Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR-γδ expression

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Abstract

Background and aims—A histopathological feature considered indicative of ulcerative colitis (UC) is the so-called basal lymphoid aggregates. Their relevance in the pathogenesis of UC is, however, unknown. We have performed a comprehensive analysis of the immune cells in these aggregates most likely corresponding to the lymphoid follicular hyperplasia also described in other colitides.

Methods—Resection specimens of UC and normal colon were analysed by immunomorphometry, immunoflow cytometry, and immunoelectron microscopy, using a large panel of monoclonal antibodies.

Results—(1) In all cases of UC, colonic lamina propria contained numerous basal aggregates composed of lymphocytes, follicular dendritic cells, and CD80/B7.1 positive dendritic cells. (2) CD4+CD28−αβ T cells and B cells were the dominant cell types in the aggregates. (3) The aggregates contained a large fraction of cells that are normally associated with the epithelium: that is, γδ T cells (11 (7%)) and αβ−γδ+ T cells (26 (13%)). The γδ T cells used Vα1 and were CD4+CD8−. Immunoelectron microscopy analysis demonstrated TcR-γδ internalisation and surface downregulation, indicating that the γδ T cells were activated and engaged in the disease process. (4) One third of cells in the aggregates expressed the antiapoptotic protein bcl-2.

Conclusions—Basal lymphoid aggregates in UC colon are a consequence of anomalous lymphoid follicular hyperplasia, characterised by abnormal follicular architecture and unusual cell immunophenotypes. The aggregates increase in size with severity of disease, and contain large numbers of apoptosis resistant cells and activated mucosal γδ T cells. The latter probably colonise the aggregates as an immunoregulatory response to stressed lymphocytes or as a substitute for defective T helper cells in B cell activation. γδ T cells in the aggregates may be characteristic of UC.

Keywords: basal lymphoid aggregates; ulcerative colitis; T cell receptor γδ; immunomorphology

Ulcereative colitis (UC) is a large intestine restricted, chronic inflammatory disease of unknown aetiology and pathogenesis. However, there are indications that immune mechanisms play an important role in the pathogenesis of the disease, for example immunosuppressive drugs have therapeutic effects. It has been suggested that UC is caused by failure to maintain homeostasis towards normal gut microbial flora, leading to an uncontrolled immune response to one or a few normally occurring gut constituents. The inflammation may be perpetuated by an autoimmune response against colonic antigens initiated by cross reactive luminal antigens. Indeed, in 1959 it was shown that autoantibodies directed against colonic components were present in the serum of UC patients. Later, immunological cross reactivity between certain microbial antigens and colonic tissue antigens was demonstrated using UC patient sera. Recently, Duchmann and colleagues demonstrated that tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). Moreover, it was shown that autoantibodies of immunoglobulin G (IgG) directed against intracellular components of epithelial cells, antineutrophil cytoplasmic antibodies, as well as antibodies directed against intestinal bacteria are produced in the colonic tissue of UC patients. Halsten and colleagues noted deposition of IgG1 and complement factors on the apical surface of epithelial cells in areas of UC colon with active disease.

A role for T lymphocytes in IBD became apparent through work with genetically manipulated mice. T cell receptor α (TcR-α) chain and TcR-β chain deficient mice lacking the majority of mature αβ T cells develop an IBD-like disease. Similarly, mice with aberrant thymus selection processes (for example, major histocompatibility complex class II gene deficient mice) and mice with human cluster of differentiation 3 (CD3) α chain transplanted with normal F1 bone marrow cells also develop IBD-like symptoms. Disruption of certain cytokine genes, such as interleukin-2 (IL-2), IL-10, and occasionally transforming growth factor β, as well as of the signal protein G subunit

Abbreviations used in this paper: UC, ulcerative colitis; Ig, immunoglobulin; IBD, inflammatory bowel disease; TcR, T cell receptor; MHC, major histocompatibility complex; IL, interleukin; mAb, monoclonal antibody; PBS, phosphate buffered saline; BSA, bovine serum albumin; FDC, follicular dendritic cells; IEL, intraepithelial lymphocytes; LPL, lamina propria leukocytes.
UC and Crohn’s disease. Several histopathological features highly characteristic of IBD, particularly those with germ free conditions. Developing colonic inflammation when kept under these conditions. Interestingly, IL-2 deficient mice did not develop colitis. In this respect, the disease in IL-2 deficient mice resembles human UC. Most of these animal models T cell-dependent functions are disrupted. The disease in IL-2 deficient mice was limited to the colon. In IL-2 deficient mice, T cell function is an important component for the onset of disease in these mice.

Therapeutic effects are achieved in corticosteroid-resistant UC patients by cyclosporin, a drug considered to act selectively on T helper function, suggesting that T cells also contribute to the disease process in UC in humans.

Previous reports, based on colonic biopsies, have described several histopathological features highly characteristic of IBD, particularly UC and Crohn’s disease. One of these features is the so-called basal lymphoid aggregates which represent large confluent nodular clusters of lymphocytes located between the bases of the crypts and the submucosa. These structures were suggested to be significant discriminators of UC. It seems likely that the aggregates histologically correspond to lymphoid follicular hyperplasia described originally in ulcerative proctitis and later in a variety of inflammatory colitides such as diversion colitis, ulcerative proctitis and later in a variety of inflammatory bowel diseases and the aggregates described originally in ulcerative proctitis and later in a variety of inflammatory bowel diseases.

Gut also causes inflammation of the gut. In all of these animal models T cell-dependent regulatory systems are disrupted. The disease in IL-2 deficient mice was limited to the colon and in this respect resembled human UC. Most interestingly, IL-2 deficient mice did not develop colonic inflammation when kept under germ free conditions. Thus failure to maintain homeostasis with the gut flora due to malfunctioning T cells is an important component for the onset of disease in these mice.

Table 1 gives the clinical characteristics of the UC patients. Control samples were obtained from 10 male and five female patients aged 58–81 years (median 69) with colon cancer (n=12), severe constipation (n=1), diverticulosis (n=1), and rectal prolapse (n=1). The disease in UC patients. Control samples were obtained from 10 male and five female patients aged 58–81 years (median 69) with colon cancer (n=12), severe constipation (n=1), diverticulosis (n=1), and rectal prolapse (n=1). Intestinal control samples were taken distant to any macroscopically detectable lesion. All patients received a single intravenous dose of antibiotics two hours before surgery. None of the control patients was or had been subjected to radiotherapy or chemotherapy, longstanding anti-biotic medication, or steroid treatment.

Methods

Tissue

Sigmoid colon specimens were obtained from patients undergoing bowel resection for UC (n=15; three analysed only by flow cytometry). Table 1 gives the clinical characteristics of the UC patients. Control samples were obtained from 10 male and five female patients aged 58–81 years (median 69) with colon cancer (n=12), severe constipation (n=1), diverticulosis (n=1), and rectal prolapse (n=1). Intestinal control samples were taken distant to any macroscopically detectable lesion. All patients received a single intravenous dose of antibiotics two hours before surgery. None of the control patients was or had been subjected to radiotherapy or chemotherapy, longstanding anti-biotic medication, or steroid treatment.

mAbs

Characteristics of mAbs used are listed in table 2.

Isolation of Leucocytes from Colonic Tissue

Lamina propria leucocytes (LPL) were isolated using the following procedure: firstly, intraepithelial lymphocytes (IEL) were removed according to a procedure established previously for isolation of IEL from normal intestine. LPL were obtained from IEL-depleted tissue pieces by treatment with 72.5 U/ml of collagenase type IV (Worthington, Freehold, New Jersey, USA) in neat, heat inactivated human AB serum with vigorous shaking at 37°C for 30 minutes followed by passage through a stainless steel sieve. Cells were thereafter suspended in 66.7% Percoll (Pharmacia, Uppsala, Sweden), overlaid with a gradient of 50%, 44%, and 20% Percoll and Tris buffered Hank’s balanced salt solution and centrifuged. Leucocytes were enriched in the interfaces between 66.7% and 50% Percoll (high density leucocytes) and between 50% and 44% Percoll (low density leucocytes). Contaminating epithelial cells were removed by incubation with goat antimouse IgG coupled magnetic beads (Dynabeads M-450, Dynal, Norway) charged with mAb BerEP4 followed by separation with a magnet. High density leucocytes constituted...
### Table 2  Monoclonal antibodies (mAb) used in this study

<table>
<thead>
<tr>
<th>Marker</th>
<th>mAb/clone</th>
<th>Isotype</th>
<th>Main reactivity</th>
<th>Source</th>
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<td>IgG1</td>
<td>Monomorphic determinant of the β chain of TcR-αβ</td>
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<tr>
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<td>V61-J61/J62 encoded determinant</td>
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<td>UCHT1</td>
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<td>T cells and subsets of thymocytes</td>
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<td>Non-lineage restricted antigen expressed on follicular dendritic cells</td>
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68 (10%) of all CD45+ cells in the preparations and low density leucocytes 32 (10%) (n=4). High and low density leucocytes were analysed separately by immunoflow cytometry. CD19/20/CD22+ cells and CD4 TcR-αβ+ cells constituted the majority of cells in the low density fraction while all lymphocyte subpopulations were present in the high density fraction. The composition of the total LPL was calculated on the basis of cell yields and the proportion of surface marker expressing cells in both fractions. With the exception of fig 4, all results from the immunoflow cytometry analysis are given as calculated percentage of marker positive cells in the total LPL preparation.

**IMMUNOFLOW CYTOMETRY**

Single and two colour staining of isolated cells were performed as described previously.25

**IMMUNOHISTOCHEMISTRY**

Fresh tissue samples were rinsed with cold phosphate buffered saline (PBS), snap frozen in isopentane precooled in liquid nitrogen, and stored at −70°C. Thick cryosections (5–7 µm) were stained by one of the following five immunoperoxidase methods.

- **Endogenous peroxidase activity**: Incubated with glucose oxidase Dakopatts, followed by incubation with mAb for 60 minutes at room temperature, and finally were incubated with horseradish peroxidase conjugated F(αβ), fragments of sheep antimouse Ig (Amersham, Buckinghamshire, UK) for 60 minutes at room temperature. Sections were developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% H2O2 in 0.05 M Tris HCl buffer (pH 7.6) and counterstained with Mayer’s haematoxylin or methyl green. In accordance with our previous studies, unfixed tissue was used to make visible TcR-γδ cells.25 Immunoperoxidase staining of γδ T cells was performed on unfixsed sections followed by blocking with PBS containing 0.2% BSA. Cells were then incubated with mAb, diluted in PBS containing 0.05% saponin for 60 minutes at room temperature, washed and fixed for 10 minutes in 1% paraformaldehyde at room temperature. Thereafter the sections were incubated with 0.2% bovine serum albumin (BSA) in PBS, followed by incubation with mAb for 60 minutes at room temperature, and finally were incubated with horseradish peroxidase conjugated F(αβ), fragments of sheep antimouse Ig (Amersham, Buckinghamshire, UK) for 60 minutes at room temperature. Sections were developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.03% H2O2 in 0.05 M Tris HCl buffer (pH 7.6) and counterstained with Mayer’s haematoxylin or methyl green. In accordance with our previous studies, unfixed tissue was used to make visible TcR-γδ cells.25 Immunoperoxidase staining of γδ T cells was performed on unfixsed sections followed by blocking with PBS containing 0.2% BSA. Cells were then incubated with mAb, diluted in PBS containing 0.05% saponin for 60 minutes at room temperature, washed and fixed for 10 minutes in 1% paraformaldehyde at room temperature. Thereafter the sections were blocked for endogenous peroxidase activity, incubated with antimouse Ig conjugate, and developed as described above.

For immunoperoxidase staining of immunoglobulin isotypes, sections were fixed for 30 seconds in acetone at −20°C, incubated with PBS containing 0.2% BSA and 0.0005% saponin, washed and incubated with mAb in
the presence of 0.0005% saponin for 60 minutes at room temperature. Finally, the sections were fixed for four minutes in acetone at $-20^\circ C$, blocked for endogenous peroxidase activity, incubated with antimouse Ig conjugate, and developed as described above.

Expression of bcl-2 was analysed in paraformaldehyde fixed tissue using an immunoperoxidase technique described by Pileri and colleagues. Expression of TcR-$\alpha\beta$, TcR-$\gamma\delta$, CD3, CD4, CD8, CD19/20/22, CD80, and CD45 was also analysed by immunofluorescence. Unfixed sections were blocked with PBS containing 0.2% BSA, incubated with mAb for 60 minutes at room temperature, washed, fixed for five minutes in acetone at room temperature, and incubated with fluorescein isothiocyanate conjugated F(ab')2 fragments of goat antimouse IgG and IgM (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA). Thereafter the sections were counterstained with Evan’s blue and mounted in 90% glycerol in PBS (pH 8.0) containing 1 mg/ml o-phenyldiamine as antifading agent.

The final concentration for optimal detection of markers ranged from 2.5 to 30 µg/ml for different mAbs. Sections incubated with isotype and concentration matched irrelevant mAbs served as negative controls, and human palatine tonsils were used as positive controls.

QUANTIFICATION OF THE LYMPHOID AGGREGATES

The total number of aggregates per section, total area of lamina propria, and the area occupied by the aggregates were determined in anti-CD45 stained sections using a 4× objective and the Leica Q500MC computer image analysis system.

QUANTIFICATION OF LEUCOCYTES IN SITU

Morphometry analysis of cells in lymphoid aggregates, solitary follicles, lamina propria outside aggregates/follicles, and the submucosa was performed according to Weibel and colleagues. A square lattice grid with 121 coarse points was superimposed on the sections using a 40× objective for immunoperoxidase and a 50× objective for immunofluorescence stained sections. Positive cells located in the coarse points were counted and expressed as a percentage of the total number of coarse points. Coarse points in empty spaces or outside the compartment under investigation were excluded. Eight to 15 ocular fields were counted for each marker and sample. In the case of the lower number of ocular fields it corresponded to all aggregates in the section. Immunoperoxidase staining was used for all markers except TcR-$\gamma\delta$. Immunofluorescence gave optimal detection of cells stained by the pan-$\alpha$-chain mAb TCR$\alpha$1 and was therefore used in morphometric analyses of TcR-$\gamma\delta$+ cells.

![Figure 1](http://gut.bmj.com/)
The area of individual aggregates was determined by morphometry in anti-CD45 stained sections. Number of aggregates was counted in an anti-CD45 stained section and the total area of lamina propria occupied by aggregates.

Severely diseased tissue: samples had flattened epithelium with low numbers of goblet cells, branched crypts and occasional disruptions. Lamina propria contained numerous scattered leucocytes. Crypt abscesses were common. The lamina propria contained numerous scattered leucocytes as well as basal lymphoid aggregates (fig 1A). Three UC specimens were classified as moderately diseased. In all the epithelium was intact with slightly reduced numbers of goblet cells and no/few branched crypts. The lamina propria contained increased numbers of leucocytes and lymphoid aggregates were present. The morphometric analysis was focused on the aggregates.

The aggregates comprised nodular merging clusters of lymphocytes without typical reactive centres. They were located between the bases of the crypts and the submucosa without apparent contact with the luminal epithelium (fig 1A). However, close proximity between cells in the aggregates and crypt epithelium was often noted (fig 1A, D). The frequency of aggregates (number/area) was similar in moderately and severely diseased tissue, ranging from 1.3 to 4.9 aggregates per mm² of lamina propria (fig 3). However, the area of the lamina propria occupied by the aggregates increased with the severity of the disease and constituted as much as 45% of the lamina propria in severely diseased colon from some patients (table 3). Normal solitary follicles in control colon were infrequent (less than 0.1 follicle/mm² of lamina propria, n=8).

Approximately 75% of cells in the aggregates were leucocytes (74 (7)% CD45+ cells (n = 9)). The proportion of leucocytes in solitary lymphoid follicles was also high (67 (6)% CD45+ cells (n=8)). Both values may be underestimated as staining with anti-CD45 mAb was heterogeneous. This heterogeneous staining may be explained, at least in part, by decreased expression of CD45 on activated B cells.

The number of leucocytes both in the lamina propria outside the aggregates (26 (7)% CD45+ cells (n=4)) and in the submucosa (8 (2)% CD45+ cells (n=4)) was increased in UC colon compared with normal colon (15 (2)% CD45+ cells in the lamina propria outside the follicles and 4 (2)% CD45+ cells in the submucosa (n=4)).

Intraepithelial leucocytes were present in lower frequencies in UC colon compared with normal colon: 28 (7) CD45+ cells/1000 epithelial cells in severely diseased colonic tissue (n=4) compared with 66 (26) CD45+ cells/1000 epithelial cells in controls (n=4, p=0.03). IEL were mainly detected within the cryptal epithelium in UC colon, partly due to erosions of the luminal surface epithelium. In normal colon, >60% of IEL were located in the luminal epithelium. Thus as the density of IEL is lower in crypt than in luminal epithelium in normal colon the lower frequency of IEL in UC colon may be explained by the fact that UC colon contains mainly cryptal epithelium.

Table 3  Frequency and area of basal lymphoid aggregates in the colonic mucosa of ulcerative colitis patients

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Disease classification#</th>
<th>Frequency of aggregates (N/mm²) *</th>
<th>Mean area of aggregates (mm²)**</th>
<th>% of lamina propria occupied by aggregates†</th>
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<tbody>
<tr>
<td>UC50</td>
<td>Severe</td>
<td>1.4</td>
<td>0.07</td>
<td>17</td>
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<td>UC68</td>
<td>Severe</td>
<td>4.9</td>
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<td>Severe</td>
<td>1.8</td>
<td>0.08</td>
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<td>1.5</td>
<td>0.14</td>
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<td>0.14</td>
<td>45</td>
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<td>Severe</td>
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<td>0.28</td>
<td>43</td>
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<tr>
<td>Mean (SD)</td>
<td>Moderate</td>
<td>2.4 (1.3)</td>
<td>0.12 (0.09)</td>
<td>29 (13)</td>
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<td>Moderate</td>
<td>1.3</td>
<td>0.13</td>
<td>17</td>
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<td>0.04</td>
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<tr>
<td>Mean (SD)</td>
<td>Severe</td>
<td>2.3 (1.3)</td>
<td>0.07 (0.05)</td>
<td>15 (7)</td>
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</table>

*Number of aggregates was counted in an anti-CD45 stained section and the total area of lamina propria in the section was determined by morphometry.
†The area of individual aggregates was determined by morphometry in anti-CD45 stained sections.
‡Total area occupied by aggregates/total area of lamina propria=x100.

Immuno-electron microscopy of TCR-a⁻β⁻ cells Colon mucosa was fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for four hours on ice. The fixed specimens were washed in the same buffer containing 3.5% sucrose and 0.05% saponin at 4°C overnight, frozen, cut into 15 µm thick cryosections, and collected on poly-L-lysine coated Thermaxon coverslips (NUNC, Rosklide, Denmark). Thereafter the sections were incubated with a mixture of mAbs TCRαβ, Vα1, and Vα51 diluted in PBS containing 0.05% saponin for 12 hours at 4°C, washed, and subsequently incubated with biotinylated F(ab)², fragments of sheep antiserum Ig (Amersham) for five hours at room temperature. Endogenous peroxidase activity was blocked by incubation in 0.02 M PBS containing 2 mM NaN₃ and 0.03% H₂O₂ for one hour at 37°C. The sections were then fixed with 1.33% OsO₄ (Sigma) for one hour, dehydrated in acetone, and flat embedded in a mixture of Epon and Araldite (Fluka, Buchs, Switzerland). Ultrathin sections were examined under a Zeiss EM 900 electron microscope. Sections incubated with irrelevant mouse IgG served as specificity controls. Three UC colon samples and two normal colon samples were analysed.

Statistical analysis Values of marker positive cells are expressed as mean (SD). Statistical analyses of differences between UC and normal colon were performed using a two tailed Student’s t test assuming unequal variance in the groups. A p value <0.05 was regarded as statistically significant.

Results LEUCOCYTES CONSTITUTE THE MAJOR CELL TYPE IN INFLAMED UC COLON AND DISTRICT DIFFERENTIALLY IN THE COLONIC MUCOSA OF UC PATIENTS COMPARED WITH CONTROLS. Twelve samples of inflamed sigmoid colon from UC patients and 15 samples of apparently normal sigmoid colon from patients with no history of IBD were subjected to phenotypic analysis by immunohistochemistry. Nine of these UC samples were classified as severely diseased. In these the epithelium was flattened with reduced numbers of goblet cells, branched crypts, and occasional ulcerations. Crypt abscesses were common. The lamina propria contained numerous scattered leucocytes as well as basal lymphoid aggregates (fig 1A).
AGGREGATES IN THE COLONIC TISSUE OF UC PATIENTS ALMOST EXCLUSIVELY COMPRISÉ T AND B LYMPHOCYTES

Aggregates were analysed for the presence of the three major types of lymphocytes: T cells (anti-CD3 mAb), B cells (a mixture of anti-CD19, anti-CD20, and anti-CD22 mAbs), and NK cells. For analysis of NK cells, anti-CD57 mAb was chosen as we have previously shown that @6 and @6 T cells expressing the classical NK cell marker CD56 are present in human gut.21 Anti-CD15 was used to detect granulocytes, anti-CD14 for blood monocytes/recently recruited macrophages, and anti-CD68 for tissue macrophages. To detect follicular dendritic cells (FDC), three different mAbs were used (table 2). Anti-CD80/B7.1 was used to detect cells with antigen presenting function and anti-CD1a was used as an additional marker for dendritic/Langerhans cells. The results of the immunomorphometric analysis are summarised in fig 3. The two major cell types were T and B cells and the sum of CD3+ cells and CD19/20/22+ cells equalled the number of CD45+ cells in most samples (77 (12)% compared with 74 (7)% CD45+ cells (n=9)). T and B cells constituted equally large proportions of the cells in the aggregates. The aggregates contained large T cell areas with no B cells and reciprocal areas dominated by B cells with only a few scattered T cells (fig 2A, B). Most aggregates consisted of densely packed cells but in some instances limited areas of more loosely packed cells were detected in the B cell zone. The proportion of B cells was significantly higher in aggregates of severely diseased UC colon compared with moderately diseased tissue (43 (8) ± 30 (6); p=0.03), suggesting increased importance of B cells in severe forms of the disease. Solitary follicles in normal colon had a slightly different appearance. The centre of the follicle contained loosely packed large B cells with few if any T cells, surrounded by a zone of both B and T cells and areas in the periphery which contained T cells only (fig 2D, E).

Follicular dendritic cells were detected in seven of nine UC samples using three different anti-FDC mAbs. No difference in staining patterns was seen between the three mAbs. The positively stained follicular dendritic cells formed a network that was located in the B cell area of the aggregates (fig 2B, C). Although every aggregate contained at least one B cell area, only about 40% contained an FDC network. FDC networks were never seen in T cell areas. In normal colon every follicle contained an FDC network which was confined to the B cell area (fig 2E, F). CD80/B7.1+ dendritic cells were also present in the aggregates of UC colon. They were most commonly seen as single dendritic cells surrounded by small CD80+ dots between lymphocytes, presumably cross sections of dendritic protrusions. Occasionally a complete network of CD80+ cells could be seen (fig 1E). This network was located in a T cell area. No CD1a+ cells with a dendritic morphology were detected in the aggregates.

Although the proportion of granulocytes, CD15+ cells, was elevated in most UC samples, such cells were generally located outside the aggregates (fig 3). Numerous tissue macrophages, CD68+ cells, were present in the lamina propria outside the aggregates. In the aggregates they were scarce and located in the outer rim (fig 3). In normal colon, most CD68+ cells were localised in the proximity of the
Characterisation of mucosal lymphoid aggregates in UC

A surprisingly large number of the cells in the aggregates were \( \gamma \delta \) T cells (11 (7%) \( \text{TcR-} \gamma \delta ^* \) cells (n=9)) (fig 1B, 3). In contrast, almost no \( \gamma \delta \) T cells were detected in follicles of normal colon (0.3 (0.3)% \( \text{TcR-} \gamma \delta ^* \) cells (n=8) (p<0.001)) (fig 3). The number of \( \text{TcR-} \gamma \delta ^* \) cells in the aggregates increased with the severity of the disease. The ratio of \( \text{TcR-} \alpha \beta ^* \) cells to \( \text{TcR-} \gamma \delta ^* \) cells in the aggregates was 2.4 (0.9) in severely diseased UC samples (n=6) but varied from 1.3 to 42 in moderately diseased samples (n=3) and was >300 in normal colon follicles. In five samples the frequencies of \( \gamma \delta \) T cells both in aggregates and in lamina propria outside the aggregates were determined. In the aggregates, the frequency of \( \gamma \delta \) T cells was 3.9 (2.5) times higher than outside the aggregates. \( \text{TcR-} \gamma \delta ^* \) cells in the aggregates mainly expressed \( \text{V}61 \) while most cells using \( \text{V}52 \) were found outside the aggregates. The total LPL fraction was analysed for \( \text{V}6 \) gene usage by immunoflow cytometry on isolated cells. In agreement with the immunohistochemical results, 86 (10)% of the \( \text{TcR-} \gamma \delta ^* \) cells expressed \( \text{V}61 \) while the remaining cells expressed \( \text{V}62 \) (table 4). In line with the ultrastructural analysis of \( \gamma \delta \) T cells (see below), staining of \( \text{TcR-} \gamma \delta ^* \) cells often showed a patchy or spotted appearance on immunohistochemistry (fig 1B, inset) and displayed a relatively low fluorescence intensity in immunoflow cytometry (fig 4). Because of the heterogeneous staining pattern for \( \text{TcR-} \gamma \delta ^* \) cells the number of \( \gamma \delta \) T cells may be an underestimate.

Two colour immunoflow cytometry analysis of isolated LPL showed that almost all \( \gamma \delta \) T cells expressed \( \text{CD45R0} \) up to 15% of the \( \gamma \delta \) T cells expressed \( \text{CD8} \) but most \( \gamma \delta \) T cells were \( \text{CD4/CD8} \) double negative.

\[ \text{CD4/CD8}^{-} \alpha \beta ^* \text{T cells constitute the major T cell subtype in the aggregates} \]

The majority of T cells in the aggregates expressed \( \text{TcR-} \alpha \beta \) and the proportion of \( \text{CD4}^+ \) cells was higher than the proportion of \( \text{CD8}^+ \) cells (fig 3) with an average \( \text{CD4/CD8} \) ratio of 1.7 (0.7) (n=8). The dominance of \( \text{CD4}^+ \) cells was even more pronounced when isolated LPL were analysed (fig 4). Two colour immunoflow cytometry analysis of LPL showed that almost all \( \text{CD4}^+ \) cells expressed \( \text{TCR-} \alpha ^* \beta \) (data not shown). \( \text{CD8}^+ \) cells were found only in the T cell area of the aggregates while \( \text{CD4}^+ \) cells were present both in the T cell area and scattered in the B cell area. In several samples the sum of \( \text{CD4}^+ \) and \( \text{CD8}^+ \) cells exceeded the number of \( \text{CD3}^+ \) cells and/or \( \text{TCR}^+ \) cells. This probably reflects underestimation of T cells because of the low level expression of the CD3/TCR complex rather than the presence of \( \text{CD4/CD8} \) double positive cells as only 2.4

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**Table 4**: \( \text{V}6 \) gene usage of \( \text{TcR-} \gamma \delta ^* \) cells in lamina propria leucocytes isolated from the colon of ulcerative colitis (UC) patients, as determined by immunoflow cytometry.

<table>
<thead>
<tr>
<th>Spec$c$ity of mAb</th>
<th>UC83</th>
<th>UC86</th>
<th>UC94</th>
<th>UC105</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{V61-J01/J02}^* )</td>
<td>71*</td>
<td>92</td>
<td>93</td>
<td>87</td>
</tr>
<tr>
<td>( \text{V62} )</td>
<td>27</td>
<td>8</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>( \text{V63} )</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

*Details for individual patients are given in table 1.

Isolated lamina propria leucocytes were analysed by indirect immunoflow cytometry using a monoclonal antibody (mAb) with the indicated specificity. For details of mAbs see table 2.

*Per cent cells stained by mAb with the indicated \( \text{V}6 \) gene specificity of all \( \text{TcR-} \gamma \delta ^* \) cells in the cell preparation.

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Figure 3: Immunomorphometric analysis of basal lymphoid aggregates in ulcerative colitis (UC) colon and solitary follicles in control colon. Bars represent mean (SD) per cent positive cells of all cells in the aggregate/follicle, as determined by morphometric counting of immunohistochemically stained cryosections. Nine UC colon samples (six severe, three moderate) were counted. Solitary follicles in 6–8 control colon samples were analysed for all markers except CD68, in which case n=2. An indirect immunoperoxidase technique was used for staining with anti-CD3, anti-TCR-\( \alpha \beta \), anti-CD4, anti-CD8, anti-CD19/20/22, anti-CD57, anti-CD15, anti-CD14, and anti-CD68 monoclonal antibodies (mAb). Indirect immunofluorescence was used for staining with anti-TCR-\( \gamma \delta \) mAb; **p<0.001, aggregates in UC compared with solitary follicles in control colon.

Luminal epithelium. Aggregates with significant numbers of CD14+ cells were seen in only two samples. These samples also had a high frequency of CD14+ cells in the submucosa (that is, 3.5% CD14+ cells compared with 1% CD14+ cells in other UC colon samples and control colon). Furthermore, CD14+ cells were concentrated near blood vessels, suggesting ongoing invasion of monocytes. There was no obvious clinical parameter that could explain why monocyte invasion had occurred, particularly in these two samples. NK cells, CD57+ cells, were rare both in UC and normal colon tissue and were only occasionally detected in aggregates (fig 3).

The lymphoid aggregates in UC colon did not differ significantly from solitary follicles in normal colon with respect to numbers of T cells, B cells, macrophages/monocytes, granulocytes, and NK cells (fig 3). Moreover, an FDC network located in the B cell zone was seen in both types of structures. However, the aggregates lacked a typical germinal centre-like B cell zone with loosely packed B cells which is seen in normal solitary follicles. Moreover, the aggregates in UC colon occupied up to 45% of the lamina propria.
determine the position of quadrant regions. Irrelevant fluorochrome conjugated mAbs served as negative controls and were used to mAb (MT310) and phycoerythrin labelled anti-CD8 mAb (DK25). Cells incubated with cells were determined by two colour immunoflow cytometry using FITC labelled anti-CD4 mAb (L28), and anti-CD45R0 mAb (UCHL-1), respectively. CD4 and CD8 positive assayed using PerCP labelled anti-CD3 mAb (SK7) and FITC labelled anti-CD45RA (TCS1) for indirect immunofluorescence. The shaded histograms superimposed on the graphs show the negative controls. Expression of CD45R0 and CD45RA by T cells was assayed using PerCP labelled anti-CD3 mAb (SK7) and FITC labelled anti-CD45RA mAb (L28), and anti-CD45R0 mAb (UCHL-1), respectively. CD4 and CD8 positive cells were determined by two colour immunoflow cytometry using FITC labelled anti-CD4 mAb (MT310) and phycoerythrin labelled anti-CD8 mAb (DK25). Cells incubated with irrelevant fluorochrome conjugated mAbs served as negative controls and were used to determine the position of quadrant regions.

Figure 4  Flow cytometry analysis of high density lamina propria leucocytes isolated from one ulcerative colitis (UC) colon sample. Cells were stained with phycoerythrin labelled anti-pan-TcR-β [1 mAb (BMA031) and with anti-Vα7,8 mAb (STCS1) for indirect immunofluorescence. The shaded histograms superimposed on the graphs show the negative controls. Expression of CD45R0 and CD45RA by T cells was assayed using PerCP labelled anti-CD3 mAb (SK7) and FITC labelled anti-CD45RA mAb (L28), and anti-CD45R0 mAb (UCHL-1), respectively. CD4 and CD8 positive cells were determined by two colour immunoflow cytometry using FITC labelled anti-CD4 mAb (MT310) and phycoerythrin labelled anti-CD8 mAb (DK25). Cells incubated with irrelevant fluorochrome conjugated mAbs served as negative controls and were used to determine the position of quadrant regions.

Table 5  Frequency of subtype non-specific markers in basal lymphoid aggregates of ulcerative colitis colon

<table>
<thead>
<tr>
<th>Marker</th>
<th>Positive cells (%)</th>
<th>n′</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45R0</td>
<td>52 (11)</td>
<td>4</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>46 (20)</td>
<td>8</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>58 (6)</td>
<td>3</td>
</tr>
<tr>
<td>uβ3 (CD103)</td>
<td>29 (13)</td>
<td>8</td>
</tr>
<tr>
<td>CD28</td>
<td>0.1 (0.1)</td>
<td>7</td>
</tr>
<tr>
<td>bcl-2</td>
<td>28 (2)</td>
<td>6</td>
</tr>
</tbody>
</table>

The monoclonal antibodies used in the study are shown in table 2. Lymphocytes present in basal lymphoid aggregates were analysed by immunoperoxidase staining and the percentage of positive cells was determined by morphometric analysis according to Weibel and colleagues. a

n, number of samples.

There were no significant differences in the frequencies and phenotypes of αβ T cell subsets between aggregates in UC colon and solitary follicles in control colon (fig 3).

**Most B cells in the aggregates express IgM on their surface**

The majority of cells in the B cell area of aggregates showed staining by anti-IgM mAb (42 (5)% IgM+ cells (n=4)) corresponding to 95 (7)% of the CD19/20/22+ cells. However, no less than 19 (2)% of the cells in the aggregates expressed IgA and a small fraction of IgG+ cells (3.3 (0.4)% (n=4)) were also detected. These data suggest that B cells in the aggregates express more than one immunoglobulin isotype and may recently have undergone class switching. No Ig+ cells showed cytoplasmic staining, indicating that plasma cells were not present in the aggregates. In contrast, plasma cells were found outside the aggregates.

IgM+ cells (28 (2)% (n=4)) outnumbered IgA+ cells (14 (1)% (n=4)) and IgG+ cells (2.2 (0.4)% (n=4)) in the follicles of control colon. However, the sum of Ig+ cells equalled the number of CD19/20/22+ cells in individual samples. More interestingly, although there was no significant difference in the number of B cells in follicles compared with aggregates (fig 3), the proportion of surface IgM+ cells, surface IgA+ cells, and surface IgG+ cells was significantly higher in aggregates of UC colon than in follicles of control colon (p<0.01 for all three isotypes).

**Expression of subtype non-restricted markers in lymphoid aggregates of UC colon**

Approximately 50% of cells in the lymphoid aggregates expressed the memory/activation marker CD45R0 (table 5). Moreover, analysis of isolated LPL showed that CD45R0 and CD45RA expressing cells each constituted approximately 50% of the population (n=4). The majority of CD3+ cells expressed CD45R0 but a significant fraction of CD45R0 expressing CD3+ cells were also present (fig 4). The majority of CD45R0 expressing cells were T cells and 20–40% of the CD45R0+ cells were B cells. In two samples the sum of CD45R0 and CD45RA expressing cells exceeded 100% suggesting that cells expressing both splice variants can be present simultaneously.

The majority of MHC class II expressing cells were located in the B cell area but scattered MHC class II positive cells were seen in the T cell area (data not shown). The proportion of cells expressing HLA-DR was approximately 50% (table 5). In five samples the number of HLA-DR+ cells exceeded the number of B cells, indicating that some T cells in the aggregates (31–89% of the CD3+ cells in these individual samples) also express HLA-DR. Similar results were obtained with three samples analysed for HLA-DQ expression (table 5).

Interestingly, the integrin αββ, which is present on a large number of IEL in normal gut, was expressed on 26 (13)% of cells in the aggregates (fig 1C, table 5). Positive cells

(1.3)% (n=4) of the total LPL were double positive in immunoflow cytometry analysis (fig 4). Although numerous cells expressing the T cell co-receptor CD28 were scattered in lamina propria outside the aggregates (13 (2)% CD28+ cells (n=3)) no CD28+ cells were detected in the aggregates (table 5; fig 1D).

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showed a heterogeneous distribution, with some areas with many positive cells and other areas with few positive cells. The $u_0$ expressing cells showed variation in the intensity of staining (fig 1C).

One third of lymphocytes in the aggregates expressed the antiapoptotic protein bcl-2 (fig 1F, table 5). Bcl-2 expressing cells were scattered over the entire aggregate. In normal solitary follicles about 10% of bcl-2 positive cells were seen. They were all located in the B cell zone.

ULTRASTRUCTURAL ANALYSIS OF γδ T CELLS IN UC COLON SHOW INTERNALISATION AND SURFACE DOWNREGULATION OF TcR-γδ

The immunoelectron microscopy investigation was focused on γδ T cells in UC colon. For comparative purposes we also analysed γδ T cells in normal colonic mucosa. TcR-γδ was detected as deposits of the electron dense reaction product.

In normal colon, the reaction product was homogeneously distributed on the cell surface of all γδ T cells found, both on those located in the lamina propria (fig 5A) and on the intraepithelial γδ T cells (fig 5B).

In contrast, γδ T cells in UC colon exhibited extreme variability in distribution of the reaction product from cell surface to cytoplasm. We identified five types of staining patterns (five morphotypes). γδ T cells of the first type (fig 6A) showed a heterogeneous distribution of the reaction product on the cell surface. Some exhibited single positively stained multivesicular bodies. In γδ T cells of the second type, the reaction product was seen as scarce linear clusters more or less randomly distributed over the cell surface (fig 6B). In addition, some clustered deposits were localised over and within surface invaginations that varied from shallow pits to deeper flask-shaped invaginations (fig 6B, inset). It was not possible to use additional counterstaining. Thus although some of the surface invaginations resembled coated pits, we could not determine whether or not these invaginations were coated. In γδ T cells of the third type the reaction product was located as cell surface clusters, and also in cytoplasmic vacuoles with morphological characteristics of endosomes (fig 6C). In γδ T cells of the fourth type the reaction product was abundantly present in the cytoplasm but barely detectable on the cell surface (fig 6D, E, F). The reaction product stained numerous cytoplasmic vesicles and vacuoles near the cell membrane and also deep in the cells close to the nucleus. Positively stained vesicles were sometimes seen in continuity with the plasma membrane and resembled coated vesicles (fig 6E). Numerous multivesicular bodies consisting of tightly packed microvesicles were also stained (fig 6F). γδ T cells of the fifth type were rare (fig 6G, H). This morphotype usually showed strong positive staining of the plasma membrane and perinuclear space. Sometimes the reaction product stained diverse cytoplasmic vacuoles of different sizes and shapes. No obvious difference between individual UC colon samples studied was seen.

In summary, the majority of γδ T cells (morphotypes 1–4) in UC colon exhibited cellular localisation of the reaction product most probably reflecting the different consecutive steps of TcR internalisation: forming of clusters, coated pits, coated vesicles, endosomes, and multivesicular bodies. A small number of cells (morphotype 5) showed active synthesis of TcR-γδ molecules.

Discussion

Our results showed that the lamina propria in UC colon tissue was characterised by a 10–50-fold increase in leucocytes compared with normal colon. More than 85% of leucocytes were lymphocytes and the majority of these lymphocytes were located in the basal lymphoid aggregates consisting of hundreds of densely packed T and B cells. In previous studies of UC colon, surprisingly little attention has been paid to lymphocyte phenotypes in these prominent aggregates. Two intriguing T cell subsets were present in the aggregates: (i) activated γδ T cells using Vδ1 and (ii) activated CD4+ αβ T cells lacking the co-stimulatory receptor CD28.

Figure 5  Immunoelectron micrographs of γδ T lymphocytes in normal colon. (A) A characteristic lamina propria γδ T cell showing homogeneous surface staining (arrows). (B) An intraepithelial γδ T cell showing diffuse surface staining (arrows). EC, epithelial cell. All ultrathin sections were examined without additional staining. Original magnification: A ×12 000; B ×11 500.
ORIGIN OF LYMPHOCYTES IN THE AGGREGATES OF UC COLON

Approximately 25% of lymphocytes in the aggregates expressed the integrin/mucosa lymphocyte marker $\alpha_E\beta_7$, suggesting a mucosal origin of a substantial fraction of the T cells in the aggregates. Furthermore, the majority of $\gamma\delta$ T cells in the aggregates used V$\delta_1$, a feature typical of intraepithelial $\gamma\delta$ T cells in normal...

Figure 6  Immunoelectron micrographs of lamina propria (A–G) and intraepithelial (H) $\gamma\delta$ T cells in ulcerative colitis colon. (A) Low power micrograph of a $\gamma\delta$ T lymphocyte with numerous surface processes which are distinctly stained by the reaction product and concentrated at one pole of the cell (arrows). The rest of the cell surface is weakly stained. Inset: High magnification of the positively stained multivesicular body indicated by the arrowhead. (B) A $\gamma\delta$ T cell showing the surface depositions of the reaction product which have the appearance of scarce clusters (arrows). One of the clusters is located over the surface flask-shaped invagination (arrowhead and in the inset at higher magnification). (C) A $\gamma\delta$ T cell displaying the clustered reaction product on the cell surface (arrows) and numerous positively stained cytoplasmic vacuoles near the cell membrane (arrowheads). (D) A $\gamma\delta$ T cell showing only numerous cytoplasmic vesicles and vacuoles of diverse sizes near the cell membrane and deep in the cell. The lumen of these structures exhibit variable intense staining by the reaction product (arrowheads). (E) A portion of the $\gamma\delta$ T cell cytoplasm showing cytoplasmic vesicles that are connected with the plasma membrane and contain the reaction product (arrowheads). In addition, the cytoplasm contains numerous positively stained vacuoles (arrowheads). (F) A portion of the $\gamma\delta$ T cell cytoplasm showing numerous multivesicular bodies mainly consisting of tightly packed positively stained microvesicles (arrowheads). Arrows depict a small cluster of the reaction product on the cell surface. (G) A $\gamma\delta$ T lymphocyte showing a strong positive staining of the cell surface (arrows) and the perinuclear space (large arrowheads). Small arrowheads indicate the positive staining of single cytoplasmic vacuoles. (H) A $\gamma\delta$ T lymphocyte showing the positive staining of the cell surface (arrows) and perinuclear space (small arrowheads). Large arrowheads indicate the positively stained cisternal structure in the cytoplasm. EC, epithelial cell. All ultrathin sections were examined without additional staining. Original magnification: A $\times 8600$, inset $\times 25 000$; B $\times 9000$, inset $\times 20 000$; C $\times 8000$; D $\times 9500$; E $\times 29 500$; F $\times 31 000$; G $\times 10 000$; H $\times 12 500$. 

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Characterisation of mucosal lymphoid aggregates in UC

Characterisation of mucosal lymphoid aggregates in UC that lack CD28. Thus it is possible that CD28− from intestinal IEL. It is also likely that CD28− models.15 17 39–42 subpopulations present (that is, CD4 + aggregates. This implies that none of the T cell lymphocytes in the aggregates. 

Firstly, almost all γδ T cells and the majority of αβ T cells have features suggestive of ligand induced TcR-γδ downregulation and endocytosis of the CD3/TcR complex, phenomena considered essential in T cell activation.35 Thirdly, approximately one third of lymphocytes had a low density suggesting that they were blasts.36 The lymphoblast fraction comprised B cells and CD4+ αβ T cells, indicating ongoing T cell dependent B cell activation. The low density lymphocyte fraction is lost in standard procedures for isolation of human intestinal lymphocytes and was therefore not reported in previous studies on isolated intestinal lymphocytes in IBD. Finally, a significant fraction of T cells seemed to express HLA-DR, suggesting activation induced MHC class II expression.

WHAT IS REFLECTED BY DOWNREGULATION OF TcR AT THE SURFACE OF γδ T CELLS IN UC COLON?

We found that TcR-γδ is actively internalised by γδ T cells in UC colon and that this internalisation involves a mechanism termed receptor mediated endocytosis.29 We also showed that internalisation induced loss of the cell surface TcR-γδ molecules and accumulation of TcR-γδ in the cytoplasmic compartment (that is, downregulation of surface TcR-γδ).

Previous ex vivo experiments demonstrated that TcR α and/or β chains are continuously internalised and recycled back to the cell surface. However, T cell stimulation by peptide antigens, superantigens, or anti-TcR antibodies leads to rapid downregulation of surface TcR. TcR downregulation is caused, at least in part, by increased receptor internalisation via the endocytotic pathway, followed by degradation in lysosomes.47 48 To our knowledge, surface downregulation of TcR-γδ caused by its internalisation has not previously been documented in vivo.

Whether TcR internalisation and concomitant surface TcR downregulation per se are required for T cell activation remains to be elucidated. Recent reports provided impressive evidence that T cell activation is correlated with the degree of TcR downregulation and that rapid internalisation of the TcR after contact with antigen is a device to remove triggered TcR molecules from the cell surface.49–52 Viola and Lanzavecchia49 demonstrated that T cells “count” the number of triggered TcR and commit themselves to activation only when this number reaches an appropriate threshold. Naturally, the capacity to reach the activation threshold becomes a problem for a cell that has only a few TcR on the surface. Another possibility is that TcR downregulation does not necessarily lead to T cell activation but is a key mechanism involved in tuning T cell function for extinction and calibration of TcR signalling.45 53 As TcR downregulation is most prominent with high affinity agonistic ligands, internalisation of TcR could be a protective measure to control the extent of T cell activation.40

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The biological consequences of surface TCR-γδ downregulation may be both physiological and pathological. On the one hand, γδ T cells with selective downregulation of surface TCR have been described in human pregnancy decidua. In this case, downregulation of TCR-γδ may contribute towards creation of transient unresponsiveness to paternal antigens and to a successful outcome of pregnancy. Interestingly, decidual γδ T cells did not show active internalisation of TCR indicating that internalisation is not the only mechanism of TCR downregulation.

On the other hand, recent experiments have shown that altered peptides (partial agonistic/antagonistic) also trigger TCR internalisation, and alter differentiation and effector functions of the responding T lymphocytes. Thus it is possible that in UC colon a few, possibly altered, peptides permanently engage and downregulate the number of TCR-γδ, dramatically reducing the number of TCR that are available for physiological agonistic ligands. This could result in alterations of the immunoregulatory functions of γδ T cells and, consequently, an abnormal mucosal immune response to enteric antigens.

POSSIBLE ROLES FOR γδ T CELLS IN UC COLON

In contrast with the normal colon, numerous γδ T cells were present mainly in the lamina propria in the lymphoid aggregates but also as scattered single cells in the lamina propria outside the aggregates and in the submucosa. γδ T cells in UC colon have been observed previously but that study focused on scattered γδ T cells with strong surface staining (morphotype 5) which constitute only a small cell population. Increased numbers of γδ T cells in peripheral blood from IBD patients has also been reported.

Previous studies have suggested a role for Vδ1 cells in the pathogenesis of inflammatory diseases. Vδ1 cells were reported to be present in the intestinal mucosa of IBD patients, the synovial tissue of patients with rheumatoid arthritis, and the epithelium of chronically inflamed parodontitis gingiva. The number of intraepithelial Vδ1 cells is elevated in active coeliac disease. Similarly, Vδ1 cells agglomerate in skin lesions of patients with leprosy and leishmaniasis. Vδ1 cells can be cytotoxic against tumour cells of epithelial origin, and can recognise stressed molecules. Aberrant immune responses causing formation of aggregates with stressed B and γδ T cells could be responsible for the observed increase in numbers of Vδ1 cells in UC colon.

Stressed malfunctioning γδ T cells are likely to develop during chronic inflammation. In this case they may be replaced by γδ T cells. γδ T cells can act as T helper cells to support antibody production and in the formation of germinal centres in γδ T cell deficient mice. However, these γδ T cell deficient mice have high levels of autoantibodies. Mice with Listeria monocytogenes infected kidneys develop an inflammatory disease with autoimmune traits. In these mice γδ T cells are recruited to the site of inflammation subsequent to γδ T cells. It is possible, therefore, that γδ T cells target the aggregates of UC colon as a response to the presence of stressed lymphocytes initially activated by infection in the colonic mucosa. Once in the aggregate, the γδ T cells may influence the development of B cells and in this way be responsible for the increased production of autoantibodies noted in UC colon.

This work was supported by grants from the Swedish Natural Science Research Council (grant No B-94-BU 11234–300, M-LH), the Swedish Medical Research Council (grant No 1994/4-11240, AD), K200-71, SH), the Swedish Cancer Foundation (SH) the County of Vasterbotten (SH and AD), Bengt Ihreís Foundation (AD), and the Swedish Labour Market Insurance Company, APA (SH). The skilful technical assistance of Elisabeth Granström and Marianne Sjöstedt is gratefully acknowledged.

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Characterisation of mucosal lymphoid aggregates in UC


