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Altered expression of cell adhesion molecules in uninvolved gut in inflammatory bowel disease

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SUMMARY

Adhesion of circulating cells to vascular endothelium occurs in the early phase of inflammation, and is mediated by specific cell adhesion molecules. Many such adhesion molecules are increased in inflamed regions of ulcerative colitis (UC) and Crohn's disease (CD) but there is limited knowledge of their expression in the uninvolved gut, adjacent to inflammation. We investigated immunohistochemically the expression of platelet endothelial cell adhesion molecule-1 (PECAM-1), intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1) on resected specimens taken at a distance of 2–4 cm from the inflamed area and without histological signs of inflammation. Compared with normal gut, we found (i) a significant increase of PECAM-1-positive vessels in the mucosa of uninvolved UC ($149{\cdot}0 \pm 24{\cdot}1$ vessels/mm^2 (mean ± s.d.); normal colon $= 123{\cdot}1 \pm 21{\cdot}6$; $P = 0{\cdot}004$); (ii) a significant decrease of ICAM-1-positive vessels in uninvolved CD ($111{\cdot}9 \pm 22{\cdot}6$ vessels/mm^2; normal ileum $= 136{\cdot}9 \pm 27{\cdot}6$; $P = 0{\cdot}04$); and (iii) a moderate but statistically insignificant increase of LFA-1-positive cells in the mucosa of uninvolved UC and Crohn's ileitis. This altered expression of cell adhesion molecules may contribute to the early lesion in inflammatory bowel disease and provide new therapeutic opportunities.

Keywords Crohn disease ulcerative colitis ICAM-1 LFA-1 PECAM-1

INTRODUCTION

Although the underlying cause of inflammatory bowel disease (IBD) remains unknown, intestinal infiltration of inflammatory and activated immunocompetent cells is characteristic of advanced ulcerative colitis (UC) and Crohn's disease (CD) [1]. Adhesion of circulating cells to the vascular endothelium occurs in the early phase of immunoactivation [2] and seems to be a prerequisite for cellular transendothelial migration into the tissue, where the recognition of an antigen and the creation of an immune response typically occur. Recently, a three-step cascade of adhesion of circulating cells to endothelial cells has been proposed, with each step involving specific groups of cell adhesion molecules [3]. This cascade involves (i) tenuous adhesion or tethering of circulating cells to endothelial cells, predominantly mediated by selectins, (ii) strong integrin-mediated adhesion, and (iii) migration of mononuclear cells through the endothelium into the surrounding tissue [3]. Some molecules involved in this adhesion cascade have been demonstrated in the normal human gut [4–8], but there is limited knowledge about cell adhesion molecules in IBD.

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Among the variety of cell adhesion molecules characterized so far, intercellular adhesion molecule-1 (ICAM-1) and its counter-receptor, lymphocyte function associated antigen-1 (LFA-1), are probably best understood. ICAM-1 (CD54) belongs to the immunoglobulin superfamily with a wide distribution on both haematopoietic and non-haematopoietic cells [9]. ICAM-1 is strongly expressed in the vascular endothelium and the molecule is involved in adhesion of neutrophils [10] and eosinophils [11] to endothelial cells. ICAM-1 also mediates co-stimulation in the activation of resting T lymphocytes [12]. One of the ligands for ICAM-1 is LFA-1 (CD11a/CD18, 180 kD), a member of the β-2 subgroup of the integrin family [9,13]. LFA-1 is present on lymphocytes, macrophages, granulocytes, lymphoid tissue and bone marrow elements. Its mediation of cell–cell adhesion is important for a wide range of leucocyte functions, including the interaction of leucocytes with endothelium, T and B cell proliferation and T cell-mediated cytolysis [9,13].

Platelet endothelial cell adhesion molecule-1 (PECAM-1; CD31, 120–130 kD) was recently characterized, and is another endothelial cell adhesion molecule of the immunoglobulin superfamily [14–16]. PECAM-1 is localized to intercellular junctions of endothelial cells and to the surface of human platelets [17]. Increased activation and aggregation of platelets

[18,19] have been proposed as a pathogenic factor in IBD [20], for example by predisposing to the multifocal intestinal microinfarction described in UC [21] and CD [22].

We have shown increased expression of ICAM-1 and LFA-1 on inflammatory cells in highly inflamed areas of both CD and UC [23]. Specifically, the percentage of mononuclear cells expressing ICAM-1 and LFA-1 is increased in colonic biopsies of active UC [24], and ICAM-1 is increased in active CD [25]. Furthermore, endothelial leucocyte adhesion molecule-1 (ELAM-1) [25,26] but not vascular cell adhesion molecule-1 (VCAM-1) [25] is up-regulated in UC and CD. However, the distribution of cell adhesion molecules in uninvolved areas of IBD has not been reported so far.

In the present study, we hypothesized that ICAM-1, LFA-1 and PECAM-1 are up-regulated in uninvolved areas of UC and CD, adjacent to sites of inflammation but not yet showing enhanced cellular infiltrate. Our aim was therefore to investigate qualitatively and quantitatively the expression of ICAM-1, LFA-1 and PECAM-1 in these areas.

PATIENTS AND METHODS

Patients and tissues

Surgically resected specimens from 21 patients with active UC and 23 patients with active CD were obtained within 30 min of removal. Tissue samples were collected from macroscopically uninvolved areas at a distance of 2–4 cm from the inflamed area. In the main part of the study, only tissues subsequently found to show no histological signs of inflammation (histograde '0', *vide infra*) were included, reducing the number of patients to 11 with UC and 13 with ileal CD (study A; one section per case). In addition, in order to study the expression of cell adhesion molecules at different distances from the main lesion within the same patient, up to seven samples per specimen were collected from uninvolved, intermediate and severely affected areas from each of a further five CD patients (study B; several sections per case). Patients with UC (male:female = 7:4; mean age 39 years, range 22–79 years) had long-standing pan-colitis or left-sided colitis requiring total colectomy ($n=8$) or subtotal colectomy ($n=3$). A total of 18 patients with CD from study A and B (male:female = 10:8; mean age 31 years, range 18–70 years) underwent ileocaecal resection ($n=12$), ileal resection ($n=2$), resection of ileo-colonic anastomosis ($n=1$) or hemicolectomy ($n=3$) for chronic stenosis and/or disease refractory to therapy. Six patients with UC and 13 patients with CD received glucocorticosteroids perioperatively. From 20 cancer patients (male:female = 12:8, mean age 58 years, range 25–82 years) non-inflamed control tissues (ileum $n=5$, colon $n=15$) were taken from hemicolectomy specimens, at least 5 cm from the cancer. In all cases, diagnosis was confirmed by histopathological examination of the resected specimen.

Histological assessment

Tissues were fixed by immersion in Zamboni's solution and rinsed in 15% (w/v) sucrose in 0·1 M PBS with 0·01% (w/v) sodium azide. Cryostat blocks were prepared and sectioned at 6-μm thickness. One section from each sample was stained with haematoxylin and eosin for histological determination of inflammation according to a previously published method grading from 0 (non-inflamed) to 3 (severely inflamed) [27].

Table 1. List of antibodies used

Antibodies to:	Dilution	Type	Source
ICAM-1 (CD54)	1:3000	m/c	Serotec, Oxford, UK
LFA-1 (CD11a)	1:800	m/c	Serotec
PECAM-1 (CD31)	1:8000	m/c	A. Mazurov [28]
Pan-T cell (CD3)	1:800	p/c	Dako, Copenhagen, Denmark
Macrophages (CD68)	1:200	m/c	Dako
Dendritic cells (CD35)	1:100	m/c	Dako
von Willebrand Factor	1:200 000	m/c	Serotec

m/c, Mouse monoclonal; p/c, rabbit polyclonal; ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; PECAM-1, platelet endothelial cell adhesion molecule-1.

Immunocytochemistry

Tissue sections were immunostained using a range of antibodies (see Table 1) by an indirect peroxidase method [29]. Sections were counterstained with neutral fast red and mounted with glycerol gelatine. Infiltrating mononuclear cells were further characterized by immunostaining for macrophages, T cells and dendritic cells (Table 1) on sections serial to those used for immunostaining of cell adhesion molecules. Microvessels were identified by staining of von Willebrand Factor (Table 1) and Ulex Europaeus Antigen-1 (biotinylated Ulex Europaeus Antigen-1; VECTOR Labs, Peterborough, UK) as conventional markers of endothelial cells [30].

Quantification

Five visual fields within the lamina mucosa propria were chosen randomly from each section for quantitative microscopic analysis for both parts of the study. Two independent observers, who were not aware of the diagnosis, counted vessels showing immunoreactivity for PECAM-1 or ICAM-1 and infiltrating cells immunoreactive for LFA-1 within an area of 0·0625 mm^2 defined by an ocular grid at ×40 objective magnification. Peyer's patches and lymphoid aggregates were excluded from quantification because the physiologically high density of lymphocytes in these areas was found to interfere with quantitative comparison.

Statistical analysis

To compare mean values of disease groups with those of control samples from the same gut region t-tests were done using the pooled estimates of variability from an analysis of variance (study A). In the random sampling series of study B an unbalanced analysis of variance was performed with factors 'localization' and 'histological grade of inflammation'. For individual patients, samples were obtained from a single localization (either ileum or colon). The effect of localization was therefore assessed relative to the variation between patients, while the effect of histological grade and its interaction with localization were assessed relative to the variation within patients. The contribution of each factor to the model was obtained from the change in the fit in the model resulting from including the factor. To avoid distortion due to the unbalanced nature of the data, the mean predicted values for each combination of grade and localization are presented by averaging the fitted values for each of the five patients [31].

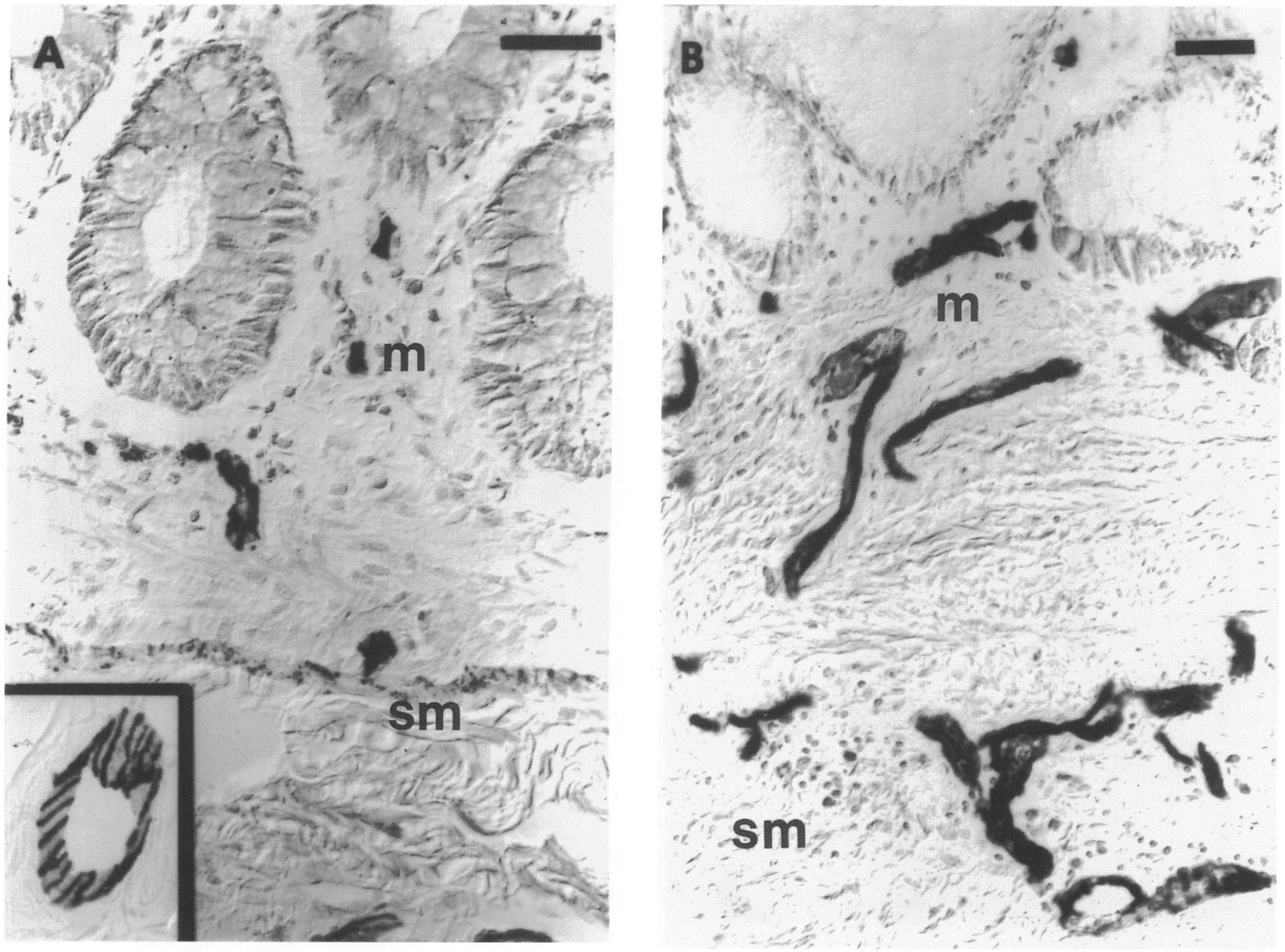

Fig. 1. Platelet endothelial cell adhesion molecule-1 (PECAM-1) expression in normal colon (a) and in histologically non-inflamed ulcerative colitis (UC) (b). Both mucosa (m) and submucosa (sm) show an increased density of PECAM-1-positive vessels in UC (b) compared with normal control colon (a). PECAM-1 staining is densest at inter-endothelial junctions (insert, a). Bars indicate 50 μm (immunoperoxidase, counterstained with neutral red).

RESULTS

Cell adhesion molecules in the normal gut

In normal ileum and colon, all identifiable endothelial cells expressed PECAM-1 strongly in each layer of the gut wall, with the densest immunostaining at intercellular junctions (Fig. 1a). Vascular density detected by PECAM-1 was higher in the ileum than in the colon (161·3 ± 21·1 vessels/mm^2 (ileum; mean ± s.d.), 123·1 ± 21·6 vessels/mm^2 (colon); $P = 0{\cdot}001$). Most PECAM-1-positive vessels co-expressed ICAM-1, so that ileum and colon also differed significantly in the frequency of vessels expressing ICAM-1 (136·9 ± 27·6 vessels/mm^2 (ileum), 104·8 ± 23·8 vessels/mm^2 (colon); $P = 0{\cdot}009$). In comparison with PECAM-1, ICAM-1 immunoreactivity revealed a more homogeneous staining pattern along the whole endothelial layer (Fig. 2a). Apart from endothelium, PECAM-1 was expressed on intravascular platelets and, rarely, on monocytes; staining for ICAM-1 was also seen on single infiltrating cells, most of them co-expressing the CD3 T cell marker. LFA-1 was present on mononuclear cells in both normal ileum and colon. The density of LFA-1-positive cells in the mucosa became greater towards the muscularis mucosae compared with the tips of villi.

Cell adhesion molecules in uninvolved gut adjacent to inflammation

In the uninvolved areas of both UC and CD (study A), PECAM-1, ICAM-1 and LFA-1 all showed a similar distribution pattern and staining intensity as in the normal gut. Quantitative analysis of cell adhesion molecules on mucosal endothelial cells showed a significantly increased density of PECAM-1-positive vessels in UC (Figs 1b and 3) and a significantly decreased density of ICAM-1-positive vessels in CD (Fig. 4). The mean density of LFA-1-positive cells in the lamina propria surpassed normal controls by approximately 20% in both UC and CD, though the difference did not reach statistical significance (Fig. 5).

The inter-individual variation for LFA-1 (coefficient of variation (CV) = 29%) was almost twice that of PECAM-1 (CV = 16%) or ICAM-1 (CV = 21%), so that a much larger sample size would have been required to detect a percentage change in LFA-1 comparable to that observed in PECAM-1.

High endothelial venules were positive for PECAM-1 and ICAM-1, dendritic cells expressed ICAM-1 (Fig. 2b) and CD68$^+$ macrophages expressed ICAM-1 and LFA-1. LFA-1 expression was largely a feature of lymphocytes, and in lymph

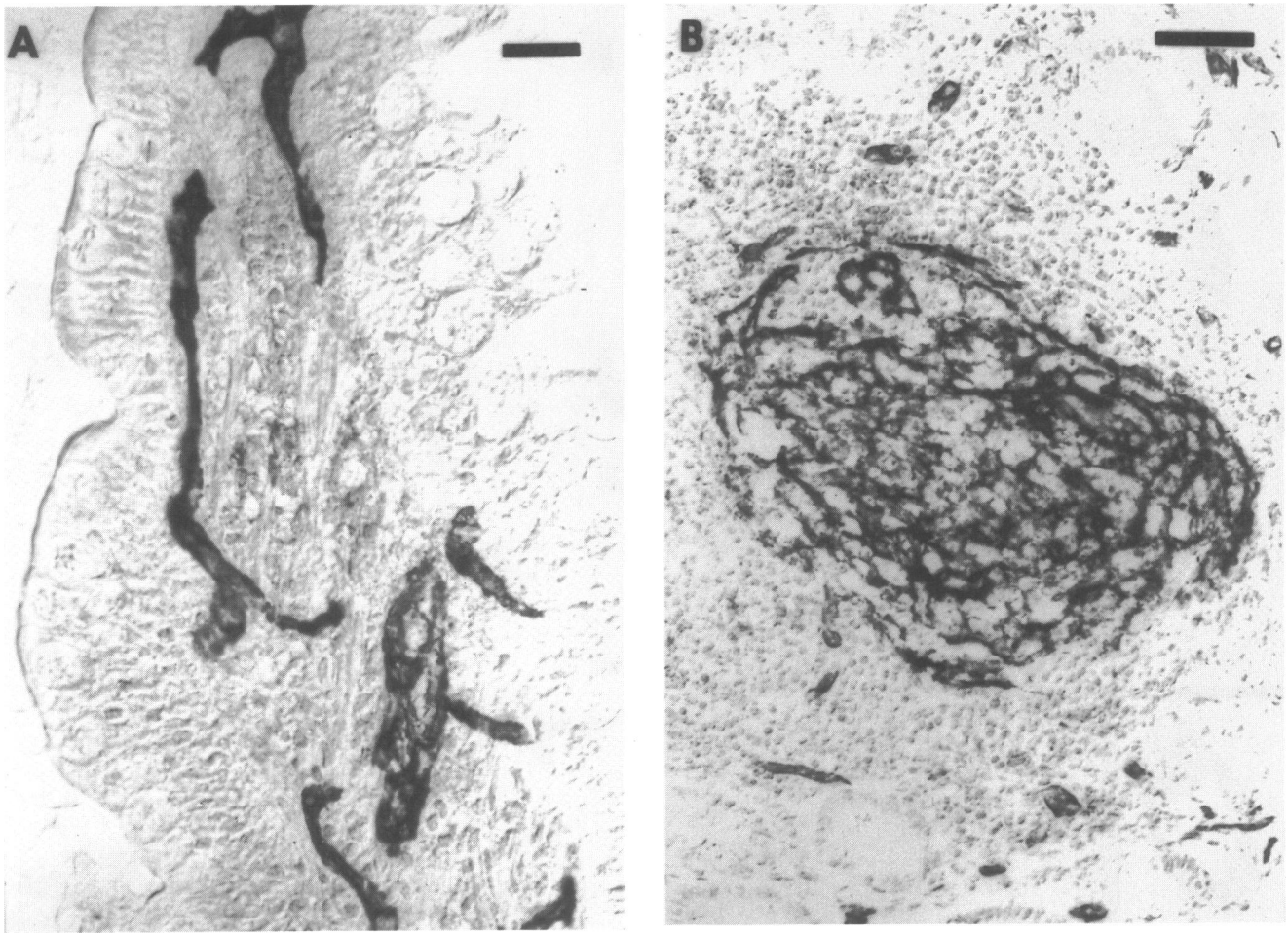

Fig. 2. Intercellular adhesion molecule-1 (ICAM-1) expression in normal ileum (a) and on dendritic cells in the centre of a lymphoid follicle in Crohn's disease (CD) (b). ICAM-1 is expressed strongly on microvascular endothelium orientated longitudinally towards the villi tips (a). Bars indicate 50 μm (immunoperoxidase, counterstained with neutral red).

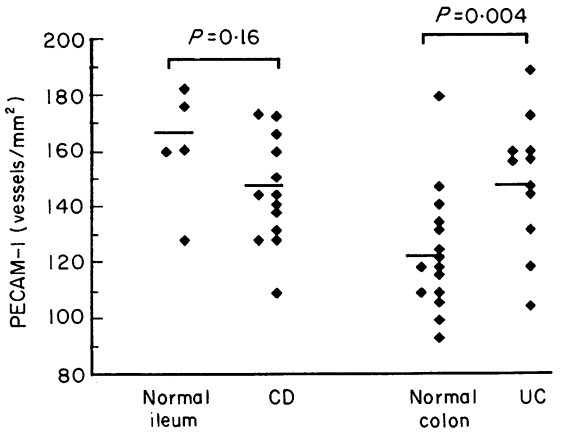

Fig. 3. Scatter plot of numbers of mucosal vessels/mm^2 expressing platelet endothelial cell adhesion molecule-1 (PECAM-1) in controls and ulcerative colitis (UC) and Crohn's disease (CD) patients (study A). Bars indicate means.

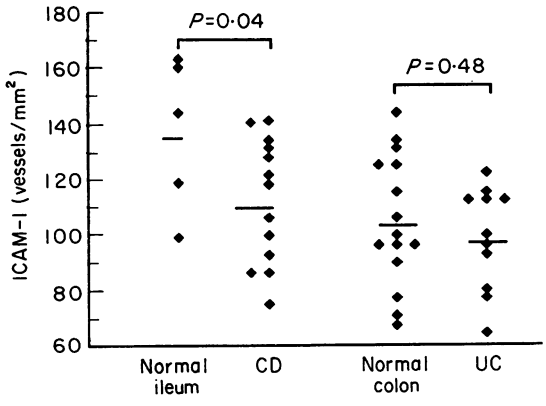

Fig. 4. Scatter plot of numbers of mucosal vessels/mm^2 expressing intercellular adhesion molecule-1 (ICAM-1) in controls and ulcerative colitis (UC) and Crohn's disease (CD) patients (study A). Bars indicate means.

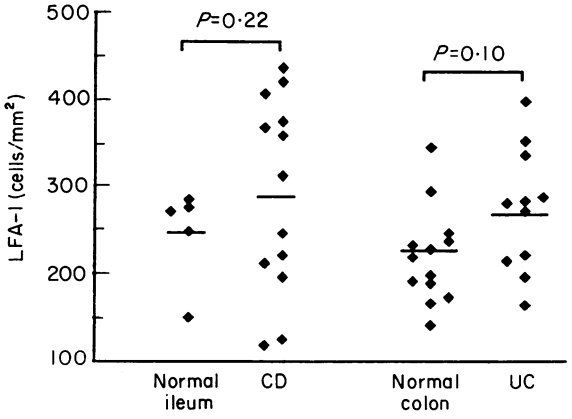

Fig. 5. Scatter plot of numbers of mucosal cells/mm^2 expressing lymphocyte function-associated antigen-1 (LFA-1) in controls and ulcerative colitis (UC) and Crohn's disease (CD) patients (study A). Bars indicate means.

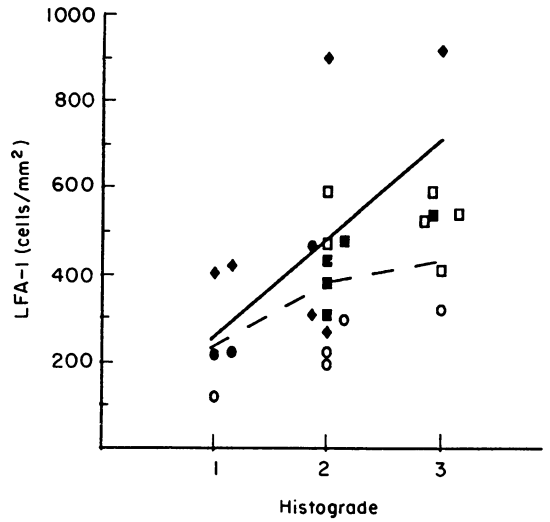

Fig. 6. Scatter plot of numbers of mucosal cells/mm^2 expressing lymphocyte function-associated antigen-1 (LFA-1) with different degrees of inflammation (study B). The closed symbols refer to three patients with Crohn's disease (CD) of the colon, the open symbols refer to two patients with CD of the ileum. Lines show the mean predicted values from the fitted model (see text; ——, ileum; - - -, colon).

follicles LFA-1 staining was found in the germinal centres. T cells of the follicular mantle zone and within interfollicular areas coexpressed ICAM-1 and LFA-1. Outside lymphoid aggregates, ICAM-1-positive infiltrating cells were apparently increased with high intra- and inter-individual variability which precluded quantitative comparison with the normal gut.

Cell adhesion molecules in inflamed bowel

Twenty-six specimens from five patients with CD were classified as histograde 1 ($n=5$), 2 ($n=14$) or grade 3 ($n=7$) (study B). Samples obtained from patients with UC were all graded as 3, and were thus not suitable for intra-individual comparison. In CD, the density of LFA-1-positive cells increased linearly with the grade of inflammation ($P=0{\cdot}01$; Fig. 6). Endothelial expression of PECAM-1 or ICAM-1 showed no significant trends with grade of inflammation. However, extensive dilation of immunoreactive vessels was noticed in severely inflamed bowel, and the altered vessel diameter may have off-set any fundamental change in numbers per unit area. The intra-individual variability of each cell adhesion molecule was smaller than its inter-individual variability (PECAM-1, $P=0{\cdot}007$; ICAM-1, $P=0{\cdot}14$; LFA-1, $P=0{\cdot}01$).

DISCUSSION

The aim of this study was to test for early changes in specific adhesion molecules in IBD by examining uninvolved gut adjacent to inflammation. We found (i) a significant increase of PECAM-1-positive vessels in UC, (ii) a significant decrease of ICAM-1-positive vessels in CD, and (iii) a moderate but not statistically significant increase of LFA-1-positive cells in UC and CD, the latter correlating with the grade of inflammation within individual CD patients.

Histologically uninvolved areas adjacent to inflammation are a useful model to investigate the role of cell adhesion molecules in the pathogenesis of IBD before infiltration of immuno-inflammatory cells and microabscesses occur. In these areas, it is unlikely that the observed changes are secondary phenomena due to the severe changes in the gut wall which characterize advanced IBD, i.e. increased mucosal permeability, loss of intestinal mucosal barrier functions and, more generally, destruction of autochthonous structures of the intestinal wall [32]. Given the susceptibility of any segment of the gastrointestinal tract to be affected by CD and the continuous spread of UC, areas in the vicinity of inflammation are likely to become infiltrated in the later course of the disease and thus may be regarded as 'early lesions'. However, previous studies of these regions have been restricted mostly to small sample sizes, because non-inflamed resected tissue is difficult to obtain, as up to 80% of macroscopically normal tissues are found subsequently to reveal histological signs of inflammation [33,34].

A variety of findings characterize the uninvolved, adjacent areas as being not entirely normal. Neural axon necrosis [35], neuronal restoration [36] and increased mucus production are present [37]. Altered distribution of vasoactive intestinal polypeptide (VIP)-containing nerves [38,39] and increased expression of MHC antigens on nerve bundles [34] and endothelial cells [40] point to increased immunoactivation in the vicinity of IBD lesions.

How do cell adhesion molecules contribute to this process? In our study, PECAM-1 was investigated in the gut for the first time and has proved to be an excellent marker of all intestinal vascular endothelial cells, giving stronger and more consistent staining than either Ulex Europaeus Antigen or von Willebrand Factor. PECAM-1 modulates endothelial cell migration [41]; it is immunostained heterogeneously on endothelial cells differentiating into capillaries and is densest on tube-forming cells [17], suggesting that it plays a role in angiogenesis. In support of this, we have been able to demonstrate consistent vascular expression of PECAM-1 in the developing human gut at 11 weeks of gestation (data not shown). Since there is constitutive expression of PECAM-1 on normal endothelial cells, our finding of increased numbers of PECAM-1-positive vessels in unin-

volved UC suggests that angiogenesis anticipates inflammatory infiltration. Increased endothelial PECAM-1 may be a target for homotypic binding with PECAM-1 on activated platelets, inducing the formation of platelet thrombi which has been reported as an early event in UC [21]. Although the factors controlling expression of endothelial PECAM-1 are not yet defined, our finding of increased expression in uninvolved gut in patients with UC but not CD underlines the different pathogenesis of the two conditions.

ICAM-1 was found to be widely expressed on small and large vessels of the normal gut. In asthmatics, ICAM-1 immunoreactivity of vascular endothelium in the bronchial mucosa is similar to normals [42], but it is increased in perennial allergic rhinitis [43] and in human cardiac allografts during rejection [44]. The observed down-regulation of endothelial ICAM-1 in the uninvolved gut of this series may be due to glucocorticoid treatment; the lowest ICAM-1 values were in patients receiving steroids. The glucocorticoid receptor regulates expression of ICAM-1 [45,46] and glucocorticoids are highly effective at inhibiting T cell secretion of a wide range of cytokines, including interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and IL-1, that are known to up-regulate ICAM-1 expression *in vitro* [46].

We found a slight increase of LFA-1-immunoreactive cells in the uninvolved gut. Stimulation of CD2 and CD3 molecules on the T cell surface induces expression of LFA-1 and enhances the affinity of LFA-1–ICAM-1 interactions [47,48]. Up-regulation of LFA-1 in these areas may be due to stimulation by local antigens, but it is also possible that intestinal LFA-1-positive cells were stimulated elsewhere (i.e. in the main lesion) and represent memory T cells which have recirculated; memory T cells are known to have a higher expression of LFA-1 than antigen-unstimulated T cells [49]. Furthermore, studies on binding of lamina propria lymphocytes from normal and inflamed bowel show a loss of selectivity of lymphocyte–endothelial interaction in IBD (M. Salmi *et al.*, unpublished data), which may contribute to the homing of LFA-1-positive lymphocytes to uninvolved areas adjacent to the lesion.

Altered expression of PECAM-1, ICAM-1 and LFA-1 in the uninvolved gut, adjacent to inflammation, suggests a contribution of these cell adhesion molecules to the early lesion in IBD. Increased PECAM-1-mediated platelet–endothelial interaction may induce the mucosal localization of platelet thrombi and contribute to multifocal microinfarction. Adhesion of LFA-1-positive circulating cells to the mucosal endothelium may precede transendothelial migration of inflammatory cells, a characteristic of advanced IBD. Thus, early inhibition of PECAM-1 and LFA-1 may form the basis of new therapeutic approaches. Studies on blocking cellular adherence with MoAbs directed against CD18, the β-2 subunit of LFA-1, have shown significant attenuation of tissue injury in models of reperfusion injury in several other organs [50,51]. These antibodies may also prove to be of benefit in the treatment of inflammatory bowel disease.

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