Virulence properties of *Escherichia coli* strains isolated from patients with inflammatory bowel disease

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Abstract

*Escherichia coli* strains cultured from 74 patients with inflammatory bowel disease at different stages of disease activity (Crohn’s disease (40), ulcerative colitis (34)) and 18 healthy controls were studied in relation to haemolysin and verotoxin production and enteroadherence. Disease activity was assessed by standard clinical and laboratory tests. Haemolytic *E coli* were isolated from 18% of patients with Crohn’s disease, 24% with ulcerative colitis, and 11% of healthy controls. None of these differences was significant. No verotoxin producing strains were detected among the 216 *E coli* isolates examined but the extract from five strains (Crohn’s (4), ulcerative colitis (1)) produced a distinctive cytopathic effect on Vero cell monolayers which was later shown not to be due to verotoxin. The adhesion indices of *E coli* isolates cultured were: mean (SEM) 42-2 (6-4) for Crohn’s disease, 43-3 (6-2) for ulcerative colitis, and 11-3 (2-0) for normal controls (p<0.0001). Adhesive *E coli* were isolated from 62% of patients with Crohn’s disease and 68% with ulcerative colitis but from only 6% of normal controls (p<0.0002). Neither haemolysin production nor enteroadherence was dependent upon disease activity, disease location, sulphasalazine treatment, or previous intestinal resection. These results indicate that only enteroadherent *E coli* were frequently associated with inflammatory bowel disease; their relation to the pathogenesis of these conditions, however, remains uncertain.

*Escherichia coli* strains are the predominant aerobic or facultative Gram negative rods in the normal human colon, where they play an important role in promoting the stability of the intestinal microbial flora and in maintaining the normal intestinal physiology. Epidemiological and volunteer studies have confirmed that certain *E coli* strains can be true intestinal pathogens in animals and in man. These strains are associated with well defined diarrhoeal syndromes. Diarrhoeagenic *E coli* belong to a small number of serotypes which differ from those normally resident in the colon in possessing distinct virulence properties of which the production of enterotoxins (enterotoxigenic *E coli*), tissue invasion (enteroinvasive *E coli*), and adherence to enterocytes (enteroadherent *E coli*) have been studied most widely. In many pathogenic strains, however, the virulence mechanism remains to be defined.

We have recently shown that patients with Crohn’s disease harboured an increased number of coliforms in their faeces, particularly during periods when the disease was active. Other workers reported increased *E coli* numbers in the stools of patients with severe ulcerative colitis. Furthermore, *E coli* strains isolated from patients with inflammatory bowel disease may also be qualitatively different from those found in the normal faeces in that they can digest mucus, produce enterotoxins, and adhere in increasing numbers to Hela cells and buccal epithelial cells. These features suggest that *E coli* may play a role in the pathogenesis of inflammatory bowel disease.

This study was aimed at determining the prevalence of haemolysin and verotoxin production and enteroadherence as established virulence properties in *E coli* strains isolated from patients with confirmed inflammatory bowel disease at different stages of activity using as control *E coli* strains cultured from the urine of patients with symptomatic urinary tract infection and the faeces of healthy volunteers.

Patients and methods

A total of 74 patients with inflammatory bowel disease was studied. In 40 patients with established Crohn’s disease (29 women; 11 men, mean age 44 years), the disease was confined to the small bowel, large bowel, and affected both sites in 13, 13, and 4 patients respectively. In 10 patients the disease had recurred at the site of a previous ileocolonic anastomosis. The disease was considered clinically active in 19 patients and quiescent in the remaining 21 – disease activity being assessed by standard clinical and laboratory tests as previously described.

Thirty four patients had ulcerative colitis (20 women: 14 men, mean age 45 years) affecting the entire colon in 11 patients, the left colon in 14, and limited to the rectum in nine. Active disease was present in 15 patients; of these nine were having their first attack of colitis. The remaining 19 had quiescent disease. Disease activity was assessed by the criteria of Truelove and Witts, and in all cases was confirmed by sigmoidoscopy and histology.

At the time of stool collection, 17 patients (Crohn’s disease (7), ulcerative colitis (10)) were taking sulphasalazine. Those who had received antibiotics or special dietary advice within the four weeks preceding the collection of faecal samples were excluded.

The control group for all experiments consisted of 18 healthy laboratory workers (10 women: 11 men, mean age 35 years). In addition, *E coli* strains from 115 patients with sympto-
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BACTERIOLOGY
Table I shows the total number of E. coli isolates cultured from each study group and the number of isolates tested for adhesiveness, haemolysin, and verotoxin production. All E. coli isolates (246) were tested for verotoxin production, but only one randomly chosen isolate from each patient was tested for both adhesiveness and haemolysin production. The identification of E. coli strains was established morphologically and confirmed by applying standard biochemical tests as previously described. Although verotoxin production may be limited to a number of E. coli serotypes, haemolysin elaboration and adhesiveness are not a function of serotype. Prior serotyping of our E. coli isolates was therefore not undertaken.

HAEMOLYSIN ASSAY
Haemolysin production was tested using blood agar containing sheep erythrocytes prepared as previously described. Coded E. coli isolates were inoculated on the blood agar and incubated aerobically at 37°C for 18-24 hours. The presence of haemolysin could be seen as a clear zone surrounding the inoculum.

VEROTOXIN ASSAY
The method of extraction of verotoxin from verotoxin producing E. coli using Polymyxin B (Sigma, Poole) has been described by Karmali et al. and is based on fact that Polymyxin B releases the usually high concentration of verotoxin present in the periplasmic space and that Polymyxin B releases 80-90% of the cytopathic activity of Shigella dysenteriae type 1 cells. Briefly, the E. coli isolates were grown aerobically on nutrient agar plates for 18 hours. A colony sweep was then inoculated into 20 ml of Isoosensitest broth (Oxoid) and incubated aerobically at 37°C for six hours before centrifugation at 4000 g for 10 minutes. The pelleted cells were resuspended in 1 ml of Polymyxin B, 0-1 mg/ml, mixed thoroughly, and incubated for a further 30 minutes. The supernate of this suspension was filter sterilised, coded, and stored at -20°C. Vero cells (kindly supplied by Dr P Chapman, Public Health Laboratory, Sheffield), were used to seed a 96 well, flat bottomed microtitration tray which was then incubated at 37°C under a cell monolayer was formed. Two dilutions of the Polymyxin B extract (neat and 1 in 100) were then added to pairs of wells in the presence of maintenance medium (Eagle’s minimum essential medium). The microtitration tray was incubated at 37°C in 5% CO2 and examined daily for the characteristic cytopathic effects produced by verotoxin (Fig 1). The Polymyxin B extract was considered to contain appreciable amounts of verotoxin only if it produced a cytopathic effect on the Vero cells both undiluted and at a 100 fold dilution within three days.

Coded E. coli isolates were tested in batches, each batch containing a positive and a negative reference E. coli strain (E.43629 and NCTC 10418 respectively). The reference strains were kindly provided by Dr R J Gross, Central Public Health Laboratory, Colindale, London, and Dr B D Hall, Centre for Vaccine Development, University of Maryland, USA. The sensitivity of the assay was such that the verotoxin from the positive E. coli strain was detectable at 1 in 1000 dilution.

ADHESION ASSAY
Mannose resistant enteroradherance was assessed using the buccal epithelial cell adhesion assay as described elsewhere. Briefly, coded E. coli isolates were cultured aerobically on blood agar at 37°C for 18 hours and a colony sweep was suspended in 10 ml of phosphate buffered saline (PBS), pH 7-3, to a concentration of about 10⁸ bacteria/ml. Buccal epithelial cells were obtained from a single donor (MHG) by gentle scraping of the buccal mucosa with a sterile wooden spatula. The cells were washed three times in PBS before being suspended in PBS containing D-mannose (28 mmol/l) to a concentration of about 10⁶ cells/ml. Equal volumes (0-5 ml) of the bacterial and buccal epithelial cell suspensions were mixed in a sterile container for 30 minutes. The non-adherent bacteria were eliminated by differential centrifugation. The cell pellet obtained after three washings in PBS was suspended in 0-05 ml of sterile PBS on a glass slide. The smear was air dried, fixed in absolute alcohol, and stained by Gram’s method. Coded E. coli isolates were tested in batches, each containing an adherent and a non-adherent reference strain, E.8517/1 and SC 13 respectively (kindly provided by Dr S Scotland, Central Public Health Laboratory). A control test without added bacteria was also included to exclude contaminated samples.

An adhesion index was determined by recording the number of unfolded epithelial cells out of

<table>
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<tr>
<th>Table I</th>
<th>Total number of Escherichia coli isolated from different study groups and the numbers of isolates tested for adhesiveness, haemolysin, and verotoxin production</th>
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<tbody>
<tr>
<td></td>
<td>Total no of E. coli isolates</td>
</tr>
<tr>
<td>Crohn’s disease (n=40)</td>
<td>135</td>
</tr>
<tr>
<td>Ulcerative colitis (n=54)</td>
<td>81</td>
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<tr>
<td>Controls (n=18)</td>
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<table>
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<tr>
<th>Table II</th>
<th>Haemolytic and adhesive properties of Escherichia coli isolates from patients with inflammatory bowel disease and controls</th>
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<tr>
<td>Disease status</td>
<td>Total no of E. coli isolates tested</td>
</tr>
<tr>
<td>Active Crohn’s disease</td>
<td>21</td>
</tr>
<tr>
<td>Quiescent Crohn’s disease</td>
<td>19</td>
</tr>
<tr>
<td>Active ulcerative colitis</td>
<td>15</td>
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<tr>
<td>Quiescent ulcerative colitis</td>
<td>19</td>
</tr>
<tr>
<td>Normal controls</td>
<td>13</td>
</tr>
<tr>
<td>Symptomatic urinary tract infection</td>
<td>115</td>
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*p<0.0002, compared with controls. NT = not tested.
Figure 1: Vero cell monolayers. (A) normal; (B) cytopathic effect (not due to verotoxin) produced by extracts from some E. coli isolates from patients with inflammatory bowel disease. (C) characteristic verotoxin effects on vero cells.

Figure 2: Adhesion indices in patients with inflammatory bowel disease (Crohn's disease (CD) and ulcerative colitis (UC)) and normal controls.

patients with Crohn's disease and 8 (24%) with ulcerative colitis compared with 2 (11%) of the control group and 38 (37%) of patients with symptomatic urinary tract infection. None of these differences was statistically significant. Disease activity in either Crohn's disease or ulcerative colitis had no influence on the frequency of isolation of haemolytic E. coli. Similarly, age, sex, disease location, previous surgery, and sulphasalazine therapy had no effect on the carriage of haemolytic strains (data not shown).

None of the 246 E. coli isolates tested for verotoxin production gave positive result. However, the extracts from five E. coli strains (Crohn's disease (4), ulcerative colitis (1)) produced a distinctive cytopathic effect on Vero cells on repeated testing (Fig 1B). This effect was reproducible and detectable at both dilutions of extract. Further testing showed that although this cytopathic effect was similar to that produced by verotoxin (Fig 1C), verotoxin production by these strains could not be confirmed.

ADHESIVENESS

The adhesion indices for E. coli isolates from patients with Crohn's disease, ulcerative colitis, and normal controls are shown in Figure 2. The mean (SEM) adhesion index of E. coli isolates from patients with Crohn's disease (42.2 (6.4)) was not different from those with ulcerative colitis (43.4 (6.2)), both were significantly higher than the index from healthy controls (11.3 (2.0)) (p=0.0001 in both cases). Adhesive E. coli were isolated from 71% of patients with active Crohn's disease, 53% with quiescent disease, 67% with active colitis, and 68% with quiescent colitis (no significant difference) but from only 6% of normal controls. The differences between each disease group and normal controls were significant (p=0.0002). The occurrence of E. coli with adhesive properties was independent of disease activity, disease site, previous surgery, or concomitant sulphasalazine therapy (data not shown).

Results

HAEMOLYSIN AND VEROTOXIN PRODUCTION

Table II shows the distribution of haemolytic and adhesive E. coli isolates in all study groups. Haemolytic strains were isolated from 7 (18%)

Discussion

The possible importance of qualitative rather than quantitative changes in the E. coli flora in patients with inflammatory bowel disease has been emphasised by the observation that enteric-adherent strains are more common in these patients than in control subjects.*"
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confirmed this and further extended the previous studies by a more detailed examination of all patient and disease factors that may influence the isolation of adhesive E. coli, and also by studying other virulence factors of E. coli that have not been examined hitherto.

Enteroadherence is a feature of many enteric pathogens such as Vibrio cholerae. This characteristic enables these strains to resist mechanical removal from the intestine. In this study a significant proportion of patients with inflammatory bowel disease harboured E. coli with adhesive properties in their stool. Only two per cent of patients with Crohn’s disease and 68% with ulcerative colitis had these strains compared with only 6% of normal controls. Adhesive E. coli were as frequently isolated during the acute exacerbations of both Crohn’s disease and ulcerative colitis as when the disease was quiescent. Furthermore, other factors such as age, sex, sulphasalazine therapy, anatomical site of the disease, or previous intestinal resection had no influence on the isolation rate of these strains. Nine patients with newly diagnosed, and therefore previously untreated, ulcerative colitis harboured adhesive strains as frequently as patients with long standing disease.

The factors favouring the acquisition of enteroadherent E. coli are not certain. This property is plasmid mediated and may therefore be gained or lost. The lack of correlation with disease activity indicates that other conditions in the intestine may enable the adhesive strains to predominate over the normal E. coli. However, the possibility that some of the acute relapses in inflammatory bowel disease might be initiated by enteropathogenic E. coli remains tenable.

The role of verotoxin producing E. coli infection in inflammatory bowel disease has not previously been reported. Hunt et al.12 described three patients with presumed ulcerative colitis from whom a verotoxin producing E. coli strain (0157:H7) was isolated. However, in only one of their cases was a final diagnosis of ulcerative colitis confirmed. In the present study, none of 246 E. coli isolates, including 216 isolates from 74 patients with inflammatory bowel disease, produced verotoxin. All of those with clinically active disease had bloody diarrhoea. Because the proportion of verotoxin producing E. coli in the faecal E. coli population may be as low as 5–20%, it has been suggested that a large number of colonies from the primary medium should be examined. Since only a limited number of E. coli serotypes are usually cultured from the faeces of patients with inflammatory bowel disease, the testing of three or four E. coli isolates from each patient for verotoxin production gives an overall estimate of the prevalence of these strains in such conditions. Therefore, our findings indicate that verotoxin producing E. coli infection plays little if any role in the pathogenesis of inflammatory bowel disease.

The extracts of five E. coli strains produced a distinctive cytotoxic effect on Vero cell similar to that produced by verotoxin. All but one were isolated from patients with Crohn’s disease. It is exceedingly unlikely that alterations in the media used – for example pH or temperature – were responsible, because these toxic effects were reproducible and evident when the E. coli strains were tested simultaneously with other positive and negative control strains. A more likely hypothesis is that these cytopathic effects resulted from an as yet unidentified toxin.

Haemolysin production is frequently associated with E. coli strains causing urinary tract infection,30 peritonitis, and appendicitis.31 Therefore, haemolysin is a feature of pathogenic E. coli. The incidence of haemolytic E. coli in inflammatory bowel disease has been studied only in ulcerative colitis.32 Similar studies in Crohn’s disease have not been reported. In this study, haemolytic E. coli strains were isolated from a small proportion of patients with inflammatory bowel disease (18% of patients with Crohn’s disease and 24% of those with ulcerative colitis). These figures were not significantly different from normal controls (11%) or from patients with urinary tract infection (37%). Our results are in agreement with those reported by Cooke,33 who found haemolytic E. coli in 20% of 50 patients with ulcerative colitis. Our data indicate that in both Crohn’s disease and ulcerative colitis, the isolation of haemolytic strains does not differ according to disease activity, for these strains were equally distributed in active and quiescent disease. No patient or disease factors affected the prevalence of these strains. This finding contrasts with an earlier report which indicated that in ulcerative colitis haemolytic E. coli were isolated more frequently from patients with active than quiescent disease. The smaller number of patients included in our study may be the reason for this discrepancy.

The significance of the isolation of haemolytic E. coli in patients with inflammatory bowel disease is not certain. Follow up over a six month period for haemolysin and necrotoxin producing E. coli isolated from patients with ulcerative colitis has shown that the acquisition of these strains tended to follow rather than precede the acute relapse.

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10 Burke DA, Axon ATR. Adhesive Escherichia coli in inflamm-
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