Association Between Intraepithelial *Escherichia coli* and Colorectal Cancer

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Background & Aims: Although multiple studies have focused on *Helicobacter pylori*, little is known about the mucosa-associated flora of the colon. The aim of this study was to detect bacteria directly in colonic mucosa from patients screened for colorectal cancer. *Methods:* Bacteria were quantified with the polymerase chain reaction and identified by comparative sequence analysis in colonoscopic biopsy specimens from 31 asymptomatic and 34 symptomatic controls with normal colonoscopic findings, 29 patients with colonic adenoma, and 31 patients with colorectal carcinoma. In 41 patients, intra- and extracellular location of bacteria was confirmed with the gentamicin protection assay. *Results:* No bacteria were detected in biopsy specimens from 97% of asymptomatic and 69% of symptomatic controls. In contrast, bacterial concentrations of 10^3–10^5 colony-forming units per microliter were detected in biopsy specimens from both malignant and macroscopically normal tissue in 90% and 93% of patients with adenoma and carcinoma, respectively. *E. coli* and *coli*-like bacteria were shown to colonize the colonic mucosa in 82% of these patients. The gentamicin protection assay indicated that *E. coli* was partially intracellular in 87% of patients with adenoma and carcinoma and in none of the controls. *Conclusions:* The colonic mucosa of patients with colorectal carcinoma but not normal colonic mucosa is colonized by intracellular *E. coli*.

The possibility that infectious agents are involved in the etiology of cancer has excited investigators since the discovery of oncogenic viruses.[1] Infection with parasites such as *Schistosoma* and liver flukes is known to increase the risk of certain cancers, and an association between hepatobiliary cancer and a typhoid carrier state was described[2]; however, only recently was a bacterial pathogen definitely connected to gastric carcinoma and lymphoma. At the World Health Organization/International Agency for Research on Cancer meeting in Lyon in 1994, *Helicobacter pylori* was added to group 1 (definite) carcinogens and thus joined other biological carcinogens such as hepatitis viruses B and C.[3] Colorectal cancer, like gastric cancer, is one of the leading neoplastic diseases worldwide. Its development occurs in a highly complex and poorly characterized bacterial environment. We investigated whether a mucosa-associated pathogen with *H. pylori*–like adhesive and invasive properties could be involved. A prospective study of patients screened for colorectal cancer was carried out. Bacteria were detected in colonoscopic biopsy specimens. The bacterial count was assessed with the quantitative polymerase chain reaction (PCR), and 16S ribosomal RNA (rRNA) sequence analysis was used to identify the bacteria involved. The gentamicin protection assay was adopted to verify whether bacteria were intra- or extracellular. Bacteria were visualized in semithick frozen sections by indirect immunofluorescence.

Patients and Methods

Patients

This study was performed at the IVth Medical Clinic, Charité University Hospital, Berlin, Germany, with prior approval of the institutional review board and the patients' informed consent. One hundred twenty-five consecutive patients underwent colonoscopy for screening or staging for colorectal malignancies. Patients with inflammatory bowel disease (IBD) or macroscopic signs of inflammation were excluded. None of the patients had a history of familial polyposis, and none received antibiotics 8 weeks before the procedure. All patients, except for 2 who were citizens of the European Union, were native Germans.

The patients were divided into four groups according to clinical diagnosis and endoscopic findings. Group 1 included 31 asymptomatic controls routinely screened for colorectal cancer without any intestinal symptoms. Group 2 included 34 symptomatic controls with abdominal complaints and normal colonoscopic findings. Group 3 included 29 patients with tubular-villous adenoma; 19 of these patients were newly

Abbreviations used in this paper: CFU, colony-forming units; PCR, polymerase chain reaction; rRNA, ribosomal RNA.

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diagnosed with tubular-villous adenoma, and 10 patients underwent colonoscopy within a year after colonoscopic polypectomy. Of these 10 patients, 4 had a normal colonoscopy and 6 had a recurrent adenoma. Group 4 included 31 patients with colorectal carcinoma; 10 patients were newly diagnosed with carcinoma and 18 patients underwent colonoscopy within 1 year after partial colectomy for carcinoma. Of these 18 patients, 6 had no relapse, 7 had developed an adenoma, and 5 had developed both adenoma and carcinoma; 3 patients without signs of relapse, who underwent colonoscopy 5, 7, and 10 years after hemicolectomy due to colonic carcinoma, were also placed in the carcinoma group.

The gender, mean age, and age range of all patients are summarized in Table 1. Patients in the adenoma, carcinoma, and asymptomatic control groups did not differ significantly in their gender, mean age, and age range. The mean age of symptomatic patients with normal colonoscopic findings was significantly lower than the mean age of the other three groups.

Between one and seven colonoscopic biopsy specimens were analyzed from each patient. Whenever possible, biopsy specimens were taken from both tumorous and normal mucosa.

Methods

Biopsy preparation. Biopsy specimens were placed in physiological saline and stored at 4°C for up to 2 hours before further steps. Each sample was vortexed for 30 seconds in a 1.5-mL Eppendorf tube and placed in 500 μL of fresh saline. This procedure was repeated eight times, after which the sample was set rotating from 14 to 18 hours at room temperature in 200 μL of 1% sodium dodecyl sulfate saline solution. DNA was extracted once with phenol, further purified using a GeneClean kit (BIO 101, La Jolla, CA), and resuspended in 30 μL of high-performance liquid chromatography water (J. T. Baker, Deventer, Holland). Ten microliters of template DNA was added to the PCR reaction.

DNA amplification. The 5′ half of the 16S rRNA genes (600 base pairs) was amplified with PCR using a primer set universal for bacteria: 5′ CTGGTTCCGGCGA AGAGTTTGAT(c/t)(c/a)TGGCTCAG-3′, 5′-CTCGCTCCGGCGA ACCGC(g/t)(a/g)CTGCTGGCAC-3′.[4] The thirteen additional nucleotides (underlined) were added at the 5′ ends for cloning (PharMingen, San Diego, CA). To prevent false-positive PCR results, pre- and post-PCR steps were carried out in different laboratories and control experiments omitting template DNA were performed in parallel. To avoid false-negative results, standard bacterial concentrations were regularly added to probes with negative PCR. No noticeable inhibition was observed throughout this study.

Table 1. Patients' Sex, Mean Age, and Age Range

	Asymptomatic controls	Symptomatic controls	Adenoma	Carcinoma
Total no. of patients	31	34	29	31
Mean age (*yr*)	57.6	39.1	60.9	61
Age range (*yr*)	37–85	20–60	34–87	37–79
Female/male	18/13	19/15	17/12	18/13

Bacterial stock solution used in quantitative PCR. For quantification of bacteria, a stock of XL1 Blue MRF′ *E. coli* cells (Stratagene, La Jolla, CA) was grown and frozen at −70°C with 20% glycerol in 1-mL aliquots. The concentration of bacteria in this stock solution was determined by counting colony-forming units (CFU) in a series of plated dilutions.

Determination of positive and negative thresholds of PCR. Before every PCR, an aliquot of frozen stock was thawed and diluted to concentrations of 10^5, 10^4, 10^3, 10^2, and 10 CFU/μL. One microliter from each dilution was directly added to the PCR. The PCR products were visualized with ethidium bromide–stained agarose gel electrophoresis. The PCR of dilutions starting at 10^3 CFU/μL was regularly positive and arranged in easily distinguishable steps. The difference between the PCR of dilutions of 10^5 and 10^6 CFU/μL could be detected only after 25 cycles. Once in a while a light band was seen in the PCR of dilution 10^2 CFU/μL, but this product was not sufficient for isolation from the agarose gel. All attempts to increase PCR sensitivity with more thermocycles, longer cycles, and higher enzyme concentrations resulted in the negative control without template DNA giving a positive signal.

Quantification of bacteria with PCR. For each set of reactions, one PCR mix was prepared for template DNA, standard bacterial dilutions of 10^5–10^1 CFU/μL, and two negative controls, one for sample preparation and DNA extraction and the other for the PCR components, and thereafter rationed into separate reactions. PCR products of template DNA were visualized simultaneously with the PCR products of bacterial dilutions and the negative controls. Because no bacterial sequences could be detected in weakly positive probe PCR signals corresponding to the 10^2 dilution after subsequent cloning and sequencing, these PCR products were considered negative. Probe PCR products with a signal stronger than the 10^2 CFU/μL dilution were considered positive and were isolated from the agarose gel for further steps.

Sequence analysis. To determine the bacterial composition of probes with a positive PCR signal, amplified 16S rRNA sequences were cloned into a pCMV-LIC vector according to a Ligation Independent Cloning Protocol (PharMingen). Between 40 and 100 clones were sequenced for each biopsy specimen on the LI-COR Model 4000 Sequencer (LI-COR, Lincoln, NE). Bacterial sequences were analyzed and compared with 16S rRNA primary structures retrieved from public databases as previously described.[5–6]

Gentamicin protection assay. The gentamicin protection assay is based on the resistance of internalized bacteria to gentamicin that does not enter the eukaryotic cell. It was used to determine whether bacteria were intra- or extracellular. Biopsy specimens were placed in 500 μL of 0.016% dithiothreitol solution for 15 minutes to deprive them of mucus. Each biopsy specimen was then cut in half. One half was placed in physiological saline and the other in physiological saline with gentamicin at concentrations of 50 mg/L. After 30 minutes of

gentamicin exposure, each half was placed in 500 µL of fresh saline and washed four times as described. After the fourth wash, the first half was put in 500 µL of saline and the second (gentamicin pretreated) half in 500 µL of distilled water, and further vortexed for 30 minutes. After this exposure, 500 µL of supernatant from both halves was plated on MacConkey agar plates. The plates were incubated aerobically at 37°C and examined at 24 and 48 hours. The number of colonies was counted and the bacteria were identified using a commercial identification system API 20E (bio Merieux, Nuertingen, France).

Fluorescence microscopy of biopsies. To visualize intracellular *E. coli*, tumor-free biopsy specimens from a patient with newly diagnosed carcinoma of ascending colon were fixed for 12 hours at 4°C in a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 100 mmol phosphate buffer (pH 7.4). Small segments of tissue were infiltrated in sucrose and 1 µm thick frozen sections were prepared on Reichert Ultracut (Reichert, Vienna, Austria), equipped with a F4 cryo attachment, as previously described.[7] Semithick frozen sections were incubated with rabbit anti–*E. coli* immunoglobulin G (diluted 1:1000; Dako, Copenhagen, Denmark) followed by rhodamine-conjugated anti-rabbit $F(ab)_2$ fragment that was preabsorbed with 10% human serum to reduce background noise. The sections were examined with a Zeiss Axiophot fluorescence microscope (Frankfurt Main, Germany).

Statistics

Where appropriate, nonparametric data were expressed by median value (range), normally distributed data were expressed as mean, and differences between or across groups were evaluated by analysis of variance. Statistical analysis was done using χ^2 and *t* tests. A *P* value of <0.05 was considered significant.

Results

No bacteria were detected in any of the mucosal biopsy specimens from 30 of 31 asymptomatic controls and 24 of 34 symptomatic controls. In contrast, bacterial concentrations between 10^3 and 10^5 CFU/µL were detected in at least one biopsy specimen from 55 of 60 patients in the adenoma and carcinoma groups. This difference between the adenoma and carcinoma groups and both normal groups was statistically significant ($P < 0.001$). In 90% of the positive biopsy specimens from the adenoma and carcinoma groups, bacterial concentrations of 10^5 CFU/µL were found (Table 2). PCR-positive biopsy specimens were also found in patients after resection of colonic carcinoma. Only biopsy specimens from the 3 patients without relapse for more than 3 years were PCR negative.

Adherent bacteria were not restricted to the site of adenoma or carcinoma. In all patients from the adenoma and carcinoma groups with positive PCR for intraepithelial bacteria, at least one biopsy specimen from normal mucosa was also positive. However, these changes did not necessarily include the whole colon. Macroscopically normal tissue of the large intestine was positive in 52% of biopsy specimens from patients with adenoma and 84% of biopsy specimens from patients with carcinoma.

Table 2. Results of PCR and Sequence Analysis

	Asymptomatic controls (%)	Symptomatic controls (%)	Adenoma (%)	Carcinoma (%)
Total no. of patients	31	34	29	31
Patients with PCR-positive biopsies	1 (3)	10 (31)	27 (93)	28 (90)
Total no. of biopsies	97	113	83	83
PCR-positive biopsies	4 (4)	25 (22)	53 (64)	71 (86)
Patients with predominantly[a] *E. coli*	1 (3)	4 (12)	18 (62)	24 (77)
Patients with predominantly[a] *coli*-like sequences	0	0	4 (14)	3 (10)
Patients with a mix of sequences	0	6 (19)	5 (17)	1 (3)

[a]Predominance was defined as >50% of all clones sequenced identical.

Fourteen patients underwent colonoscopy again within a week of the initial procedure for reasons of incomplete preparation and fecal remains in the colon. Seven additional patients underwent colonoscopy repeatedly in the following 6 months. All 11 patients in the adenoma and carcinoma groups who were initially positive were also positive after the second colonoscopy, and all 10 of the initially negative patients from both normal groups remained negative.

Two patterns of bacterial sequences were observed. Some biopsy specimens analyzed contained a mixture of sequences with no sequence repeating in more than 20% of all the clones. This mixture most often consisted of different clostridia, *Bacteroides*, *Pseudomonas*, and *E. coli* sequences. In the second pattern more than 80% of all the clones sequenced were identical. The predominance of a single sequence was found in biopsy specimens from 22 of 29 patients with adenoma and 27 of 31 patients with carcinoma. The repeating sequence in these biopsy specimens was either *E. coli* or *coli*-like (3× *Hafnia alvei*, 3× *Citrobacter freundii*, and 1× *Kluyvera*). Biopsy specimens from the remaining 4 patients with adenoma and 1 patient with carcinoma had a mixture of sequences. Out of the 11 patients with PCR-positive biopsy specimens from normal colonoscopy groups, 5 had a predominance of *E. coli* sequences and 6 had a mixture of sequences (Table 2).

One hundred sixty-nine biopsy specimens from 41 of the above patients were tested with the gentamicin

protection assay to differentiate between intra- and extracellular location of bacteria (Table 3). From every patient, four biopsy specimens from standard locations in the cecum, transverse colon, ascending colon, sigmoid colon and, whenever possible, a fifth biopsy from the tumor were tested. No more than three *E. coli* colonies were observed in the fourth wash of PCR-negative biopsy specimens from all four groups. In the fourth wash of PCR-positive biopsy specimens from normal symptomatic patients, patients with adenoma, and patients with carcinoma, 468 ± 592, 289 ± 290, and 230 ± 307 colonies were observed, respectively. After gentamicin treatment, the number of *E. coli* colonies dropped in the normal symptomatic group to less than one but remained at 80.1 ± 106 and 46.7 ± 83 in the adenoma and carcinoma groups, respectively. This indicates intracellular bacteria in 87% of patients with adenoma and carcinoma.

Figure 1 shows the results of indirect immunofluorescence. Numerous bacteria were observed on the surface of the mucosa. In some sections, granular immunofluorescence with this antibody was also observed in the basal or intermediate part of the mucosa. These granules were apparently associated with enterocytes and not with goblet cells, although a precise distinction between these two cell types was difficult to obtain on the specimens used.

Table 3. Results of the Gentamicin Protection Assay

	Asymptomatic controls	Symptomatic controls	Adenoma	Carcinoma
Total no. of patients	11	14	7	9
Patients with positive[a] *E. coli* culture	0	4	7	8
Patients with positive gentamicin protection assay	0	0	5	8
Total no. of biopsies	44	56	30	39
% of PCR-positive biopsies	0	29	53	79
% of gentamicin protection assay–positive biopsies	0	0	28	56
Mean no. ± SD of *E. coli* colonies grown from PCR-negative biopsies	0.69 ± 0.56	1.1 ± 2.4	0.9 ± 2.0	1.3 ± 2.3
Mean no. ± SD of *E. coli* colonies grown from PCR-positive biopsies		468 ± 592	289 ± 290	230 ± 307
Mean no. ± SD of *E. coli* colonies grown after gentamicin treatment	0.1 ± 0.18	0.6 ± 0.9	80.1 ± 106	46.7 ± 83

[a]Growth of more than five *E. coli* colonies in the fourth biopsy wash plated on MacConkey agar was considered positive.

Discussion

In the early 1970s, the observation of marked geographic variations in the incidence of colorectal cancer, which could not be explained by racial and dietary differences, lead to the hypothesis that intestinal bacteria might play a key role in cancerogenesis.[8] However, the etiologic relationship of intestinal bacteria and colorectal cancer could not be definitively documented. In the 1980s and 1990s, extensive investigations linked a permanent colonization of the gastric mucosa with *H. pylori* to gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[9] At the same time, the mucosa-associated colonic flora was overlooked. Until now it was not even known whether specific bacteria could colonize the human colonic mucosa. This disparity was the result of methodological problems that accompany the evaluation of the complex intestinal flora with classical bacteriologic methods. The colon contains between 10^9 and 10^{13} bacteria per gram of luminal contents. The composition of colonic microflora is highly heterogeneous and unpredictably influenced by factors such as age, diet, and geography. Studies of fecal specimens, although time and work intensive, are not representative of the microecology of epithelial and cryptal communities throughout the digestive tract.[10]

Testing the 1970s hypothesis with methods of the 1990s, we had surprising results. Despite high concentrations of intraluminal microorganisms in the large intestine, the colonic mucosa of asymptomatic controls proved not to be colonized by bacteria. The cleanliness of the colon at the time of investigation did not affect this result. In contrast, high concentrations of bacteria were detected in at least one biopsy specimen from 90% of patients with carcinoma and 93% of patients with adenoma. These findings were reproducible in patients who repeatedly underwent colonoscopy.

Explanations of high concentrations of bacteria in colonic biopsy specimens of patients in the carcinoma and adenoma groups include: compromised host immunity at different levels that leads to the invasion of the mucosa by intraluminal bacteria, expression of abnormal surface antigens in cancer patients that promotes adhesion of bacteria, and a microorgan-

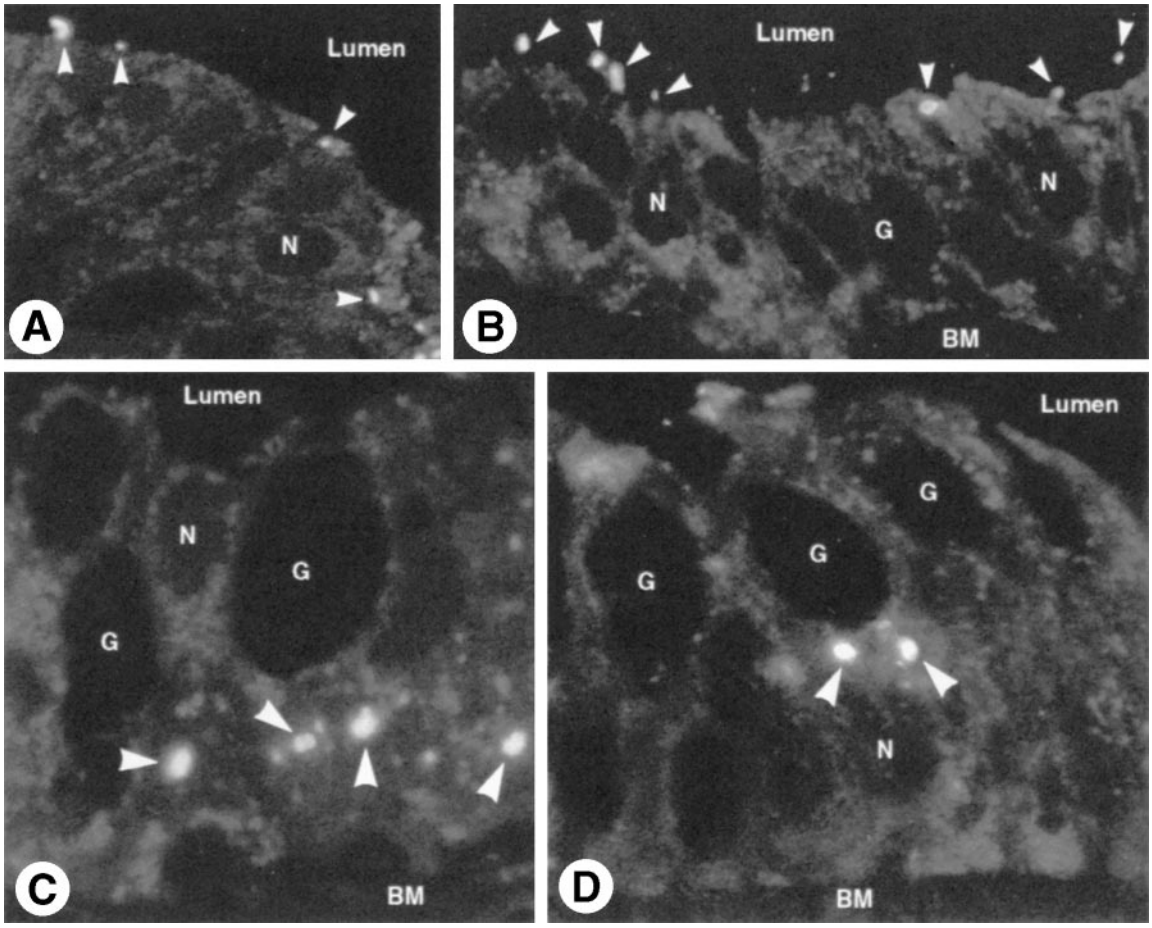

Figure 1. Immunofluorescence of frozen sections from normal mucosa of patient with carcinoma of the ascending colon. *Arrowheads* indicate *E. coli*. N, nucleus; G, goblet cell; BM, basement membrane. Original magnification: *A* and *B*, 400×; *C* and *D*, 1200×.

ism specifically colonizing the colonic mucosa before adenoma or carcinoma development.

A local effect of tumor-damaged tissue allowing an unspecific invasion is unlikely because high bacterial counts were not only seen in tumors, but in adjacent and distant, normal tissue and in biopsy specimens from relapse-free patients within 3 years after partial colectomy. It was even the case that the bacterial count at the site of the tumor was often below that of adjacent tissue, possibly indicating hindered bacterial adhesion to neoplastic epithelia.

An unspecific mucosal barrier breakdown throughout the colon is also unlikely. In such a case one should see a variety of bacteria. However, the most striking finding of the sequence analysis was the predominance of one *E. coli* and sometimes *coli*-like sequence. A mixture of different sequences was the exception in 3% of cancer patients and 17% of patients with adenoma. Although *E. coli* is generally considered to be a normal constituent of the fecal flora, this statement refers exclusively to intraluminal and not to mucosa-adherent *E. coli* strains. No reliable data are available about the prevalence of these pathogens in the general population or about pathogen clearance, because nonpathogenic *E. coli*, the major constituent of the human aerobic flora, hinders the detection of pathogenic strains.[11]

Twelve percent of patients with digestive disturbances and normal colonoscopic findings also had high concentrations of *E. coli* in biopsy specimens and in the culture of the wash fluid from the biopsy surface. However, these *E. coli* differed from the *E. coli* in cancer patients. Strains isolated from symptomatic controls with normal colonoscopy were gentamicin protection assay negative and possibly came from a prolonged infection with adherent but noninvasive bacteria. A significantly lower mean age of patients in this group is consistent with the proposal of a subclinical infection. More than 20% of the *E. coli* population isolated from the biopsy specimens taken from patients with adenoma and carcinoma after hypotonic lysis was positive in the gentamicin protection assay, indicating a partially intracellular mechanism of colonization, similar to that described for some enteropathogenic *E. coli* strains. Although the majority of the enteropathogenic *E. coli* population is located on the

epithelial cell layer, a small proportion of adherent organisms invades. Known enteroinvasive *E. coli* organisms do not adhere well, but a large proportion of bacteria that adhere invade. The number of intracellular enteropathogenic *E. coli* bacteria is comparable with the number of intracellular enteroinvasive *E. coli* strains.[12] The results of our study cannot be directly compared with the in vitro results obtained on epithelial cell cultures. However, the data of gentamicin protection assay and of the fluorescence microscopy of biopsy specimens from carcinoma patients are implicative for a specific intracellular pathogen.

The next challenge lies in determining whether an intracellular *E. coli* colonization of the colonic mucosa is primary or secondary to the malignant pathology. The present data are not sufficient to answer this question. Nevertheless, even if intracellular *E. coli* is later proved to be insignificant, its high incidence in patients with adenoma and carcinoma opens new perspectives in screening for colorectal cancer.

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