Immune status in Crohn's disease¹

3. Peripheral blood B lymphocytes, enumerated by means of F(ab)₂-antibody fragments, Null and T lymphocytes

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SUMMARY In the peripheral blood of patients with Crohn's disease (CD) the numerical distribution of the three major B lymphocyte subsets was determined by the identification of surface immunoglobulins using F(ab)₂-antibody fragments. T cell counts were also obtained and the number of null cells was calculated. Twenty-eight patients with Crohn's disease including 14 patients with previously untreated and very short-standing disease (group CD 1) and 14 patients with long-standing and/or previous drug treated disease (group CD 2) were compared with 28 sex and age-matched normals as well as with 13 patients with acute inflammatory bowel disease (group D). Patients in group D and inactive patients of group CD 1 showed a significant absolute lymphocytosis due to an increase in both the three B cell subsets and the T cells, without changes in the null cells. While the proportion of T cells was normal, there was a significant relative B lymphocytosis and a relative null cytopenia in these patients. Active CD 1 patients, however, showed significantly lower absolute lymphocyte and T cell numbers. In group CD 2, there was a significant absolute lymphopenia caused by an equal decrease in B and T cells. Highly active CD 2 patients showed higher absolute null cell counts than inactive patients. With increasing disease duration there was a significant decrease of the relative and absolute B cell concentrations. The data obtained suggest that T and B cell populations in the peripheral blood are reduced in certain patients with Crohn's disease and that this occurs secondarily to activity of disease, chronicity of disease, and the effects of therapy.

In patients with Crohn's disease (CD) the appearance of circulating immune complexes (Hodgson *et al.*, 1977), increased catabolism of immunoglobulins (Ig; Jensen *et al.*, 1970), and the transferability of an *in vitro* cytotoxic effect against colonic epithelial cells onto peripheral blood lymphocytes of normal subjects by means of Ig containing CD-serum fractions (Shorter *et al.*, 1971), suggest an involvement of the humoral immune response in the pathogenesis of Crohn's disease. However, controversy surrounds the numerical distribution of the cellular carriers of this response, the B lymphocytes, in the peripheral blood in Crohn's disease (Strickland *et al.*, 1974;

Høj and Sørensen, 1976; Thayer *et al.*, 1976b; Sachar *et al.*, 1976; Auer *et al.*, 1978b).

It has recently been established that human peripheral blood lymphocytes bearing membrane-incorporated surface membrane immunoglobulin (SmIg)—that is, B lymphocytes—comprise only about one-third of peripheral blood lymphocytes bearing easily detectable surface immunoglobulin (Winchester *et al.*, 1975; Lobo and Horwitz, 1976; Sedlacek *et al.*, 1976). Binding of both cytophilic human Ig (Lobo and Horwitz, 1976; Winchester *et al.*, 1976; Sedlacek *et al.*, 1976) and complexed heterologous conjugated antibody (Winchester *et al.*, 1975; Sedlacek *et al.*, 1976; Alexander and Sander, 1977) via the Fc part to Fc receptor positive, SmIg negative lymphoid cells will falsely increase the B cell counts. Furthermore, autoantibodies to lymphocytes previously demonstrated in Crohn's disease (Lancet, 1976) might mimic SmIg (Winchester *et al.*,

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1974).

Thus, the validity of the conflicting data on the distribution of B cells in Crohn's disease can hardly be assessed. Therefore, and because improved techniques for determination of B cells are now available, we re-evaluated peripheral blood B lymphocytes in this disease, using F(ab)₂-antibody fragments and preincubation of cells at 37°C (Winchester *et al.*, 1974, 1975; Lobo and Horwitz, 1976; Sedlacek *et al.*, 1976; Winchester *et al.*, 1976). As T lymphocytes were simultaneously determined, the fraction of peripheral mononuclear blood cells lacking both surface characteristics could be calculated, and referred to as null cells (Winchester *et al.*, 1975; Schlossmann and Chess, 1976).

The group of CD patients consisted of (1) a selected consecutive series of newly diagnosed patients who had short-standing disease and who had not received any specific therapy (group CD 1), and (2) a group of patients who had long-standing disease and were on drug treatment before being tested (group CD 2).

Methods

PATIENTS

At the time of testing all patients had been free from any drugs such as salicylazosulphapyridine (SASP), steroids, or azathioprine (AZA) for at least three weeks.

Twenty-eight patients (group CD) were studied in whom the disease had been diagnosed on clinical, radiological, and histological ($n = 15$) grounds. None of the patients had had previous bowel resections or had undergone any other surgery within the last six months. Disease activity was graded according to the Crohn's disease activity index (CDAI; Best *et al.*, 1976). Group CD as a whole comprised the subgroups CD 1 and CD 2. Group CD 1 consisted of 14 Crohn's disease patients whose disease was of short duration (mean ± 1 standard deviation [SD]: 12.7 ± 8.5 months) and who had never been treated by SASP, steroids, or AZA (age mean: 27.4 ± 11.1 years). Seven of the patients had mild disease (CDAI ≤ 150); seven had active disease (CDAI > 150). Group CD 2 consisted of 14 patients, whose disease had already been treated by drugs and/or was of long duration (mean ± 1 SD: 61.3 ± 31.8 months). Eleven patients had received SASP and eight of them were also on steroids. The age mean was 33.6 ± 10.2 years. The CDAI of six patients was ≤ 150 , of eight patients > 150 .

CONTROLS

Group D comprised 13 patients suffering from salmonella gastroenteritis ($n = 6$) including typhoid

fever ($n = 1$), non-specific acute gastroenteritis ($n = 3$), bacterial overgrowth ($n = 2$), and acute gastroenteritis not otherwise specified ($n = 2$). The age mean was 45.9 ± 14.7 years.

Groups NCD and ND consisted of sex and age-matched normal control subjects who were tested simultaneously with the corresponding patients (age mean ± 1 SD, NCD 1: 26.7 ± 4.4 years, NCD 2: 29.4 ± 7.3 years, and ND: 40.0 ± 19.0 years).

TECHNIQUES

Separation of mononuclear cells

Heparinised venous peripheral blood was incubated with poly-L-lysine coated carbonyl iron spherules for 90 minutes at 37°C under constant rotation. Thereafter, mononuclear cells were isolated by means of a Ficoll gradient at 20°C as outlined previously (Auer *et al.*, 1978b).

SmIg-positive lymphocytes (B lymphocytes)

Mononuclear cells were suspended in RPMI 1640 containing HEPES buffer (25 mM) and heat-inactivated foetal calf serum (FCS, 10%) and were incubated overnight at 37°C in 5% CO₂-95% air atmosphere. Viability (Trypan blue exclusion) thereafter was $\geq 98\%$. Dilutions of the fluorescein isothiocyanate (FITC)-F(ab)₂ fragments³ (Sedlacek *et al.*, 1976) of rabbit IgG of hyperimmune sera monospecific against human IgM (F/P 1.5, total protein 22 mg/ml, antibody content about 6 mg/ml), IgG (F/P 1.0, total protein 32 mg/ml, antibody content about 3.5 mg/ml) and IgA (F/P 1.0, total protein 28 mg/ml, antibody content about 2.0 mg/ml) were made in PBS, pH 7.8, supplemented with 5% foetal calf serum. 0.1 ml lymphocytes ($20-30 \times 10^6$ /ml in RPMI 1640 plus HEPES [25 mM], NaN₃ [10 mM], and foetal calf serum [10%]), were sedimented, re-suspended in the FITC-F(ab)₂-fragments, and incubated for 30 minutes at 4°C. Cells were washed three times in RPMI 1640 (containing NaN₃ and 5% foetal calf serum) at 4°C. Cell suspensions were examined as described previously (Auer *et al.*, 1978b). At least 500 cells were evaluated. Residual macrophages were recognised by surface bound or ingested iron particles. The sum of lymphocytes staining for the three B cell subsets was used as the value for total B cells.

Lymphocytes binding sheep red blood cells

T lymphocytes were determined as described previously (Auer *et al.*, 1978b) with the slight modification of a final three hour rather than one hour incubation.

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Table 1 Lymphocyte subpopulations in Crohn's disease and controls (mean $\pm$ SD)

	Crohn's disease							Diseased controls				
	CD		NCD	CD 1		NCD 1	CD 2		NCD 2	D	ND	
Lymphocytes no./mm ³	1858 ^a ± 788	<0.1 ⁺	2296 ± 728	2202 ^{b, c} ± 785		2253 ± 836	1515 ^{c, d} ± 649	<0.05	2339 ± 632	2971 ^{a, b, d} ± 840	<0.05	2472 ± 542
%	4.8 ± 2.8		4.3 ± 2.3	6.0 ^e ± 3.4	<0.05	4.0 ± 1.9	3.4 ^e ± 1.0	<0.1 ⁺	4.5 ± 2.0	5.4 ± 2.9		4.2 ± 1.5
B cells												
no./mm ³	88 ^g ± 65		92 ± 46	101 ^h ± 75		86 ± 43	54 ^{f, h} ± 29	<0.05	98 ± 47	144 ^{f, g} ± 58		101 ± 48
%	72.5 ± 7.7		73.8 ± 8.5	73.7 ± 5.0		73.6 ± 8.7	69.8 ± 9.7		74.1 ± 8.5	70.4 ± 9.5		70.5 ± 7.9
T cells												
no./mm ³	1339 ^j ± 576		1718 ± 626	1622 ^k ± 592		1736 ± 669	1057 ^{i, k} ± 408	<0.05	1698 ± 603	2093 ^{i, j} ± 684	<0.05	1721 ± 374
%	22.9 ± 7.3		20.3 ± 7.6	20.5 ± 4.8		21.2 ± 8.2	25.5 ± 8.7		19.2 ± 7.1	21.2 ± 5.2		20.4 ± 6.8
Null cells no./mm ³	473 ± 282		443 ± 244	454 ± 227		473 ± 290	418 ± 341		410 ± 183	590 ± 165		489 ± 185

a: CD vs. D, $P < 0.001$. b: CD 1 vs. D, $P < 0.05$. c: CD 1 vs. CD 2, $P < 0.005$. d: CD 2 vs. D, $P < 0.0005$. e: CD 1 vs. CD 2, $P < 0.05$. f: CD 2 vs. D, $P < 0.0005$. g: CD vs. D, $P < 0.05$. h: CD 1 vs. CD 2, $P < 0.01$. i: CD 2 vs. D, $P < 0.0005$. j: CD vs. D, $P < 0.005$. k: CD 1 vs. CD 2, $P < 0.05$. + < 0.1 $\pm$ 0.1 > $P > 0.05$.

Null cells

The proportions of Null cells were calculated by subtracting the sum of the percentage of B plus T cells from 100.

There is much debate whether the absolute numbers of lymphocyte subsets in mononuclear cell suspensions obtained by Ficoll gradient are representative of those in the peripheral blood. Nevertheless, we calculated absolute numbers of B, Null, and T cells per mm³ blood by relating the proportion of these cells to the absolute lymphocyte counts/mm³ peripheral blood, so that the data of this study could be compared with those of other workers.

Statistics

Results are given as mean ($\bar{x}$) $\pm$ 1 standard deviation (SD) unless otherwise stated. The various patients and the corresponding simultaneously tested normal controls were compared by Wilcoxon's matched pairs signed rank test (WMPSR). All other significances of difference were tested by Wilcoxon's two sample rank test (U-test). Correlation studies were done using Spearman's rank correlation test. All tests were two-tailed.

Results

LYMPHOCYTES

The means of the absolute lymphocyte counts are given in Table 1. They were virtually normal in group CD 1, but significantly decreased in CD 2 compared with normal subjects, group CD 1, and

diseased controls, the latter showing a significant lymphocytosis.

PROPORTIONS OF LYMPHOCYTE SUBPOPULATIONS

B lymphocytes

In group CD 1 there was a significant relative total B lymphocytosis ($n = 13$), whereas group CD 2 ($n = 12$) showed a strong tendency, which barely missed significance, to subnormal proportions of B cells (Table 1). This led to a significant difference in the percentages of total B cells between group CD 1 and CD 2.

The significant relative total B lymphocytosis in group CD1 and the decrease in CD 2 was caused by an approximate equal increase and decrease, respectively, of each of the three major B cell subsets (Fig. 1). The proportion of B cells bearing SmIgM was significantly higher in CD 1 than in CD 2 ($P < 0.05$).

T lymphocytes and Null cells

No differences were observed except for a slight T cell decrease in CD 2 (Table 1).

ABSOLUTE CONCENTRATIONS OF LYMPHOCYTE SUBPOPULATIONS

B lymphocytes

Total absolute B lymphocyte concentrations were significantly decreased in group CD 2 compared with

Fig. 1 Proportions of the three major B lymphocyte subsets in the various groups of Crohn's disease patients and controls. Dotted area indicates mean $\pm$ 1 SD of the corresponding sex and age-matched normal controls. Thick horizontal bars: mean of the indicated patient group. Thin horizontal bars: $\pm$ 1 SD. WMPSR: Wilcoxon's matched pairs signed rank test.

Fig. 2 Absolute concentrations of the three major B lymphocyte subsets in the various groups of Crohn's disease patients and controls. Dotted area indicates mean $\pm$ 1 SD of the corresponding sex and age-matched normal controls. Thick horizontal bars: mean of the indicated patient group. Thin horizontal bars: $\pm$ 1 SD. WMPSR: Wilcoxon's matched pairs signed rank test.

group NCD 2, group D (n = 7), and also group CD 1 (Table 1). Group D had clearly increased B cell concentrations; however, no statistical significance was achieved, perhaps because of the small

numbers of patients involved (Table 1). All these changes in total B lymphocytes were due to approximately equal changes in all three major B cell subsets (Fig. 2).

Fig. 3 Absolute numbers of T and B lymphocytes in inactive patients with Crohn's disease and controls. Dotted area: mean $\pm$ 1 SD of the corresponding sex and age-matched normals. Thick horizontal bars: mean of the indicated patient group. Thin horizontal bars: $\pm$ 1 SD. WMPSR: Wilcoxon's matched pairs signed rank test.

Table 2 Influence of disease activity (CDAI) on B, Null, and T lymphocytes in group CD 1

	CDAI $\leq$ 150				CDAI > 150			
	NCD 1 n = 7		CD 1 n = 7		CD 1 n = 7		NCD 1 n = 7	
B lymphocytes								
%	3.35 $\pm$ 2.21		6.6 $\pm$ 4.7		4.63 $\pm$ 1.51		5.35 $\pm$ 1.74	
no./mm ³	74 $\pm$ 45	< 0.05	154 $\pm$ 95	< 0.1	99 $\pm$ 41		87 $\pm$ 34	
SmIgM								
%	1.21 $\pm$ 1.38		3.99 $\pm$ 2.74		2.89 $\pm$ 1.43		3.66 $\pm$ 1.45	
no./mm ³	49 $\pm$ 29	= 0.05	94 $\pm$ 54		59 $\pm$ 24		58 $\pm$ 25	
SmIgG								
%	0.73 $\pm$ 0.82		1.8 $\pm$ 1.3		1.03 $\pm$ 0.83		1.09 $\pm$ 0.43	
no./mm ³	15 $\pm$ 17	< 0.05	41 $\pm$ 27		27 $\pm$ 29		19 $\pm$ 9	
SmIgA								
%	0.30 $\pm$ 0.11	= 0.05	0.85 $\pm$ 0.76		0.43 $\pm$ 0.26		0.56 $\pm$ 0.43	
no./mm ³	7 $\pm$ 2	< 0.1	20 $\pm$ 17		12 $\pm$ 11		9 $\pm$ 8	
Null lymphocytes								
%	25.08 $\pm$ 4.85		19.87 $\pm$ 4.4		20.83 $\pm$ 5.5		18.07 $\pm$ 9.32	
no./mm ³	578 $\pm$ 262	< 0.05	537 $\pm$ 216		383 $\pm$ 227		381 $\pm$ 300	
T lymphocytes								
%	70.02 $\pm$ 7.42		73.14 $\pm$ 4.49		73.01 $\pm$ 5.9		77.65 $\pm$ 8.44	
no./mm ³	1610 $\pm$ 256	< 0.05	1952 $\pm$ 489	< 0.05	1299 $\pm$ 513	0.1 $\geq$ P > 0.05	1832 $\pm$ 915	

T lymphocytes

In CD 1 the absolute T cell counts did not differ significantly from NCD 1 and were not significantly lower than in group D (n = 13), which exhibited a significant T lymphocytosis (Table 1). In contrast, a significant absolute T lymphopenia was present in CD 2 (Table 1).

INFLUENCE OF DISEASE PARAMETERS

Disease activity

Inactive CD 1 patients (CDAI $\leq$ 150) showed a significant absolute lymphocytosis (CD 1 [2641 $\pm$

599] vs. NCD 1 [2283 $\pm$ 481], $P < 0.05$) which was caused equally by all B cell subsets and the T lymphocytes (Fig. 3; Table 2). This pattern was similar to that of group D (Figs. 3a and b; Table 1). While the proportions of T cells were normal in these CD 1 patients, the proportions of B cells were significantly increased at the expense of Null cells (Table 2). However, in active CD 1 patients (CDAI > 150) the absolute lymphocyte ($P < 0.05$), T cell ($P < 0.05$), and absolute B cell counts were lower than in the inactive patients of group CD 1 (Table 2).

Serum immunoglobulin levels in these inactive CD 1 patients were significantly above normal for

Table 3 Serum immunoglobulin levels in active and inactive patients of group CD 1

	Immunoglobulin concentrations (mg%) [*]			
	IgM	IgG	IgA	Total
Group CD 1				
CDAI $\leq$ 150	197 $\pm$ 27†	1444 $\pm$ 156‡	251 $\pm$ 32	1925 $\pm$ 208‡
CDAI > 150	164 $\pm$ 23	1318 $\pm$ 117	294 $\pm$ 88	1855 $\pm$ 167‡
Normal	139 $\pm$ 12	1198 $\pm$ 26	209 $\pm$ 16	1546 $\pm$ 39

^{*}Mean $\pm$ standard error. †CD 1 vs. N, $P = 0.05$.

‡CD 1 vs. N, $P < 0.05$.

Table 4 Influence of disease duration on B lymphocytes in Crohn's disease

	Disease duration (months)			
	≤ 12 $n = 7$		≥ 49 $n = 7$	
B lymphocytes				
%	5.82 $\pm$ 2.33	< 0.05	3.51 $\pm$ 1.31	
no./mm ³	111 $\pm$ 55	< 0.05	55 $\pm$ 31	
SmIgM				
%	3.72 $\pm$ 1.70	$= 0.05$	1.98 $\pm$ 1.05	
no./mm ³	67 $\pm$ 34	< 0.05	33 $\pm$ 24	
SmIgG				
%	1.41 $\pm$ 0.78		0.98 $\pm$ 0.57	
no./mm ³	30 $\pm$ 20		14 $\pm$ 9	
SmIgA				
%	0.66 $\pm$ 0.38		0.54 $\pm$ 0.3	
no./mm ³	12 $\pm$ 8		7 $\pm$ 5	

IgG, IgM, and total Ig; however, they were only slightly above those of the active CD 1 patients (Table 3). In active patients (CDAI > 150) of groups CD 2 and CD, the proportions of Null cells were significantly raised (CD 2 [28.8 $\pm$ 11.71%] vs. NCD 2 [17.34 $\pm$ 7.95%], $P < 0.05$; CD vs. NCD, $P < 0.05$). The absolute Null cell counts, however, were not significantly different from the control group (CD 2 [521 $\pm$ 465] vs. NCD 2 [345 $\pm$ 200]). CD 2 patients with severe, active disease (CDAI > 200) had significantly higher absolute Null cell counts than CD 2 patients with quiescent disease (CDAI $\leq$ 110; $P < 0.05$).

Disease duration

Both relative and absolute B lymphocyte concentrations decreased significantly with increasing disease duration (Table 4).

Site of disease

Significantly lower proportions of T cells were found in Crohn's disease patients with colonic disease than in those with the disease restricted to the ileum ($P < 0.01$). In group CD 2 the proportions of Null cells were significantly higher in patients whose disease involved the colon than in those with disease restricted to the ileum ([27.89 $\pm$ 10.8%] vs. [18.1 $\pm$ 0.42%], $P < 0.05$).

Discussion

The present study has shown that patients with inactive Crohn's disease of short duration and without previous treatment have a relative and absolute B lymphocytosis. This is due to an increase in each of the three subsets of B cells. The absolute T cell counts were likewise increased in this group. Similar changes were also observed in a miscellaneous group of patients with gastrointestinal infections (group D) but were in marked contrast with patients with active Crohn's disease (short history, no treatment) who had lower T and B cell counts.

As the rise in T and B cells in the inactive, untreated Crohn's disease patients is similar to that in the disease control group, it is suggested that there may be continuing immunological activity within the bowel wall even during a clinical remission. The rise in B and T cells in these patients (CD 1) may be due to systemic immune challenge or may be due to an increased output of cells from the gut associated lymphoid tissue following stimulation with intestinal antigens (Craig and Cebra, 1971; Robertson and Cebra, 1976). Either of these possibilities could result from stimulation by an aetiological agent or from an increased absorption of antigens through the intestinal epithelium secondary to inflammation and increased permeability. That such absorption occurs in inflammatory bowel disease is suggested by the presence of endotoxin in peripheral blood (Auer *et al.*, 1978b) and by increased titres of antibodies to bacterial and dietary antigens (Thayer *et al.*, 1969; Falchuck and Isselbacher, 1976; Schüssler *et al.*, 1976; Wensinck, 1976).

The fall in absolute T and B cell numbers in patients in group CD 1 who had active disease might be due to antigen-induced sequestration of lymphocytes in the bowel wall and/or to increased luminal loss from the inflamed gut (Douglas *et al.*, 1976). The presence of an increased number of faecal lymphocytes and of B and T cells within the inflamed gut (Strickland *et al.*, 1975; Baklien and Brandtzaeg, 1976) would support both hypotheses but direct evidence is lacking.

Similar mechanisms may explain the reduced sub-populations of lymphocytes seen in the patients in group CD 2 who had longer histories than patients in group CD 1, although it is likely that the cell populations were also influenced by the effects of therapy (Thayer *et al.*, 1976a; Cooper *et al.*, 1977). Nevertheless, the present data suggest that the sub-normal absolute counts of T and B cells in patients with long-standing disease are secondary to either the disease process itself and/or the effects of therapy and they are not seen in the early stages of Crohn's

disease. This is supported by the finding that peripheral blood lymphocytes rise to normal levels after resection of diseased bowel (Stickland *et al.*, 1975) and by the results of *in vitro* cell-mediated immune responses in Crohn's disease patients (Auer *et al.*, 1978a). Absolute B and T lymphopenia in patients similar to those in group CD 2 has also been reported by Høj and Sørensen (1976). However, the use of complete antibody conjugates to detect B cells in this latter study makes direct comparison with the present results difficult.

Finally, our data indicate that a relative expansion of Null cells occurs in active patients with long-standing disease. How this relates to the *in vitro* cytotoxicity to colon epithelial cells, claimed to be mediated by a subset of Null cells (Stobo *et al.*, 1976), is unclear at present.

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