Intraepithelial and lamina propria leucocyte subsets in inflammatory bowel disease: an immunohistochemical study of colon and rectal biopsy specimens

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Abstract

Aims—To gain new insights into the pathogenesis and differential diagnosis of ulcerative colitis and colonic Crohn's disease.

Methods—Immunohistochemistry for different leucocyte subsets was performed in biopsy specimens of the sigmoid colon and rectum from 55 patients with inflammatory bowel disease and 11 healthy controls.

Results—Colonic biopsy specimens from patients with active ulcerative colitis had significantly higher numbers of CD45+ and CD3+ leucocytes compared with those from patients with inactive disease, and higher numbers of total leucocytes and macrophages than those from patients with Crohn's disease. Rectal biopsy specimens from patients with Crohn's disease had greater numbers of intraepithelial leucocytes (CD45, CD3 and CD8 cells) than specimens from patients with active or inactive ulcerative colitis, or from healthy controls.

Conclusions—Because of the phenotypic differences in the inflammatory infiltrate in the mucosa from the sigmoid colon and the rectum, the segment of the intestine to be biopsied should be specified. Assessment of the leucocytic component of the intraepithelial infiltrate in rectal biopsy specimens was more useful than examination of colonic biopsy specimens in the differential diagnosis of ulcerative colitis and Crohn's disease.

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CD4/CD8 ratio is similar to that in the normal mucosa.^{5,7,10,11} Greater numbers of macrophages have also been found in comparison with control tissues.¹²⁻¹⁴ One study has shown that intraepithelial leucocyte numbers are slightly increased compared with control values¹⁰; at this site, as in normal mucosa, the CD8+ phenotype predominated.^{5,10,15,16}

In the diagnosis of IBD intestinal biopsy, used to distinguish between ulcerative colitis and Crohn's disease, plays an important role.^{17,18} Although rectal biopsy has been considered less informative in the histological diagnosis of Crohn's disease than in ulcerative colitis,¹⁹ some authors do not share this assumption, despite the normal gross appearance of the mucosa in the former.¹⁷

The two objectives of the present study were to quantitatively assess the inflammatory infiltrate (with the exception of plasma cells) in the mucosa of the large intestine of patients with ulcerative colitis and Crohn's disease; to compare sigmoid colon and rectal biopsy specimens for histological features that might aid the differential diagnosis; and to gain information on local immunological changes in IBD.

Methods

Fifty five patients with either ulcerative colitis or Crohn's disease were included in this prospective study. The patients were classified on the basis of clinical, endoscopic and histopathological findings.¹⁹⁻²¹ Five patients with ileal Crohn's disease sparing the colon were excluded. Cryostatic sections of tissue from the sigmoid colon and rectum were also obtained from 11 healthy subjects; all were normal on histopathological examination.

Both the patients with IBD and the controls gave their informed consent. Ethical approval was obtained from the committee responsible for human experimentation at the Virgen de las Nieves Hospital.

The clinical and biochemical characteristics used to assess disease activity are given in table 1.

On endoscopy, two biopsy specimens of the sigmoid colon and two of the rectum were obtained from each patient. All specimens were processed for light microscopic observation and immunohistochemical analysis. Histopathological lesions were analysed in B5 fixed, paraffin wax embedded sections.

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Ulcerative colitis and Crohn's disease are inflammatory diseases of unknown aetiology, grouped together under the term inflammatory bowel disease (IBD). Immune factors (among others) have been implicated in the cause of these disorders.¹⁻⁴ Earlier studies reported increased inflammatory infiltrate in the intestinal mucosa of patients with IBD.^{23,5} The lamina propria contains greater numbers of plasma cells,⁶ together with increased numbers of T lymphocytes.^{5,7-9} Of these, CD4+ cells predominate over CD8+ cells,^{5,8} although the

Table 1 Mean age and analytical values for patients with IBD

	Normal values	Active ulcerative colitis*	Inactive ulcerative colitis*	Crohn's disease**
Age (years)		44 ± 3.2	38 ± 3.6	28 ± 2.6
Albumin	3.5–5 g/dl	4 ± 0.16	44 ± 0.3	4 ± 0.2
Orosomucoid	40–100 mg/dl	111 ± 11	59 ± 3.8	128 ± 26
α -1-antitrypsin	135–300 mg/dl	196 ± 17	152 ± 9.7	227 ± 22
Iron	59–158 g%	46 ± 5.3	71 ± 10	35 ± 8
Platelets	(130–400) × 10 ³	(337 ± 26) × 10 ³	(264 ± 22) × 10 ³	(433 ± 57) × 10 ³

* Histological activity. ** Only one case of Crohn's disease showed activity.

For the immunohistochemical studies, the avidin-biotin peroxidase complex (ABC) method was used to stain frozen sections (4 μ m) with a panel of monoclonal antibodies (table 2). Briefly, sections were air-dried, fixed in acetone for 10 minutes at 4°C, air-dried for a second time and postfixed in chloroform for 30 minutes. After 20 minutes' incubation in normal rabbit serum (1 in 10 dilution), sections were incubated with appropriate dilutions of the monoclonal antibodies for 16 hours at 4°C, washed in Tris buffered saline (TBS) and incubated with the biotinylated second layer antibody (1 in 400 dilution) and the avidin-biotin/horseradish peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes each at room temperature. After extensive washing in TBS, sections were developed with 3,3-diaminobenzidine tetrahydrochloride, dehydrated in a graded series of ethanols and mounted.

Lamina propria cellularity was evaluated using a 1 cm eyepiece grid divided into 100 1 mm² squares, placed in the $\times 10$ ocular of an Olympus BH2 light microscope. Slides were observed at $\times 40$ and the number of positive cells per field was counted in 10 randomly chosen fields; the results were expressed as the number of cells per mm². The immunophenotype of the intraepithelial leucocytes was quantified in 20 transversally sectioned glandular crypts from each biopsy specimen.

STATISTICAL ANALYSIS

Normality of the variables was analysed using the Kolmogorow-Smirnov test. Clinical parameters and immunohistochemical data were correlated using the Student's *t* test, the χ^2 test, analysis of variance, and Pearson's linear regression analysis.

Results

The main clinical and analytical findings in patients with IBD are summarised in table 1. Eight patients were diagnosed with Crohn's disease and the other 47 with ulcerative colitis;

of this latter group, 31 had evidence of histological activity (active ulcerative colitis), while the remaining 16 patients were considered to have inactive disease.

Mean age differed between the groups (active ulcerative colitis, inactive ulcerative colitis, Crohn's disease) ($p < 0.01$, analysis of variance) with the lowest mean age being found in the Crohn's disease group ($p < 0.001$ v the active ulcerative colitis group, Student's *t* test). Mean values for serum orosomucoid, iron and platelets also differed between groups (table 1). Serum concentrations of orosomucoid ($p < 0.001$) and α -1-antitrypsin ($p < 0.05$) were significantly lower in patients with histologically active ulcerative colitis compared with the inactive ulcerative colitis group (Student's *t* test).

Disease was limited to the rectum in nine patients (five with active and four with inactive ulcerative colitis) and affected the left colon in 30 (17 with active and nine with inactive ulcerative colitis, and four with Crohn's disease). Pancolitis was found in the 16 remaining patients (nine with active and three with inactive ulcerative colitis, and four with Crohn's disease). Both the ileum and the colon were affected in 50% of the patients with Crohn's disease.

IMMUNOHISTOCHEMICAL FINDINGS

Data for the sigmoid colon and rectal mucosa will be considered separately, as will the findings for leucocytes in the lamina propria and epithelium.

Leucocytes in the lamina propria of sigmoid colon mucosa

In the sigmoid colon the numbers of CD45+ ($p < 0.01$) and CD68+ cells (macrophages) ($p < 0.05$) differed between all four groups studied (active and inactive ulcerative colitis, Crohn's disease and controls) (analysis of variance; table 3), with highest numbers present in the active ulcerative colitis group and the lowest in patients with Crohn's disease.

When patients with active ulcerative colitis were compared with controls (table 3), the total leucocyte count was higher in the patients (Student's *t* test; fig 1). The differences for CD8+ lymphocytes and macrophages approached, but did not reach, significance. The CD45+ ($p < 0.001$) and CD3+ ($p < 0.05$) cell counts were higher in patients with active than in those inactive ulcerative colitis. Cell counts per mm² were lower in the Crohn's disease than in the active ulcerative colitis group (CD45+, $p < 0.001$; CD68+, $p < 0.05$). Fewer CD25+

Table 2 Monoclonal antibodies used and their specificities

Monoclonal antibodies	Specificity	Source	Dilution
CD45	All leucocytes	Dako	1 in 50
CD20	Pan-B cells	Dako	1 in 20
CD3	Pan-T cells	Becton Dickinson	1 in 10
CD4	T helper/inducer cells	Becton Dickinson	1 in 10
CD8	T suppressor/cytotoxic cells	Becton Dickinson	1 in 10
CD25	T cells, B cells, macrophages	Becton Dickinson	1 in 10
CD68	Monocytes/macrophages	Dako	1 in 10
CD57	Natural killer cells	Becton Dickinson	1 in 10

Dako, Glostrup, Denmark; Becton Dickinson, San José, CA, USA.

Table 3 Leucocyte subsets in colonic biopsy specimens from patients with IBD and healthy controls

Leucocytes	Active ulcerative colitis	Inactive ulcerative colitis	Crohn's disease	Controls	Analysis of variance
Lamina propria*					
CD45	3010 ± 305	1325 ± 209	1470 ± 203	1429 ± 114	p<0.01
CD3	1166 ± 113	742 ± 101	836 ± 177	918 ± 119	p<0.10
CD4	835 ± 82	681 ± 87	532 ± 116	716 ± 49	
CD8	374 ± 45	376 ± 67	258 ± 41	229 ± 55	
CD4/CD8	2.8 ± 0.5	2.5 ± 0.6	2 ± 0.4	4.5 ± 1	
CD20	82 ± 26	60 ± 23	30 ± 13	41 ± 1	
CD25	168 ± 44	117 ± 35	81 ± 21	143 ± 16	
CD57	20 ± 6	14 ± 3	13 ± 6	14 ± 3	
CD68	1074 ± 95	872 ± 67	668 ± 131	830 ± 74	p<0.05
Intraepithelial**					
CD45	33.2 ± 4	35.7 ± 6	34 ± 8.5	34.2 ± 3.5	
CD3	31 ± 3	34.9 ± 5	32 ± 4.2	41.7 ± 3.8	
CD4	8.8 ± 1.8	4.9 ± 1.4	3.4 ± 1.7	2.3 ± 0.6	p<0.05
CD8	15 ± 2.5	16 ± 3.5	12.5 ± 2.5	19.4 ± 3.8	
CD4/CD8	0.6 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.07	
CD20	0	0	0	0	
CD25	0	0	0.5 ± 0.3	0	
CD57	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	
CD68	4.6 ± 0.8	2 ± 1	2.3 ± 0.7	2.6 ± 0.8	

* Cells/mm² ± SE. ** Cells/20 glandular crypts.

cells were found in the control group than in the patients with Crohn's disease ($p<0.05$). There were no significant differences in cell counts between patients with Crohn's disease and those with inactive ulcerative colitis (table 3). The CD4/CD8 cell ratio was greater than 1 in both patients and controls (table 3), and was 4.5 ± 1 in the latter group.

Intraepithelial leucocytes in sigmoid colon mucosa

Leucocyte counts were similar in patients and controls, with the exception of CD4+ cells (analysis of variance, $p<0.05$) (table 3). Macrophages were found in the intraepithelial compartment in all groups, with highest counts appearing in patients with active ulcerative colitis. Only biopsy specimens taken from patients with Crohn's disease were CD25+, although

the numbers of these cells were low. The CD4/CD8 ratio was less than 1 in both patients and controls, with values ranging from 0.2 to 0.6 (table 3). The highest ratio was found in the active ulcerative colitis group.

Leucocytes in the rectal lamina propria

No significant differences were found between groups in the numbers of cells belonging to the different leucocyte subtypes (table 4). Leucocyte subtype counts in the rectal lamina propria did not differ significantly between biopsy specimens from patients with active and inactive ulcerative colitis, or between the latter and controls. When patients with active ulcerative colitis and controls were compared, the only significant difference was in the number of CD4+ leucocytes present ($p<0.05$).

Fewer B lymphocytes (CD20+) ($p<0.05$) and natural killer cells (CD57) ($p<0.01$) were found in patients with Crohn's disease than in those with active ulcerative colitis. These subtypes were also less prevalent in patients with Crohn's disease than in the healthy controls ($p<0.1$ for B lymphocytes and $p<0.01$ for natural killer cells). The CD4/CD8 ratio was greater than 1 in all groups (table 4).

Intraepithelial leucocytes in rectal mucosa

As in the lamina propria of the sigmoid colon, the counts for CD45+ ($p<0.05$), CD3+ ($p<0.05$) and CD8+ cells ($p<0.01$) differed between the groups (table 4). Average numbers of CD8+ ($p<0.01$) (fig 2) and natural killer cells ($p<0.05$) were lower in patients with active ulcerative colitis than in controls (table 4).

The most relevant finding was that specimens of rectal mucosa from patients with Crohn's disease contained more CD45+ ($p>0.01$), CD3+ ($p<0.01$) and CD8+ cells ($p<0.05$) than those from patients with active ulcerative colitis. The findings were similar when patients with Crohn's disease and those with inactive ulcerative colitis were compared: Crohn's disease biopsy specimens contained higher numbers of total leucocytes ($p<0.05$) and CD8+ cells (NS) than tissues from patients with inactive ulcerative colitis (table 4). No B lymphocytes were found in the epithelial

Figure 1 Sigmoid colon mucosa from a patient with active ulcerative colitis. Numerous CD45+ leucocytes are seen in the lamina propria, and some positive leucocytes are present in the glandular crypts of the epithelium (ABC; original magnification $\times 50$).

Table 4 Leucocyte subsets in rectal biopsy specimens from patients with IBD and health controls

Leucocytes	Active ulcerative colitis	Inactive ulcerative colitis	Crohn's disease	Controls	Analysis of variance
Lamina propria*					
CD45	1635 ± 149	1494 ± 249	1797 ± 264	1552 ± 101	p<0.10
CD3	1505 ± 128	1101 ± 148	1120 ± 167	1143 ± 133	
CD4	1100 ± 116	877 ± 206	976 ± 181	796 ± 88	
CD8	600 ± 66	505 ± 99	465 ± 61	462 ± 51	
CD4/CD8	2.1 ± 0.3	1.9 ± 0.3	2.3 ± 0.3	1.8 ± 0.1	p<0.10
CD20	271 ± 43	302 ± 85	129 ± 38	430 ± 82	
CD25	328 ± 46	349 ± 86	237 ± 42	417 ± 59	
CD57	287 ± 44	194 ± 63	110 ± 33	245 ± 56	
CD68	878 ± 65	924 ± 106	738 ± 89	867 ± 61	
Intraepithelial**					
CD45	27.2 ± 3.7	26.4 ± 5.6	47.9 ± 4.2	30.2 ± 4	p<0.05
CD3	24.2 ± 3.2	31.9 ± 5	43.1 ± 4.7	25.3 ± 4.3	p<0.05
CD4	8.8 ± 1.7	9.3 ± 3	8.2 ± 3.5	4.7 ± 1.2	p<0.01
CD8	6 ± 1.1	12.1 ± 4.5	23.9 ± 7.4	13.4 ± 2.1	
CD4/CD8	0.7 ± 0.15	0.7 ± 0.2	0.7 ± 0.3	0.3 ± 0.09	
CD20	0.3 ± 0.16	1 ± 0.04	0	1.3 ± 0.05	
CD25	0.3 ± 0.15	0.8 ± 0.3	0.3 ± 0.3	0.2 ± 0.14	
CD57	1.1 ± 0.4	3 ± 0.1	1.2 ± 0.1	4.8 ± 1.4	
CD68	5.4 ± 0.9	4.8 ± 0.8	5 ± 1.4	7.3 ± 1.4	

* Cells/mm² ± SE. ** Cells/20 glandular crypts.

compartment of the rectal mucosa from patients with Crohn's disease.

Similar numbers of macrophages were found in glandular crypts in all groups. As in the sigmoid colon, the CD4/CD8 ratio was less than 1 (table 4).

Comparison of colon and rectal mucosa

When the values in tables 3 and 4 were compared, similar figures for the different leucocyte subtypes in rectal mucosa were found in both controls and patients with IBD. The only exceptions were the higher numbers of CD45+ and CD68+ cells in colonic lamina propria, and the higher numbers of intraepithelial CD8+ cells in patients with active ulcerative colitis.

Figure 2 Rectal mucosa from a patient with Crohn's disease. Numerous CD8+ leucocytes are seen among the epithelial cells of the glandular crypts, and some positive cells are present in the lamina propria (ABC; original magnification × 50).

Discussion

Intestinal biopsy provides information that complements clinical and endoscopic findings in the diagnosis of IBD and makes the study of local immunological alterations in this disease possible. Despite the many available diagnostic tools, including immunohistochemical analysis, the differential diagnosis between ulcerative colitis and Crohn's disease can still present problems.²² Our findings are in agreement with previous reports that T cells of the CD4+ phenotype constitute the predominant cell type in the lamina propria of the intestinal mucosa^{5 7-9 11 23-25} and CD8+ T cells in the epithelia^{5 7-9 11 23 26-28} in both healthy subjects and patients with IBD.

Most studies of leucocyte subsets in the normal or diseased intestine do not state whether biopsy specimens were obtained from the sigmoid colon or from the rectum. The present results illustrate the qualitative and quantitative differences in the inflammatory infiltrate at these two sites. The greater cell density in the rectum may be because of its proximity to the external environment, which may involve differences in antigenic stimulation.

Of particular importance is our finding of greater numbers of intraepithelial CD45+ leucocytes in glandular crypts of the rectum in patients with Crohn's disease compared with those with ulcerative colitis and with healthy controls. These differences can potentially be used as a morphological parameter to distinguish between these two conditions. Most of these cells were of the CD8+ phenotype and can act as suppressor lymphocytes; this would account for the lack of epithelial lesions in the rectum of patients with Crohn's disease. By contrast, this type of cell was significantly less prevalent in patients with active ulcerative colitis than in those with Crohn's disease or in healthy controls.

We were unable to locate any studies that separately analysed biopsy specimens displaying and those not displaying histological activity. Only one study¹⁰ compared diseased mucosa with normal mucosa from the margins of the lesion in patients with IBD. Our findings in biopsy specimens from patients with ulcerative colitis show that in the lamina propria, the inflammatory infiltrate (CD45+ and

CD3+ cells) in ulcerative colitis is more abundant in patients with active than in those with inactive disease. By contrast, intra-epithelial CD3 cells were more numerous in mucosa of patients with inactive disease, making analysis of this antibody potentially useful in differential diagnosis.

We found no differences in the leucocyte subtypes present in patients with inactive ulcerative colitis and controls, and therefore believe that this condition should continue to be diagnosed on the basis of conventional histopathological studies.

Macrophage numbers were increased in the lamina propria of the sigmoid colon,¹²⁻¹⁴ and these cells were more numerous in active than in inactive ulcerative colitis. The fact that macrophages were more numerous than CD4+ cells at this site is probably related to the more widespread glandular destruction in this area than is seen in inactive ulcerative colitis. Similar relations between macrophage numbers were found when we compared active ulcerative colitis with control¹⁴ and Crohn's disease biopsy specimens. The mean number of macrophages in the patients with ulcerative colitis was higher than in the groups studied by Allison and Poulter,¹² a difference that may be due to the different counting methods used. Intraepithelial macrophages have been found in healthy mucosa,¹⁵ but not in mucosa from patients with IBD. The highest mean numbers of CD68+ cells were found in glandular crypts of the rectum from healthy subjects, where they were more numerous than CD4+ cells. Although slightly more macrophages were counted in the colonic mucosa of patients with active ulcerative colitis, the difference between this figure and the count in controls was not significant. These findings suggest that macrophages participate in the modulation of the local immune response in these diseases.

The CD4/CD8 ratios in the lamina propria and epithelium of the colon and rectum were similar to previously published figures,^{4,5,7,10,11} except in the lamina propria of the sigmoid colon in healthy controls. We found a higher ratio (4.5 ± 1), with CD4+ cells noticeably outnumbering CD8+ cells, a finding noted previously in patients with IBD.¹⁰ In the rectal lamina propria, however, the CD8+ cell counts were similar in patients with ulcerative colitis and in those with Crohn's disease. We do not know whether the type of biopsy used by Hirata *et al*¹⁰ (healthy margins in colon cancer) influenced their results.

We noted similarities between our findings for B lymphocytes and earlier studies. In patients with IBD, as in normal subjects, B lymphocytes were scarce in the lamina propria and most of them were differentiated plasma cells.²⁹ No B lymphocytes were found in the colonic epithelium,^{27,30} although a few were present in the rectal epithelium of patients with ulcerative colitis and healthy controls.^{5,10,15}

Few natural killer cells were found in the colonic mucosa.^{10,15} In the rectal mucosa the largest numbers of these cells were found in control specimens, suggesting that these cells have a limited role in the pathogenesis of IBD.

At both sites, natural killer cells were scarcest in patients with Crohn's disease, a finding that apparently contradicts the results of a recent functional study.³¹

The only marker of leucocyte activation examined in this study was interleukin-2 receptor (CD25+). Cells of the lamina propria showed intense expression of CD25+,^{5,7} although positivity was greater in the rectum than in the sigmoid colon, both in control subjects and in those with ulcerative colitis. We found that CD25 expression was lower in Crohn's disease than in normal mucosa of sigmoid colon, in contrast to an *in vitro* study³² and to an earlier one in children.³³ In the epithelial compartment, this receptor was scarce, if present at all.^{5,33} Most activated cells in colonic biopsy specimens from patients with active ulcerative colitis were CD4+ cells and macrophages, whereas in the rectum of those with Crohn's disease, we found only CD4+ cells. Our results concur for the most part with those of earlier studies.^{16,33}

In conclusion, an immune response occurs in the epithelial compartment in IBD.²⁶ This response, like that seen in the lamina propria, is more intense in the rectum than in the sigmoid colon. To elucidate the local immunological events in IBD, findings in different sites of the intestine should be compared. Our results suggest that (1) the exact location of the intestinal biopsy should be specified in future immunopathological studies of IBD, given the differences in the phenotypic characteristics of the inflammatory infiltrates present in the rectum and the sigmoid colon; and (2) that biopsy of the rectum is more informative than that of the colon in the differential diagnosis between ulcerative colitis and Crohn's disease. The epithelial component is more useful than the lamina propria, and is easier to evaluate.

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