

Lymphocyte subpopulations in adult coeliac disease

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SUMMARY Rosetting techniques were used to estimate T and B cell subpopulations in the peripheral blood in patients with treated and untreated adult coeliac disease and in control subjects. In patients with untreated coeliac disease, T cell numbers were significantly lower than in controls or treated patients, although there was no difference in total lymphocyte counts. There was no significant difference in B cell numbers between treated and untreated patients, and the subpopulation which increased to replace the T cells in untreated patients comprised cells not identified by B or T cell markers. Total lymphocyte counts and lymphocyte subpopulations were affected by splenic atrophy. It is suggested that these effects might be caused by the loss of lymphocytes from the gastrointestinal tract in untreated coeliac disease.

Impaired function of peripheral blood lymphocytes (Blecher *et al.*, 1969; Asquith, 1974; Scott and Losowsky, 1976) and abnormalities of lymphocyte distribution (Ferguson and Murray, 1971) have been described in untreated coeliac disease. Alteration in numbers of lymphocytes in certain subpopulations might explain the impaired function of peripheral blood lymphocytes, and reduction in numbers of circulating thymus dependent lymphocytes (T cells), has been described (O'Donaghue *et al.*, 1976). Since total lymphocyte counts are not reduced, there must be a compensatory increase in the numbers of non-T lymphocytes.

The purpose of this study was to measure lymphocyte subpopulations in treated and untreated patients with coeliac disease and control subjects in order to identify the cell type or types replacing T cells in the peripheral blood. Furthermore, it is known that some patients with coeliac disease have splenic atrophy (McCarthy *et al.*, 1966) and that some patients who have had a splenectomy develop a persistent lymphocytosis (Lipson *et al.*, 1959), and thus additional alterations in lymphocyte populations might occur in association with splenic atrophy in some patients with coeliac disease. Therefore treated coeliac patients with the blood film features of splenic atrophy, and healthy controls who had had a splenectomy because of trauma were also studied in order to separate out these phenomena.

Methods

SUBJECTS

Lymphocyte subpopulations were studied in 21 patients with untreated coeliac disease, 32 patients

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with treated coeliac disease, 34 healthy control subjects of age distribution similar to the coeliac patients, and 22 control patients who had had a small bowel biopsy to exclude the diagnosis of coeliac disease.

The diagnosis of coeliac disease was based on abnormal jejunal histology (subtotal or severe partial villous atrophy) with histological evidence of improvement on gluten withdrawal. All treated patients had been on a gluten free diet for at least six months, and none was taking steroids. The final diagnoses in the patients who were submitted to biopsy but found not to have coeliac disease were irritable bowel syndrome (five), post-vagotomy malabsorption (five), chronic pancreatitis (three), nutritional anaemia (two), anorexia nervosa (one), lactose intolerance (one), mast cell disease (one), giardiasis (one), hypobetalipoproteinaemia (one), Crohn's disease (one) and radiation ileitis (one).

Ten treated coeliac patients with the blood film features of splenic atrophy—that is, Howell-Jolly bodies, acanthocytes, giant platelets, and target cells were considered separately, and compared with 10 healthy subjects who had undergone splenectomy because of trauma and who also had typical blood film features.

LYMPHOCYTE SUBPOPULATIONS IDENTIFIED

The subpopulations identified were lymphocytes forming rosettes with sheep red cells (E rosettes, putative T cell count), lymphocytes forming rosettes with sheep red cells sensitised with horse haemolytic serum and exposed to complement (EAC rosettes, complement receptor bearing lymphocytes, putative B cell count), and cells unclassified by these rosetting techniques (unclassified cells, non-E non-EAC rosette forming cells).

LYMPHOCYTE SEPARATION

Eight millilitres of venous blood was collected in EDTA and the total and differential leucocyte count determined. Four millilitres of blood was layered on to 5.5 ml Ficol-sodium metrizoate and centrifuged at 400 *g* for 40 minutes at 10°C. The separated lymphocytes were removed immediately, washed twice in normal saline, and resuspended in saline to give an approximate concentration of 8×10^6 cells/ml. The lymphocyte yield was consistently above 75%.

SHEEP RED CELL PREPARATION

Sheep red cells (no preservative, not more than 3 weeks old) were washed three times in normal saline and used as a 1 in 40 suspension in saline for identifying E rosette forming lymphocytes. For EAC rosettes, washed sheep red cells were sensitised with horse haemolytic serum (final concentration 1 in 4000 in saline) for one hour at 37°C, washed twice, and exposed to freshly thawed human serum as a source of complement (final concentration 1 in 80 in saline) for 20 minutes at 37°C, washed a further three times, and used as a 1 in 40 suspension in saline. As a control for the latter technique, to detect rosettes formed with sheep red cells sensitised only with antibody (EA rosettes), sheep red cells were prepared as for EAC rosettes but omitting exposure to complement.

ROSETTE PREPARATION

In each tube, 0.1 ml of the appropriate sheep red cell suspension and 0.1 ml of lymphocyte suspension were mixed, incubated for 10 minutes at 37°C, and centrifuged for five minutes at 125 *g*.

For E rosettes, the tube was immersed in ice for 1½ hours, and the contents mixed by turning the tube gently. The suspension was mounted on chilled haemocytometer chambers.

For EAC rosettes, and EA controls, the tubes were returned to the incubator for 20 minutes at 37°C, and the contents mixed on a blood wheel for three minutes. The suspension was mounted on haemocytometers at room temperature.

All tubes were prepared in duplicate and 600 cells were counted for each preparation of E and EAC rosettes. All free cells with the morphology of lymphocytes were counted, but cell clumps were ignored. Lymphocytes with three or more adherent red cells were counted as rosettes. Occasional free cells with the morphology of monocytes, some showing erythrophagocytosis were excluded from the count, but these amounted to less than 5% of the free nucleated cell population, since the monocytes tended to clump (Anthony *et al.*, 1975). Occasional free monocyte rosettes were seen in EAC prepara-

tions, being identified by the greater amount of cytoplasm visible around the nucleus, with a peripheral ring of erythrocytes; these rosettes were ignored. Monocyte rosettes were not noted in E preparations. Non-specific esterase staining (Koski *et al.*, 1976) to identify monocytes confirmed that there were few free monocytes in rosette preparations, although up to 15% of mononuclear cells in the original lymphocyte suspension were monocytes. The percentage of EA rosetting cells was never more than 3% and no correction was made for these cells.

The mean percentage of rosetting cells for each preparation was determined, and the percentage of unclassified cells was calculated by subtraction of the sum of E and EAC rosette percentages from 100%.

The absolute numbers of each cell type/mm³ of blood were calculated from the total lymphocyte count, based on counting 200 leucocytes.

Preparations from patients and controls were examined concurrently.

The significance of differences in cell populations between the groups studied was analysed by Student's *t* test for data relating to absolute cell numbers, and by the Mann-Whitney U test for data relating to percentage cell numbers. In each group of subjects, the mean \pm SE for both percentage and absolute cell numbers was calculated and tabulated.

Results

Total lymphocyte counts in the six groups of subjects are shown in Table 1.

There were no significant differences between controls, biopsied controls, patients with untreated coeliac disease, and patients with treated coeliac disease without the peripheral blood film changes of hyposplenism. Total lymphocyte counts were significantly higher in treated coeliacs with hyposplenism than in both controls ($p < 0.001$) and treated coeliacs without the blood film changes of hyposplenism ($p < 0.005$). Lymphocyte counts were significantly higher in asplenic controls than in normal controls ($p < 0.001$) and tended to be higher than in patients with treated coeliac disease and hyposplenism, but the difference was not significant ($0.05 < p < 0.1$). Because of the increase in lymphocyte count in the patients with hyposplenism, which affects the absolute numbers of rosette forming cells/mm³ of blood, the results of rosette tests in treated coeliacs with hyposplenism are shown separately.

The percentage and absolute numbers/mm³ blood of E rosette forming cells (E-RFC), EAC rosette forming cells (EAC-RFC), and unclassified cells in controls, biopsied controls, and untreated and treated coeliacs are shown in Table 2. There were no signi-

Table 1 Total lymphocyte counts in six groups of subjects

	Controls	Biopsied controls	Coeliac disease		Treated coeliac disease and hyposplenism	Asplenic controls
			Untreated	Treated		
Subjects (no.)	34	22	21	22	10	10
Total lymphocytes/mm ³ mean \pm SE	2157 \pm 184	2229 \pm 180	2050 \pm 138	2354 \pm 121	3427 \pm 359	4482 \pm 323

Table 2 E rosette forming cells, EAC rosette forming cells, and unclassified cells in six groups of subjects

	Controls	Biopsied controls	Coeliac disease		Treated coeliac disease and hyposplenism	Asplenic controls
			Untreated	Treated		
E-RFC %	63.36 \pm 1.03	63.00 \pm 1.22	48.89 \pm 2.32	61.49 \pm 1.09	50.04 \pm 2.84	51.78 \pm 2.72
Number/mm ³	1368 \pm 69	1396 \pm 110	1016 \pm 100	1464 \pm 76	1713 \pm 203	2297 \pm 554
EAC-RFC %	24.86 \pm 1.17	25.34 \pm 1.35	30.62 \pm 2.25	24.44 \pm 1.60	23.90 \pm 3.29	32.69 \pm 2.84
Number/mm ³	533 \pm 35	564 \pm 52	610 \pm 56	569 \pm 41	778 \pm 103	1514 \pm 210
NON-E, EAC-RFC %	11.79 \pm 1.06	11.65 \pm 1.07	20.49 \pm 2.08	14.07 \pm 1.54	26.06 \pm 2.38	15.53 \pm 2.44
Number/mm ³	257 \pm 26	268 \pm 35	424 \pm 54	342 \pm 40	936 \pm 153	671 \pm 87

All results shown as mean \pm SE.

ficant differences in percentage or absolute number of any cell type in controls, biopsied controls, or patients with treated coeliac disease.

In untreated coeliac disease, both percentage ($P < 0.001$) and absolute numbers ($P < 0.01$) of E-RFC were significantly lower than in controls, and both percentage ($P < 0.001$) and absolute numbers ($P < 0.005$) of unclassified cells were significantly higher than in controls. The percentage of EAC-RFC was also significantly higher than in controls ($P < 0.05$) but there was no significant difference in absolute numbers.

Results were available in 10 patients with coeliac disease before and after treatment (Figure). Using the Wilcoxon matched-pairs signed-ranks test, percentage and absolute E-RFC were significantly higher ($P < 0.01$, $P < 0.05$) and the percentage of unclassified cells significantly lower ($P < 0.05$) after treatment. Absolute numbers of unclassified cells fell in eight out of 10 patients after treatment but the difference did not reach significance. Neither percentage nor absolute EAC-RFC numbers were significantly different after treatment.

Peripheral blood lymphocyte subpopulations in treated coeliacs with the blood film features of hyposplenism and asplenic controls are also shown in Table 2. Although treated coeliacs with hyposplenism had significantly lower percentage E-RFC than other treated coeliacs ($P < 0.002$), since the total lymphocyte count was higher in those with hyposplenism, absolute numbers of E-RFC were not significantly different. Whereas percentage EAC-RFC were not significantly different in the two groups, in those

with hyposplenism absolute numbers were just significantly higher ($P < 0.05$). However, both percentage ($P < 0.001$) and absolute numbers ($P < 0.001$) of unclassified cells were significantly higher in treated coeliacs with hyposplenism than in other treated coeliacs. There was no significant difference between percentage E-RFC or EAC-RFC in asplenic controls and treated coeliacs with hyposplenism, but the percentage of unclassified cells was significantly higher ($P < 0.02$) in the coeliacs. Since total lymphocyte counts were higher in the asplenic controls than in the coeliacs with hyposplenism, higher absolute numbers of all cell types might be expected in the asplenic controls, and were found for E-RFC and EAC-RFC, but, notwithstanding this, the number of unclassified cells was higher in the coeliacs with hyposplenism than in the asplenic controls, though the difference was not significant.

Discussion

In interpreting our results, cells forming spontaneous rosettes with sheep red blood cells at 4°C were considered to be T cells and complement receptor bearing cells were considered to be mainly B cells, although any monocytes which were undetected and some lymphocytes capable of antibody dependent cell mediated cytotoxicity (K cells) would also form EAC rosettes (Schlossman and Chess, 1976). Cells forming neither E nor EAC rosettes, unclassified cells, probably represent a heterogeneous subpopulation, some of which are true null cells.

Our results suggest that in untreated coeliac disease

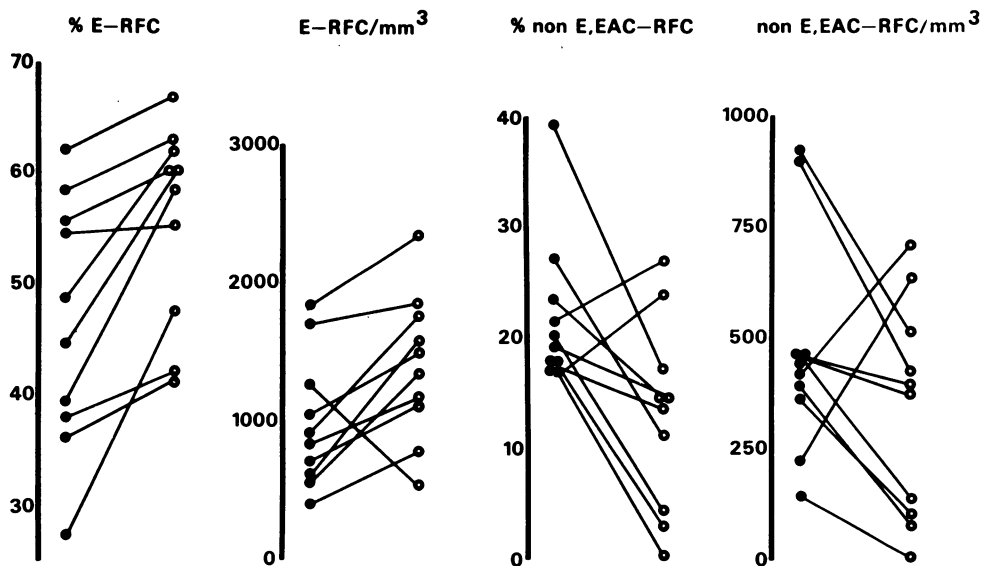


Figure Percentage and absolute numbers of E-RFC and non-E non-EAC-RFC in the peripheral blood of 10 coeliac patients before (●) and after (○) at least six months on a gluten free diet.

there is T cell depletion in the peripheral blood, but the total lymphocyte count is maintained by an increase in non-E non-EAC rosette forming cells; these abnormalities return towards normal after treatment in patients without splenic atrophy.

The finding of T cell depletion in the peripheral blood confirms the observations of O'Donoghue *et al.* (1976) and might explain impaired blast transformation with non-specific mitogens (Scott and Losowsky, 1976; Blecher *et al.*, 1969; Asquith, 1974), and the association of malignancy and coeliac disease (Austad *et al.*, 1967; Harris *et al.*, 1967). Scott and Losowsky (1976) have shown that impaired lymphocyte responses to phytohaemagglutinin in untreated coeliac disease improve after treatment with a gluten free diet; this may be related to increase in T cell numbers. However, malignancy may develop in treated coeliac patients, in whom T cell numbers are presumably normal, suggesting that the relationship of T cell numbers to the risk of development of malignancy is not direct. Perhaps the long-term depletion of T cells during the period of time when patients are untreated increases the risk of malignancy developing later, in spite of subsequent return of lymphocyte subpopulations to normal. Alternatively, treated patients who develop malignancy may not have achieved normal lymphocyte subpopulations; further study is necessary to clarify this.

T cell depletion in the peripheral blood in untreated coeliac disease might be secondary to in-

creased sequestration of lymphocytes in the small bowel or loss into the gut lumen (Douglas *et al.*, 1976). Whether the majority of lymphocytes sequestered or lost are T cells is not known, although evidence suggests that interepithelial lymphocytes are mainly T cells (Guy Grand *et al.*, 1974; Meuwissen *et al.*, 1976). However, it has been calculated (Weiden *et al.*, 1972) that chronic gastrointestinal loss of lymphocytes would have a greater effect on peripheral blood concentrations of long-lived circulating cells, as most T cells are thought to be, than on short-lived cells, as most B cells are thought to be, and this too might contribute to T rather than B cell depletion in the peripheral blood.

If the abnormalities of lymphocyte subpopulations in the peripheral blood in untreated coeliac disease are a secondary effect of lymphocyte loss, similar immunological abnormalities should be found in other illnesses associated with chronic gastrointestinal loss of lymphocytes. In intestinal lymphangiectasia there is increased lymphocyte loss (Weiden *et al.*, 1972) and it has been shown that peripheral blood lymphocytes have impaired *in vitro* transformation to non-specific mitogens, and there is an increased incidence of malignancy in this condition (Waldmann *et al.*, 1972), features which would be compatible with T cell depletion.

Furthermore, there may be an analogy between patients with chronic gastrointestinal loss of lymphocytes and animals with large losses of lymphocytes due to experimental chronic thoracic duct drainage.

Bohs *et al.* (1976) have shown that thoracic duct drainage in sheep causes little change in total peripheral blood lymphocyte counts, but marked alterations in blood lymphocyte subpopulations consisting of decrease in the numbers of T cells and increase in the numbers of cells not identified by T or B lymphocyte markers. Fish *et al.* (1970) have shown that chronic thoracic duct drainage in calves produces lymphoreticular atrophy, and their description of histological changes in the spleen is similar to those of splenic atrophy in coeliac disease. Thus, chronic lymphocyte loss from the gastrointestinal tract may contribute to the development of splenic atrophy in coeliac disease.

The replacement of T cells with increased numbers of non-E non-EAC rosetting cells in the blood in untreated coeliac disease or after other lymphocyte loss is unexplained, since the origin and significance of the latter cells is not known. True null cells which carry neither T nor B cell markers must form part of this subpopulation, but their relationship to T and B cells and monocytes is unresolved. It has been suggested that some null cells are immature T cells, since they can be induced to develop T cell marker characteristics on treatment with thymosin (Bach, 1976; Thomas *et al.*, 1976). In association with general reticuloendothelial atrophy, abnormalities in the thymus may occur, causing decreased ability to confer T cell characteristics. Some cells which do not form E or EAC rosettes may be immature B cells, or even cells of the monocyte line, without phagocytic or enzymatic activity or complement receptors. Alternatively, adherence of immune complexes to the surface of lymphocytes might prevent rosette formation.

Peripheral blood lymphocyte subpopulations in treated coeliacs with splenic atrophy differ from those of other treated patients. The increase in total peripheral blood lymphocytes after splenectomy is well documented, although not well understood, and this accounts for some of the differences. The measurement of lymphocyte subpopulations in the post-splenectomy control group gives an indication of the effects of splenectomy only, but the coeliacs with splenic atrophy differ from this group in that they may have some residual splenic function, and they may also have general reticuloendothelial atrophy (McCarthy *et al.*, 1966). The finding of lower total lymphocyte counts in the coeliacs with splenic atrophy than in splenectomised controls may reflect residual splenic function; the finding of increased numbers of non-E non-EAC rosetting cells, in spite of the lower total lymphocyte counts, might be partly due to impaired conversion of null cells to other forms perhaps owing to changes in the thymus, in association with general reticuloendo-

thelial atrophy.

It is well known that some lymphocytes carry more than one marker and within any simple classification there are subsets of cells of different types. We suggest that further studies of lymphocyte subpopulations in coeliac disease using different techniques may provide helpful information in understanding the mechanisms and manifestations of the disease.

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