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Escherichia coli antibody-secreting cells in the human intestine

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SUMMARY

Mononuclear cells were isolated from the mucosa and submucosa of small intestine and colon of 22 subjects with localized, anatomically remote disease and four subjects with Crohn's disease (nine specimens) by sequential treatment with EDTA and collagenase. The effects of isolation techniques on cell yields and viability were examined. Secretion of specific IgA, IgM and IgG antibodies to common faecal *Escherichia coli* strains by individual mononuclear cells was studied using a haemolytic plaque assay. A majority of specific antibody secreting cells secreted IgA antibody. This response was greatest and most consistent in the distal colon but extended from stomach to rectum. There was no evidence of a primary defect in IgA antibody response in the few subjects with Crohn's disease available for study.

INTRODUCTION

Certain strains of *Escherichia coli* form an important component of the gut flora of Western populations (Cooke, 1974). From the age of 2 years virtually all Western subjects have substantial serum antibody titres to common *E. coli* strains and this appears to result from intestinal colonization (Kunin, 1962). Using agglutination techniques, with their inherent bias toward detection of IgM antibody, serum antibodies to many strains of *E. coli* appear to be increased in subjects with ulcerative colitis (Tabaqchali, O'Donoghue & Bettelheim, 1978; Heddle & Shearman, 1979) and Crohn's disease (Tabaqchali *et al.*, 1978). One hypothesis which might explain such results is that an impaired local antibody response allows increased entry to bacterial antigens from the gut. The local intestinal antibody responses to commensal *E. coli* have not been measured in either health or disease. The aim of this study was to examine such responses using isolated intestinal mononuclear cells and a haemolytic plaque assay.

MATERIALS AND METHODS

Subjects and specimens of intestine. Specimens of intestine were obtained from 22 subjects with localized anatomically remote, disease ('control subjects'). Of these, 13 underwent surgery for carcinoma (separated by at least 10 cm from the site of sampling), two for peptic ulceration, two for angiodysplasia and three for obesity. Rectal biopsy specimens were obtained from a subject with melanosis coli and another with rectal polyps. Specimens were obtained from four subjects with Crohn's disease, three at surgery for intractable disease and one at rectal biopsy. Diagnoses were

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based upon clinical, radiological and histological findings. Three of the subjects were receiving azathioprine and/or small doses of systemic prednisolone. A member of the team was in theatre at the time of surgical excision. Samples were divided for immediate processing and for routine histopathology.

Isolation of intestinal mononuclear cells. The technique of Bull & Bookman (1977) was followed. Modifications were:

(a) a different preparation of collagenase (Type I; Sigma Chemical Co., St Louis, Missouri) was used and satisfactory tissue dispersal was achieved only by using a 10-fold higher concentration by weight of the enzyme preparation;

(b) 10% heat-inactivated (56°C, 30 min) fetal calf serum (Commonwealth Serum Laboratories, Melbourne) was used instead of pooled human blood group A B serum in the complete medium;

(c) 20 mM HEPES buffer was added to the complete medium. The other constituents (RPMI 1640, Commonwealth Serum Laboratories, Melbourne; antibiotics) remained as specified by Bull & Bookman (1977). The pH, adjusted initially to 7·2, remained in the range 7·2–7·5 in an atmosphere of 5% CO_2 in air.

The principal steps involved: (1) dissection of mucosa and submucosa from muscularis; (2) weighing of pieces of mucosa–submucosa, after blotting on filter paper and addition to a pre-weighed container of Hanks' balanced salt solution; (3) washing in dithiothreitol (1 mM, 22°C, 15 min) in Hanks' balanced salt solution in order to disperse mucus; (4) agitation in calcium, magnesium free Hanks' balanced salt solution in the presence of 0·75 mM EDTA (37°C, 90 min, then repeat in fresh medium) to disperse epithelial cells; (5) incubation in complete medium containing 0·5 mg/ml collagenase (37°C, 16 hr with agitation in an atmosphere of 5% CO_2 in air); (6) separation of mononuclear cells by centrifugation over a layer of Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) (Böyum, 1968).

Cells were counted in a haemocytometer after incubation for 15 min at 22°C with an equal volume of 0·5% trypan blue in saline. Cells which excluded trypan blue were considered viable. Cytocentrifuge preparations were air-dried, fixed in absolute methanol and stained with Leishman's stain. Differential counts were made using a 100 × oil immersion objective.

Antigens. Lipopolysaccharides (LPS) of smooth *E. coli* strains O1, O2 and O75 were selected for the study because these strains are frequently found in faeces of healthy Western subjects (Grüneberg, Leigh & Brumfitt, 1968). Sources, culture conditions and the method of confirming O serotypes have been published (Heddle & Shearman, 1979). LPS was extracted by the phenol/water technique of Westphal, Lüderitz & Bister (1952). The LPS was precipitated from the aqueous phase by the addition of 5 vol of absolute ethanol. LPS were prepared for coating sheep red blood cells (SRBC) by exposure to 0·02 M NaOH for 16 hr at room temperature (Crumpton, Davies & Hutchinson, 1958). SRBC were obtained from a single animal and washed three times in physiological saline before use as controls or for coating with the LPS preparations. Equal volumes of 5% SRBC and of physiological saline containing 100 μg/ml each of the *E. coli* O1, O2 and O75 LPS preparations were mixed and incubated at 37°C with gentle stirring for 1 hr. Coated cells were washed thrice immediately before use. These concentrations of LPS gave consistent responses when coated SRBC were used in a direct haemolytic plaque assay with spleen cells from an unimmunized inbred strain of mice (F_1, C57Bl × BALB/c; Central Animal House, University of Adelaide). Experiments with spleen cells obtained from mice pre-immunized with O1, O2 or O75 LPS demonstrated that SRBC coated polyvalently with the three LPS were equally able to detect a haemolytic plaque response to O2 or O75 LPS as SRBC monovalently coated with the respective antigens. Polyvalent coating appeared to inhibit moderately the detection of responses to O1 LPS.

Assay for antibody secreting cells. A haemolytic plaque assay (Dresser & Greaves, 1973) was used. Final concentrations of components were: agarose 1% in the complete medium; antibiotics nil; viable mononuclear cells approximately 1×10^6/ml and SRBC (with or without LPS coating) approximately 1×10^8/ml. Plates were incubated at 37°C for 45 min following which they were incubated at 37°C for 30 min with an equal volume of either the complete medium (for 'direct' IgM plaques) or of appropriate enhancing antiserum diluted in the complete medium. Enhancing antisera were commercial rabbit antisera monospecific for human (serum) IgA and IgG (Hoechst

Australia, Melbourne) which, following preliminary experiments, were used at dilutions of 1 in 50 and 1 in 100 respectively. The enhancing antiserum was then poured off and the plates inspected macroscopically for 'false' plaques which were marked appropriately. Finally plates were incubated at 37°C for 45 min with an equal volume of fresh guinea-pig serum, diluted 1 in 20 in the complete medium, as a source of complement. It was found necessary to pre-absorb guinea-pig serum by exposure, on ice for 1 hr, to an equal volume of 5% O1-O2-O75 LPS coated SRBC.

Plaques were read macroscopically and confirmed microscopically. Only 'plaques' demonstrating a mononuclear cell centrally in a zone of clearing were accepted. 'False' plaques were fewer and usually showed non-cellular debris or bubble artefact. Spleen cells from the inbred strain of mice were included in all experiments as a positive control for cell handling during the plaque assay, red cell coating with LPS and complement activity.

RESULTS

Isolation of mononuclear cells

Mechanical methods (Clancy, 1976; Clancy & Pucci, 1978) gave poor yields and viabilities in our laboratory. Using the enzyme-based technique, yields of mononuclear cells were variable at all levels of the intestine but tended to be lower in the colon than the more proximal intestine (Fig. 1). Greater than 90% of the mononuclear cells were viable as assessed by trypan blue exclusion.

It has been suggested that prolonged exposure to proteolytic enzymes decreases the yield of viable mononuclear cells (Crofton, Cochrane & McClelland, 1978). Incubating colonic mucosa with collagenase for 16 hr at 37°C was at least as effective as allowing enzyme to diffuse into tissues at 4°C for 15·5 hr followed by a 30 min incubation at 37°C (water-bath), as suggested by Crofton *et al.* (1978) (Table 1). In two experiments with isolated mononuclear cells from spleens of inbred mice, incubation with dithiothreitol and collagenase, according to the usual protocol, yielded as many

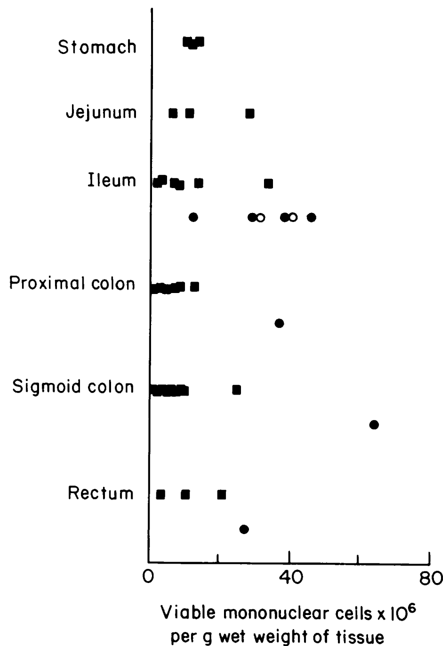

Fig. 1. Yields of viable mononuclear cells from mucosa plus submucosa according to region of intestine. Closed and open circles represent specimens from subjects with Crohn's disease that were, respectively, diseased and 'normal' by routine microscopy. (■ = Controls).

Table 1. Comparative results of short and prolonged incubation in collagenase

Tissue	Incubation with collagenase: 37°C 16 hr	Incubation with collagenase: 4°C 15·5 hr followed by 37°C 0·5 hr	Yield of viable mononuclear cells $\times 10^6$ per g tissue	% Mononuclear cells failing to exclude trypan blue	IgA anti-*E. coli* plaques per g tissue
Proximal	+	–	7·8	5	320
colon	–	+	5·3	6	180
Sigmoid	+	–	2·5	4	500
colon	–	+	1·7	10	410

viable mononuclear cells as incubation in medium alone. In contrast, incubation of these isolated mouse cells in EDTA for 3 hr produced a marked fall in numbers of viable mononuclear cells. Treatment of human tissues with EDTA for 3 hr did not reduce the yield of viable mononuclear cells (Table 2). This limited EDTA treatment reduced the proportion of epithelial cells in final cell suspensions from approximately 60% to approximately 30%.

The limited number of specimens obtained from subjects with Crohn's disease gave high yields of mononuclear cells, irrespective of whether the specimen appeared normal or diseased on routine histopathological examination (Fig. 1).

Table 2. Comparative results of mononuclear cell isolation with and without EDTA treatment

Tissue	EDTA	Yield of viable mononuclear cells $\times 10^6$ per g tissue	% Mononuclear cells failing to exclude trypan blue	IgA anti-*E. coli* plaques per g tissue
Proximal	+	6·6	2	Not
colon	–	11·2	1	done
Proximal	+	5·0	1	Not
colon	–	3·0	4	done
Sigmoid	+	8·3	0	13,000
colon	–	3·0	2	5,500
Sigmoid	+	2·5	4	500
colon	–	3·6	10	1,500

Antibody-secreting cells

A substantial specific antibody response to these common faecal coliforms was observed in nearly all specimens (Fig. 2). This response was particularly prominent in the distal colon. The few jejunal specimens yielded relatively large numbers of both total mononuclear cells (Fig. 1) and cells secreting the anti-*E. coli* antibodies (Fig. 2). Most specimens of intestine yielded more cells secreting IgA than IgG specific antibody (Fig. 2). Specific IgM antibody responses varied greatly and were usually minimal or undetected.

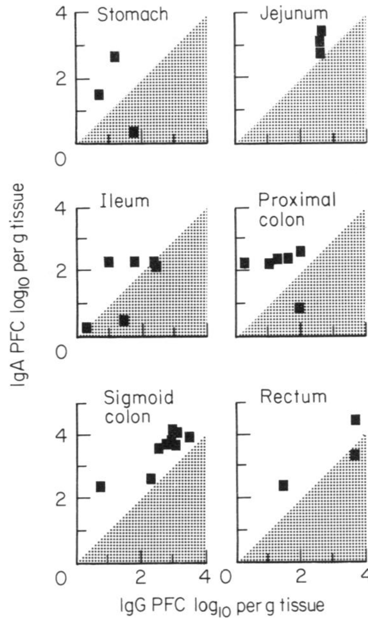

Fig. 2. Regional yields of cells secreting IgG and IgA anti-*E. coli* antibodies in control subjects. Results indicate the number ($\log_{10}$) of mononuclear cells secreting IgG or IgA specific antibody per gram of wet mucosa plus submucosa. Target antigens were pooled *E. coli* O1, O2 and O75 lipopolysaccharides. The shaded areas correspond to an excess of IgG antibody secreting cells.

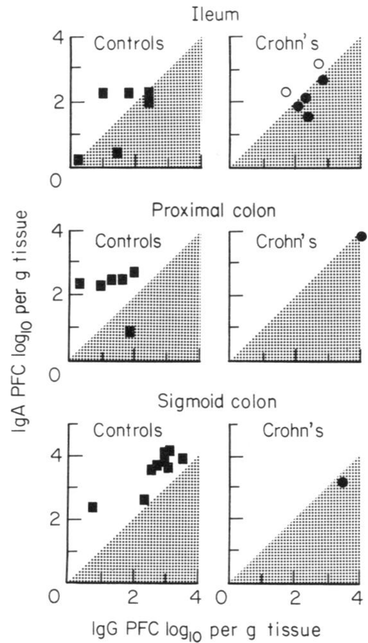

Fig. 3. Yields of cells secreting IgG and IgA anti-*E. coli* antibodies in control subjects and subjects with Crohn's disease. The manner of indicating results and the target antigens are the same as for Fig. 2. Closed and open circles represent specimens from subjects with Crohn's disease that were, respectively, diseased and 'normal' by routine microscopy.

Eight specimens from three subjects with Crohn's disease yielded normal or high-normal numbers of mononuclear cells secreting IgA specific antibody (Fig. 3). This applied irrespective of whether the specimens appeared normal or diseased on routine microscopy (Fig. 3). Correction for the higher yields of mononuclear cells in Crohn's disease by calculating haemolytic IgA plaques per 10^6 viable mononuclear cells indicated that the proportions of mononuclear cells secreting IgA anti-*E. coli* antibody were similar in Crohn's disease and control subjects. The ninth Crohn's disease specimen was a rectal biopsy and yielded insufficient cells for haemolytic plaquing.

The immunoglobulin class distribution of anti-*E. coli* plaques from Crohn's disease specimens was examined. Rather more IgG than IgA plaques were obtained from each of the six specimens of intestine which appeared diseased on routine histopathology (Fig. 3). Specimens of 'normal' intestine from two of the same Crohn's disease subjects yielded more cells secreting IgA than IgG specific antibody (Fig. 3). As in control specimens, IgM specific antibody responses varied greatly and were often not measurable.

DISCUSSION

Clancy (1976) and Goodacre *et al.* (1979) reported useful yields of mononuclear cells from human ileum and colon using mechanical methods of cell dispersal. Other groups (Bull & Bookman, 1977; Crofton *et al.*, 1978), including the present authors, have not obtained satisfactory results with such techniques. Direct comparison of yields between studies is difficult because yields are variously related to surface area or mass of mucosa and submucosa and some authors include results of specimens involved by inflammatory bowel disease with those of reference specimens. Nevertheless, yields of mononuclear cells obtained in the present study can be compared with and appear similar to those obtained by Crofton *et al.* (1978), Goodacre *et al.* (1979), Eade *et al.* (1980) and MacDermott *et al.* (1980). The latter two groups used dithiothreitol–EDTA–collagenase sequences similar to that used in the present study.

Crofton *et al.* (1978) reported that incubating peripheral blood lymphocytes at 37°C in pronase for more than 30 min reduced viabilities, and recommended that tissues be held in enzyme solutions at 4°C overnight followed by a brief (30 min) incubation at 37°C. This recommendation did not appear to be applicable to the collagenase system used in the present study (Table 1). The relatively high viability of cells after the more prolonged incubation was demonstrated not only by the trypan blue exclusion results but also by the capacity to form IgA antibody plaques.

Two key issues in such studies are the preservation of the isolated cells and whether or not they are representative of the *in situ* cell population. T and B cell markers (Bartnik *et al.*, 1980), HLA-antigens (Eade *et al.*, 1980), Fc receptors and cytotoxic activities (MacDermott *et al.*, 1980) all appear to survive sequential treatment with dithiothreitol, EDTA and collagenase. There is less evidence regarding the second question but similar proportions of eosinophils (Bull & Bookman, 1977) and of total immunoglobulin and IgA bearing cells (Bartnik *et al.*, 1980) have been demonstrated in mucosal tissues and final cell suspensions.

Another problem is the sources of intestine. In the studies cited and the present study the most frequent sources of colon were surgical specimens removed for neoplasia. It cannot be assumed that such specimens were immunologically normal.

The effects of prolonged EDTA treatment on isolated mouse spleen cells led us to restrict the duration of EDTA treatment. Approximately 30% of the final cell suspension comprised epithelial cells and it is likely that intraepithelial lymphocytes were present in significant numbers.

E. coli and related aerobic Gram-negative bacteria are found in greatest numbers in the colon and are uncommonly found in the jejunum (Cooke, 1974). That the proportions and absolute numbers of mononuclear cells synthesizing antibody to the pooled *E. coli* antigen were greatest in the distal colon is less interesting than the considerable commitment of jejunal cells to specific antibody synthesis. The numbers of cells secreting the specific antibodies and the class of those antibodies varied more in ileum than colon (Fig. 2). Although macroscopically observable Peyer's patches were avoided, one explanation for this observation could be the regional variations in ileal lymphoid tissues. Monteiro *et al.* (1971) were unable to detect antibodies to faecal aerobes,

including Enterobacteriaceae, in extracts of colonic mucosae from control subjects (and subjects with ulcerative colitis). The apparent discrepancy between their results and ours can probably be explained on the basis of the sensitivities of techniques for the detection of specific antibody. The ready demonstration by Monteiro *et al.* (1971) of antibodies in mucosa to faecal anaerobes stresses the need to extend studies of local antibody responses to antigens of other bacteria.

Despite the restricted availability of specimens, the present study is of interest in relationship to Crohn's disease because specific antibody responses were studied at a cellular level. Results obtained using this unique approach support those of studies of cell associated immunoglobulins by immunofluorescence (Söltoft, 1969; Persson & Danielsson, 1973; Skinner & Whitehead, 1974; Baklien & Brandtzaeg, 1975; Green & Fox, 1975; Meijer, Bosman & Lindeman, 1979) and of immunoglubulin synthesis (McClelland *et al.*, 1976; Bookman & Bull, 1979) in finding no evidence of a primary impairment of local IgA production in subjects with Crohn's disease. Most of these studies demonstrated, in parallel with results obtained in this study of specific anti-*E. coli* responses, increased proportions and absolute numbers of IgG-containing or secreting cells in intestine overtly involved by Crohn's disease. Comparisons of qualitatively normal and diseased Crohn's intestine (Söltoft, 1969; Skinner & Whitehead, 1974; McClelland *et al.*, 1976; Bookman & Bull, 1979; Meijer *et al.* 1979; Fig. 3) suggest that the increased commitment to IgG in overtly diseased intestine is secondary rather than primary. A high local concentration of IgG specific antibodies may contribute to on-going inflammation through immune complex or other mechanisms (Skinner & Whitehead, 1974; Baklien & Brandtzaeg, 1975).

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