

# Circulating antibodies against human colonic extract enriched with a 40 kDa protein in patients with ulcerative colitis

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## Abstract

We have previously described a 40 kDa colonic protein(s) which is specifically recognised by tissue-bound immunoglobulin G obtained from the colon of patients with ulcerative colitis. We now report the presence of circulating antibodies against this antigen using an enzyme-linked immunosorbent assay with a highly enriched preparation of the 40 kDa protein from normal colon extracts. Serum was collected from 79 patients with ulcerative colitis, 36 with Crohn's disease, 16 with specific diarrhoeal syndromes, and from 19 normal subjects. Twenty nine of 79 patients with ulcerative colitis, 21 of 36 with Crohn's disease, and all patients with diarrhoea were symptomatic during the collection of sera. The difference in optical density values between patients with symptomatic ulcerative colitis and each of the other groups, including patients with ulcerative colitis in remission, was highly significant ( $p < 0.01$ ). Seventy nine per cent of patients with symptomatic ulcerative colitis had optical density values above the means for all other groups. Fifty five per cent of sera from patients with symptomatic ulcerative colitis had optical densities beyond two SDs of the values for all other groups and only two of 71 sera from non-ulcerative colitis patients (one Crohn's disease and one normal subject) had values in this range. These results show the presence of anti-colon antibodies against the 40 kDa protein(s) in the sera of many patients with symptomatic ulcerative colitis.

Idiopathic ulcerative colitis is a chronic inflammatory disease of the large intestine, the aetiology and pathogenesis of which are unclear. Autoimmunity has been suggested in the pathogenesis of ulcerative colitis because of the presence of circulating and local antibodies that react with unknown alimentary tract antigens and because of the striking increase in immunoglobulin G (IgG) secreting plasma cells observed in the mucosa of patients with this disorder.<sup>1-6</sup> These antibodies, however, were heterogeneous and belonged to IgG, IgA, and IgM classes and were present in other diseases.<sup>3,4</sup>

We recently identified a 40 kDa protein(s) in extracts of both normal and diseased colon that is specifically recognised by the colon tissue bound IgG antibodies from patients with ulcerative colitis and not by colon tissue eluted IgG from patients with Crohn's disease affecting the colon or by control serum IgG.<sup>7</sup> Non-colonic tissue extracts, including those from the small

intestine, did not react with ulcerative colitis colon-associated IgG.<sup>7</sup> Using an anti-40 kDa monoclonal antibody the 40 kDa protein has been localised to the colonic epithelial cell membrane and its organ specificity is further shown.<sup>8</sup> These findings suggest that the 40 kDa protein may represent an autoantigen in ulcerative colitis.

In this study, we assessed in patients with ulcerative colitis the presence and specificity of circulating antibodies against the 40 kDa colonic protein by an enzyme-linked immunosorbent assay (ELISA).

## Methods

### SERA

Sera were collected (mostly at outpatient clinics) from 79 patients with ulcerative colitis, 36 with Crohn's disease, 16 with diarrhoeal syndromes caused by specific pathogen(s) and other causes (Table), and from 19 normal subjects. During collection of sera, the physician recorded the patients' symptoms according to a specific protocol modified from Harvey and Bradshaw.<sup>9</sup> The physicians also recorded whether the patients were symptomatic or in remission.

Patients with diarrhoea (three or more loose bowel movements per day) and with mucus or blood, or both, in the stool were considered symptomatic. Twenty nine of 79 ulcerative colitis and 21 of 36 Crohn's disease patients and all 16 patients with specific diarrhoeal syndromes were symptomatic during collection of sera. Since most of the patients were attending the outpatient clinic, no further classification of severity of the disease - for example, mild, moderate, or severe - was made. Most of the patients with ulcerative colitis were taking sulphasalazine. Twenty patients with ulcerative colitis were taking systemic steroids and five were treated with steroid enema. None of the patients were treated with azathioprine or 6-mercaptopurine. All sera were stored frozen at  $-80^{\circ}\text{C}$  until use, and were clarified by centrifugation before dilution and use for the ELISA.

### Demographic features of patients and control subjects

	Normal	Specific diarrhoeas*	Crohn's disease	Ulcerative colitis
Total no	19	16	36	79
Men	16	6	14	40
Mean age (yrs)	31	44	34	45
Age range (yrs)	25-46	23-81	12-80	15-75

\*Infectious diarrhoea (8), radiation colitis (2), ischaemic colitis (2), lymphogranuloma (1), appendical abscess with diarrhoea (1), and lactose intolerance (2).

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## TISSUE ANTIGEN

*Enrichment of the 40 kDa protein*

The method of preparing a soluble colon extract with enriched 40 kDa protein has been described previously.<sup>7</sup> Briefly, operative specimens of histologically normal segments of colon were obtained from three patients undergoing resection for colonic carcinoma. Tissue was received within half an hour of surgery, separated from serosal fat, washed in phosphate buffered saline (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.5) and stored at  $-80^{\circ}\text{C}$  in 10 g portions.

Portions of tissue were thawed, minced with a sterile carbon steel blade, and washed in volumes of PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2% sodium azide, and 2 mM phenyl methyl sulfonyl fluoride (PMSF). Washings were repeated five times, minced tissue being collected by centrifugation at 1000 g for three minutes. The washed tissue was then homogenised with a polytron (5 $\times$  speed for four times at 10 second intervals) in 10 volumes of PBS with EDTA, sodium azide, and PMSF as above. The soluble extract was then prepared by centrifugation at 2000 g for 20 minutes followed by a further centrifugation of the supernatant fraction at 20 000 g for one hour. All steps were performed at  $4^{\circ}\text{C}$  or on ice. The high speed supernatant fraction (PBS colon extract) was adjusted to 5–10 mg/ml protein, as measured by the Bio-Rad protein assay with bovine serum gammaglobulin as the standard. The extract was stored in 10% glycerol at  $-80^{\circ}\text{C}$ , until used. The PBS colon extract was dialysed against 25 mM potassium phosphate buffer, pH 7.5 containing 0.02% sodium azide. The dialysed extract (with 200 mg protein) was applied to a 30 ml column of diethylaminoethanol (DEAE) cellulose (Whatman DE-52) that had been equilibrated with the same buffer. The column was then washed with six bed volumes of the same buffer and then a KCl step gradient was used to elute proteins bound to the column (0.05 M KCl steps of two column volumes each). Collected fractions were dialysed against equilibration buffer and concentrated 10–12 fold by ultrafiltration with a YM-10 membrane (Amicon). Immunoreactivity of each of the eluates was examined by ulcerative colitis colon eluted-IgG using the transblot technique.<sup>7</sup> Because the 40 kDa protein(s) is highly enriched in the 0.35 M KCl eluate, this fraction is used for the ELISA against human sera.

## ELISA

To avoid any interexperimental variation, all 150 sera were tested simultaneously. Ninety six well polystyrene plates were treated with the enriched human 40 kDa colonic protein(s) antigen (0.35 M KCl eluate from DEAE column) in coating buffer (85 mM sodium carbonate, pH 9.6) at 1.5  $\mu\text{g}$  per well (100  $\mu\text{l}$ ), at  $20^{\circ}\text{C}$  for 7.5 hours. Each well was then washed five times with 200  $\mu\text{l}$  PBS with 0.5% Tween 20 (this washing buffer and procedure were also used between all subsequent incubations). Any remaining protein binding sites in the wells were blocked with 100  $\mu\text{l}$  per well of 5% bovine serum albumin (BSA) in coating buffer for one hour at  $37^{\circ}\text{C}$  and with 2%

horse serum for one hour at  $37^{\circ}\text{C}$ . Clarified sera were diluted in 5% bovine serum albumin in PBS and applied to wells in triplicate. All sera were diluted 1:5 and 1:10 before their use in ELISA.

Blanks consisted of antigen coated wells that were incubated with no human sera and BSA coated wells incubated with human sera. After an incubation of one hour with sera at  $37^{\circ}\text{C}$ , plates were washed, incubated with alkaline phosphatase conjugated anti-human IgG (Cappel, CA) applied in 100  $\mu\text{l}$  per well at a dilution of 1/500 for one hour at  $37^{\circ}\text{C}$ . Unbound second antibody-enzyme conjugate was removed by washing. Bound alkaline phosphatase was assayed at  $37^{\circ}\text{C}$  with the Sigma substrate with 100  $\mu\text{l}$  per well in 1 mM magnesium chloride, 50 mM sodium carbonate pH 9.8. Optical density at 405 nm was determined in an Artek V beam reader at one, and three hours. All samples showed linear optical density in response to dilution, and were within the linear response of the plate reader at one and three hours. The data shown are the means for the three hour readings on the 1/5 dilutions of sera. The BSA blank controls are deducted from each of the values.

## STATISTICAL ANALYSIS

Ulcerative colitis and Crohn's disease patients were initially analysed in two groups – patients who were symptomatic and those in remission. As there was no difference in the two Crohn's disease groups or in the subgroup of Crohn's colitis, all the data for these patients were treated as one group. The data from all groups were subjected to a one way analysis of variance and were also analysed by Newman-Keuls multiple comparisons. In the symptomatic ulcerative colitis group, there was a broad spectrum of values that was not as normally distributed as in the other groups (see results). Therefore, all groups were also compared by the non-parametric rank sum test and the Kruskal-Wallis analysis of variance. The statistical analysis was performed with the True Epistat programme library.

## Results

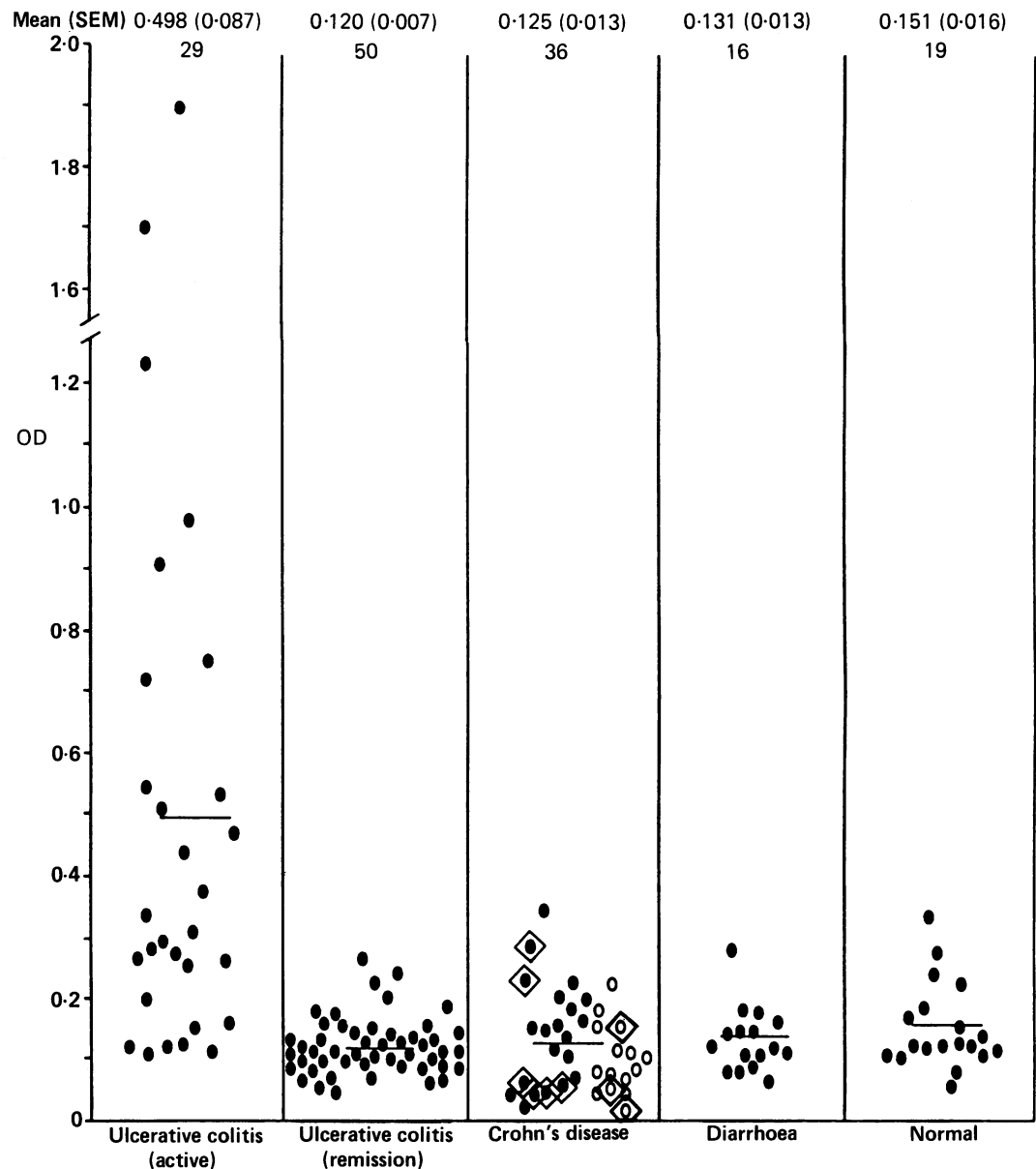
The demographic features of all the patients with inflammatory bowel disease and control subjects are shown in the Table. The results of the ELISA for each of the 150 subjects are shown in the Figure. Sera from patients with symptomatic or active ulcerative colitis had a mean optical density value three to four times greater than those of all the other groups – Crohn's disease (active and in remission), normal subjects, and patients with specific diarrhoeas. One way analysis of variance and Newman-Keuls multiple comparisons analysis indicated that the active ulcerative colitis group was significantly different from all the other groups at  $p < 0.01$ . Using non-parametric comparison tests, values of  $p < 0.0001$  were obtained (see discussion section). The mean optical density of ulcerative colitis patients in remission was in the same range as that of the non-ulcerative colitis groups. There was no difference in the mean optical density of Crohn's patients with disease that was

Scattergram showing the optical density (OD) for each serum from various groups of patients and normal subjects when tested against the highly enriched 40 kDa protein in the direct ELISA.

In patients with Crohn's disease closed circles indicate symptomatic patients, open circles patients in remission, and circles enclosed in diamonds are Crohn's colitis patients. Since there were no differences between symptomatic and remission patients, they are shown as a single Crohn's disease group.

The horizontal bar indicates the mean value for each of the groups.

The active ulcerative colitis group was significantly different from all the other groups at the 0.01 level (Newman-Keuls multiple comparison analysis). See text for medians, and non-parametric test results.



either active or in remission, or Crohn's colitis (Fig).

The mean optical density values plus 2 SDs for Crohn's disease patients, for patients with specific diarrhoeas, and for normal subjects were 0.281, 0.235, and 0.291 respectively. Seventy nine per cent of patients with active ulcerative colitis had an optical density value above the mean values of the other groups. Fifty five per cent of the patients with symptomatic ulcerative colitis had values beyond 2 SDs of all the other groups. Only one Crohn's disease patient, one normal subject, and none of the diarrhoeal control subjects had an optical density above 0.291. When the optical densities were compared with the age, sex, duration of disease, and treatment in patients with symptomatic ulcerative colitis, there seemed to be no significant differences.

Additional serum samples were obtained from five symptomatic patients with ulcerative colitis. Two of them had had a total colectomy and three were in remission during subsequent collection of sera. One patient had a preoperative optical density value of 0.684 and postoperatively (five months later) the value dropped to 0.093. The

other patient had a preoperative value of 0.140 and postoperatively (eight months later) it was 0.088. In three other patients with active ulcerative colitis, the optical density values declined after successful treatment.

## Discussion

In previous studies circulating antibodies in ulcerative colitis were described that reacted with human fetal and adult colon cells, epithelial cell-associated components from murine small intestine, rat colonic epithelial glycoproteins, intestinal bacterial polysaccharide, and antigens from germ free rat faeces.<sup>1-5</sup> These heterogeneous antibodies belonged to IgG, IgA, and IgM classes and were present in patients with both ulcerative colitis and Crohn's disease.<sup>4</sup> The extraction of disease specific colonic mucosal-bound IgG antibodies (CCA-IgG) in our earlier studies<sup>7,10</sup> led to the identification of the 40 kDa protein(s) present in both normal and diseased colon.<sup>7</sup> CCA-IgG extracted from autologous ulcerative colitis colon also reacted with the 40 kDa protein from the same colon, further supporting autoantigenicity of the 40 kDa protein.



With the development of murine monoclonal anti-40 kDa antibody, the 40 kDa protein has been localised exclusively in colonic epithelium, mainly along the plasma membrane.<sup>8</sup> The organ specificity of the 40 kDa protein to human colon has been shown by two sensitive techniques, namely immunotransblot analysis using CCA-IgG<sup>7</sup> and the immunoperoxidase method using the anti-40 kDa monoclonal antibody.<sup>8</sup>

The current study shows that patients with ulcerative colitis have a circulating IgG antibody immunoreactive to the enriched preparation of the 40 kDa protein when compared with patients with Crohn's disease, normal subjects, and patients with diarrhoeal syndromes due to other causes. Since the tissue eluted anti-40 kDa Ig belonged to the IgG isotype,<sup>7,10</sup> we did not examine for circulating IgA or IgM antibodies against 40 kDa protein(s). The immunoreactivity was significantly higher in patients with symptomatic ulcerative colitis than in patients with active Crohn's disease, other diarrhoeal syndromes, ulcerative colitis in remission, and normal subjects ( $p < 0.01$ ). Seventy nine per cent of active ulcerative colitis sera had optical density values above the means for all other groups and 55% had values beyond 2 SD of the mean values of active Crohn's disease, normal subjects, or patients with specific diarrhoeal syndromes. A value at 2 SD incorporates 95% of the values in a distribution. This implies that with a confidence of  $>95\%$ , one can identify about 55% of all active ulcerative colitis patients using this ELISA. Only two sera of patients who did not have ulcerative colitis (one with Crohn's disease and one normal subject) had an optical density greater than 0.291, the highest mean optical density plus 2 SD for a non-ulcerative colitis group. This implies the specificity of this assay is 98% when the optical density is at or above 0.291.

The distribution of the symptomatic ulcerative colitis group is very broad – about two thirds of the individual values within this group are below the group mean and the remaining third are above. The means for all other groups were essentially identical to their medians. The median values are: symptomatic ulcerative colitis, 0.310; ulcerative colitis in remission 0.113; all Crohn's disease, 0.110; patients with diarrhoeas, 0.119; normal control subjects, 0.120. Thus the symptomatic ulcerative colitis group does not seem as normally distributed as the other groups. Because of this the data were subjected to non-parametric comparison tests, rank sum and Kruskal-Wallis analysis of variance. These tests gave  $p$  values even more significant, at  $p < 0.0001$  in all comparisons, indicating that the symptomatic ulcerative colitis group was different from all the other groups. Furthermore, when medians rather than means are compared, similar conclusions are reached. The median for the symptomatic ulcerative colitis group is 2.6 times higher than that of the other groups. Only two values from the other groups are above the symptomatic ulcerative colitis median. Eighty three per cent of the symptomatic ulcerative colitis values are above the other group medians. Thus, if the ELISA were scored positive for values at or above the symptomatic

ulcerative colitis median (0.310), the calculated specificity would be 52%, the sensitivity would be 98%, and the false positive values would be 1.7%. These values are essentially identical to those obtained when the highest mean plus 2 SD for a non-ulcerative colitis group (0.291) is used as the point at or above which the ELISA is scored positive.

The background optical density values of normal and all diseased sera in this ELISA may be partly the result of non-specific binding of human IgG with colon extract containing proteins other than the 40 kDa protein. The significantly high titre measured for active ulcerative colitis is most likely against the 40 kDa protein. Previous studies using a limited number of purified serum IgG from patients with ulcerative colitis showed the presence of antibody directed against the 40 kDa protein by the immunotransblot analysis.<sup>7</sup>

It is possible that in this direct ELISA, ulcerative colitis serum containing immune complexes will not react or react poorly to the 40 kDa protein on the plate because of the bound antibody with the antigen(s) in the complexes. This may account for the finding that some of the ulcerative colitis sera did not show high optical density. Although circulating immune complexes have been described in patients with inflammatory bowel disease,<sup>11</sup> it is not known whether 40 kDa protein constitutes an antigen component(s) in these complexes. Further studies are needed to examine for specific antigen(s) in the immune complexes isolated from patients with ulcerative colitis and appropriate controls.

Specific autoantibodies against various target cell components have contributed to our understanding of the pathogenesis of various autoimmune diseases. Double stranded DNA in systemic lupus erythematosus, insulin receptor in juvenile diabetes mellitus, thyroglobulin in autoimmune thyroiditis, and acetylcholine receptor in myasthenia gravis act as organ specific autoantigens in these diseases.<sup>12-16</sup> Many patients who are acutely ill have high titres of these specific autoantibodies, and monitoring these circulating autoantibodies was very helpful in the diagnosis and in assessing the clinical course.<sup>13-16</sup>

In the current study, a positive correlation of the optical density values with activity of the disease state was evident. While ulcerative colitis patients with active disease had significantly higher optical densities, patients in remission did not. Future studies are needed to evaluate critically the relation between clinical severity and the titre of the anti-40 kDa antibodies in the same group of patients when they undergo remission after treatment. It is interesting that two patients with ulcerative colitis had a higher preoperative optical density value which dropped during the postoperative period. Three patients who had sera collected at symptomatic and remission stages also showed a decline in optical density values after remission. Since the patients in this series were followed as outpatients and most of them did not have barium enema or colonoscopy during the time of collection of serum, the precise extent of the disease and its

relation with the antibody titre could not be evaluated.

This study supports the notion that the 40 kDa protein acts as at least one autoantigen in patients with ulcerative colitis. The polymorphism of the 40 kDa protein(s), if any, from normal and diseased colon and identification of epitopes immunoreactive with various ulcerative colitis sera, may provide further understanding of the role of this protein in ulcerative colitis. Future ELISA studies using a purified 40 kDa protein (preferably immunoreactive epitopes) should increase the sensitivity and specificity of the assay. This assay may be a valuable test in diagnosing and objectively assessing the clinical course of the disease and its response to various treatments.

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