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Flow cytometric analysis of peripheral blood lymphocytes in ulcerative colitis and Crohn's disease

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Abstract

Using two colour immunofluorescence with fluorescein isothiocyanate and phycoerythrin labelled monoclonal antibodies, multiparameter flow cytometry was used to examine the antigenic characteristics of peripheral blood lymphocytes in whole blood of patients with ulcerative colitis and Crohn's disease who were not taking immunosuppressive drugs. The numbers of CD4+ and CD8+ lymphocytes in patients with ulcerative colitis and Crohn's disease remained unchanged so that the CD4/CD8 ratio was the same as that of normal control subjects. In Crohn's disease there were many activated T cells (CD3+, CD25+). Although natural killer cells in active Crohn's disease were lower than in normal control subjects, cytotoxic T lymphocytes, as defined by CD3+, CD16+, did not differ in patients with inflammatory bowel disease compared with normal control subjects. For B cell subsets, there were differences in Leu-1+ B cells, Leu-8+ B cells, FcεR+B cells (Leu-16+, Leu-20+), and activated B cells (Leu-12+, Leu-21+) between patients with inflammatory bowel disease and normal control subjects. These differences are compatible with local activation of B cells in the inflamed colon.

Although the aetiology and pathogenesis of inflammatory bowel disease are still unknown, immunological factors seem to be involved.[1] Inflammatory bowel disease shows a variety of prominent immunological abnormalities.

The development of monoclonal antibodies to cell surface antigens has allowed detail characterisation of subpopulations of lymphoid cells. Surface marker identification can be performed by immunofluorescence and immunoperoxidase techniques on tissue sections or on isolated lymphoid cells. Flow cytometry utilises rapid automated counting of large numbers of cells, which permits multiple surface markers to be analysed. Flow cytometry can also provide a quantitative measurement of fluorescence intensity which may be related to receptor density.[2] Monoclonal antibodies and immunofluorescence analysis by flow cytometry provide a highly sensitive and specific approach to phenotypic analysis of human lymphocyte subsets. The use of simultaneous two colour immunofluorescence that utilises two different antibodies, one labelled with fluorescein isothiocyanate, the other with phycoerythrin, has added a new dimension to this type of analysis. Furthermore, the use of a whole blood assay avoids the use of density gradient centrifugation, which might potentially alter the ratios of lymphocyte subsets.

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The aim of the present study was to investigate lymphocyte subpopulations in whole blood of patients with ulcerative colitis and Crohn's disease, who were not taking immunosuppressive treatment. Two colour immunofluorescence staining and multiparameter flow cytometry were used.

Methods

PATIENT POPULATION

Peripheral blood was obtained from 60 patients with ulcerative colitis and 31 patients with Crohn's disease. The diagnosis was based on clinical, endoscopic, histological, and radiological features of the disease. The mean age of the patients with ulcerative colitis (30 men, 30 women) was 47 years (range 17–85 years). Disease activity of ulcerative colitis was assessed by the method of Truelove and Witts.[3] The colitis was active in 41 patients and inactive in 19. The mean age of the 31 patients with Crohn's disease (12 men, 19 women) was 48 years (range 22–81 years). The criteria of de Dombal *et al* were used to determine disease activity.[4] Twelve patients had active disease, while in 19 patients it was quiescent. No patient had been treated with steroids within one month of entry to the study nor had immunosuppressive treatment within six months of entry.

NORMAL CONTROL SUBJECTS

Twenty three healthy subjects (15 men, 8 women) with a mean age of 33 years (range 20–47 years) were studied.

TABLE I *Specificity of monoclonal antibodies*

Monoclonal antibodies (FITC/PE)	*Antigen cluster designation*	*Target subset*
HLe-1/Leu-M3	CD45/CD14	For electronic gate setting for lymphocytes
IgG1/IgG2		For negative control and non-specific binding
Leu-3/Leu-2	CD4/CD8	CD4/CD8 ratio
Leu-3/Leu-8	CD4/-	Suppressor-inducer and helper-inducer T cells
Leu-18/Leu-3	CD45R/CD4	Suppressor-inducer T cells
Leu-2/Leu-8	CD8/-	CD8+ T subsets
Leu-4/HLA-DR	CD3/-	Activated T cells
Leu-4/IL-2R	CD3/CD25	Activated T cells
Leu-7/Leu-2	-/CD8	CD8 subsets
Leu-7/Leu-4	-/CD3	CD3 subsets
Leu-4/Leu-11+19	CD3/CD16, -	Natural killer cells and cytotoxic CD3+T lymphocytes
Leu-16/Leu-1	CD20/CD5	Leu-1+B cells
Leu-16/Leu-8	CD20/-	Leu-8+ and Leu-8−B cells
Leu-16/Leu-20	CD20/CD23	FcεR+B cells
Leu-21/Leu-12	-/CD19	Activated B cells

FITC/PE=fluorescein isothiocyanate/phycoerythrin.

MONOCLONAL ANTIBODIES

Fifteen pairs of monoclonal antibodies conjugated with fluorescein isothiocyanate and phycoerythrin were used. All monoclonal antibodies were prepared at the Becton Dickinson Monoclonal Center (Mountain View, CA). The specificities of the reagents used in this study are given in Table I. They were all directly conjugated with fluorescein isothiocyanate or phycoerythrin.

TWO COLOUR DIRECT IMMUNOFLUORESCENCE STAINING OF WHOLE BLOOD

Whole blood collected by venepuncture with ethylenediamine tetra-acetate was used for staining; 100 µl of anticoagulated whole blood was placed into 12×75 mm polystyrene tubes (Falcon Plastics, Oxnard, CA); 20 µl of each monoclonal reagent was added to each tube. The tubes were incubated for 15 minutes at room temperature in the dark. 2 ml of 1×FACS Lysing Solution (Becton Dickinson) was added to each tube. The samples were then agitated and incubated for 10 minutes. The samples were washed once in Dulbecco's modified phosphate buffered saline without calcium and magnesium (Gibco, UK) containing 0·1% sodium azide. The cell sediment was resuspended in 0·5% paraformaldehyde in phosphate buffered saline. The fixed cells were stored at 4°C in the dark until analysis.

TWO COLOUR FLOW CYTOMETRY

Analysis by two colour flow cytometry was performed with a FACScan (Becton Dickinson, FACS Division, Sunnyvale, CA). SimulSET software (Becton Dickinson) was used for the flow cytometric data acquisition, data analysis, and report generation in two colour flow cytometric experiments and lymphocyte subset analysis. SimulSET software uses a LeucoGATE (HLe-1 FITC+Leu-M3 PE, Becton Dickinson Immunocytometry Systems, Mountain View, CA) sample to compute the optimum lymphocyte gate for an individual patient. Subsequent samples for the same patient are analysed using this gate. In determining the quadrant markers, SimulSET software uses the second sample in the panel of monoclonal antibodies, stained with Simultest Control reagent (IgG1 FITC+IgG2 PE, Becton Dickinson) to compute the optimum fluorescence quadrant markers for the patient. Each sample following LeucoGATE and Simultest Control is automatically gated, and the data are analysed and reported. Percentages are calculated based on the number of lymphocytes found in each quadrant and corrections are made to account for non-lymphocyte contamination within the lymphocyte gate.

STATISTICAL METHODS

Statistical analysis of results was performed by using Student's paired or unpaired *t* test, and a probability of less than 5% was considered to be significant.

TABLE II *Percentage of T cells, B cells, and the CD4/CD8 ratio (% of total lymphocytes; mean (SD))*

Group	CD3+	CD4+	CD8+	CD19+	CD4/CD8
Normal (n=23)	72 (6)	43 (7)	30 (7)	14 (4)	1·5 (0·6)
Ulcerative colitis					
Active (n=41)	73 (8)	44 (8)	31 (9)	14 (4)	1·6 (0·7)
Inactive (n=19)	76 (9)	45 (7)	35 (8)	11 (4)*	1·4 (0·5)
Crohn's disease					
Active (n=12)	74 (8)	44 (10)	31 (11)	13 (5)	1·6 (0·7)
Inactive (n=19)	76 (10)	45 (12)	31 (9)	10 (4)**	1·6 (0·7)

*p<0·05, **p<0·001.

TABLE III *Numbers of white blood cells (WBC), lymphocytes, T cells, and B cells in peripheral blood in inflammatory bowel disease (all ×10⁶/l; mean (SD))*

Group	WBC	Lymphocytes	CD3+	CD4+	CD8+	CD19+
Normal	6224 (1826)	2125 (610)	1535 (423)	915 (320)	641 (219)	306 (109)
Ulcerative colitis						
Active	7839 (1975)**	1792 (628)*	1334 (499)	782 (290)	566 (293)	243 (114)*
Inactive	7042 (1425)	1848 (563)	1426 (500)	815 (260)	663 (290)	199 (75)***
Crohn's disease						
Active	9367 (2074)***	1940 (843)	1440 (651)	847 (394)	599 (316)	262 (189)
Inactive	7437 (2190)	1895 (674)	1453 (604)	860 (412)	592 (276)	176 (83)***

*p<0·05, **p<0·01, ***p<0·001.

TABLE IV *CD4+ and CD8+ lymphocyte subsets in inflammatory bowel disease (% of total lymphocytes; mean (SD))*

Group	CD4+, Leu-8+	CD4+, Leu-8−	CD4+, CD45R+	CD4+, CD45R−	CD8+, Leu-8+	CD8+, Leu-8−
Normal	33 (7)	10 (3)	17 (6)	25 (6)	14 (5)	16 (5)
Ulcerative colitis						
Active	35 (8)	9 (3)	18 (9)	27 (8)	13 (4)	18 (9)
Inactive	35 (7)	10 (4)	13 (5)*	31 (7)**	13 (4)	23 (8)**
Crohn's disease						
Active	33 (11)	11 (5)	18 (9)	26 (8)	11 (4)	20 (11)
Inactive	33 (11)	12 (5)	14 (8)	31 (9)**	12 (4)	19 (9)

*p<0·05, **p<0·01.

Results

Tables II and III show the percentages and absolute numbers (mean (SD)) of singly fluorescent peripheral blood lymphocytes expressing monoclonal antibodies, Leu-4 (CD3), CD4, CD8, Leu-12 (CD19), and the CD4/CD8 ratio in normal control subjects and patients with ulcerative colitis and Crohn's disease. There was a significant decrease in the proportion of B cells in patients with inactive disease. There was a higher proportion of CD8+ lymphocytes in inactive ulcerative colitis but the CD4/CD8 ratio did not change significantly.

The phenotype of CD8+ cells and CD4+ cells was further investigated using two colour immunofluorescent staining (Table IV). The proportion of CD8+, Leu-8− cells was higher in patients with inactive ulcerative colitis ($p<0·01$). CD8+, Leu-8+ cells collaborate with CD8+, Leu-8− cells to generate suppression of B cell differentiation.[5]

The percentages of CD4+, Leu-8+ cells (suppressor-inducer T) and CD4+, Leu-8− cells (helper-inducer T) remained unchanged in inflammatory bowel disease. These subpopulations of CD4+ cells were also detected using the CD4, Leu-18 (CD45R) combination. CD4+, CD45R+ lymphocytes (suppressor-inducer T) were lower in inactive ulcerative colitis. The percentage of CD4+, CD45R− cells (helper-inducer T) in inactive disease groups was significantly increased ($p<0·01$) (Table IV).

The percentage of activated T cells (CD3+, IL-2R+) in Crohn's disease was significantly increased compared with control subjects ($p<0·05$). Although CD3+, HLA-DR+

TABLE V *Activated T cells in inflammatory bowel disease (% of total lymphocytes; mean (SD))*

Group	*CD3+, HLA-DR+*	*CD3+, IL-2R+*
Normal	8 (5)	15 (6)
Ulcerative colitis		
Active	11 (7)	17 (9)
Inactive	11 (6)	17 (9)
Crohn's disease		
Active	11 (9)	20 (7)*
Inactive	10 (5)	19 (7)*

*p<0·05.

lymphocytes in inflammatory bowel disease were increased compared with control subjects, this difference was not significant (Table V).

Cytotoxic lymphocyte subsets identified by monoclonal antibodies Leu-7, Leu-11 (CD16), and Leu-19 were also investigated (Table VI). There was no significant difference in the proportion of cytotoxic T lymphocytes between control subjects and patients with inflammatory bowel disease. Natural killer cells (CD3−, CD16+, Leu-19+) were significantly decreased in patients with active Crohn's disease (p<0·05). The proportion of Leu-7+, CD8+ lymphocytes and Leu-7+, CD3+ cells was significantly higher in inactive disease groups than in control subjects (inactive ulcerative colitis: p<0·001, inactive Crohn's disease: p<0·05).

Examination of B cell subsets showed a decrease in the proportion of Leu-1+ (CD5) B cells (Table VII). A significantly lower proportion of Leu-8+ B cells was present in inactive Crohn's disease compared with control subjects (p<0·01). Loss of Leu-8 antigens has been shown to be associated with initial activation of B cells. In active inflammatory bowel disease, there was no difference in the proportion of B cells expressing FcεR (CD23), but in inactive disease the proportion of these cells was significantly decreased compared with that in control subjects (inactive ulcerative colitis: p<0·05, inactive Crohn's disease: p<0·01). Leu-21 recognises an antigen expressed by activated B cells. There was no significant difference in the percentage of Leu-21+ B cells in inactive ulcerative colitis and Crohn's disease, but in ulcerative colitis in active stage the proportion of these cells was decreased (p<0·05).

TABLE VI *Leu-7+ cell subsets, natural killer (NK) cells (CD3− CD16+ Leu-19+), and cytotoxic T lymphocytes (CD3+ CD16+ Leu-19+) in inflammatory bowel disease (% of total lymphocytes; mean (SD))*

Group	*Leu-7+, CD8+*	*Leu-7+, CD3+*	*NK cells*	*Cytotoxic T lymphocytes*
Normal	9 (5)	7 (5)	14 (5)	6 (4)
Ulcerative colitis				
Active	12 (8)	9 (7)	14 (7)	6 (4)
Inactive	16 (7)**	12 (7)**	14 (5)	7 (5)
Crohn's disease				
Active	13 (7)	11 (8)	11 (4)	7 (5)
Inactive	14 (7)*	12 (7)*	16 (8)	6 (5)

*p<0·05, **p<0·001.

TABLE VII *B cell subsets in inflammatory bowel disease (% of total lymphocytes; mean (SD))*

Group	*CD20+, CD5+ (CD5+ B)*	*CD20+, Leu-8+ (Leu-8+ B)*	*CD20+, CD23+ (Fc ε R+ B)*	*CD12+, Leu-21+ (activated B)*
Normal	4 (2)	11 (4)	9 (3)	5 (3)
Ulcerative colitis				
Active	3 (2)*	10 (4)	8 (3)	4 (2)*
Inactive	2 (2)*	9 (3)	7 (3)*	4 (7)
Crohn's disease				
Active	2 (3)	10 (4)	8 (4)	5 (4)
Inactive	3 (2)*	7 (3)**	6 (3)**	4 (2)

*p<0·05, **p<0·01.

Discussion

Lymphocyte subsets in whole blood of normal subjects and of patients with active and inactive ulcerative colitis and Crohn's disease were investigated by using monoclonal antibodies and multiparameter flow cytometry. There have been several studies of peripheral blood lymphocyte subsets in patients with ulcerative colitis and Crohn's disease. The initial study by Selby and Jewell showed that, although the absolute numbers of T lymphocytes might be low, there was no change in the proportion of CD4+ and CD8+ cells.[6] Subsequently, not all studies have agreed with this, as shown in Table VIII. The reasons for the discrepancies have been attributed to the small numbers of patients studied in some series, the different methods used, and the possible effect of treatment. Therefore, the aim of this study was to use a whole blood assay (to avoid artefacts created by density gradient solution procedures), to use a highly sensitive technique with double labelling, and to investigate patients who had not received corticosteroid or immunosuppressive drugs within a defined period of time. B cell markers were also investigated.

In many patients with autoimmune disorders, the CD4/CD8 ratio is increased, reflecting a relative or absolute deficiency of CD8+ cells.[16,17] Although CD8+ lymphocytes were low in inactive ulcerative colitis, the CD4/CD8 ratio did not change.

In previous studies cells with the CD4+, Leu-8− phenotype have been shown to contain the majority of helper function involved in B cell differentiation into plaque forming cells.[5] In patients with inflammatory bowel disease the ratio of CD4+ Leu-8− to CD4 Leu-8+ cells remained unchanged. The CD4+ cells can be subdivided by monoclonal antibodies reacting with different epitopes of the high molecular weight T200 common leucocyte antigen (CD45R). These monoclonal antibodies are Leu-18,[18] 2H4,[19] and 3AC5 and G1-5.[20] The CD4+ cells expressing the CD45R antigen have been reported to possess a T suppressor-inducer function, whereas those helper cells lacking CD45R show a CD4+ inducer function.[19] The percentage of cells with the CD4+, CD45R+ phenotype from patients with inactive ulcerative colitis was decreased with a corresponding increase in the CD4+, CD45R− phenotype. The slightly different results between CD4, Leu-8 and CD4, CD45R phenotypes of the present study may be explained by the different distribution of the Leu-8 antigen and CD45R antigen.[19] Our findings differ slightly from those of James *et al*,[12] who found no major differences of CD4 subpopulations defined by 2H4 (CD45RA+) comparing Crohn's disease with control subjects.

The number of T cells expressing HLA-DR class II molecules (CD3+, HLA-DR+) in inflammatory bowel disease was increased, but not significantly. Selby and Jewell[6] found that HLA-DR antigens were detected on circulating T lymphocytes in nine of 16 normal subjects, and

TABLE VIII *Peripheral blood lymphocyte subsets*

Study	*Patients*	*Specimens*	*Methods*	*Monoclonal antibodies*
Pepys *et al*[15]	Crohn's disease	PWB	AP	CD3
Selby and Jewell[6]	Inflammatory bowel disease	PBMC	IF	CD3, CD4, CD8, CD4/CD8
Yuan *et al*[7]	Crohn's disease	PBMC	IF	CD3, CD4, CD8, CD4/CD8
Pallone *et al*[8]	Crohn's disease	PBMC	IF	CD3,† CD8†
James *et al*[9]	Crohn's disease	PBMC	FACS	CD3, CD4,† CD8, CD4/CD8
Godin *et al*[10]	Inflammatory bowel disease	PBMC	IF	CD4, CD8,† CD4/CD8*
Auer *et al*[11]	Inflammatory bowel disease	PBMC	IF	CD4, CD8, CD4/CD8
James *et al*[12]	Crohn's disease	PBMC	FACS	CD4/Leu-8, CD4/CD45R, CD8/CD11
Davidsen and Kristensen[13]	Inflammatory bowel disease	PBMC	IF	CD2, CD4, CD8, CD4/CD8
Gossum *et al*[14]	Crohn's disease	PBMC	IF	CD3, CD4, CD8, CD4/CD8

PWB=peripheral whole blood; PBMC=peripheral blood mononuclear cells; IF=immunofluorescent method; AP=alkaline phosphatase-labelled reagents method; FACS=fluorescence activated cell sorter.
*Increase; †decrease.

only three patients with inflammatory bowel disease had a higher proportion of HLA-DR+ T cells than controls. Yu *et al*[21] found a similar increase in four of seven patients with Crohn's disease. This difference might be ascribed to the sensitivity between fluorescence microscopy and flow cytometry. CD3+, IL-2R+ T lymphocytes from patients with inflammatory bowel disease were significantly increased, suggesting that circulating T lymphocytes in this disorder are activated.

The Leu-7 cell subset comprises two essentially non-overlapping subpopulations, depending on whether cells are coexpressing the natural killer cell marker CD16 (Leu-7+, CD16+ phenotype) or the pan-T cell marker CD3 (Leu-7+, CD3+ phenotype).[22] It is possible to split Leu-7+, CD3+ cells into two subsets as follows: about 80% of Leu-7+, CD3+ express CD8 antigen, whereas 20% express CD4 antigen.[22] A rise in these phenotypes is associated with aging.[23] In inactive inflammatory bowel disease both Leu-7+, CD3+ cells and Leu-7+, CD8+ cells were increased compared with normal control subjects. The mean age of the patients with inactive inflammatory bowel disease is greater than that of the control population so these results might be attributed to aging. Although natural killer cells are heterogeneous with respect to expression of cell surface differentiation antigens, it has been clearly established that the majority of peripheral blood lymphocytes mediating non-major histocompatibility complex restricted cytotoxicity express the CD16[24] and Leu-19 (NKH-1) antigens.[23] The CD3+, CD16+, Leu-19+ population is a unique subset of non-major histocompatibility complex restricted cytotoxic CD3+ T lymphocytes, and CD3−, CD16+, Leu-19+ natural killer cells are the most abundant and the most efficient cytotoxic effectors.[25,26] Cytotoxic CD3+ T lymphocytes from patients with inflammatory bowel disease showed no change, but natural killer cells in active Crohn's disease were decreased.

A detailed study of the phenotype of lymphocyte subpopulations in ulcerative colitis and Crohn's disease has shown only minor changes in patients compared with normal control subjects when the disease is in remission. The importance of the decrease in suppressor-inducer cells (CD4+, Leu8+) in patients with inactive ulcerative colitis can only be fully assessed when the results of functional studies are known. In active disease the phenotypic changes are compatible with T and B cell activation.

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