Lymphocyte subpopulations of intestinal mucosa in inflammatory bowel disease

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SUMMARY Lymphocyte subpopulations in peripheral blood (PBL) and intestinal mucosa (IML) of 10 patients with inflammatory bowel disease (IBD) were compared with those of 11 non-IBD controls. PBL were separated on Ficoll/Hypaque gradients, and IML were isolated by incubations in dithiothreitol, EDTA, and collagenase. These methods yielded cells of good viability and with intact HLA A and B-antigens. T-cells, identified by neuraminidase-treated sheep RBC rosettes and non-specific esterase staining, comprised approximately 91% of the IML from normal mucosa of all groups. B-cells, identified by erythrocyte-antibody-complement rosettes and surface immunoglobulins, were only 7% of these IML populations. Cell yields were two-fold or more greater from abnormal IBD mucosa, with T-cells ranging from 55 to 95% and B-cells from 2 to 36%. The percentage of Fc receptor bearing cells was low in all specimens. By these methods, T-lymphocytes predominated in intestinal mucosa of both IBD and non-IBD patients, but there is marked increase in the percentage of B-cells isolated from abnormal mucosa in IBD.

There is a growing body of evidence that lymphocytes play an important role in the pathogenesis of inflammatory bowel disease (IBD). Most studies supporting such a role have been conducted with peripheral blood lymphocytes, whereas the most important site of immunological reaction is likely to be in the gut itself. Accordingly, it is appropriate to define the subpopulations of intestinal lymphocytes and to investigate their role in the inflammatory response of IBD and other disorders.

Intestinal lymphocyte populations have been enumerated by immunofluorescent studies of frozen tissue sections and by the study of purified lymphoid cells isolated from intestinal resections. Immunofluorescent studies of frozen sections indicate a predominance of T-cells in epithelium and lamina propria in normal tissue and in unaffected bowel from patients with Crohn's disease but a preponderance of B-cells in all but the epithelial layer in areas of IBD.[1–4] These studies yielded semiquantitative results because precise morphological interpretation is difficult and a variable loss of diffusable immunoglobulin occurs in frozen sections.[5]

Recently, methods have been described for isolating lymphocytes from intestinal resections which permit more precise identification and quantification.[6–12] In studies of IBD, ileal T lymphocytes have ranged from 63% to 68·5% compared with 35·4% to 83% for controls, and affected colonic tissue contained 49% T-cells compared with 64% T-lymphocytes in controls.[13 14] B-cell populations were reciprocally increased in diseased tissue from both ileum and colon.

In the present study, we have examined subpopulations of lamina propria lymphocytes from patients with IBD and other disorders using both a stabilised erythrocyte rosetting technique and alpha-naphthyl acetate esterase staining to identify T-cells. Surface immunoglobulin and formation of erythrocyte-IgM antibody-complement rosettes (EAC) were used to identify B-cells, and erythrocyte-antibody (EA) rosettes to identify Fc receptor bearing cells. We found a higher percentage of T-cells than previously reported in both normal and diseased mucosa, and a variable increase in B-lymphocytes in affected tissue.

Methods

PATIENTS AND SPECIMENS

Ten patients with IBD (seven Crohn's disease and three ulcerative colitis) and 11 non-IBD controls

Table 1   *Patient data and histopathology of specimens studied*

Patient no. and diagnosis	Age (yr)		Sex	Specimen—Histology			
				Ileum		Colon	
				Normal	Abnormal	Normal	Abnormal
IBD							
Crohn's disease	1	30	M	+			+
	2	30	F	+	+		
	3	23	M		+		
	4	24	F		+		
	5	41	M		+	+	
	6	29	F				+
Ulcerative colitis	7	24	M				+
	8	73	M				+
	9	28	F				+
	10	19	F	+			+
Non-IBD							
Large bowel cancer	1	47	M			+	
	2	50	M			+	
	3	56	F			+	
	4	57	F			+	
	5	68	F			+	
	6	75	F			+	
	7	76	F			+	
	8	81	F			+	
	9	64	M			+	
Cancer of bladder	10	48	M	+			
Neurogenic bladder	11	38	F	+			

(nine of whom had colorectal cancer) were studied (Table 1). Indications for resection in IBD patients were obstruction in three and intractable disease in the remaining seven, including relentless perianal disease in one, and perforation in another. All were taking steroids shortly before or at the time of operation. Intestinal resections were obtained immediately after surgical removal. Specimens measuring approximately 2×4 cm were placed directly into Eagle's Minimal Essential medium (MEM) containing 10% fetal calf serum (FCS), penicillin (10 000 U/ml), streptomycin (10 000 μg/ml), and chlortetracycline (5000 μg/ml). Specimens were examined by light microscopy and the histopathology from each patient is indicated in Table 1.

PERIPHERAL BLOOD LYMPHOCYTE
ISOLATION (PBL)
Lymphocytes were separated from heparinised blood on a Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, Md.) density gradient.[15]

INTESTINAL MUCOSAL LYMPHOCYTE
ISOLATION (IML)
A modification of the method of Bull and Bookman[10] was used. The mucosa was dissected from muscularis and washed in Hank's balanced salt solution, immersed in 1 mM dithiothreitol (DTT) for 10 minutes and then in calcium-magnesium free Hank's balanced salt solution. The washed pieces were incubated in 0·75 mM ethylene-diaminotetracetate (EDTA) for two 90 minute periods to remove surface epithelial cells. The exposed lamina propria was then incubated in type IV collagenase (Sigma Chemical Co, St Louis, Mo, Lot 55C 6810), 0·075 mg/ml MEM at 37°C with gentle stirring overnight. The lymphocyte suspension was washed twice and post-collagenase digestion debris was removed by filtration through a stainless steel wire mesh (300 size). The lymphocytes were separated by Ficoll-Hypaque density gradient and resuspended in Eagle's Minimal Essential medium fetal calf serum.

VIABILITY TESTING AND COUNTING
Viability was assessed by complete exclusion of 0·4% trypan blue. Lymphocytes were counted in a haemacytometer, monocytes being excluded by size and morphology.

T CELL IDENTIFICATION
Neuraminidase-treated sheep erythrocyte (EN) rosettes[16]
Washed sheep erythrocytes (Becton Dickinson & Co., Cockeysville, Md) were incubated with neuraminidase (Sigma), 0·16 μ/ml final concentration, for 40 minutes at 37°C. After incubation, the erythrocytes were washed three times with Hank's balanced salt solution and kept at 4°C until used. Lymphocytes were incubated with neuraminidase-treated erythrocytes at 37°C for 15 minutes, centrifuged at

50 g for 10 minutes, chilled for 20 minutes, and incubated overnight at 4°C. Cells were gently resuspended to avoid disrupting the rosettes, stained with 0·1% toluidine blue in 90% methanol, and the percentage of rosetting lymphocytes determined by counting 200 cells.

Alpha-naphthyl acetate esterase activity (ANAE)[17]
Cytocentrifuge (Shandon-Southern Instruments, Inc, Sewickly, Pa) preparations were made from resuspensions of the neuraminidase-treated sheep erythrocyte pellets. Slides were air dried for at least two hours and stained with ANAE in pararosaniline and sodium nitrite (Sigma) by overnight incubation. Slides were counterstained with 1% methyl green or 1% toluidine blue and examined for ANAE staining.
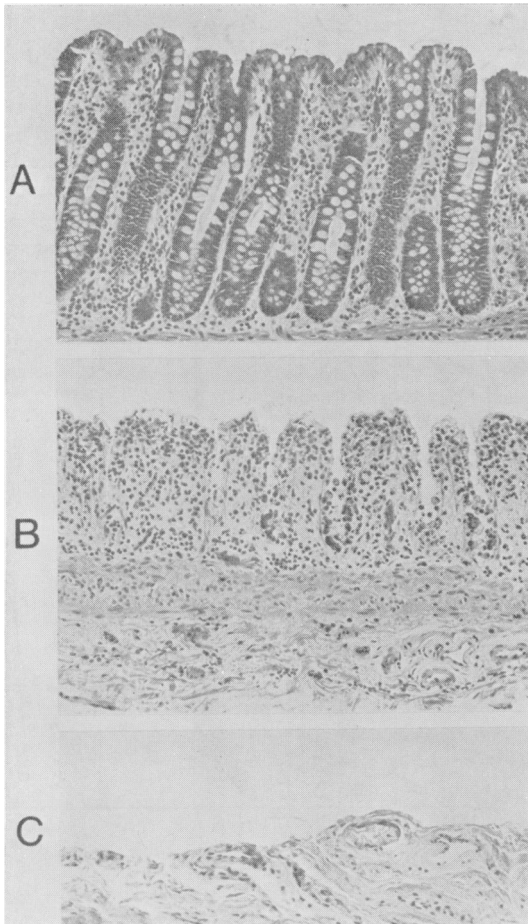
B CELL IDENTIFICATION
EAC rosettes[18]
To identify C3 receptor bearing lymphocytes, rosettes were prepared with sheep erythrocytes coated with a 1:75 dilution of IgM antibody (Cordis Laboratories, Miami, Fla). Fresh frozen mouse serum was used as the source of complement. Incubations were for 30 minutes at 37°C. Rosettes forming without addition of complement (E IgM) and with uncoated sheep erythrocytes (EU) served as controls.

Surface immunoglobulins
Surface immunoglobulins were detected using fluoresceinated antihuman polyvalent goat immunoglobulin with high fluorescein to protein ratio (Meloy Laboratories Inc, Springfield, Va). 4×10^5 lymphocytes were incubated with 0·1 ml of a 1:2.5 dilution of fluoresceinated immunoglobulin for 45 minutes at 4°C, resuspended in 1 ml Hank's balanced salt solution, centrifuged at 50 g for 10 minutes, resuspended in equal volumes of HBSS-2% paraformaldehyde in PBS for 15 minutes in the dark, centrifuged at 50 g for 10 minutes, and resuspended in PBS. Cells were centrifuged again and examined for surface fluorescence with a Leitz-Wetzlar fluorescence microscope fitted with an HBO 200 mercury vapor lamp.
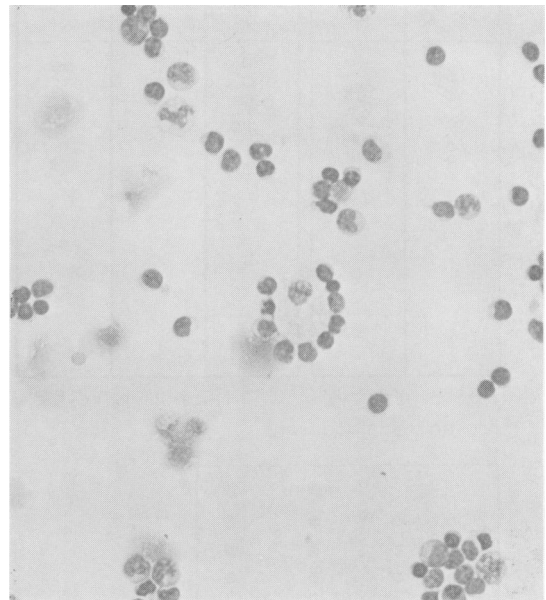

Fig. 1   *A. Colonic mucosa after dithiothreitol treatment. B. Colonic mucosa with epithelium stripped by incubation in EDTA. C. Muscularis after denudation of lamina propria by collagenase (original magnification × 100).*

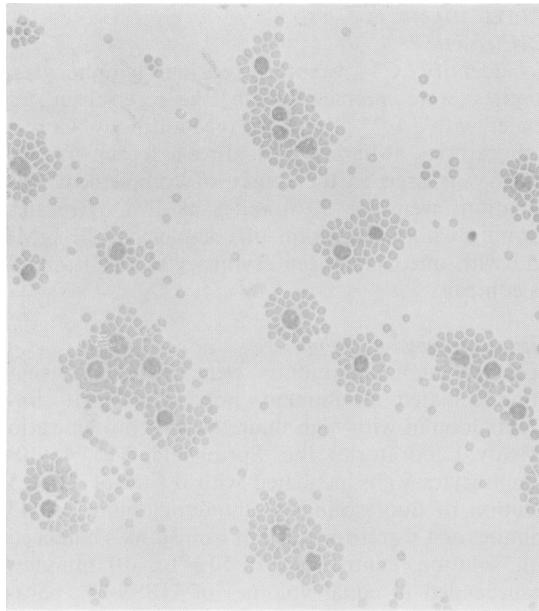Fig. 2   *Cytospin preparation of lamina propria mononuclear cells (original magnification × 250).*

Fig. 3 *EN rosettes of intestinal mucosa lymphocytes (original magnification × 250).*

EA ROSETTES

To identify Fc receptor bearing lymphocytes, rosettes were prepared by incubation with sheep erythrocytes coated with 7S (IgG) antibody (Cordis Labs.) at a 1:600 dilution for 30 minutes at 21°C.

HISTOCOMPATIBILITY ANTIGEN TESTING (HLA)

To further test the surface integrity of intestinal mucosal lymphocytes, HLA antigens were deter-

Table 2 *HLA antigens of intestinal mucosa lymphocytes (IML) and peripheral blood lymphocytes (PBL) from five additional patients undergoing bowel resection*

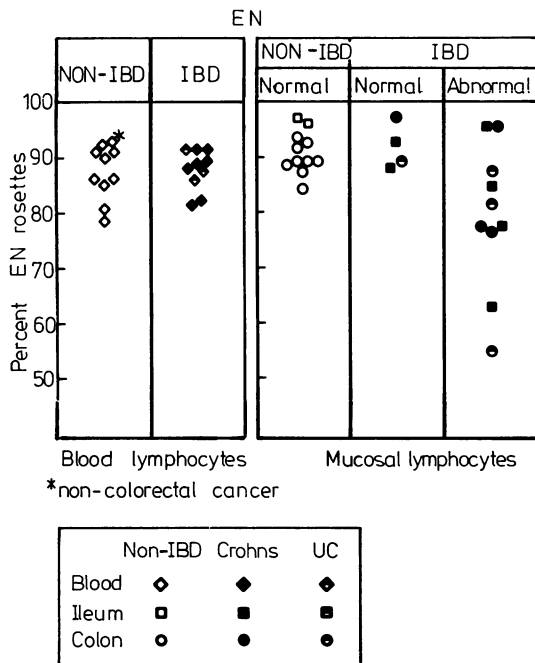| Patient		A Locus	B Locus
1	IML	2, W24	5, 7.
	PBL	2, W24	5, 7.
2	IML	3, 11	W35, –
	PBL	3, 11	W35, –
3	IML	3, 29	7, 17
	PBL	3, 29	7, 17
4	IML	1, 3	5, 15
	PBL	1, 3	5, 15
5	IML	1, W24	5, 12
	PBL	1, W24	5, 12

Fig. 4 *Percentages of lymphocytes from blood and normal and diseased intestinal mucosa identified as T-cells by EN rosette formation.*
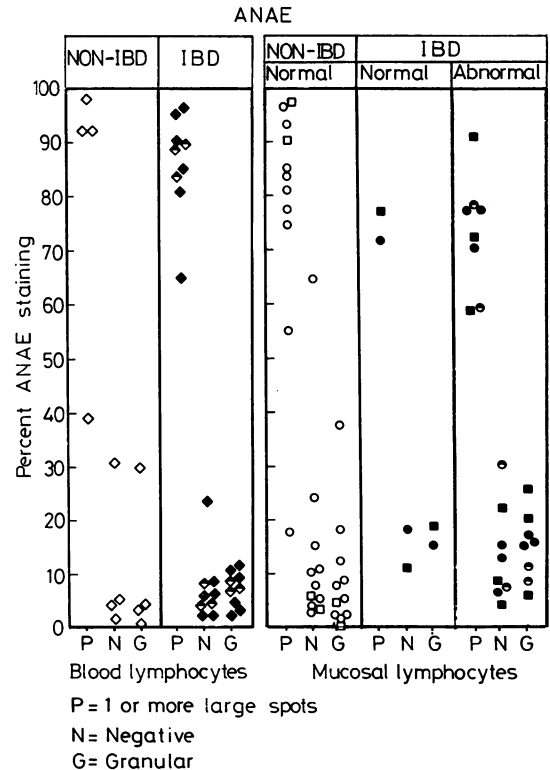

Fig. 5 *Alpha naphthyl acetate esterase staining of lymphocytes of blood and normal and diseased intestinal mucosa. Symbols as in Fig. 4.*
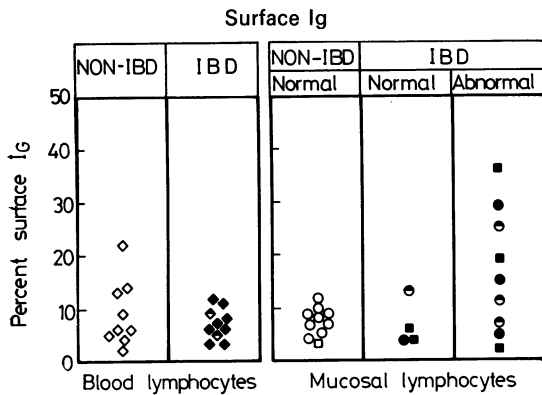
Surface Ig

Fig. 6   *Percentages of B-lymphocytes identified by surface immunoglobulin in blood and normal and diseased intestinal mucosa. Symbols as in Fig. 4.*
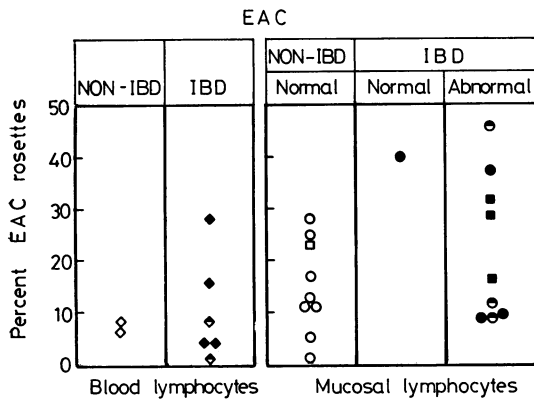
EAC

Fig. 7   *Percentages of blood and mucosal lymphocytes demonstrating erythrocyte-antibody-complement rosetting. Symbols as in Fig. 4.*

mined on peripheral blood lymphocytes and corresponding IML from five additional patients by the Amos modification of the microlymphocytotoxicity technique.[19]

Results

The histology of an intestinal specimen at various stages in the isolation procedure is shown in Fig. 1 and a cytospin preparation of isolated lamina propria mononuclear cells is shown in Fig. 2. The total numbers of IML isolated from 2 to 8 g of tissue varied from 2 to 200×10^6 and tended to be higher with inflamed mucosa. Mean viabilities exceeded 90%. The HLA antigens in 5 IML suspensions were identical to those of the mucosal lymphocytes (Table 2). Macrophages, plasma cells, mast cells, eosinophils, neutrophils, and epithelial cells were seen in small numbers, as were naked nuclei and other tissue debris.

Percentages of T, B and Fc receptor bearing lymphocytes identified in intestinal mucosal preparations are shown in Figs. 4–10. In normal tissue from IBD and non-IBD resections, T-lymphocytes predominated, averaging 91·6% and 90·6% respectively by the EN rosetting technique (Figs. 3 and 4). The less specific ANAE staining results supported this finding (Fig. 5). Correspondingly low numbers of B-cells were identified in the IBD and non-IBD specimens by SIg, 6·8% and 7·4% respectively (Fig. 6). The slightly higher percentages of EAC rosetting lymphocytes (Fig. 7) are explained in part by non-specific rosetting in control tests (EIgM and EU) seen with IML (Figs. 8 and 9). Percentages of lymphocytes with Fc receptors were uniformly low (Fig. 10).

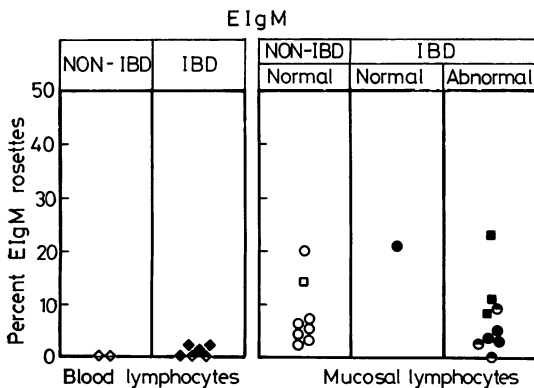In abnormal IBD tissue, T-lymphocytes also pre-

E IgM

Fig. 8   *Percentages of blood and mucosal lymphocytes rosetting in the presence of erythrocytes and antibody (E IgM) without complement. Symbols as in Fig. 4.*

E U
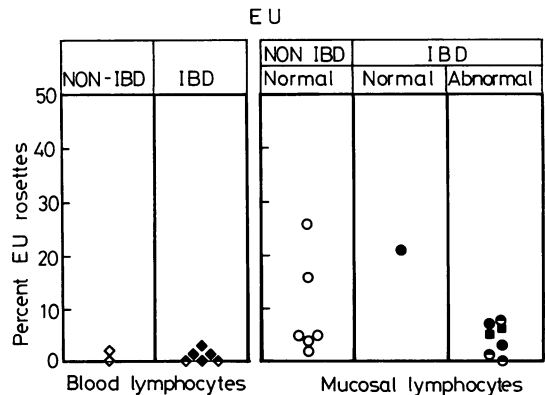

Fig. 9   *Percentages of blood and mucosal lymphocytes rosetting with erythrocytes not coated with IgM (EU). Symbols as in Fig. 4.*
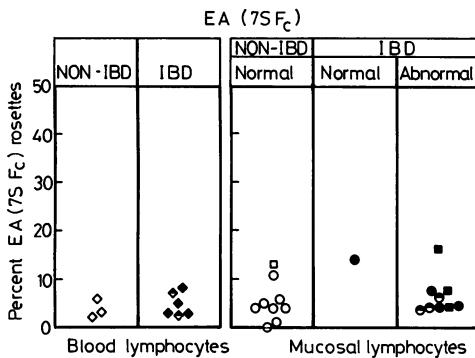
EA (7S F$_c$)

Fig. 10   *Percentages of blood and mucosal lymphocytes demonstrating Fc receptors. Symbols as in Fig. 4.*
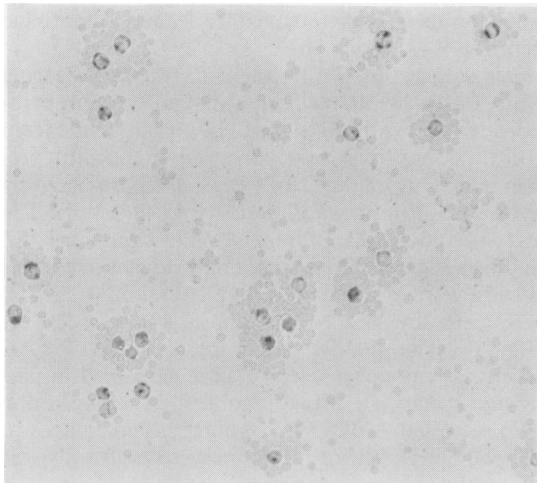

Fig. 11   *Patterns of alpha naphthyl acetate (ANAE) esterase staining observed in mucosal lymphocyte preparations—that is, one or two spots—granular (original magnification × 250).*

dominated, but the percentages were more variable. T-cells identified by EN rosetting ranged from 54·5% to 95%, with B-cell percentages ranging from 2% to 36% by the SIg technique. Cell yields were usually higher from diseased areas compared with normal regions. In one case of Crohn's disease, percentages in the muscular layers were similar to those in mucosa.

The majority of IML were ANAE positive, the predominant pattern being one or two darkly staining spots as with PBL (Fig. 11). Some lymphocytes contained finer, more numerous granules, and macrophages stained diffusely. No major staining differences were observed between IML from inflamed and non-inflamed gut from IBD patients. Unfortunately, ANAE preparations were not

available from the IBD patient showing the most striking increase in B-lymphocytes in the region of disease. Two patients with colon cancer demonstrated greater numbers of cells which either stained with a granular pattern or were ANAE negative. In both instances, the percentage of EN rosetting cells in IML preparations exceeded 80% (Fig. 5).

Discussion

The high T-lymphocyte counts observed with EN rosetting were supported by ANAE positive cells in IML suspensions and the fact that B-lymphocyte counts were correspondingly low. The percentages of both populations were similar to those seen in peripheral blood. As in the present study, Bull and Bookman[10] found that the percentage of T-cells in peripheral blood was similar to that in intestinal mucosa. PBL-IML comparisons were not reported by Goodacre *et al.*[12] The preparations of T- and B-cells in our study differed somewhat from those of other studies. Breucha *et al.*[20] found 30–60% T-cells in small intestinal and appendiceal specimens. Bull and Bookman[10] found a mean of 64% T-cells in colon using the E rosetting method of Jondal *et al.*[21] and 36% B-cells by SIg. Goodacre *et al.*[12] reported 38% T-cells identified by E rosettes. These studies, however, did not use rosettes stabilised with neuraminidase or 2-aminoethyl isothiouronium bromide (AET). Such stabilisation appears to be essential for detecting the total T-cell population.[22]

Numerous studies document ANAE staining of T-cells.[23–30] In the present study, the majority of ANAE positive IML showed at least one dense localised spot of ANAE activity, the pattern most often observed with T-cells in the previous studies. Macrophages are also ANAE positive, but are readily distinguished from lymphocytes morphologically and by their diffuse staining pattern. The literature suggests that helper T-lymphocytes with Fc receptors for IgM (TM-cells) stain with one or two large spots, whereas suppressor T-cells with IgG Fc receptors (TG-cells) either do not stain or stain with a granular pattern.[27 30] In two cases of colon cancer, the percentage of T-cells showing single spot staining was low, suggesting that the number of helper cells was diminished and that suppressor cells were increased.

It is theoretically possible that our high percentage of T-cells was due to a selective loss of B-lymphocytes during FH separation. However, when the cells sedimenting through the FH gradient were examined, only a few lymphocytes were found and they had the same proportion of EN rosetting cells as those from the interface. Conceivably, B-lymphocytes could be more fragile and disrupt

during the isolation procedure, but we know of no evidence for this. In fact, studies have shown that the FH separation technique is non-selective for PBL.[31] A third possible explanation for our finding of low B-lymphocyte numbers in IML suspensions might be that B-lymphocytes of the gut rapidly differentiate into plasma cells. We have not enumerated plasma cells. Although plasma cells identified on morphological grounds were present in only small numbers as previously observed, they were present in somewhat greater numbers in the FH separation pellet. Naked plasma cell nuclei were sometimes observed, indicating that some disruption had occurred.

We found no evidence of a large null cell population in the human gut mucosa and here, our findings are in agreement with those of Bull and Bookman.[10 14] Moreover, the low percentages of IgG Fc receptor bearing cells, which included the K-cells, are compatible with the absence of ADCC as reported by some investigators for guinea-pig[32] and human[11 14] intestinal lymphocyte preparations.

There were no consistent differences between IML from ileum and colon. Although results for T and B IML from IBD patients were variable, there was a tendency towards higher B-cell counts in inflamed tissue. This is in agreement with the results of Meijer, Bosman, and Lindeman[4] and Bookman and Bull.[14] In one case, we were unable to confirm any major differences in subpopulations of lymphocytes derived from mucosa from deeper layers, a finding which contrasts with the results of others.[1 3] Furthermore, there were no consistent differences between IML from patients with Crohn's disease and those with ulcerative colitis.

In conclusion, we found a striking predominance of T-lymphocytes in preparations from histologically normal human intestinal mucosa. Although there was a tendency for the B-cell population to increase in inflamed tissue of patients with IBD, T-lymphocytes remained the predominant cell type. Additional functional studies of IML from patients with IBD will be required to identify any specific role for mucosal lymphocytes in the pathogenesis of this disorder.

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