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Serum antibodies to *Escherichia coli* in subjects with ulcerative colitis

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SUMMARY

It has been proposed that in ulcerative colitis the intestinal flora stimulates autoimmune reactions to colonic epithelium through shared specificities exposed in a 'common antigen' found in most *Enterobacteriaceae*. The present experiments aimed to resolve conflicting data as to whether patients with ulcerative colitis have selectively increased serum antibody titres to enterobacterial common antigen or *E. coli* 014, which is rich in enterobacterial common antigen. Antibody titres to enterobacterial common antigen and lipopolysaccharides of *E. coli* 014 and of five serotypes of *E. coli* which occur frequently in human faeces were measured by passive haemagglutination. Sera were obtained from patients with ulcerative colitis, age- and sex-matched controls and subjects with other gastrointestinal disorders. Serum titres to enterobacterial common antigen and *E. coli* 014 lipopolysaccharide were not increased significantly in subjects with ulcerative colitis but significant increases were observed in subjects with chronic liver disease without colitis. Patients with active ulcerative colitis, patients with chronic liver disease and subjects convalescent from *Salmonella* or *Shigella* infections all had significantly increased serum titres to the antigens as a group. Class-specific enhancement of passive haemagglutination indicated that the class distribution of serum antibodies was similar in subjects with ulcerative colitis and controls.

INTRODUCTION

It has been proposed that antigens of the intestinal flora stimulate or perpetuate autoimmune reactions to colonic epithelium in predisposed individuals (Weinstein, 1961; Lagercrantz *et al.*, 1968). Immunological cross-reactivity has been demonstrated between epithelial cells of germ-free rat colon and enterobacterial 'common antigen' (ECA) (Lagercrantz *et al.*, 1968). The latter is present in most *Enterobacteriaceae* (Kunin, Beard & Halmagyi, 1962; Mäkelä & Mayer, 1976). It has been reported that patients with ulcerative colitis have raised serum antibody titres (in passive haemagglutination) to ECA (Bull & Ignaczak, 1973; Eckhardt, Heinisch & Meyer zum Büschenfelde, 1976) and to the lipopolysaccharide (LPS) of *E. coli* 014 (Lagercrantz *et al.*, 1968; Thayer *et al.*, 1969; Lagercrantz, Perlmann & Hammarström, 1971; Carlsson, Lagercrantz & Perlmann, 1977), and in this respect LPS preparations of *E. coli* 014 contain large quantities of ECA (Kunin, 1963; Lagercrantz *et al.*, 1968). Although these findings are consistent with the preceding hypothesis, they have not been substantiated by others (Fink *et al.*, 1967; Anselm, Lo Grippo & Hayashi, 1971). Furthermore, ECA is only one component of *E. coli* 014 LPS preparations; most studies included only one other bacterial antigen (Thayer *et al.*, 1969; Lagercrantz *et al.*, 1971; Bull & Ignaczak, 1973; Eckhardt *et al.*, 1976; Carlsson *et al.*, 1977); only Lagercrantz *et al.* (1968) and Lagercrantz *et al.* (1971) used age- and sex-matched controls.

In the present study antibodies to the following antigens were measured: ECA, *E. coli* 014 LPS,

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lipopolysaccharides of five 0 serotypes of *E. coli* (02, 04, 06, 07 and 075) which occur frequently in human faeces (Grüneberg, Leigh & Brumfitt, 1968), and the LPS of *E. coli* 0119 which occurs less frequently. Subjects studied included subjects with ulcerative colitis, 'normal' controls, subjects convalescent from *Salmonella* or *Shigella* infection (in whom recent disruption of the intestinal mucosa appeared likely) and subjects with chronic liver disease. The latter were included because raised serum antibody titres to gut associated bacteria have been shown in such individuals (Bjørneboe, Prytz & Ørskov, 1972; Triger, Alp & Wright, 1972).

Tabaqchali, O'Donoghue & Bettelheim (1978) reported that few sera from subjects with inflammatory bowel disease had agglutinating antibody to *E. coli* 014 at titres which exceeded an arbitrarily chosen value of 1 in 100. High antibody titres to a variety of other *E. coli* were found more frequently in sera from subjects with inflammatory bowel disease than in sera from normal subjects.

The passive haemagglutination and agglutination techniques used in the preceding studies are biased towards the detection of IgM antibody (Steele, Chaicumpa & Rowley, 1974). Passive haemagglutination techniques were supplemented in this study by an anti-globulin enhanced passive haemagglutination technique in order to avoid this bias (Steele *et al.*, 1974; Heddle & Rowley, 1975). The same technique allowed the identification of IgA, IgM and IgG antibodies.

MATERIALS AND METHODS

Subjects. Sera were obtained from thirty-five consecutive adult patients with clinical, radiological, endoscopic and biopsy findings typical of ulcerative colitis. Four subgroups were selected for study (Table 1). Patients with 'active' disease had clinical and biopsy evidence of activity. Subjects who had received corticosteroids in the 2 months prior to study were classified as 'treated'. Most 'untreated' subjects had never received steroids. One 'treated' subject had also received azathioprine.

TABLE 1. Subjects with ulcerative colitis

	Number
Active 'untreated' disease*	11
Active treated disease	10
Inactive, 'untreated' disease	4
Inactive, treated disease	10
Total subjects	35

* The respective criteria are explained in the Materials and Methods section.

Eleven of the twenty 'treated' subjects but only three of the fifteen 'untreated' subjects were receiving salazopyrine. Seven of the thirty-five subjects had received antibiotics during the illness but were included in the study because their history, biopsy findings and clinical features supported a diagnosis of ulcerative colitis.

Control sera were obtained from healthy laboratory and hospital staff or from patients whose clinical conditions and therapies were not expected to alter antibody titres to *E. coli*. The following factors excluded patients from the control group—present or recent infection or antibiotic therapy, receipt of steroid or immunosuppressive agents, illness of suspected immune or autoimmune origin, debilitating illness, diabetes mellitus, malignancy, renal failure and liver disease. Each serum from a subject with ulcerative colitis was matched with a serum from a control subject of the same age ($\pm$ 5 years) and sex. Similar numbers of control and ulcerative colitis subjects were hospital inpatients.

Serum was obtained from thirteen patients with chronic liver disease (twelve alcoholic, one chronic active hepatitis) and eighteen patients who had suffered a recent, bacteriologically proven *Salmonella* (thirteen) or *Shigella* (five) infection. These sera were matched with sera from age- and sex-matched controls in the same way as the sera from patients with ulcerative colitis.

Antigens. E. coli serotypes were obtained from the Department of Microbiology and Immunology, University of Adelaide or the Institute of Medical and Veterinary Science, Adelaide. 0 serotypes were confirmed by the typing of boiled suspensions prepared from the final broth cultures (Institute of Medical and Veterinary Science—reference laboratory).

Bacteria were grown for 18 hr at 37°C in enriched nutrient broth containing $CaCl_2$, 56 μM; $FeSO_4$, 30 μM; KH_2PO_4, 18·3 mM; K_2HPO_4, 35·7 mM; $MgSO_4$, 337 μM; $(NH_4)_2SO_4$, 18·8 mM; Na citrate, 1·41 mM; acid hydrolysate of casein

(Calbiochem, San Diego, California), 20·7 g/l; peptone (Oxoid, London, England) 41·5 g/l and water soluble yeast extract (Difco, Detroit, Michigan), 8·3 g/l. Lipopolysaccharide (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 five volumes 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).

Enterobacterial common antigen (ECA) was prepared by treating *E. coli* 014 LPS with 0·25 M NaOH for 3 hr at 50°C in order to selectively destroy 014-specific antigen (Hammarström *et al.*, 1971). An extract of *E. coli* 08 was used in haemagglutination inhibition studies designed to confirm the common antigen content of the ECA preparation. A suspension of washed *E. coli* 08 was heated (100°C, 1 hr). The supernatant obtained by centrifugation at 23,000 *g* for 20 min of this suspension (Schmidt *et al.*, 1976) was used for the coating of SRBC without further treatment.

Techniques. Sera were heated at 56°C for 30 min to inactivate complement and were stored, in small aliquots, at −20°C. Sera were absorbed with equal volumes of 10% (v/v) washed SRBC (22°C, 30 min) immediately prior to titration.

Sheep red blood cells (SRBC). These were obtained from a single animal and coated with LPS (Auzins, 1968). Bacterial antigens were used for coating SRBC at concentrations which resulted in maximum passive haemagglutination titres when coated SRBC were titrated against sera from six healthy subjects.

Passive haemagglutination. Serial doubling dilutions of sera were titrated against coated and uncoated SRBC (Auzins, 1968). Paired test and control sera were titrated simultaneously and in duplicate, together with an aliquot of a large pool of serum of known t itre. All sera were titrated against all antigens.

Enhanced passive haemagglutination. This was performed using an immunoglobulin class specific antiglobulin enhancement technique (Steele *et al.*, 1974; Heddle & Rowley, 1975). Paired test and control sera were titrated simultaneously against coated and uncoated SRBC. Enhancing antisera were commercial rabbit antisera to human (serum) IgA, IgM and IgG (Hoechst Australia Ltd., Melbourne, Victoria, batch numbers 2721G, 2716D and 2733B). The antisera were employed at a dilution of 1 in 200, which provided optimal enhancement of passive haemagglutination. At this dilution the antisera enhanced monospecifically and lacked activity against the sensitized SRBC.

Inhibition of passive haemagglutination. This was performed in order to establish that the anti-ECA antibodies were directed against ECA rather than antigens specific for *E. coli* 014. Use was made of the observation that the somatic antigens of *E. coli* 08 and *E. coli* 014 cross-react immunologically through ECA but not *E. coli* 014 specific antigen (Lagercrantz *et al.*, 1968). Serum was incubated (37°C, 60 min) with an equal volume of saline or of saline containing *E. coli* 08 extract at 1 g/l. Absorbed and unabsorbed sera were titrated against SRBC and SRBC which had been coated with *E. coli* 014 LPS, ECA or (as controls) *E. coli* 08 extract or *E. coli* 07 LPS.

Statistical methods. Endpoint titres were expressed logarithmically in order to minimize skew (Fig. 1). All means were calculated as mean log titres. 'D' represents the difference in mean titres to an antigen of a test serum and serum from the age- and sex-matched control. The significance of differences in serum titres between patients and controls was assessed using the values of D for the respective antigen in the paired *t*-test (Bailey, 1968). Values of D for all antigens were used in assessing differences in responses to the antigens as a group. This approach minimized the effects of variations in absolute titres between antigens, from one day to another and with age and sex. Results of the *t*-test are presented without correction for the effects of testing several subgroups of subjects and a number of antigens on the likelihood of obtaining a 'significant' result by chance alone. Allowance for such effects was made in interpreting results.

Analysis of variance (Bailey, 1968) was used to determine whether D differed significantly between the various groups of subjects with ulcerative colitis ,between subjects within groups, between antigens or with interactions between groups of subjects and antigens. If the variance ratio for a given factor was significant at the 5% level, differences in the mean values of D for the appropriate groups of data were assessed for significance using an unpaired *t*-test (Bailey, 1968).

RESULTS

Serum antibody titres in ulcerative colitis

When compared with age- and sex-matched controls neither the total population nor any subgroup of subjects with ulcerative colitis showed significantly increased titres to *E. coli* 014 LPS or ECA ($P > 0{\cdot}10$). There was marked overlap in titres of 'normal' and 'colitic' sera to these antigens (Fig. 1). Similar overlap was observed with the other antigens (Fig. 2). *P* values for differences in titres between 'colitic' and control populations were not less than 0·01 for any antigen and were always greater than 0·05 when comparing the total populations. None of these values can be considered significant because of the large number of comparisons which were made in this analysis.

Antibody titres to the antigens as a group were greater in subjects with active 'untreated' colitis than their age- and sex-matched controls ($P < 0{\cdot}01$). Differences between the other groups of subjects with ulcerative colitis and their respective controls were less significant ($P > 0{\cdot}05$). Both groups of subjects with inactive ulcerative colitis had mean titres to the antigens as a group which were less than those of their respective controls.

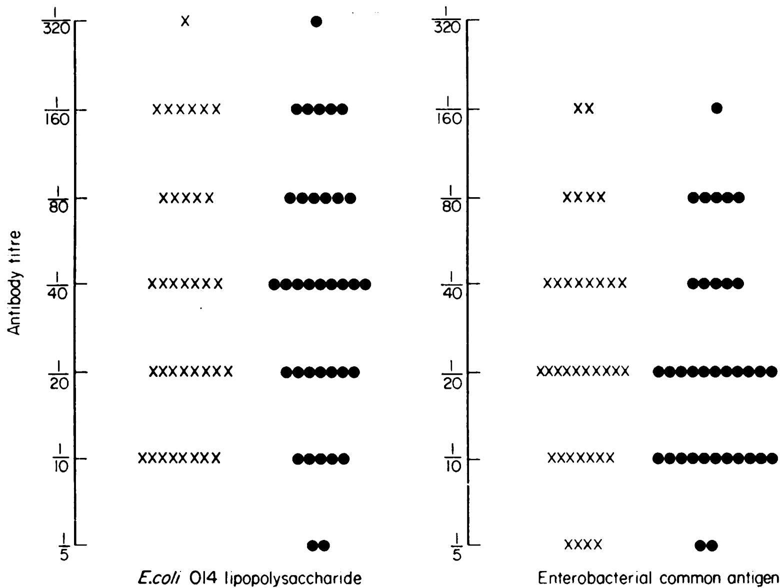

Fig. 1. Serum antibody titres to *E. coli* 014 lipopolysaccharide and enterobacterial common antigen in ulcerative colitis. Each point represents the mean titre in passive haemagglutination of serum from a different subject. Ulcerative colitis subjects (X), control subjects (●). (Mean titres have been rounded to the nearest whole number.)

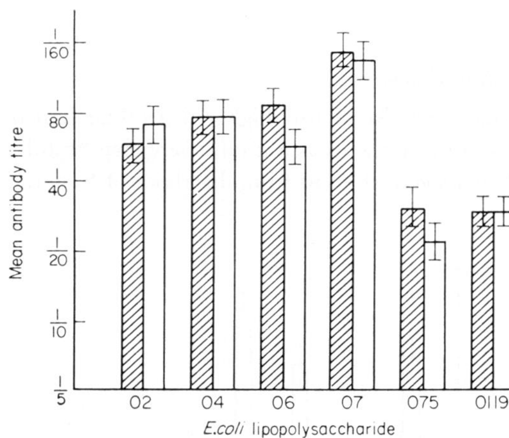

Fig. 2. Serum antibody titres to 'control' strains of *E. coli* in ulcerative colitis. Sera of thirty-five subjects with ulcerative colitis (▨) and thirty-five control subjects (□) were titrated in passive haemagglutination. Vertical bars represent s.e.m.

Analysis of variance demonstrated that D varied significantly between groups of subjects ($P < 0{\cdot}02$). In particular, D was considerably greater when comparing subjects with active 'untreated' colitis with their controls than when comparing subjects with inactive 'untreated' colitis with their controls ($P < 0{\cdot}005$). Variations in D between subjects within groups were significant at $P < 0{\cdot}001$. Variations in D between antigens and with interactions between subject groups and antigens were of doubtful significance ($P = 0{\cdot}05$ and $P < 0{\cdot}10$, respectively). In particular, D was not increased significantly when comparing results obtained with *E. coli* 014 LPS and ECA with results for other antigens.

When subjects were grouped according to disease activity alone, subjects with active ulcerative colitis had significantly higher titres to the antigens as a group than controls ($P < 0{\cdot}01$). D was significantly greater for subjects with active ulcerative colitis and their controls than for subjects with inactive ulcerative colitis and their controls ($P < 0{\cdot}01$). No significant differences in titres to ECA or *E. coli* 014 LPS were found in this analysis ($P > 0{\cdot}10$).

Verification of the specificity of ECA

Absorption of six colitic and six control sera with *E. coli* 08 extract caused marginal reductions in titres of most sera to *E. coli* 014 LPS but consistent and significant reductions in titres to ECA (Table 2). The effects of the absorption on titres to SRBC which had been coated with *E. coli* 08 extract or *E. coli* 07 LPS indicated that the absorption was specific.

TABLE 2. Specificities of antibodies to *E. coli* 014 LPS and ECA*

	Mean antibody titre† to:			
	E. coli 014 LPS	ECA	*E. coli* 08 extract	*E. coli* 07 LPS
Unabsorbed sera	32	21	160	160
Sera absorbed with *E. coli* 08 extract	15 (3/12)‡	< 5 (12/12)	< 5 (8/8)	160 (0/3)

* Enterobacterial common antigen.
† Determined by passive haemagglutination.
‡ Proportion of sera showing a fall in titre of two dilutions or greater.

Serum antibody titres in other clinical conditions

Sera of patients with chronic liver disease had higher titres than control sera to each antigen ($P = 0{\cdot}025$ or less) (Fig. 3). Titres to the antigens as a group were significantly higher in the subjects with liver disease ($P < 0{\cdot}001$). Patients convalescent from diarrhoea of bacterial aetiology had raised serum

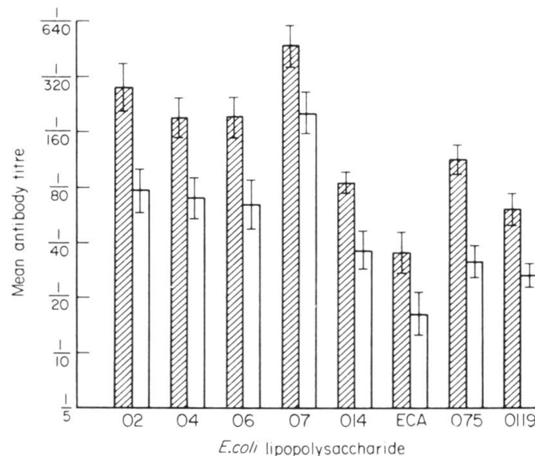

FIG. 3. Serum anti-*E. coli* antibody titres in chronic liver disease. Sera of thirteen subjects with chronic liver disease (▨) and thirteen control subjects ( ) were titrated in passive haemagglutination. ECA Enterobacterial common antigen. Vertical bars represent s.e.m.

antibodies to the antigens as a group ($P = 0{\cdot}005$). Titres to any individual antigen were not significantly greater than in the age-sex matched controls (P, 0·10 or greater). The increased titres in both conditions appeared to be directed against many *E. coli* because D did not vary significantly between antigens ($P > 0{\cdot}10$, analysis of variance).

Immunoglobulin class distribution of anti-E. coli antibodies

Sera from eleven patients with active 'untreated' disease were titrated in the enhanced passive haemagglutination assay against SRBC and SRBC coated with *E. coli* 06, 07 or 014 LPS or ECA. The immunoglobulin class distribution of serum antibody was similar in subjects with ulcerative colitis and controls. Representative results are shown in Fig. 4.

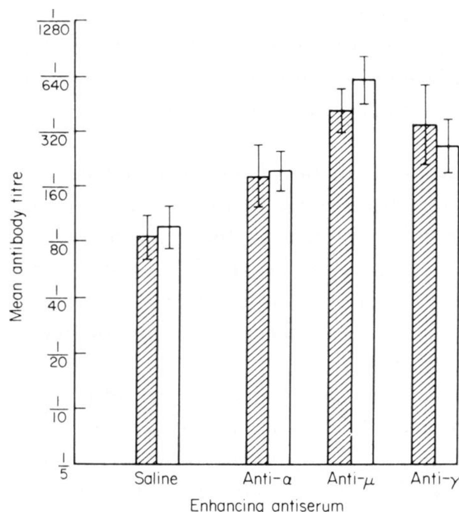

FIG. 4. Immunoglobulin class of serum anti-*E. coli* 07 antibodies in ulcerative colitis. Sera of eleven subjects with active 'untreated' ulcerative colitis (▨) and eleven age- and sex-matched controls (□) were titrated in antiglobulin enhanced passive haemagglutination against *E. coli* 07 lipopolysaccharide coated sheep erythrocytes. Vertical bars represent s.e.m.

DISCUSSION

In contrast to several previous passive haemagglutination studies (Lagercrantz *et al.*, 1968; Thayer *et al.*, 1969; Lagercrantz *et al.*, 1971; Bull & Ignaczak, 1973; Eckhardt *et al.*, 1976; Carlsson *et al.*, 1977), patients with ulcerative colitis did not show a significant or selective increase in serum haemagglutinating antibodies to *E. coli* 014 LPS or to enterobacterial common antigen (ECA). Furthermore, subjects with chronic liver disease, in whom there was no clinical evidence of colitis, had increased serum antibody titres to these antigens ($P = 0{\cdot}025$ or less). It is arguable that the use of the other six antigens can be ignored when considering the significance of differences in titres to *E. coli* 014 LPS and ECA. The latter antigens were preselected as being of special interest following consideration of the results of other workers. Shigellosis and chronic urinary tract infection have also been associated with high serum anti-ECA titres (Mäkelä & Mayer, 1976). These observations do not support the hypothesis (Lagercrantz *et al.*, 1968) that ulcerative colitis develops because members of the intestinal flora containing ECA stimulate or perpetuate autoimmune reactions to colonic epithelium in predisposed individuals.

The findings regarding titres of agglutinating antibody to *E. coli* 014 LPS support the results of Fink *et al.* (1967), Anselm *et al.* (1971) and Tabaqchali *et al.* (1978). It has not been reported previously that subjects with ulcerative colitis and controls have similar serum antibody titres to ECA. The use of ECA prepared by alternative methods (Bull & Ignaczak, 1973) might have yielded different results, but the specificity of our ECA preparation was demonstrated using the cross-reaction between *E. coli* 014 and 08 somatic antigens (Lagercrantz *et al.*, 1968). The same cross-reaction was used by Lagercrantz *et al.* (1968) to demonstrate the shared specificities of ECA and germ-free rat colon.

Use of the paired *t*-test as described is likely to have minimized interference with the demonstration of a 'significant' result by such variables as age and sex and daily variations in the assay. It seems unlikely that a colitis-related difference in antibody titres to *E. coli* 014 LPS or ECA was obscured by responses to other stimuli (e.g. unreported urinary tract infections in women). Separate analysis of results for each sex failed to demonstrate a significant difference between subjects with ulcerative colitis and controls in serum antibody titres to these antigens ($P > 0{\cdot}10$).

This study does not exclude the possibility that unusual local antibody or cellular responses to ECA may contribute to the pathogenesis of ulcerative colitis. Any possible differences between control and ulcerative colitis subjects in local antibody responses were not reflected in the class distributions of serum antibodies. Eckhardt *et al.* (1976) suggested that a reciprocal relationship between cellular and humoral immunity to ECA existed in individual patients. Nevertheless, the same authors and Bull & Ignaczak (1973) reported increases in both cellular and humoral immunity to ECA in their groups of subjects with ulcerative colitis. Studies of cellular immunity to colon antigens and *E. coli* 014 LPS in ulcerative colitis have yielded conflicting results (Watson & Shorter, 1975; Astrup, Rasmussen & Binder, 1977).

Tabaqchali *et al.* (1978) questioned whether *E. coli* 014 contained more accessible ECA than other strains of *E. coli* because few sera from either healthy subjects or subjects with inflammatory bowel disease gave positive (titre 1 in 200 or greater) agglutination reactions with this strain. The same observation applied to most other strains of *E. coli*. ECA, as found in most strains of *E. coli*, is weakly immunogenic (Kunin & Beard, 1963). It is doubtful, therefore, whether this observation of Tabaqchali *et al.* (1978) can be used to dispute other evidence concerning the relationship of ECA and *E. coli* 014 (Kunin, 1963; Mäkelä & Mayer, 1976).

Subjects with active ulcerative colitis had higher serum antibody titres to the antigens as a group than age- and sex-matched controls ($P < 0{\cdot}01$). Two observations suggest that this resulted from a diffuse increase in titres to the various *E. coli* antigens. Neither the total population nor any subgroup of subjects with ulcerative colitis had significantly increased serum titres, relative to control subjects, to any individual antigen. Analysis of variance failed to demonstrate that differences (D) in titres between subjects with ulcerative colitis and controls varied significantly between antigens or as a result of interactions between subgroups of subjects and antigens.

Do the preceding results represent specifically increased titres to different strains of *E. coli*, as observed in ulcerative colitis by Tabaqchali *et al.* (1978), or an increased titre to cross-reacting antigen(s)? The somatic antigens of the strains selected for study do not show strong serological cross-reactions except through ECA (Kauffmann, 1944; Kunin & Beard, 1963; Kauffmann, 1969). Sera were titrated from high starting concentrations and some cross-reactions cannot be excluded, but antibodies to ECA were present in lower titre (Fig. 1) than antibodies to most of the lipopolysaccharides (Fig. 2). *E. coli* 02, 04, 06, 07, 075 and 0119 contain ECA in its 'non-immunogenic' form (Kunin & Beard, 1963) which is almost completely soluble in the concentration of ethanol which was used to precipitate LPS in this study (Suzuki, Gorzynski & Neter, 1964). Treatment of LPS with a dilute alkali is likely to have impaired the binding to SRBC of any remaining ECA (Mayer *et al.*, 1972). These comments are not applicable to 'immunogenic' ECA as present in *E. coli* 014 (Suzuki *et al.*, 1964; Mayer *et al.*, 1972). It is probable that the results reflect increased serum antibody titres to a variety of *E. coli* antigens in ulcerative colitis.

The increased serum antibody titres to *E. coli* 014 and ECA found in some studies of ulcerative colitis may have been part of a broader trend. This was unlikely to have been shown in those studies which measured antibodies to not more than one other bacterial antigen (Thayer *et al.*, 1969; Lagercrantz *et al.*, 1971; Bull & Ignaczak, 1973; Eckhardt *et al.*, 1976; Carlsson *et al.*, 1977). Lagercrantz *et al.* (1968) did not find a non-selective increase in serum antibody titres to a panel of five *E. coli* antigens and it is difficult to explain the discrepancy between their results and those obtained in this study. Their study differed in the inclusion of many sera from children.

There are a number of factors which could cause increased serum antibody titres to many *E. coli* in ulcerative colitis. Primary immune abnormalities have been sought unsuccessfully in subjects with

ulcerative colitis using non-specific criteria such as serum immunoglobulin concentrations and proportions of cells containing immunoglobulins of different classes (Brandtzaeg *et al.*, 1974). Contrary to a previous report (Brown & Lee, 1973), the class distribution of serum antibodies to selected *E. coli* antigens was found to be similar in subjects with ulcerative colitis and controls (Fig. 4). That bacteraemias secondary to damage to the colon might cause increased serum anti-*E. coli* titres in ulcerative colitis is suggested by findings of increased serum antibody titres to the panel of *E. coli* antigens following *Salmonella* and *Shigella* infections ($P = 0{\cdot}005$) and in subjects with active but not inactive colitis. Many patients with ulcerative colitis have subclinical liver disease (Sherlock, 1975) which might explain a non-selective increase in titres to antigens of gut-associated bacteria (Triger *et al.*, 1972; Bjørneboe *et al.*, 1972; Fig. 3).

Cooke *et al.* (1974) reported increased carriage of haemolytic *E. coli* strains during active ulcerative colitis. Haemolytic strains are particularly frequent in *E. coli* 0 groups 2, 4 and 6 (Kauffmann, 1975), but serum titres of subjects with active ulcerative colitis to these strains were unremarkable in the present study.

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