Phenotyping of peripheral blood lymphocytes in adult coeliac disease

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SUMMARY

In coeliac disease immunological abnormalities are not confined to the small bowel and it has been suggested that changes in peripheral blood lymphocytes may predispose to autoimmune or malignant complications. Using dual-colour immunofluorescence with labelled monoclonal antibodies, multiparameter flow cytometry was used to analyse peripheral blood lymphocytes in 32 untreated coeliacs, 29 treated coeliacs and 20 healthy volunteers. When the absolute numbers were considered, a decrease of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ lymphocytes was found in untreated coeliacs compared with treated coeliacs and healthy volunteers. The proportion of $CD3^+$ was significantly higher in untreated coeliacs ($P<0.05$) than in healthy volunteers. No differences were observed in $CD4^+$, $CD8^+$ and $CD19^+$ subsets between the three groups studied. The proportion of $CD3^+$ $CD25^+$ and $CD3^+$ HLA-DR$^+$ cells were higher in untreated coeliacs ($P<0.001$ and $P>0.005$) and in treated coeliacs ($P<0.005$ and $P<0.05$) than in healthy volunteers. On the contrary, natural killer cells and cytotoxic cells were lower in untreated and treated coeliacs than in healthy volunteers. As regards B-cell subsets, the only difference was the increase in FcεR$^+$ B cells in untreated coeliacs. The absolute reduction of peripheral lymphocytes in coeliac disease probably reflects their compartimentalization in intestinal mucosa. The decrease of natural killer cells and cytotoxic cells may be in keeping with the increased prevalence of malignancy in this condition. Finally, the phenotypic changes found in untreated coeliacs indicate T-cell activation.

INTRODUCTION

Coeliac disease (CD) is caused by an abnormal immune reaction to gliadin and related prolamines within the mucosa of the small intestine.[1] However, immunological abnormalities are not confined to the small bowel in this condition, and it has been suggested that changes in peripheral blood lymphocytes may be involved in its pathogenesis and may predispose in adult patients to autoimmune and malignant complications.[2–6]

In the present study we have made a flow cytometric analysis[7] of lymphocyte subpopulations in peripheral blood samples taken from patients with untreated and treated CD and controls. Abnormal proportions of natural killer (NK) cells, activated and cytotoxic T lymphocytes (CTL) were found.

Abbreviations: CD, coeliac disease; CTL, cytotoxic T lymphocytes; GFD, gluten-free diet; NK, natural killer; PBL, peripheral blood lymphocytes.

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MATERIALS AND METHODS

Patients

Peripheral blood was obtained from 61 patients with biopsy proven CD. Thirty-two patients (mean age 36·5 years, range 18–64) were untreated whereas 29 patients (mean age 37·2 years, range 20–68) had been on gluten-free diet (GFD) for at least 12 months at the time of the study. For all treated coeliac patients histological improvement of jejunal mucosa following gluten withdrawal was shown.

Twenty healthy volunteers, sex and age matched (mean age 36·8 years, range 20–66) with the patients, were also studied. Informed consent was obtained from all patients and control subjects.

Monoclonal antibodies

Fifteen pairs of monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE) were used. All monoclonal antibodies were prepared at the Becton Dickinson Monoclonal Centre (Mountain View, CA). The specificities of the reagents used in this study are given in Table 1. They were all directly conjugated with fluorescein isothiocyanate and 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

Table 1. Specificity of monoclonal antibodies

Monoclonal antibodies (FITC/PE)	Antigen cluster designation	Target subset
III.c-1/I.cu.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 cells
Leu-4/HLA-DR	CD3/–	Activated T cells
Leu-4/IL-2R	CD3/CD25	Activated T cells
Leu-4/Leu-11 + 19	CD3/CD16,–	Natural killer cells and cytotoxic CD3$^+$ 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	FC$_\varepsilon$R$^+$ B cells
Leu-21/Leu-12	–/CD19	Activated B cells

FITC/PE = fluorescein isothiocyanate/phycoerythrin.

whole blood was placed in 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 min at room temperature in the dark. Two ml of 1 × fluorescence-activated cell sorting (FACS) Lysing Solution (Becton Dickinson) was added to each tube. The samples were washed once in Dulbecco's modified phosphate-buffered saline without calcium and magnesium (Gibco, Paisley, 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° in the dark until analysis.

Two-colour flow cytometry
Analysis by two-colour flow cytometry was performed with FACScan (Becton Dickinson, FACS Divitin, 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 lymphocytes 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 were analysed using this gate. In determining the quadrant markers, SimulSET software uses the second sample in the panel of monoclonal antibodies, stained with Simultest Contro Reagent (immunoglobulin G1; 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.

Soluble interleukin-2 (IL-2) receptor detection
Soluble IL-2R was detected by capture enzyme-linked immunosorbent assay (ELISA) using ligand-coated wells, ligand-labeled monoclonal anti-sIL-2R antibody, horseradish peroxidase-labelled monoclonal anti-sIL-2R antibody, and

multivalent antiligand antibodies (DPC, Los Angeles, CA). The normal range was established at 280 ± 80 (mean ± SD) units/ml (based on 20 healthy blood donors).

Statistical analysis
Data are expressed as mean ± SD. Statistical comparisons between mean values were carried out using the Mann–Whitney U-test for non-parametric data.

RESULTS

Tables 2 and 3 show, respectively, the absolute numbers and percentages of singly fluorescent peripheral white blood cells, and lymphocytes expressing monoclonal antibodies, Leu-4 (CD3), CD4, CD8, Leu-12 (CD19) in untreated and treated CD patients and in healthy volunteers.

Table 2 shows that the absolute numbers of white blood cells, total lymphocytes, CD3$^+$, CD4$^+$ and CD8$^+$ lymphocytes were significantly decreased in untreated coeliac patients compared to treated and controls, whereas no differences between the latter two groups were found. The absolute number of CD19$^+$ lymphocytes was significantly lower in untreated coeliacs than in controls, whereas no difference was found between untreated and treated patients and between the latter and the controls.

Table 3 shows that the percentage of CD3$^+$ lymphocytes was significantly higher in untreated than in treated coeliacs and controls. No differences were found in the percentage of CD4$^+$, CD8$^+$ and CD19$^+$ T cells between the groups.

As shown in Table 4, the percentages of activated T cells (HLA-DR$^+$ and CD25$^+$) and sIL-2R levels were significantly higher in coeliac patients compared with controls, irrespective of treatment. No difference was found between untreated and treated CD patients as far as the percentage of activated T cells, whereas sIL-2R levels were significantly higher in untreated than in treated coeliacs.

The percentages of NK cells and CTL were also investigated (Table 5). Both were significantly lower in untreated and treated coeliac patients than in healthy subjects. Evaluation of B-cell subsets (Table 6) showed only a significant increase of the percentage of cells expressing FcɛR II (CD23) in coeliac patients. No significant difference was found in this respect between untreated and treated coeliac patients.

DISCUSSION

There have been many attempts over the years to study peripheral blood lymphocytes in CD. In fact, although CD is primarily a small bowel disease, its immunological abnormalities are not confined to the intestinal tract, and associated conditions, especially those of autoimmune or neoplastic nature, may in some way be related to these abnormalities.

First of all, our study confirms by flow cytometric analysis the reduction in the absolute number of total[8,9] and T lymphocytes[3,9], shown in untreated CD by earlier studies. This reduction was considered to be secondary to the compartmentalization of gluten-sensitive lymphocytes within the intestinal mucosa[9,10] and/or to their loss into the gut lumen.[11] Although it cannot be excluded that malnutrition *per se* may be a main factor for this reduction,[12] the lack of a correlation between nutritional status and delayed cutaneous response led

Table 2. Peripheral white blood cells, lymphocytes, T and B cells (mean number (SD) $\times 10^6$/l)

	Untreated coeliac disease	Treated coeliac disease	Healthy volunteers
White blood cells	6800 (2400)	7500 (2200)	7265 (1800)
	$\overline{}$ $P < 0.005$ $\overline{}$	$\overline{}$ NS $\overline{}$	
		$P < 0.005$	
Total lymphocytes	1480 (400)	2190 (700)	2204 (570)
	$P < 0.01$	NS	
		$P < 0.001$	
CD3$^+$ lymphocytes	1117 (156)	1630 (400)	1573 (526)
	$P < 0.001$	NS	
		$P < 0.001$	
CD4$^+$ lymphocytes	582 (178)	810 (280)	741 (266)
	$P < 0.005$	NS	
		$P < 0.05$	
CD8$^+$ lymphocytes	513 (140)	811 (290)	704 (340)
	$P < 0.001$	NS	
		$P < 0.05$	
CD19$^+$ lymphocytes	215 (152)	244 (101)	281 (140)
	NS	NS	
		$P < 0.005$	

Table 3. Percentages of T and B cells in the peripheral blood (mean % of total lymphocytes (SD))

	Untreated coeliac disease	Treated coeliac disease	Healthy volunteers
CD3$^+$ lymphocytes	76.2 (6.6)	74.6 (6.5)	70.5 (11.1)
	$P < 0.05$	NS	
		NS	
CD4$^+$ lymphocytes	37.6 (7.3)	39.0 (9.2)	40. (9.2)
	NS	NS	
		NS	
CD8$^+$ lymphocytes	35.0 (7.0)	36.8 (8.7)	36.9 (9.3)
	NS	NS	
		NS	
CD19$^+$ lymphocytes	12.8 (4.9)	12.9 (3.6)	12.1 (4.6)
	NS	NS	
		NS	

Table 4. Percentages of activated T cells and serum levels of soluble interleukin-2-receptor in the peripheral blood

	Untreated coeliac disease	Treated coeliac disease	Healthy volunteers
CD3$^+$, HLA-DR$^+$	12.8 (7.4)	10.2 (7.7)	6.2 (3.0)
	NS	$P < 0.005$	
		$P < 0.001$	
CD3$^+$, CD25$^+$	16.0 (8.0)	14.0 (7.2)	9.7 (7.2)
	NS	$P < 0.05$	
		$P < 0.005$	
sIL-2R (U/ml)	1131 (430)	734 (180)	262 (50)
	$P < 0.005$	$P < 0.001$	
		$P < 0.001$	

Table 5. Percentages of natural killer cells (CD3$^-$CD + Leu-19$^+$) and cytotoxic T lymphocytes (CD3$^+$CD16$^+$Leu-19$^+$)

	Untreated coeliac disease	Treated coeliac disease	Healthy volunteers
Natural killer cells	9.0 (4.3)	9.9 (5.2)	15.7 (7.3)
	NS	$P < 0.001$	
		$P < 0.001$	
Cytotoxic T lymphocytes	5.2 (3.0)	14.0 (7.2)	9.7 (7.2)
	NS	$P < 0.01$	
		$P < 0.01$	

Table 6. Percentages of B-cells subsets in the peripheral blood

	Untreated coeliac disease	Treated coeliac disease	Healthy volunteers
CD20$^+$, CD5$^+$	3·5 (2·4)	4·0 (3·0)	4·8 (3·7)
	NS ———————— NS		
	————————— NS —————————		
CD20$^+$, Leu-8$^+$	9·3 (4·1)	8·0 (3·2)	8·7 (3·8)
	NS ———————— NS		
	————————— NS —————————		
CD20$^+$, CD23$^+$ (FCεR$^+$B)	7·9 (3·4)	7·4 (2·6)	6·3 (3·2)
	NS ——————— $P < 0.05$		
	——————— $P < 0.05$ ———————		
CD19$^+$, Leu-21$^+$	4·5 (2·4)	5·0 (2·0)	12·1 (4·6)
	NS ———————— NS		
	————————— NS —————————		

to the suggestion that in untreated CD peripheral lymphopenia should not be related to nutritional deficiencies.[13] Our results differ from those of a study in which the reduction of circulating lymphocytes in 14 untreated coeliac patients did not reach statistical significance.[14] Because 4 out of 14 patients of this series were children, the lack of significance may be ascribed to their shorter duration of CD.

It is conceivable that in untreated CD the increased percentage of CD3$^+$ cells reflects the increase of their activated subset. In fact both HLA-DR$^+$ and CD25$^+$ T lymphocytes are significantly increased and the percentage of activated T cells remains significantly elevated despite GFD. This persistent activation may be a result of a minimal amount of gluten still present in the diet, to the action of follicular dendritic cells within primary follicles and germinal centres which can retain gluten in its native form for a long time and continue to stimulate lymphocytes,[15] or, finally, to the maintenance of an immunological memory towards gluten. Evidence supporting this latter hypothesis comes from the study by Kerttula et al.,[14] which shows an enhanced T-cell 'memory activity' in the peripheral blood of untreated and treated coeliac patients in the form of an increased percentage of peripheral CD45RO$^+$ T cells. Because the expression of CD45RO can be considered a marker of cell activation, as well as of memory, the results of Kerttula et al.[14] confirm our results, also as regards the persistence of a state of immunological activation after GFD.

Unlike what occurs for the percentage of activated T cells, the serum levels of sIL-2R, which are raised in untreated CD, decrease significantly after gluten withdrawal, although some overlaps between our untreated and treated CD patients cast some doubts on the usefulness of this measurement in the assessment of response to treatment as it had been proposed.[16] Because sIL-2R is mainly shed by activated T lymphocytes, its level is a marker of gluten-related T-cell activation, although whether these activated T lymphocytes are mucosal or peripheral in origin is still not clear.[17]

A new result of the present study is the reduction in the absolute number of CD19$^+$ cells (B lymphocytes) found only in untreated coeliac patients and this was the only finding to be normalized after GFD.

In our study the proportion of CTL and NK cells were significantly lower in untreated and treated coeliac patients than in controls. GFD has a very little effect on these subsets and this is at variance with the results of a previous non-cytofluorimetric study which showed a normal proportion

of NK cells in treated CD patients.[18] It may be that this persistent abnormality is linked to a constitutive deficiency of this particular subset in CD or, again, to the effect of a minimal amount of gluten in the diet. It is probable that the low levels of CTL are, in turn, secondary to a decrease in NK cells. Recent studies have, in fact, shown that in addition to their cytotoxic properties NK cells may provide help for the development of CD8$^+$ CTL-activity[19] and that the generation of CTL requires direct cell contact with NK cells.[20] Moreover, as NK cells provide help for CD8$^+$ T cells to become suppressor cells, i.e. antigen-non-specific suppressor of antibody production,[21] the decrease in the number of NK cells may therefore favour the increased antibody production in CD. A mechanism of this type has been demonstrated in other autoimmune diseases characterized by impaired downregulatory T-cell function, such as systemic lupus erythematosus.[22] NK cells may have an equally important role in protecting against malignancy[23] and their decrease in CD is in keeping with the observed increased prevalence of malignancy in this condition. Although, in this regard, a protective effect of GFD has been shown[24] our results suggest that treated patients still carry a theoretical immunological predisposition to malignancy.

Finally, cells expressing the low-affinity receptor for the Fc region of IgE (FcεR II) were found to be raised both in untreated and treated CD patients. These cells may play a major role in IgE-related immune responses[25] and the increase of their proportion may be responsible for the established association between atopy and CD.[26,27] Other studies have suggested that FcεR II may be involved in the regulation of growth and differentiation of B cells[28] and so may be considered an activation marker. Accordingly, our results could be interpreted as an increased activation of B cells in untreated CD.

In conclusion, our results show that in CD absolute numbers and proportions of peripheral blood lymphocytes are greatly altered. In particular the reduction of T lymphocytes may in some way be related to their increased activation. In fact, in addition to the hypothesis already put forward,[9–12] their peripheral reduction could be the result of the apoptosis of these cells, triggered precisely by their state of greater activation[29] that we have shown. Apoptosis, in fact, can act as a homeostatic mechanism to limit the expansion of activated lymphocytes following antigenic stimulation, ensuring the clearance of primed lymphocytes that are no longer required.[30]

It is likely that the increased propensity of CD lymphocytes to undergo apoptosis is the consequence of an unbalanced and continuous activation of the immune system. It would thus be interesting to verify this hypothesis in the light of the recent discoveries regarding the mechanisms of peripheral lymphocyte Fas-mediated apoptosis,[31] and in the light of our preliminary results which implicate the Fas–FasL system in triggering increased enterocyte apoptosis in CD.

ACKNOWLEDGMENTS

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