. coli (Table 2). But 2 invasive strains (strains LF16 and LF54) isolated from chronic lesions from CD patients hybridized with an intragenic probe corresponding to the afaD gene encoding the invasin AFA of diffusely adhering E. coli, and strain LB13 isolated from colonic control hybridized with the tia probe.

Intracellular survival and replication within macrophages. The nonpathogenic E. coli K-12 C600 was efficiently killed after phagocytosis by J774-A1 macrophages, indicating efficient bactericidal activity of the macrophages (Table 2). All of the invasive E. coli strains isolated from CD or UC patients and from controls were
Table 2. Characteristics of Invasive E. coli Strains Isolated From Patients With CD or UC and From Controls

<table>
<thead>
<tr>
<th>Origin of specimen</th>
<th>Strain</th>
<th>% invasiona</th>
<th>Residual invasionb</th>
<th>Adhesion indexc</th>
<th>% survival at 24 h with J774-A1 macrophages</th>
<th>Known invasive determinantsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>ileal specimens of CD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Chronic lesion</td>
<td>LF16</td>
<td>0.75 ± 0.28</td>
<td>2.34 ± 0.95</td>
<td>3.4</td>
<td>8.5</td>
<td>14 ± 5</td>
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<tr>
<td></td>
<td>LF31</td>
<td>0.39 ± 0.07</td>
<td>0.82 ± 0.39</td>
<td>3.6</td>
<td>13.7</td>
<td>14 ± 1</td>
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<td></td>
<td>LF54</td>
<td>0.79 ± 0.20</td>
<td>1.12 ± 0.28</td>
<td>14.8</td>
<td>9.1</td>
<td>5 ± 1</td>
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<td></td>
<td>LF71</td>
<td>0.18 ± 0.01</td>
<td>0.83 ± 0.76</td>
<td>18.1</td>
<td>21.7</td>
<td>4 ± 1</td>
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<td></td>
<td>LF82</td>
<td>1.29 ± 0.55</td>
<td>1.62 ± 0.72</td>
<td>19.8</td>
<td>28.5</td>
<td>4 ± 1</td>
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<td></td>
<td>LF123</td>
<td>0.30 ± 0.14</td>
<td>0.33 ± 0.13</td>
<td>2.3</td>
<td>8.9</td>
<td>12 ± 1</td>
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<td></td>
<td>LF138</td>
<td>1.41 ± 0.80</td>
<td>0.46 ± 0.11</td>
<td>7.9</td>
<td>13.3</td>
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<td>Early recurrent lesion</td>
<td>LF9</td>
<td>0.15 ± 0.02</td>
<td>0.71 ± 0.23</td>
<td>2.3</td>
<td>6.1</td>
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<td></td>
<td>LF15</td>
<td>0.59 ± 0.13</td>
<td>0.45 ± 0.14</td>
<td>2.9</td>
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<td></td>
<td>LF28</td>
<td>0.12 ± 0.05</td>
<td>0.27 ± 0.12</td>
<td>9.4</td>
<td>20.7</td>
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<td></td>
<td>LF50</td>
<td>0.30 ± 0.05</td>
<td>2.32 ± 1.03</td>
<td>4.1</td>
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<td></td>
<td>LF65</td>
<td>0.38 ± 0.03</td>
<td>0.29 ± 0.07</td>
<td>1.0</td>
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<td></td>
<td>LF119</td>
<td>0.37 ± 0.07</td>
<td>0.42 ± 0.14</td>
<td>18.9</td>
<td>18.9</td>
<td>6 ± 1</td>
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<td>Healthy mucosa</td>
<td>LF128</td>
<td>0.19 ± 0.11</td>
<td>0.11 ± 0.07</td>
<td>6.7</td>
<td>17.6</td>
<td>10 ± 5</td>
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<td></td>
<td>LF130</td>
<td>0.31 ± 0.04</td>
<td>0.25 ± 0.24</td>
<td>6.0</td>
<td>11.5</td>
<td>3 ± 2</td>
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<td></td>
<td>LF73</td>
<td>0.46 ± 0.15</td>
<td>0.39 ± 0.10</td>
<td>4.4</td>
<td>4.5</td>
<td>2 ± 1</td>
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<tr>
<td>ileal specimen of controls</td>
<td>LF100</td>
<td>0.14 ± 0.07</td>
<td>0.10 ± 0.05</td>
<td>17.6</td>
<td>21.6</td>
<td>1 ± 0</td>
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<tr>
<td></td>
<td>LF110</td>
<td>0.42 ± 0.30</td>
<td>1.03 ± 0.44</td>
<td>2.0</td>
<td>6.6</td>
<td>4 ± 2</td>
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<tr>
<td></td>
<td>LF134</td>
<td>0.23 ± 0.05</td>
<td>0.19 ± 0.04</td>
<td>11.0</td>
<td>10.6</td>
<td>1 ± 0</td>
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<tr>
<td>Colonic specimens of CD patients</td>
<td>LF49-2</td>
<td>0.16 ± 0.08</td>
<td>0.25 ± 0.04</td>
<td>1.8</td>
<td>13.5</td>
<td>5 ± 2</td>
</tr>
<tr>
<td></td>
<td>LF50-2</td>
<td>0.15 ± 0.08</td>
<td>0.74 ± 0.23</td>
<td>3.9</td>
<td>21.0</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Controls</td>
<td>LB11</td>
<td>0.34 ± 0.11</td>
<td>1.05 ± 0.44</td>
<td>2.5</td>
<td>5.8</td>
<td>19 ± 3</td>
</tr>
<tr>
<td></td>
<td>LB13</td>
<td>0.14 ± 0.04</td>
<td>1.79 ± 1.06</td>
<td>91.0</td>
<td>93.3</td>
<td>5 ± 2</td>
</tr>
<tr>
<td></td>
<td>LB29</td>
<td>0.12 ± 0.05</td>
<td>0.32 ± 0.12</td>
<td>100.4</td>
<td>143.2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>LF452</td>
<td>0.70 ± 0.36</td>
<td>3.38 ± 1.92</td>
<td>2.1</td>
<td>23.4</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>Reference strain</td>
<td>K-12</td>
<td>0.0005</td>
<td>0.0005</td>
<td>ND</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>

NOTE. For more accurate assessment of the results, we listed in this table only 1 invasive E. coli strain for each patient or control, although the bacteria were invasive for 2 or 3 colonies analyzed for a given biopsy specimen.
aPercentage of inoculum surviving after 1 hour of gentamicin treatment.
bPercentage of intracellular bacteria with cells treated with cytochalasine D or colchicine relative to the number with untreated cells, defined as 100%.
cMean number of bacteria per cell after 3 hours of incubation.
dPercentage of intracellular bacteria at 24 hours postinfection relative to the number after 1 hour of gentamicin treatment, defined as 100%.
eResults of colony blot hybridization of intragenic tia, afaD, eae, and ipaC probes.
fNot determined.

Prevalence of AIEC Associated With Intestinal Mucosa of CD and UC Patients and of Controls

Based on the criteria used to define AIEC strains, as stated in the Patients and Methods section, the following invasive E. coli strains do not belong to the AIEC pathovar: strains LF16 and LF54, isolated from chronic lesions of CD because they harbor the afaD gene of diffusely adhering E. coli; strain LF50-2, isolated from a colonic specimen of a UC patient because it was not able to replicate within J774-A1 macrophages; and strains LB13 and LB29, whose invasive processes do not involve host cell actin polymerization and microtubules. Thus, the prevalence of AIEC associated with ileal mucosa was 21.7% in CD chronic lesions. The prevalence of AIEC associated with the neoterminal ileal mucosa was 36.4% in CD early lesions and 22.2% in healthy mucosa of CD patients. The prevalence of AIEC associated with ileal mucosa of controls was 6.2% (Table 3). A very low prevalence or absence of AIEC was observed in colonic specimens of CD patients (3.7%), UC patients (0%), and

able to survive. All invasive strains but one isolated from a UC patient (strain LF50-2) also showed high intracellular replication. At 24 hours postinfection, the percentages of intracellular bacteria able to replicate ranged from 147% ± 44% to 3537% ± 418%, indicating that the number of phagocytozed bacteria increased 1.47- to 35.37-fold greater than the number at initial infection.
controls (1.9%). Statistical analysis indicated that the prevalence of AIEC associated with early CD lesions of ileal mucosa was significantly higher in patients than in controls ($P = 0.034$).

**Discussion**

The main result of this study was that pathogenic AIEC are associated with ileal CD. AIEC strains were isolated from almost one third of ileal specimens in CD as compared with 6% in ileal controls and less than 5% in colonic samples from both IBD patients and controls. AIEC strains were found more frequently in early recurrent lesions after surgery. This led us to propose that AIEC are not only secondary invaders but that they could be involved in the initiation of the inflammatory process. However, the presence of AIEC in 22% of the patients having no or minimal endoscopic recurrence suggests that recurrence of CD may occur at different time points in patients colonized with AIEC or that other factors are required to trigger new onset of inflammation.

**Table 3. Prevalence of Invasive E. coli Strains Associated With the Ileal and Colonic Mucosa of Patients With CD and of Controls**

<table>
<thead>
<tr>
<th>Origin of the strains</th>
<th>Total no. of subjects</th>
<th>No. of subjects (%) positive</th>
<th>$P$ value$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ileal specimens of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD patients with chronic lesion</td>
<td>23</td>
<td>7 (30.4)</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td>CD patients with early lesion</td>
<td>22</td>
<td>8 (36.4)</td>
<td>8 (36.4)</td>
</tr>
<tr>
<td>CD patients with healthy mucosa</td>
<td>18</td>
<td>4 (22.2)</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td>Controls</td>
<td>16</td>
<td>1 (6.2)</td>
<td>1 (6.2)</td>
</tr>
<tr>
<td>Colonic specimens of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD patients</td>
<td>27</td>
<td>1 (3.7)</td>
<td>1 (3.7)</td>
</tr>
<tr>
<td>UC patients</td>
<td>8</td>
<td>1 (12.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Controls</td>
<td>102</td>
<td>4 (3.9)</td>
<td>2 (1.9)</td>
</tr>
</tbody>
</table>

$^a$E. coli strain was defined as invasive when a mean percentage superior or equal to 0.1% of the original inoculum was recovered after 1 hour of gentamicin treatment.

$^b$AIEC were defined as described in the Materials and Methods section.

$^c$Statistical analysis of the prevalence of AIEC in ileal or colonic specimens of CD patients and in controls.

$^d$Significant higher prevalence of AIEC in early ileal lesions of CD patients compared with controls.
AIEC strains do not represent a specific pathogen exclusively found in CD because we observed their presence in a few ileal or colonic control specimens. This suggests that AIEC strains may belong to transient normal flora but preferentially could colonize CD ileal mucosa. Bacterial adherence to the intestinal mucosa occurs through fimbrial structures and/or other bacterial surface proteins known as adhesins. These act as lectins, recognizing glycosyl motifs expressed by host cell-surface glycolipids or glycoproteins, and play a key role in bacterial pathogenicity. Aberrant glycosylation of mucosal glycoconjugates has been shown in UC and CD, and bacterial pathogenicity. Aberrant glycosylation of mucosal glycoconjugates has been shown in UC and CD,42 and such modified glycolipids or glycoproteins could be involved in AIEC colonization of CD ileal mucosa.

The present study has failed to find a high prevalence of AIEC in UC patients. This result was confirmed with E. coli strains isolated from colonic specimens of German UC patients (data not shown). E. coli strains with invasive properties were found on rectal biopsy specimens from UC patients43 and optic microscopic assays showed the presence of adhesive or invasive E. coli in the fecal samples of 35% of patients with active UC and 27% of patients with quiescent UC.44 But these results are open to criticism because of the difficulties in differentiating between extracellular and intracellular bacteria by optic microscopic examination.

The role of AIEC in the initiation or perpetuation of the inflammatory disease is not yet defined. AIEC strains, which are able to adhere to and to invade intestinal epithelial cells and to trigger uptake into and survival within macrophages, could translocate across the human intestinal barrier, move to deeper tissues, continuously activate macrophages, and, thus, potentially induce the formation of granulomas. Immunohistochemical studies have shown that intramucosal E. coli in CD are found in macrophages within the lamina propria, in the germinal centers of mesenteric lymph nodes, and in granulomas. It is well established that bacterial pathogens that have the ability to penetrate the intestinal epithelial barrier and to resist macrophage killing trigger a strong host inflammatory response. The hypothesis regarding the participation of invasive bacteria to the pathogenesis of CD was strengthened with the identification of mutations in the NOD2/CARD15 encoding gene in CD patients.24,25 Indeed, NOD2/CARD15 is a cytosolic receptor responding to the presence of bacterial components and recent data indicated that intestinal epithelial cells expressing a NOD2/CARD15 variant associated with CD were unable to constrain bacterial replication. Further clues may be provided by the expression pattern of the CARD15 protein: CARD15 mutations have been associated consistently with ileal involvement in CD. Originally thought to be confined to myelomonocytic and dendritic cells, CARD15 expression recently has been found in intestinal epithelial cells and Paneth cells. The discovery of CARD15 expression in Paneth cells is particularly interesting because these cells have long been known to play an antibacterial role in the gut, secreting potent antimicrobial substances such as lysozyme, phospholipase A2, and α- and β-defensins. Furthermore, Paneth cells are highly concentrated in the terminal ileum. Recent data have shown that CD is characterized by multiple defensin deficiencies and that HBD-5 and HBD-6 expression, which predominates in the ileum, is down-regulated in patients with NOD2 mutations.

In summary, an abnormally high prevalence of AIEC is observed in ileal CD. Further work is needed to understand which factors play a crucial role in the colonization of the ileum by these pathogenic strains.

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Received June 6, 2003. Accepted April 22, 2004.
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Supported by a grant from the Ministère de la Recherche et de la Technologie (PRFMMIP 2000) and grants from the Association F. Aupetit, Société Nationale Française de Gastro-Entérologie, and the Institut de Recherche des Maladies de l’Appareil Digestif (to J.B.). The authors thank Michael Donnenberg for providing enteropathogenic E. coli strain E2348/69, Dolores Evans for providing enterotoxigenic E. coli strain H10407, Chantal Le Bouguenec for providing AFA strain A30, and Philippe Sansonetti for providing Shigella flexneri strain SC301. The authors also thank Chantal Rich and Laurence Nakusi for their technical help, and Herbert Van Kruiningen for helpful discussion and critical reading of the manuscript.