Increased macrophage subset in inflammatory bowel disease: apparent recruitment from peripheral blood monocytes

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Abstract
Mucosal specimens from active Crohn's disease (ileum, n=6; colon, n=6), active ulcerative colitis (n=9), normal ileum (n=6), and normal colon (n=6) were subjected to paired immunofluorescence staining for characterisation of macrophage subsets in situ. In the normal state, only few CD68+ macrophages (<10%) expressing the myelomonocytic L1 antigen (calprotectin) were seen. In inflamed mucosa, especially near small vessels, the CD68"L1" fraction increased with the degree of inflammation, near ulcers to median 65% (range 35–91%). Cells reactive with the monoclonal antibody RFD7 were also increased in inflammation but less than 5% of them contained for L1 antigen. It was concluded that L1 producing macrophages are distinct from the RFD7+ subset and probably recently recruited from peripheral blood monocytes. Like granulocytes, L1+ macrophages may be important in non-specific defence, providing calprotectin with putative anti-microbial and anti-proliferative properties.

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Dense accumulations of large HLA class II positive macrophages are found beneath the surface epithelium in normal gut mucosa. They can apparently function both as phagocytes and antigen presenting cells and may play an important part in the inflammatory process of ulcerative colitis and Crohn's disease. In this study we used the monoclonal antibody (mAb) RFD7 as well as antibodies to CD68 (mAb KP1) and the leucocyte L1 antigen (calprotectin) to characterise macrophage subpopulations in inflammatory bowel disease (IBD). KP1 was raised against a lysosomal fraction of human lung macrophages and has been shown to detect CD68 in most types of tissue macrophages and neutrophilic granulocytes but does not generally react with normal dendritic cells or eosinophilic granulocytes. RFD7 specifically recognises mature tissue macrophages. L1 or calprotectin is an anti-microbial myelomonocytic 36 kDa cytosol protein complex containing light (8 kDa) and heavy (14 kDa) polypeptide chains, which have been named differently. L1 is strongly expressed by all monocytes in peripheral blood and by a subset of infiltrating macrophages in pathological lymph nodes, whereas various dendritic cells are generally negative. We have recently shown that the macrophage population in normal ileal mucosa and Peyer's patches does not express substantial amounts of L1. This study aimed at examining if the same is true in the normal colonic mucosa and if this pattern is changed in IBD.

Methods

Tissue specimens

Colon
Surgical specimens from nine patients with ulcerative colitis (seven men and two women; median age 34 years, range 20–64) and from six patients with Crohn's disease (two men and four women; median age 30–5 years, range 24–39) were included. In ulcerative colitis the indication for surgery was epithelial dysplasia, malignant transformation, or intractable diarrhoea, and in Crohn's disease fistulas or stenosis. Duration of symptoms was 2–19 years. Inflammation varied from slight to severe (also for samples taken from different sites in the same specimen) as deemed by conventional histological examination; none of the selected samples were from areas with epithelial dysplasia. At operation four of the patients with ulcerative colitis received sulphasalazine, one sulphasalazine and prednisolone. Two patients with Crohn's disease received sulphasalazine, one sulphasalazine and prednisolone, and one mesalazine. None of the patients were treated with prednisolone at higher doses than 10 mg/day.

Surgical specimens from three patients with colonic carcinoma and one organ donor with maintained peripheral circulation together with specimens from one patient with colonic adenoma and one with irritable bowel syndrome (six women; median age 69 years, range 10–77) served as controls. These specimens were taken at least 6 cm away from any tumour and were all histologically normal.

Ileum
Surgical specimens from six patients with Crohn's disease were included (two men, four women; median age 24 years, range 16–48); apart for one, they were not the same as the donors of the colonic specimens. The clinical signs of inflammation were usually mild at operation, which was performed 2–11 years after the initial symptoms. At operation three patients received sulphasalazine, one sulphasalazine and prednisolone, and two mesalazine and prednisolone. None of the patients were treated with prednisolone at higher doses than 10 mg/day.

Surgical specimens from five organ donors with maintained peripheral circulation (three
men and two women; median age 25 years, range 7–41) served as controls and were histologically normal.

TISSUE PREPARATION
The tissue specimens were brought to the laboratory in ice chilled isotonic phosphate buffered saline (PBS), pH 7.5, and trimmed immediately, fixed in 96% ethanol for 20 hours at 4°C, and prepared for paraffin wax embedding.10 Serial sections were cut at 5 μm perpendicular to the mucosal surface. Preliminary studies showed that CD68, RFD7 antigen, and L1 antigen were better preserved after this tissue preparation method than in routinely formalin fixed material.

Immunohistochemistry
PAIRED IMMUNOFLUORESCENCE STAINING FOR CD68 AND L1
L1 and CD68 are expressed both by monocytes and granulocytes,12 whereas lactoferrin is present only in the second cell type.13 We wanted to inhibit immunofluorescence detection of L1 and CD68 selectively in granulocytes by an initial immunoperoxidase staining for lactoferrin. After dewaxing, the sections were immersed in paraformaldehyde (2%) lysine periodate fixative (PLP, 2%) for 10 minutes at 4°C to reduce leaching of cytoplasmic L1 antigen during the subsequent immunohistochemical procedures.14 Endogenous peroxidase was blocked by incubation for 30 minutes with 0-3% H2O2 in absolute methanol, and the sections were reacted with rabbit antisem to lactoferrin (1:500)15 for 30 minutes at room temperature followed by swine antirabbit IgG (Dakopatts, Glostrup, Denmark) and peroxidase anti-peroxidase complexes (Dakopatts, Glostrup, Denmark). The enzyme reaction was developed with 0.5% diaminobenzidine and 0.0015% H2O2 for 10 minutes. After washing in PBS, the sections were next incubated in normal rabbit serum for 15 minutes at room temperature before a mix of tetramethylrhodamine isothiocyanate (TRITC) conjugated rabbit IgG (0-035 g/l) specific for L116 and mAb KPI at 1:50 (supernatant, courtesy Dr K Pulford and Dr D Mason, Oxford, UK) was added for 20 hours at room temperature.10 This step was followed by biotinylated horse IgG mouse IgG (0-025 g/l) (Vector Laboratories, CA, USA) for three hours at room temperature, and subsequently by fluorescein isothiocyanate (FITC) labelled streptavidin at 1:50 (Boehringer Mannheim, Mannheim, Germany) for 30 minutes, mainly according to a two colour method described previously.17

Sections from inflamed mucosa stained for lactoferrin by immunoperoxidase were compared with adjacent sections stained for chloroacetate esterase that selectively appears in neutrophilic granulocytes and mast cells.13 Sections stained for lactoferrin by immunoperoxidase followed by chloroacetate esterase reaction were further used to evaluate the reliability of the former detection method with regard to inclusion of all neutrophilic granulo-

cytes. To find out if pretreatment with dimamino-benzidine and H2O2 decreased subsequent immunostaining for L1 or CD68, serial sections were subjected to paired immunofluorescence staining for these two markers with or without such pre-treatment. One serial section from each tissue specimen was stained with haematoxylin and eosin for evaluation of the degree of inflammation.

PAIR IMMUNOFLUORESCENCE STAINING WITH ANTI-CD68 (KP1) AND RFD7
Sections were incubated with mAb RFD7 (supernatant, obtained from Dr L W Poulter, London, UK) at 1:20 for 20 hours, followed by biotinylated horse antinouse IgG and FITC labelled streptavidin as described earlier. After PLP fixation (1%, 10 minutes, 4°C) the sections were incubated with KPI (1:50) for 20 hours, followed by biotinylated horse antinouse IgG and streptavidin Texas red (Bethesda Research Laboratories, MD, USA) at 1:400 for 30 minutes.

PAIR IMMUNOFLUORESCENCE STAINING WITH ANTI-L1 AND RFD7
Satisfactory immunofluorescence for RFD7 in combination with minimal leakage of L1, was achieved by applying RFD7 twice, first as a mix of this mAb (1:20) and TRITC conjugated rabbit IgG specific for L1 (0-09 g/l) for 15 minutes followed by immersion of the section in 96% ethanol for 30 minutes at 4°C before PLP fixation (2%, 10 minutes, 4°C), and then reincubation with RFD7 for 20 hours, which was followed by biotinylated horse antinouse IgG and FITC labelled streptavidin as described above.

MICROSCOPY AND HISTOMORPHOMETRY
The immunostained sections were examined with an ×25 immersion objective and an ×10 ocular in a Leitz Orthoplan fluorescence microscope. A Ploem type vertical illuminator afforded selective observations of red and green emissions; immunoperoxidase staining of granulocytes could be shown in the same field by conventional light microscopy. This permitted evaluation of individual cells with regard to phenotype and at the same time evaluation of the granulocyte density in the actual field and the efficacy of blocking immunofluorescence in cells stained for lactoferrin by immunoperoxidase.

Comparative enumeration of macrophage subgroups and granulocytes was performed in every section with an optical grid (0-06 mm2) in two different areas of the mucosa, 2–4 grids large, including 100–200 macrophages. In an adjacent section stained with haematoxylin and eosin two independent observers (blinded) graded inflammation as light, moderate, and severe, based on evaluation of inflammatory cell infiltration, crypt abscesses, crypt destruction, and crypt atrophy. The degree of inflammation, however, often differed between adjacent visual fields. The distinction between moderate and severe inflammation in an area was hence often difficult based on serial section stained by haematoxylin and
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Increased macrophage density stained for lactoferrin by immunoperoxidase in the actual field evaluated for L1 increased with increasing degree of inflammation, an upper limit of 15 granulocytes/grid was arbitrarily chosen as an additional criterion to distinguish moderate from severe inflammation.

The height of the colonic mucosa from the muscularis mucosae to intact surface epithelium was measured in the areas evaluated for macrophage density. Based on this height and the grid size the total number of macrophages/mm of muscularis mucosae was determined to give an absolute estimate in relation to an individually defined intestinal length unit. Similar measurements in ileal mucosa were difficult because of the villous structure.

REPRODUCIBILITY

Different amounts of extracellular L1, especially in fields with dense granulocyte infiltrates, made it necessary to count more than 200 macrophages in the evaluated areas in most of the specimens to reach reproducibility within the limits of 10% variation between three consecutive calculations.

STATISTICS

Student’s t test for paired samples was used when comparing numbers of immunostained cells in serial sections subjected to different staining techniques. In the other statistical calculations the data were treated as non-parametric and described by median and observed range. Comparisons between groups were based on the two tailed Mann-Whitney U test. Correlations were calculated as Spearman’s rank correlation coefficient.

Figure 1: Two colour immunofluorescence staining (double exposure) for CD68 and L1 antigen in normal colonic mucosa showing accumulation of CD68+ L1+ macrophages beneath the surface epithelium; only one CD68+ L1+ cell (arrow) and no CD68+ L1+ cells were found in this field. The basement membrane zone is indicated by dashed line (original magnification: ×400).

Results

METHODOLOGICAL ASPECTS

The number of neutrophilic granulocytes detected by staining for chloroacetate esterase in three inflamed mucosal tissue specimens did not differ significantly from that in adjacent sections subjected to immunoperoxidase staining for lactoferrin (data not shown).

Sections from the same three specimens stained for lactoferrin and then for chloroacetate esterase showed only some scattered cells in the submucosa and a few in the lamina propria positive for esterase (probably mast cells), in contrast with the large number of peroxidase positive cells. It was concluded that immunoperoxidase staining for lactoferrin detected most of the neutrophils in inflamed mucosa. Moreover, in sections from inflamed tissue triple stained for lactoferrin, L1, and CD68, immunofluorescence for the two latter markers was undetectable in the immunoperoxidase stained cells concurrently examined by conventional light microscopy and fluorescence microscopy. This accorded with our previous results showing masking of immunofluorescence by the benzidine product. Moreover, the number of CD68+ cells seen in sections of histologically normal mucosa (containing very few granulocytes) did not differ (p=0.6) after blocking of endogenous peroxidase and immunoperoxidase staining for lactoferrin compared with that in corresponding areas of adjacent sections not subjected to similar pretreatment (data not shown). This result confirmed the reliability of staining for CD68 in relation to L1 when such pretreatment was necessary.

NORMAL MUCOSA

Accumulations of CD68+ L1+ macrophages were seen beneath the surface epithelium in ileal and colonic mucosa, forming a dense subepithelial band in the latter (Fig 1). Only a small fraction of CD68+ L1+ cells were seen, both in the ileum (median 1-3%, range 0-3-0%) and colon (median 4-5%, range 0-9-2%). Virtually all CD68+ macrophages were co-stained with RFD7 (median 100%, range 92-100%).

IBD MUCOSA

Beneath intact surface epithelium CD68+ L1+ macrophages prevailed both in the ileum and colon, even in severe inflammation (Fig 2). In other parts of the lamina propria an increasing fraction of CD68+ L1+ macrophages were detected with increasing degree of inflammation, reaching median 70% in severely inflamed areas of colonic mucosa (range 35-90%) and median 60% in severely inflamed ileal mucosa (range 50-70%). The fraction of CD68+ L1+ macrophages showed strong correlation with the number of granulocytes identified by immunoperoxidase staining for lactoferrin in the same visual field both in ileal and colonic mucosa (r=0.90 and 0.86, respectively) (Figs 3A and B, respectively).

When relying exclusively on the grading of inflammation in adjacent sections stained with haematoxylin and eosin, IBD specimens considered to be un inflamed did not differ significantly from those with slight inflammation in terms of CD68+ L1+ macrophages, and specimens with moderate inflammation did not differ from those with severe inflammation. Specimens with moderate or severe inflammation, however, had a significantly increased (p=0.019) fraction of CD68+ L1+ macrophages compared with those with no or slight inflammation (Fig 3C). The same was true with regard to immuno-
Figure 2: Two colour immunofluorescence staining for CD68 and L1 antigen in Crohn's colitis. (A) Double exposure showing an increased number of CD68+ L1+ macrophages (arrows) beneath the surface epithelium; (B) selective filtration for L1 fluorescence. Note that the CD68+ L1 cells are the dominating subepithelial macrophage population also in inflamed colonic mucosa. The basement membrane zone is indicated by dashed line (original magnification: x400).

Figure 3: (A) Correlation between the fraction (%) of CD68+ macrophages positive for L1 and the topical density of granulocytes in two fields of ileal lamina propria from six Crohn's disease patients; (B) correlation between the fraction (%) of CD68+ macrophages positive for L1 antigen and the topical density of granulocytes in two fields of colonic lamina propria from six Crohn's disease patients (filled circles) and seven ulcerative colitis patients (open circles); (C) fraction (%) of CD68+ macrophages positive for L1 in ileal and colonic IBD mucosa related to the histopathological degree of inflammation in serial tissue section. (D) Crohn's disease, + ulcerative colitis. Medians indicated by horizontal bars.

Figure 4: Two colour immunofluorescence staining for CD68 and L1 antigen in ulcerative colitis. (A) Double exposure; (B) selective filtration for L1 fluorescence (lumen of a small vein indicated by broken line). Luminal CD68+ monocytes were all positive for L1 antigen, as were most of the perivascular macrophages (A) small arrow). Only one CD68+ L1+ cell (A) large arrow) can be seen in this field, presumably an eosinophilic granulocyte (original magnification: x400).

peroxidase stained granulocytes (p=0.009).

Using the number of granulocytes in the actual field as an additional criterion for the degree of inflammation (see earlier), the fraction of CD68+ L1+ macrophages did not differ when Crohn's disease was compared with ulcerative colitis of a similar degree of inflammation; the median fraction of CD68+ L1+ macrophages in severely inflamed lesions was 70% in Crohn's disease (range 50–90%) and 60% in ulcerative colitis (range 35–80%). Accumulations of CD68+ L1+ macrophages were seen particularly near small vessels in both diseases (Fig 4). The percentage of CD68+ L1+ macrophages in severely inflamed areas of ileal and colonic submucosa (median 75%, range 60–90%) was not significantly different (p=0.38) from that in severely inflamed areas of the lamina propria.

The number of CD68+ macrophages/grid in colonic mucosa was median 42 (range 25–60) in light to moderate inflammation and 38 (range 23–41) in severe inflammation, compared with median 38 (range 15–45) in control mucosa. The height of the mucosa in areas under intact epithelium was increased (p<0.05) in IBD, significantly more so (p<0.05) in moderate (median 2.0-fold, range 1.5–2.5-fold) than in severe (median 1.5-fold, range 1.3– to 2.0-fold) inflammation. Taking this change into account, the number of macrophages/mm muscularis mucosae was raised 2.2-fold (range 1.7– to 2.8-fold) in the moderately inflamed areas but only 1.5-fold (range 1.3– to 2.0-fold) in severely inflamed areas compared with normal.

The fraction of CD68+ macrophages reactive with RFD7 was median 85% in colonic (range 40–100%) and 75% in ileal (range 55–95%)
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mucosa with light to moderate inflammation. The percentage of CD68+ RFD7+ macrophages in severely inflamed areas was significantly lower than in the best controlled areas (<0.01), in contrast to the control areas. These data suggest that tumour cell lines apparently because of inhibition of casein kinase II. This might be of significance in control of epithelial dysplastic to neoplastic development in IBD. Further functional studies are necessary to elucidate the differences in the CD68+ L1+ macrophages in the pathogenesis of IBD.

Inflamed submucosa in Crohn’s disease was dominated by a CD68+ L1+ RFD7+ macrophage subpopulation similar to comparably inflamed lamina propria. Moreover, by means of CD68 and antibodies to CD68 and L1 we could not find differences in macrophage populations between ulcerative colitis and Crohn’s disease when areas of similar degree of inflammation were compared. Such previously reported differences were based on another combination of phenotypic markers. Our results suggested that the topographical extent of inflammation rather than the disease category influences the size and change in the various macrophage subpopulations in IBD.

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Discussion

A dense subepithelial accumulation of CD68+ L1+ macrophages was present in histologically normal as well as inflamed mucosa from both the ileum and colon, and more than 90% of them were costained with RFD7. This subepithelial population probably represented the CD4+ HLA class II+ macrophages that have been described in this location and have been suggested to function both as phagocytes and antigen presenting cells. The total number of CD68+ macrophages/length unit of the colon was increased in IBD. This might partly result from proliferation of the resident macrophage population but more probably reflects recruitment of new monocytes/macrophages from peripheral blood. The good correlation we saw between granulocytes and CD68+ L1+ macrophages in IBD mucosa supports the second possibility.

Several studies have shown the appearance of macrophage subpopulations with a changed phenotype in IBD. Moreover, in IBD mucosa many macrophages have been reported to be positive for the monocyte marker CD14. In accordance with the second finding we found an increased fraction of CD68+ L1+ monocyte-like macrophages in inflamed ileal and colonic mucosa. This subpopulation dominated in severely inflamed areas, particularly near vessels. Less than 5% of P: E RFD7+ mature macrophages costained for L1 in any section. The accumulation of CD68+ L1+ cells near blood vessels and in the areas mostly densely infiltrated with granulocytes, and the fact that mature macrophages have poor proliferative ability, support the idea that CD68+ L1+ macrophages have been recently recruited from blood. The CD68+ L1+ fraction of macrophages in moderately to severely inflamed areas showed wide variation (35-90%) which, however, might be at least partly artefactual, explained by masking resulting from large amounts of intermittent L1 derived from granulocytes and macrophages.

Recently recruited ‘inflammatory’ macrophages may have an activation potential and a cytokine profile different from resident tissue macrophages. Compared with macrophages from IBD mucosa, those from normal intestinal mucosa show minimal responsiveness to inflammatory stimuli such as interferon γ and lipopolysaccharide in vitro as determined by their production of oxygen radicals and interleukin 1β, respectively. The change we saw in the mucosal macrophage composition might explain such differences and hence be important in the pathogenesis of IBD. The large content of L1 protein (calprotectin), however, in these cells could support defence against microbial invasion in IBD mucosa. L1 has also been claimed to act as a primer for other monocyte/macrophage functions.

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