GUT IMMUNOLOGY

B cell attracting chemokine 1 (CXCL13) and its receptor CXCR5 are expressed in normal and aberrant gut associated lymphoid tissue

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Background and aims: In mice, the B lymphocyte chemoattractant (BLC) CXC chemokine ligand 13 (CXCL13) is sufficient to induce a series of events leading to the formation of organised lymphoid tissue. Its receptor, CXCR5, is required for normal development of secondary lymphoid tissue. However, the human counterpart, B cell attracting chemokine 1 (BCA-1) has only been detected in the stomach and appendix and not in other parts of normal or diseased gut. Hence to elucidate the potential role of this chemokine and its receptor in human gut associated lymphoid tissue (GALT), we analysed their expression in normal intestine and ulcerative colitis (UC).

Methods: Frozen sections of surgical specimens were studied by multicolour immunofluorescence staining, in situ mRNA hybridisation, and reverse transcription-polymerase chain reaction.

Results: BCA-1 mRNA was detected in all normal colonic and UC specimens. BCA-1 was produced and accumulated in relation to peripheral dendritic elements of lymphoid follicles in Peyer's patches and normal colon, as well as in irregular lymphoid aggregates in UC lesions. BCA-1 was partially associated with the traditional follicular dendritic cell phenotype but also with extracellular fibrils in GALT structures. CXCR5 protein was expressed by mantle zone B cells and appeared at a high level on scattered germinal centre T cells.

Conclusions: BCA-1 and CXCR5 are expressed in normal GALT structures as well as in irregular lymphoid aggregates in UC. This strongly suggests that BCA-1 plays an important role not only in the formation of normal GALT but also in the generation of aberrant lymphoid tissue in inflammatory bowel disease.

Chemokines have recently emerged as crucial orchestrators for lymphocyte trafficking and activation.[1] These secreted polypeptides exert their function by binding to specific cell surface receptors and can be divided into two categories: homeostatic and inflammatory. The former category is constitutively expressed in a certain tissue or organ, which suggests a specific function that involves normal cell migration. Conversely, the inflammatory chemokines are strongly upregulated by proinflammatory or immune stimuli and most likely participate in reactive tissue processes by targeting effector leucocytes.[2] Several chemokines constitutively expressed in organised lymphoid tissue (so-called lymphoid chemokines) have been identified as major cues for homeostatic lymphocyte trafficking and positioning within such organs.[3 4] The CXC chemokine BCA-1 (B cell attracting chemokine 1)/CXCL13 (CXC chemokine ligand 13) is an efficacious attractant of naive B cells in vitro and has been shown to be produced constitutively by stromal cells in lymphoid follicles of human lymph nodes.[5] This chemokine was concurrently described in mice[6] and called B lymphocyte chemoattractant (BLC). Several lines of evidence suggest that BLC is strongly involved in the formation of organised lymphoid tissue. Thus ectopic expression of BLC can induce a series of events leading to the formation of extranodal B cell follicles.[7] Also, mice deficient in its receptor, CXCR5 (BLR1), show impaired development of Peyer's patches (PPs), inguinal lymph nodes, and splenic follicles. Furthermore, B cells from mice lacking CXCR5 fail to enter lymphoid follicles of PPs and the spleen after transfer to wild-type littermates, despite normal B cell extravasation in the T cell zones.[8] BLC deficient mice also show severe deficiency of PPs.[9] Together, these findings indicate a fundamental requirement for the BLC-CXCR5 ligand-receptor pair in the development of murine gut associated lymphoid tissue (GALT).

GALT is normally comprised of numerous scattered solitary lymphoid follicles, particularly in the distant gut, together with PPs, and the appendix. In the diseased gut, so-called basal lymphoid aggregates (BLAs), defined as located between the muscularis mucosae and the crypts, are indicative of inflammatory bowel disease (IBD) and considered to be a highly significant discriminator of IBD versus acute self limited colitis in rectal biopsies.[10] BLAs are a purely histopathological feature and, to our knowledge, there are no available data to discriminate BLAs in functional terms from other lymphoid aggregates in IBD lesions or in the normal colon. Therefore, here we prefer to use the term "irregular lymphoid aggregates" to include all lymphoid aggregates in mucosal IBD lesions.

In the original work of Legler and colleagues,[5] BCA-1 mRNA transcripts were found in the human appendix and stomach but not in the colon or small intestine. Such disparity suggested that this homeostatic chemokine, so crucial for the

Abbreviations: BCA-1, B cell attracting chemokine (in humans); BLA, basal lymphoid aggregate; BLC, B lymphocyte chemoattractant (in mice); CXCL13, CXC chemokine ligand 13; CXCR5, CXC chemokine receptor 5; DIG, digoxigenin; DRC, dendritic reticulum cell; FDC, follicular dendritic cell; GALT, gut associated lymphoid tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC-Th, germinal centre T helper; H&E, haematoxylin and eosin; IBD, inflammatory bowel disease; MAdCAM-1, mucosal addressin cell adhesion molecule 1; MALT, mucosa associated lymphoid tissue; PP, Peyer's patch; RT-PCR, reverse transcription-polymerase chain reaction; vWF, von Willebrandt factor; UC, ulcerative colitis.

Table 1 Clinicopathological information on subjects providing tissue specimens and a summary of immunohistochemical findings

Specimen category*	Age (y)/ sex	Histological grade of inflammation	Disease duration (y)	Lymphoid aggregates†	No of BCA-1$^+$ CD21$^+$ aggregates‡	No of BCA-1$^+$ CD21$^-$ aggregates	No of BCA-1$^+$ DRC$^+$ aggregates	No of BCA-1$^+$ DRC$^-$ aggregates
UC 1	28/F	3	10	Confluent¶	0	0	0	0
UC 2	60/F	3	14	7–8	1	1	1	0
UC 3	32/M	2	14	8–7	5	0	5	0
UC 4	44/F	2	2	3–4	4	0	4	0
UC 5	12/M	3	1	12–24	13	7	9	8
NC 1	38/F	0		0–1	1	0	0	1
NC 2	23/F	0		0–3	3	0	3	0
NC 3	33/F	0		0–3	2	0	2	0

*UC, ulcerative colitis; NC, normal colon.
†Number of lymphoid aggregates per section judged by haematoxylin and eosin staining. Parallel sections from the same tissue block were evaluated (cut a distance of approximately 750 μm apart) and the numbers from the two sections are presented as a range.
‡Parallel sections 8 μm apart were examined for BCA-1$^+$ follicles: one section was stained with polyclonal anti-BCA-1 in combination with anti-CD21; the other section was stained with monoclonal anti-BCA-1 in combination with the DRC antibody. Follicles were described as BCA-1$^+$ when a reticular staining meshwork was seen; CD21$^+$ or DRC$^+$ aggregates were always surrounded by a BCA-1$^+$ reticular meshwork.
¶Extensive cell infiltration was seen but separate lymphoid aggregates were difficult to identify.
BCA-1, B cell attracting chemokine 1.

development of murine GALT, might be restricted to certain parts of the human GALT, perhaps having a different and less important role in humans than in mice. To address this possibility, we examined normal human GALT specimens for BCA-1 and CXCR5 expression. Although BCA-1 was primarily described as a constitutively expressed chemokine, recent data have suggested that it might also contribute to the formation of malignant and inflammatory lymphoid aggregates.[11 12] Hence we further examined the occurrence of BCA-1 and its receptor CXCR5 in colonic lesions of ulcerative colitis (UC) to reveal possible differences in the expression patterns between normal and aberrant lymphoid tissue in the human gut. We found that BCA-1 and CXCR5 are expressed in normal GALT structures as well as in irregular lymphoid aggregates in UC lesions. Our findings provide the first evidence to suggest an important role of this chemokine-receptor pair in the formation of both normal and aberrant lymphoid tissue in the human gut.

MATERIAL AND METHODS

Patient characteristics

The characteristics of the subjects are listed in table 1. Surgical specimens from 15 UC patients were screened for the presence of lymphoid aggregates by haematoxylin and eosin (H&E) staining of one section from each tissue block, and five specimens were selected for this study by their large number of lymphoid aggregates. These selected UC specimens were from two men and three women (median age 32 years (range 12–60); median disease duration 10 years (range 1–14)). All except one patient had received corticosteroids while two had received azathioprine and mesalazine (an aminosalicylic acid preparation) in addition to steroids. One patient had received only mesalazine. All UC patients had their colon removed because of therapy resistant colitis. Colonic and small bowel specimens were also obtained from one patient with Crohn's disease (a 28 year old male; disease duration 15 years) who underwent bowel resections because of stenoses. He had received azathioprine and mesalazine. Histologically normal colonic control specimens were obtained from macroscopically healthy mucosa of three females (median age 33 years (range 23–38)) operated on for long term chronic obstipation. The PP specimens were obtained from three organ transplant donors (median age 30 years (range 3–40)) whose circulation was artificially maintained.

Tissue specimens

Small mucosal tissue blocks (approximately 4×15 mm^2) were excised from fresh surgical specimens and embedded in OCT compound (Miles Labs, Elkhart, Indiana, USA), snap frozen in liquid nitrogen, and stored at −70°C until cutting. Cryosections were cut at 8 μm perpendicular to the mucosal surface and stained with H&E for evaluation of histological grade of inflammation, number of follicles, and to ensure an acceptable morphology of the sections before further serial cutting for immunohistochemistry. These sections were air dried overnight, fixed in acetone (10 minutes, 22°C), and stored at −20°C until use.

In addition, five 150 μm thick sections from each tissue block were cut from the normal colon and UC specimens and subjected to RNA extraction followed by new sections stained with H&E. Thereafter, 8 μm thick sections were cut and fixed in 4% paraformaldehyde/diethyl pyrocarbonate treated phosphate buffered saline (15 minutes) for in situ hybridisation. The Crohn's disease specimens were only prepared for immunohistochemistry.

Histological grading of inflammation in the UC specimens was performed according to the following 0–4 scale: 0, normal mucosa; 1, no active inflammation in the lamina propria; 2, mild active inflammation; 3, moderate active inflammation; and 4, severe active inflammation.[13] All UC specimens showed histologically active inflammation (table 1).

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from 5×150 μm cryosections by means of QIAshredder columns and the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA primed with oligo (dT) and reverse transcribed with Superscript II (Gibco BRL, Life Technologies Inc., Rockville, Maryland, USA) was used for 20 μl reactions. Specific mRNA was amplified by real time RT-PCR with the Light Cycler (Roche Diagnostics, Indianapolis, Indiana, USA) and Fast-Start DNA master SYBR Green reagents, as described previously.[14] Primers used were (5′ to 3′) BCA-1: TGCTAATGAGCCTGGAC, AGGGATAAGGGAAGAATG; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): AAATCCCATCACCATCTTCC, CATGAGTCCTTCCACGATACC. The annealing temperature for both primer pairs was 60°C, and 35 cycles of PCR amplification were performed. The PCR products were separated by electrophoresis in 1.6% agarose gel and stained with ethidium bromide.

In situ hybridisation

A 436 bp digoxigenin (DIG) labelled riboprobe was generated from the coding region of human BCA with the DIG RNA labelling kit according to the manufacturer's instructions

Table 2 Characteristics of the immunoreagents used in the study

Specificity	Clone	Immunoglobulin class/subclass	Origin of antibody	Conjugate/labelling	Producer/source
BCA-1	53610.11	IgG1	Mouse	None	R&D Systems, Oxon, UK
BCA-1		IgG	Goat	None	R&D Systems
CXCR5	51505.111	IgG2b	Mouse	None	R&D Systems
CD3		Ig	Rabbit	None	Dako, Glostrup, Denmark
CD20	L26	IgG2a	Mouse	None	Dako
CD21	1F8	IgG1	Mouse	None	Dako
CD23	EBV CS-5	IgG1	Mouse	None	Becton-Dickinson, San Jose, CA, USA
CD35	To5	IgG1	Mouse	None	Dako
MAdCAM-1	10G3	IgG2a	Mouse	None	Courtesy of Michael Briskin
MAdCAM-1	10A6	IgG1	Mouse	None	Courtesy of Michael Briskin
DRC	R4/23	IgM	Mouse	None	Dako
IgD	IgD26	IgG1	Mouse	None	Dako
vWF		Ig	Rabbit	None	Dako
Muscle actin	HHF35	IgG1	Mouse	None	Dako
SMA	1A4	IgG2a	Mouse	None	Dako
Fibronectin		Ig	Rabbit	None	Dako
Collagen IV	CIV 22	IgG1	Mouse	None	Dako
Mouse IgG1		IgG	Goat	Cy3 (red)	Southern Biotechnology, Birmingham, AL, USA
Mouse IgG2a		IgG	Goat	Biotinylated	Southern Biotechnology
Mouse IgM		IgG	Goat	Biotinylated	Southern Biotechnology
Goat IgG		Ig	Donkey	Alexa 546 (red)	Molecular Probes, Eugene, OR, USA
Rabbit IgG		IgG	Goat	Alexa 488 (green)	Molecular Probes
Mouse IgG1		Ig	Rabbit	Alexa 488	Zymed Laboratories, San Francisco, CA, USA. Molecular Probes
Mouse IgG		IgG	Horse	Biotinylated	Vector Laboratories, Burkingame, CA, USA
Mouse IgG		Ig	Donkey	Cy2 (green)	Jackson Immuno Research, West Grove, PA, USA
				Cy3 labelled streptavidin	Jackson
				Cy2 labelled streptavidin	Ammersham, Life Sciences, UK
				Alexa 350 labelled (blue) streptavidin	Molecular Probes

BCA-1, B cell attracting chemokine 1; CXCR5, CXC chemokine receptor 5; DRC, dendritic reticulum cell; vWF, Von Willebrandt factor; MAdCAM-1, mucosal addressin cell adhesion molecule 1; SMA, smooth muscle actin.

(Boehringer Mannheim, Mannheim, Germany). All incubations took place at room temperature unless otherwise stated. Briefly, after fixation, sections were washed twice (15 minutes each) in phosphate buffered saline containing 0.1% active diethyl pyrocarbonate (Sigma-Aldrich, St Louis, Missouri, USA). After 15 minutes of equilibration in 5× SSC, sections were hybridised overnight at 59°C with 250 ng/ml of riboprobe in hybridisation solution (50% formamide, 5× SSC, 50 µg/ml yeast tRNA, 100 µg/ml heparin, 1× Denhardt solution, 0.1% Tween 20, 0.1% CHAPS, and 5 mM EDTA) followed by high stringency wash. Detection of the hybridised probe was performed according to a protocol kindly provided by Bradley St Croix (Johns Hopkins Oncology Center, Baltimore, Maryland, USA).[15] The sections were incubated (45 minutes) with horseradish peroxidase conjugated rabbit anti-DIG (1/50; Dako, Glostrup, Denmark) in blocking buffer (0.1% Boehringer blocking agent dissolved in 100 mM Tris HCl (pH 7.5), 150 mM NaCl) followed by signal amplification with biotin-tyramide deposition (GenPoint kit; Dako). Subsequently, sections were incubated (20 minutes) with horseradish peroxidase conjugated rabbit antibiotin (1/50 in blocking buffer; Dako), followed by an additional cycle of biotin-tyramide deposition. Signal was detected by incubation (20 minutes) with alkaline phosphatase conjugated rabbit antibiotin (Dako), followed by the alkaline phosphatase substrate Fast Red according to the manufacturer's instructions (Ventana Medical Systems, Tucson, Arizona, USA). Finally, the sections were counterstained with haematoxylin.

Two and three colour immunofluorescence staining

Immunostaining on cryosections was performed as described previously,[16] and primary as well as secondary antibody reagents are listed in table 2. Briefly, acetone fixed sections were first incubated with a mixture of primary reagents for one hour at room temperature. Either the monoclonal or polyclonal anti-BCA-1 was used at a concentration of 10 µg/ml in combination with different primary reagents detecting either follicular dendritic cells (FDCs), B cells, T cells, adhesion molecules, vessels, or extracellular matrix proteins. This combination was followed by incubation with the appropriate mixture of secondary antibody reagents and, when a biotinylated antibody was used, a final application with labelled streptavidin. The monoclonal anti-BCA-1 reagent was always visualised with a subclass specific secondary antibody reagent (goat antimouse IgG1; 1/2000). The monoclonal antibody to CXCR5 was applied at 5 µg/ml and mixed with anti-CD3 followed by horse IgG antimouse IgG (1/200) and, subsequently, Alexa 488 conjugated goat IgG antirabbit IgG together with Cy3 labelled streptavidin.

As negative controls, we used tissue sections incubated in the first step with irrelevant isotype and concentration matched monoclonal antibodies, and concentration matched normal goat IgG (Sigma-Aldrich) and rabbit IgG (authors' laboratory) purified from serum of non-immunised animals. As an additional control, recombinant BCA-1 (R&D Systems, Oxon, UK) was incubated at different concentrations together with antibody reagents against BCA-1 before the mixtures were applied to tissue sections in an attempt to block the staining reactions.

RESULTS

Distribution of BCA-1 expressing cells in Peyer's patches and normal colon

BCA-1 was detected in the mantle zone of all lymphoid follicles in the gut (fig 1). The staining patterns obtained with the polyclonal and monoclonal antibody reagents were similar, often resembling a meshwork associated with cells that extended dendrites. Although this expression pattern was generally seen throughout the mantle zone, it was often most prominent peripherally, with positive dendrites outlining the

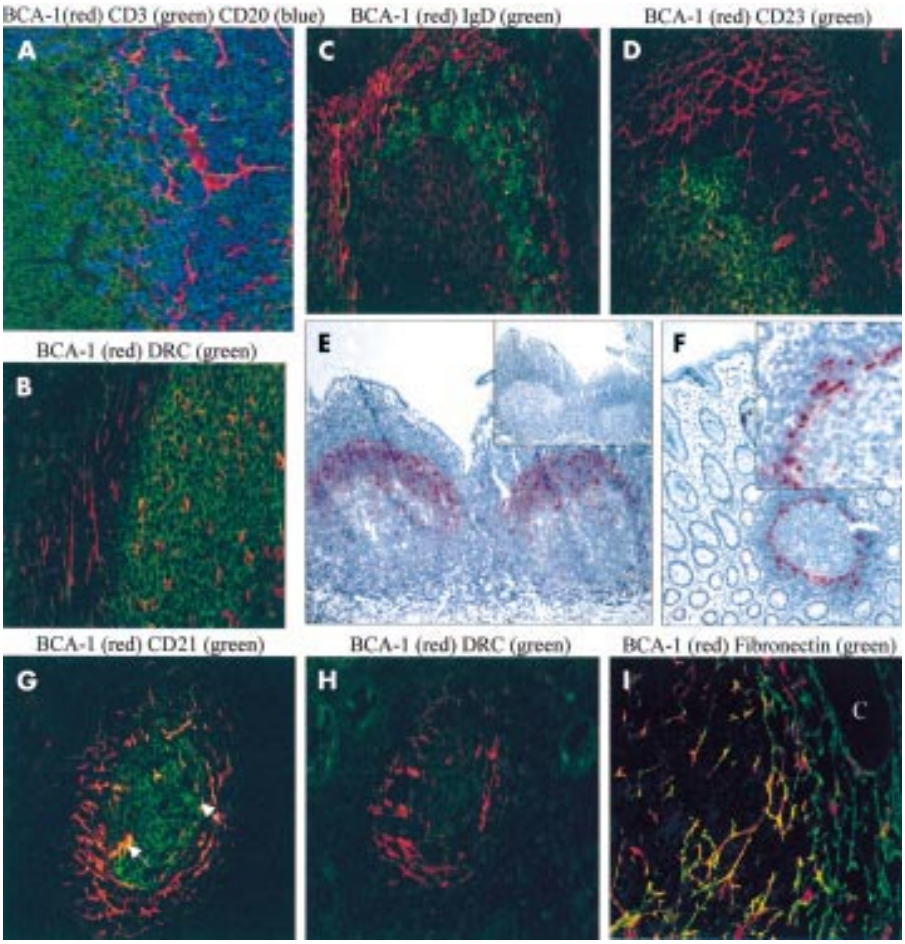

Figure 1 Expression of B cell attracting chemokine 1 (BCA-1) in normal gut specimens. (A–D, G–I) Immunofluorescence and (E, F) in situ hybridisation analysis of frozen tissue sections with BCA-1 visualised by red signals in all panels. (A) Three colour staining of Peyer's patch (PP) showing BCA-1 expressing elements between $CD3^+$ T cells (green) and $CD20^+$ B cells (blue). (B–D) Two colour staining of PP showing BCA-1 expressing cells detected by monoclonal (B) or polyclonal (C, D) antibody reagent combined with either anti-dendritic reticulum cell (DRC) (green) (B), anti-IgD (green) (C), or anti-CD23 (green) (D). BCA-1 protein is predominantly associated with a meshwork in the mantle zone while disparate staining patterns are produced in the germinal centre by monoclonal and polyclonal anti-BCA-1 (scattered central cells positive with the former, and weak positivity corresponding to follicular dendritic cells with the latter reagent). (E, F) Expression of BCA-1 mRNA in PP (E, inset: sense probe control) and solitary colonic follicle (F, inset: enlarged details) with predominantly dendritic distribution of message in the mantle zone. (G, H) Two colour staining of BCA-1 in solitary follicle of normal colon detected by polyclonal (G) or monoclonal (H) antibody reagent combined with anti-CD21 (green) (G) or anti-DRC (green) (H) to reveal follicular dendritic cells shows some costaining (yellow) of dendrites in the inner part of the mantle zone (arrows). The two sections were cut in parallel at a distance of 8 μm; some dendrites positive for BCA-1 but negative for DRC appear centrally as the follicle is cut closer to the mantle zone and therefore appears smaller (weak unspecific green staining of surrounding epithelial goblet cells is seen in (H)). (I) Two colour staining of BCA-1 and fibronectin (green) in PP shows extensive dendritic colocalisation (yellow) in the mantle zone (C=epithelial crypt). Original magnifications: ×200 (A–D, G, H); ×100 (E, F, and inset F); and ×400 (inset C, H, and I).

B cell zone (fig 1A). Conversely, in the germinal centres, a disparate staining pattern was obtained with the polyclonal versus the monoclonal antibody reagent. The latter often stained scattered cells in the germinal centres which appeared most prominently in PPs (fig 1B). This feature seemed to be related to the frequent large size of the germinal centres in these GALT structures. The polyclonal reagent did not decorate these scattered germinal centre cells but produced a relatively weak staining corresponding to the FDC network in some parts of the germinal centres, possibly representing the light zones (fig 1C, 1D).

Given the secretory nature of the chemokine BCA-1 and the disparate germinal centre staining pattern obtained with the two antibody reagents, we compared the distribution of BCA-1 protein to that of BCA-1 encoding mRNA transcripts. By means of in situ hybridisation, we detected BCA-1 transcripts in the mantle zone of all lymphoid follicles in PPs and normal colon (fig 1E, 1F). Thus the cellular nature of at least some of the reticular pattern observed with immunostaining was also supported by observing mRNA in a similar distribution (fig 1F, inset). Some scattered weak signals were furthermore observed in large germinal centres (fig 1E), corresponding to the scattered cells detected by means of the monoclonal antibody reagent (fig 1B). The presence of BCA-1 mRNA at various concentrations in all normal colonic and UC specimens was confirmed by RT-PCR performed on extracted RNA (fig 2).

BCA-1 is associated with, but not restricted to, the traditional FDC phenotype in GALT

In an effort to phenotype the BCA-1 expressing cells, we performed paired staining with two colour immunofluorescence for several FDC markers. In the inner part of the mantle zone, some colocalisation of BCA-1 with these markers was seen in both PPs and normal colonic follicles: dendritic reticulum cell (DRC) (fig 1B), CD23 (fig 1D), CD21 (fig 1G), CD35 (data not shown, but see fig 4D), and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (not shown). Thus the polyclonal reagent to BCA-1 produced costaining with the different FDC

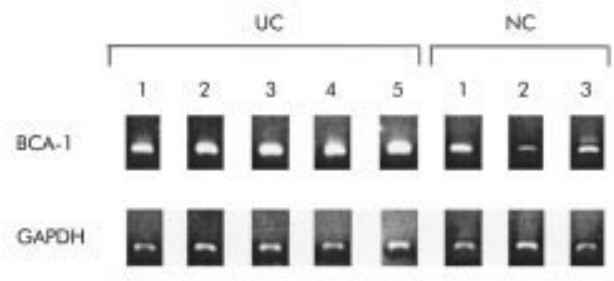

Figure 2 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of B cell attracting chemokine 1 (BCA-1) expression in normal and inflamed colonic mucosa. BCA-1 mRNA is present in all samples of ulcerative colitis (UC) and normal colon (NC). Total RNA was isolated from frozen tissue samples of colonic mucosa, reversed transcribed, and subjected to RT-PCR with specific primers and a standardised numbers of cycles. PCR products were separated by electrophoresis in agarose gel stained with ethidium bromide. Comparable PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers is shown as a control for the cDNA content of the individual samples.

markers centrally in the germinal centres but generally not more peripherally (fig 1D). Even cells with the strongest expression of the two most restricted FDC markers, CD23 (fig 1D) and MAdCAM-1 (not shown), displayed only partially overlapping staining for BCA-1.

FDCs were detected in the centre of most but not all follicles decorated for BCA-1 (table 1). Parallel sections were investigated for some specimens, and these revealed that the FDCs disappeared while prominent staining for BCA-1 remained in follicles when cut closer to the mantle zone (fig 1G, 1H). This finding showed that the appearance of BCA-1 in follicles without detectable FDCs could, at least partially, be explained by the differential localisation of BCA-1 and FDC markers within the follicle.

In contrast with the limited colocalisation of BCA-1 and FDC markers seen in the inner B cell zone, extensive overlapping fluorescence for BCA-1 and fibronectin occurred throughout the mantle zone meshwork (fig 1H). Conversely, immunostaining with antimuscle actin or antismooth muscle actin did not show any overlap with the BCA-1 positive meshwork but only occasional peripheral intermingling of separately decorated elements (data not shown). Based on the description of BCA-1 expressing vessels in human tonsils,[17] we also costained for BCA-1 and von Willebrandt factor (vWF) and observed a small proportion of BCA-1 positive vessels both in the germinal centres and mantle zones of all specimens. In the mantle zones, the BCA-1 positive vessels were often bordered by strongly BCA-1 positive dendrites (see fig 5A, C).

The dendritic mantle zone pattern seen with both BCA-1 antibody reagents, and also the disparate staining features described for the germinal centres, were blocked by incubation with recombinant BCA-1. The monoclonal antibody was neutralised at an antibody: chemokine molar ratio of 1:2.5 while the polyclonal reagent was neutralised at a molar ratio of 1:0.25.

Distribution of CXCR5 expressing B and T cells in GALT

Expression of CXCR5 was seen mainly in lymphoid aggregates, both in the normal and diseased gut, while only scattered CXCR5$^+$ cells appeared in the T cell zones of PPs as well as outside of the lymphoid aggregates in UC lesions. In an attempt to identify these positive elements as B cells, we used an IgG2B specific secondary antibody reagent to detect CXCR5 concurrently with staining for CD20. However, with this relatively insensitive costaining protocol, CXCR5 expression was restricted to lymphoid follicles and aggregates (data not shown).

CXCR5 was detected mainly on mantle zone cells in PPs and normal colonic and UC follicles; the overall staining pattern clearly represented a general surface expression on B cells in this lymphoid compartment (fig 3A, 3B). When serial tissue sections were examined, CXCR5 expression was found in all follicles investigated. Such expression was also often seen in the centre of lymphoid follicles with no apparent germinal centre reaction; some of this staining reflected the fact that lymphoid follicles were cut close to the mantle zone. However, CXCR5 expression could also be found in follicles where no germinal centres appeared on serial sectioning, thus reflecting a general surface expression on B cells in primary follicles.

High expression of CXCR5 on T cells was restricted to lymphoid follicles. When CXCR5 staining occurred in overt germinal centres, it was not of even intensity but appeared particularly strong on T cells scattered throughout this compartment (fig 3C) and occasionally in the inner part of the mantle zone (fig 3A). This was the case in both the normal and diseased gut but most prominent in PPs, apparently being mainly associated with large germinal centres.

Expression of BCA-1 in UC lesions

By means of serial section analysis, we detected BCA-1 protein and transcripts in all aberrant lymphoid aggregates in UC (fig 4). Additional colonic and small bowel sections from a patient with Crohn's disease were also investigated for BCA-1 protein, and a similar follicular meshwork positive for BCA-1 was

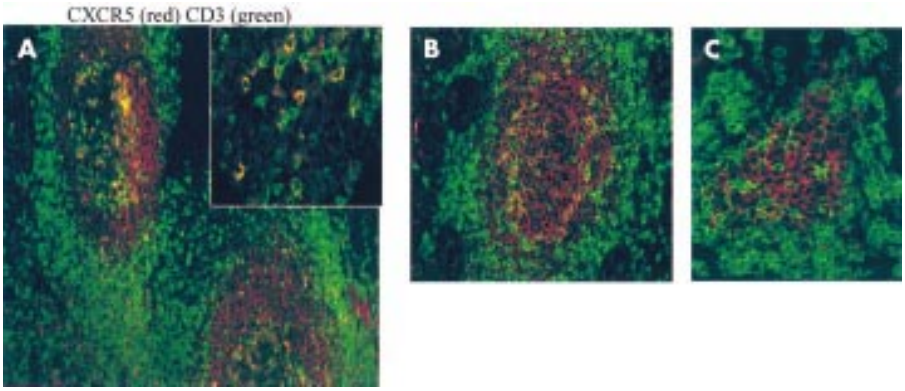

Figure 3 Localisation of CXC chemokine receptor 5 (CXCR5) protein in normal and inflamed gut. Two colour immunofluorescence staining of cryosections for CXCR5 (red) and CD3 (green). (A) Prominent CXCR5 expression is seen on B cells in the mantle zone of the Peyer's patch and (B) solitary colonic follicle in normal mucosa. Strongly CXCR5$^+$ T cells are seen scattered in the germinal centre (see inset in (A)) and the inner part of the mantle zone shown in (B). (C) CXCR5 is also abundantly expressed on B cells in irregular lymphoid aggregate in ulcerative colitis. Original magnifications: ×100 (A); ×600 (inset A and C); and ×200 (B).

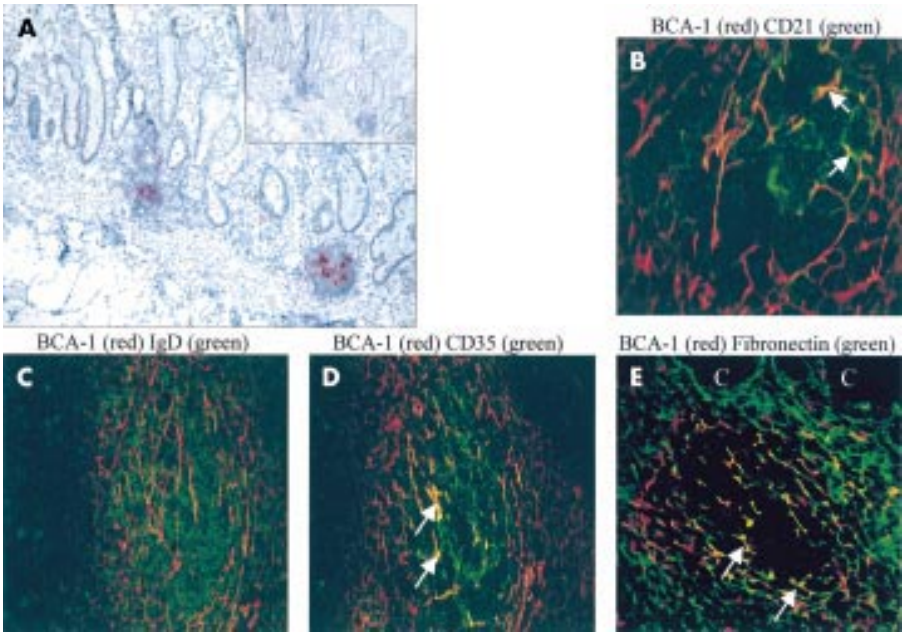

Figure 4 Expression of B cell attracting chemokine 1 (BCA-1) in ulcerative colitis lesions. (A) In situ hybridisation and (B–E) two colour immunofluorescence staining of frozen tissue sections with BCA-1 visualised by red signals in all panels. (A) BCA-1 transcripts in irregular lymphoid aggregates (inset: sense probe control). (B–D) Two colour staining with polyclonal anti-BCA-1 combined with (green) anti-CD21 (B), anti-IgD (C), or anti-CD35 (D). Two sections (C, D) were cut in parallel at a distance of 8 μm; the entire B cell area expresses surface IgD, reflecting the mantle zone where BCA-1$^+$ dendrites are abundant (C); in the inner mantle zone there is colocalisation (yellow, arrows) of BCA-1 with (D) CD35 and (B) CD21. (E) Monoclonal anti-BCA-1 combined with antifibronectin (green) shows extensive mantle zone dendritic colocalisation (yellow, arrows) in a follicle situated between crypts (labelled C) and muscularis mucosae. Original magnifications: ×100 (A, inset); ×400 (B, E); and ×200 (C, D).

found (data not shown). As in the normal gut, there was partial colocalisation of BCA-1 and FDC markers in the inner part of the mantle zone in UC aggregates (fig 4B, 4D), and the meshwork positive for BCA-1 was most prominent at the follicle periphery (fig 4C). Serial sections revealed that some of the aggregates appeared to be without detectable FDCs because they were cut close to the mantle zone, similar to what was seen by parallel sections in the normal colon. However, serial section analysis did not reveal detectable FDCs in all UC aggregates positive for BCA-1 but at least some colocalisation with fibronectin was seen in all meshworks (fig 4E). Costaining with the monoclonal anti-BCA-1 reagent and anti-vWF revealed some vessels positive for the chemokine, which were not restricted to germinal centres but also occasionally occurred outside of lymphoid follicles in IBD lesions (fig 5B, 5D).

DISCUSSION

This study showed that BCA-1 and its receptor CXCR5 are expressed in lymphoid follicles of human GALT in the small and large intestine. As an additional novel finding, aberrant lymphoid aggregates were shown to produce BCA-1. Our data thus suggested that this chemokine-receptor pair plays an important role in the formation of human GALT, in keeping with experimental studies of PPs in mice.[8 9] The phenotype of the CXCR5 expressing cells was clearly defined in this study, and that of the BCA-1 expressing dendritic elements mainly overlapped with extracellular fibrils and to some extent with FDCs.

General expression of CXCR5 on mantle zone B cells agrees with the notion that this chemokine is a selective and highly efficacious chemoattractant for circulating naïve human B lymphocytes.[5] The level of CXCR5 on these B cells was relatively low, perhaps paralleling the moderate in vitro potency of BCA-1 on peripheral blood B cells.[5] The CXCR5 positive T cells that localise to B cell follicles have been functionally described as "follicular B helper T cells" (T_{FH} cells)[17 18] because they show all the characteristics required for efficient B cell help within the lymphoid follicles. A subpopulation of T_{FH} cells has been identified as CD57$^+$ CXCR5$^+$ T cells and termed germinal centre T helper (GC-Th) cells because they appear to be essential for B cell differentiation and antibody production in lymphoid tissue[19]; these cells are localised only within the germinal centres. We observed quite prominent expression of CXCR5 on scattered T cells restricted to germinal centres and the inner part of the mantle zone in GALT, and such cells were also detected in tonsillar follicles (our unpublished observations). FACS analysis of isolated cells from murine PP[8] and human tonsils[17–19] has revealed a much higher proportion of CXCR5$^+$ T cells, implicating the presence of a substantial fraction of such cells in the T cell zone of these tissues. Our observation of CXCR5$^+$ T cells being restricted to germinal centres in both PPs and tonsils most probably reflected the lower sensitivity of the applied in situ immunostaining method. In this location, the observed scattered T cells should belong to the GC-Th subset. Their prominent CXCR5 expression was probably a result of local receptor upregulation, which could explain why GC-Th cells reportedly show a stronger chemotactic response to BCA-1 than other CXCR5$^+$ T cells isolated from tonsils.[19]

The fibrillar meshwork positive for BCA-1 protein detected in GALT mantle zones was a striking observation. Its colocalisation with the extracellular matrix protein fibronectin was extensive, suggesting that BCA-1 is deposited on reticular fibres in follicles where the fibrillar network is looser than in other compartments of lymphoid tissue.[20] Also, BCA-1 mRNA was expressed in a pattern that strongly suggested a dendritic morphology of the cells producing BCA-1 in this compartment. BLC has been reported to be produced by non-lymphoid cells present in murine lymphoid follicles[6]; its origin was implicated to be FDCs, but this was not explicitly shown.[21] The observed partial overlapping of our immunostaining for the chemokine with several traditional FDC markers might result from BCA-1 secreted from another cell type and deposited on peripherally located FDCs. However, electron microscopy has

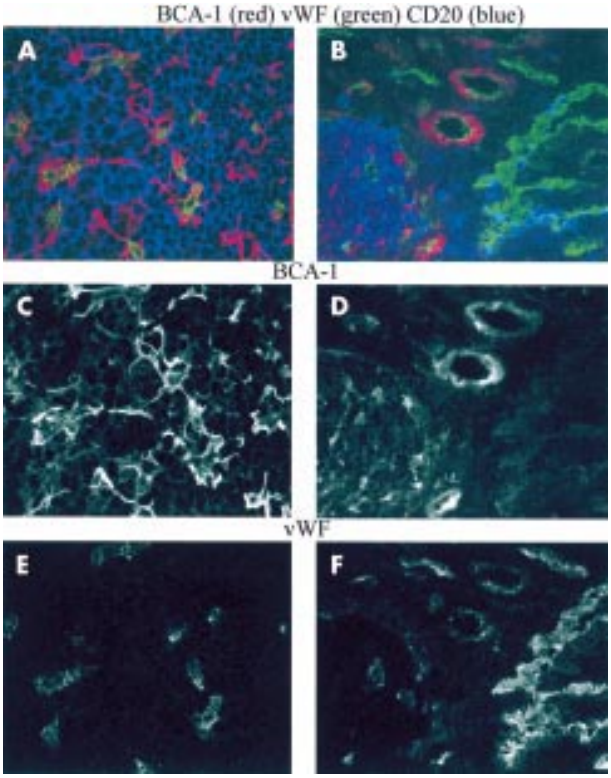

Figure 5 Vessel wall localisation of B cell attracting chemokine 1 (BCA-1) protein in normal gut associated lymphoid tissue and ulcerative colitis lesions. Three colour immunofluorescence staining of BCA-1 (red), von Willebrandt factor (vWF) (green), and CD20 (blue) on frozen tissue sections from (A, C, and E) Peyer's patch and (B, D, and F) ulcerative colitis lesion with single exposures identifying BCA-1 (B, D) and vWF (C, E). Scattered vWF$^+$ vessels in the mantle zones (identified by densely packed CD20$^+$ B cells) are weakly positive for BCA-1 and sometimes bordered by strongly BCA-1$^+$ dendrites. In ulcerative colitis, larger BCA-1$^+$ vessels are occasionally seen outside of follicles. Original magnifications: ×600 (A, C, and E); and ×400 (B, D, and F).

suggested considerable heterogeneity of FDCs in tonsillar germinal centres[22]; possibly FDC related, peripherally located so-called germinal centre bordering cells with long extensions have been described in rat lymph nodes.[23] Therefore, an FDC related subset of dendritic cells remains a potential but yet not proved source of BCA-1 in lymphoid tissue. This accords with in vitro stimulation of isolated human FDC-like cells in which no BCA-1 protein could be detected.[24]

BCA-1 protein expression observed on scattered cells in GALT germinal centres was detected only by the monoclonal antibody but was supported by mRNA signals in the same compartment. In contrast, a more extensive germinal centre staining was only produced by the polyclonal reagent. Both antibodies were raised against recombinant human BCA-1 and selected for their ability to recognise BCA-1 in direct ELISAs and western blots. Neither antibody showed any cross reactivity with a range of other chemokines, as reported by the manufacturer. Thus no obvious explanation could be found for the observed disparity. The dissimilar germinal centre staining was seen only in some areas, possibly representing the light zones, and in this zone murine FDCs have been suggested to produce BLC.[21] We found some colocalisation of the FDC marker CD23 and BCA-1 in this zone with the polyclonal antibody reagent. Therefore, the possibility exists that epitopes of BCA-1 were partially masked by immune complexes on FDCs in germinal centres, hence being difficult to detect by the more restricted monoclonal antibody.

Our data on UC lesions elucidated the relationship between BCA-1 expression and FDCs in the aberrant development of human lymphoid follicles. BCA-1 expressing cells could develop concurrently with the formation of lymphoid follicles, and before the occurrence of classical FDCs in these structures. Single cells expressing BCA-1 protein in GALT germinal centres did not have the characteristics of FDCs but were scattered within the FDC meshwork. In the inner mantle zone of UC follicles however, some colocalisation of BCA-1 protein and the FDC markers CD21, CD23, CD35, DRC, and MAdCAM-1 was found while BCA-1 expressing dendritic elements mainly overlapped with extracellular fibrils. Hence the distribution of BCA-1 in UC follicles corresponded to our findings in normal GALT, thus suggesting a similar role of this chemokine in aberrant and normal GALT.

The so-called BLAs are a histopathologically defined feature indicative of IBD,[10 25] although their relevance in the pathogenesis remains unknown.[26] Experimental inflammation in mice can induce ectopic expression of BLC (and another lymphoid chemokine, namely SLC, also called 6Ckine, exodus-2, or CCL21).[27] The first report on BCA-1 expression in human disease was from *Helicobacter pylori* induced gastric mucosa associated lymphoid tissue (MALT) in chronic gastritis and gastric B cell (MALT) lymphoma.[11] The distribution of BCA-1 producing cells in such aberrant MALT was found to resemble that observed in human tonsils. Also, the reticular appearance of the immunostaining for BCA-1 was taken to suggest that it was produced by FDCs.[11] In MALT lymphoma however, prominent expression of BCA-1 was seen even in the absence of detectable FDCs.[11] Rare expression of BCA-1 was also noted in the normal stomach in keeping with the original work of Legler and colleagues[5] in which BCA-1 transcripts were found in the human stomach and appendix.

There are data on BCA-1 expression in other human diseases such as rheumatoid arthritis[12 28] and Sjögren's syndrome.[29 30] Contrary to our findings in normal GALT and UC lesions, immunostaining for BCA-1 was reported to be associated only with FDCs in germinal centres in rheumatoid arthritis while the mantle zones were negative.[12 28] In situ transcripts for BCA-1 and the cytokine lymphotoxin β suggested that BCA-1 and lymphotoxin β were necessary, but not sufficient, for the occurrence of FDCs in rheumatoid arthritis.[28] This would be in keeping with our results in normal GALT and UC lesions where FDCs without exception were surrounded by a BCA-1 positive reticular meshwork.

The findings in experimental models and human diseases suggest that BLC/BCA-1 also has a function in aberrant lymphoid tissue. Our detection of both BCA-1 and CXCR5 in lymphoid aggregates in IBD lesions strengthens this notion. It has been proposed that induction of BCA-1 and other lymphoid tissue inducing chemokines at sites of inflammation could convert the lesion from an acute to a chronic state, and that blocking of chemokine activity therefore might be of therapeutic value.[31] If this is true, our results point to IBD as a potential candidate for such chemokine blockade in the future.

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B cell attracting chemokine 1 (CXCL13) and its receptor CXCR5 are expressed in normal and aberrant gut associated lymphoid tissue

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