Distribution of macrophages and granulocytes expressing L1 protein (calprotectin) in human Peyer's patches compared with normal ileal lamina propria and mesenteric lymph nodes

K Bjerke, T S Halstensen, F Jahnsen, K Pulford, P Brandtzaeg

Abstract

Antibodies to the cytosolic leucocyte L1 protein (or calprotectin) were examined for reactivity with macrophages, neutrophils, and eosinophils identified by paired immunofluorescence staining in sections of normal human ileal mucosa, including Peyer's patches. Macrophages were recognised by expression of the myelomonocytic antigen CD68 (monoclonal antibody KP1). Neutrophilic granulocytes were identified by their content of neutrophil elastase, and eosinophilic granulocytes by monoclonal antibody EG2. Virtually all CD68⁺ macrophages in normal lamina propria and Peyer's patches were L1⁻ and the same was true for most extravasated macrophages in normal peripheral lymph nodes. Some mesenteric lymph nodes, however, and all peripheral lymph nodes with overt pathological processes (malignant lymphoma) contained many CD68⁺L1⁺ macrophages. Numerous L1⁺ cells were also localised to the crypt region and to some extent beneath the villous epithelium in normal lamina propria, but they were mainly identified as EG2⁺ eosinophils. Such cells were remarkably scarce or absent beneath the follicle associated epithelium in the dome region of Peyer's patches, where CD68⁺L1⁻ macrophages were abundant. Also subepithelial and interfollicular CD68⁻ interdigitating dendritic cells in Peyer's patches (recognised by antibody to S-100 protein) were usually unreactive with L1 antibody. The L1 protein shows a broad spectrum of antimicrobial activities in vitro, and its putative anti-proliferative properties are interesting in relation to the immunosuppression postulated to take place in lamina propria. The virtual absence of L1 producing cells beneath the follicle associated epithelium in Peyer's patches may support the immunostimulatory function of these macrophage rich structures, which are held to be crucial for induction of specific mucosal immunity.

(Gut 1993; 34: 1357-1363)

these cell types. Specificity and memory are properties of the adaptive immune system, depending on B and T lymphocytes acting with accessory cells.

The monocyte/macrophage cell lineage is remarkably heterogeneous, including classic macrophages and various dendritic antigen presenting cells.¹ They originate from pluripotent bone marrow stem cells and migrate through peripheral blood to tissues and body cavities where they differentiate into specialised cells showing great diversity of phenotypic and functional characteristics.² After emigration, mononuclear phagocytes may remain as immobile tissue macrophages for several months until they are stimulated or activated.³

It remains uncertain whether the precursors of different dendritic cells are found among the peripheral blood monocytes⁴ or represent a discrete circulating cell type. By the time the dendritic cells appear in peripheral lymph, however, they have become irreversibly differentiated and may terminate as interdigitating dendritic cells in T cell zones of organised lymphoid tissue. Interdigitating dendritic cells are characterised by a highly irregular surface, strong constitutive expression of major histocompatibility complex class II molecules, and low phagocytic ability. Interdigitating dendritic cells are therefore believed to function primarily as antigen presenting cells,⁴ although they are often included in the generic term 'macrophages'.

Previous studies of the distribution and nature of macrophages in human intestinal mucosa and Peyer's patches have shown a bewildering heterogeneity, probably reflecting both developmental and functional overlaps (see Discussion). The formalin resistant myelomonocytic L1 antigen detected by polyclonal and monoclonal antibodies has previously been proposed as a marker for reactive macrophages and certain monocyte derived histiocytes (reviewed in ref 5). Because of the interesting anti-microbial and anti-proliferative properties of this cytosolic protein, which is also called calprotectin,⁵ we decided to examine its distribution in macrophages and granulocytes of the normal human gut, focusing on ileal mucosa and Peyer's patches. Virtually all peripheral blood monocytes and neutrophilic granulocytes are L1 positive.⁵ Conversely, circulating eosinophils have been shown to be negative, whereas they apparently may express L1 after extravasation into inflamed tissue.⁵ It has been reported that there is very little overlap between major histocompatibility complex class II (HLA-DR) and

Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, University of Oslo, The National Hospital, Rikshospitalet, Oslo, Norway
K Bjerke
T S Halstensen
F Jahnsen
P Brandtzaeg

The Nuffield Department of Pathology, John Radcliff Hospital, Oxford
K Pulford

Correspondence to:
Dr P Brandtzaeg, LIIPAT,
Rikshospitalet, N-0027 Oslo,
Norway.

Accepted for publication
2 February 1993

The immune system has evolved to defend the individual against a diverse array of micro-organisms. Apart from the physical protection afforded by the surface epithelium, the innate defence system includes primarily monocytes/macrophages, neutrophilic and eosinophilic granulocytes, and natural killer cells along with various anti-microbial factors produced by all

L1 expressing lamina propria cells in normal human gut mucosa⁵ but the nature of those producing L1 at this site has not been determined. L1 expression was therefore in this study related to the distribution of various other cell markers identifying macrophages (CD68),⁶ neutrophils (neutrophil elastase), and eosinophils (eosinophil cationic protein)⁷ by two or three colour immunofluorescence staining *in situ*. For comparison, similar marker studies were performed on sections of peripheral and mesenteric lymph nodes.

Methods

COLLECTION AND PREPARATION OF TISSUE SPECIMENS

Specimens of distal ileal mucosa containing Peyer's patches identified macroscopically, and mesenteric lymph nodes, were obtained from seven organ donors with maintained peripheral circulation (age 10–47 years) and from one patient (age 57 years) with cancer of the ascending colon. Additional ileal biopsy specimens from eight patients (age 15–78 years) and eight peripheral lymph nodes removed for diagnostic purposes were included. Histological examination of the material showed normal mucosa with frequent appearance of secondary (activated) lymphoid follicles in Peyer's patches. Slight reactive changes were seen in the lymph nodes, dominated by follicular hyperplasia in the peripheral and sinus histiocytosis in the mesenteric ones. Peripheral lymph nodes with high grade

malignant non-Hodgkin's B cell lymphoma (n=4) were also included.

The specimens from the organ donors were immediately placed in ice cold isotonic saline. Small tissue blocks of Peyer's patches and the surrounding mucosa at some distance were excised as soon as possible (less than two hours) after collection, and were fixed in cold ethanol (96%) and then embedded in low melting paraffin wax.⁸ Additional specimens were fixed in buffered formalin (10%, pH 7.4) at room temperature for 48 to 72 hours before routine paraffin embedding. Serial sections were cut at 4–6 µm perpendicular to the mucosal surface.

After dewaxing, one section from each series was stained with haematoxylin and eosin for histological evaluation; the remainder were subjected to paired immunohistochemical staining (see below). Sections of formalin fixed tissue were pretreated with trypsin (1.0 g/l) for 10–15 minutes at 37°C before application of the primary antibodies.⁹ Sections of ethanol fixed tissue were prefixed in periodate lysine (2%) paraformaldehyde for 10 minutes at 4°C and briefly washed in isotonic phosphate buffered saline (pH 7.5) before use.

TWO AND THREE COLOUR IMMUNOFLUORESCENCE STAINING

Table I lists the different monoclonal and polyclonal primary antibody reagents as well as the secondary reagents. In paired stainings (Table II) the reaction sites of monoclonal antibody to CD68, HLA-DR, eosinophil cationic protein, or L1 were detected by a three step method employing biotinylated horse anti-mouse IgG and fluorescein isothiocyanate (FITC) labelled Streptavidin.⁹ The first step also included tetramethylrhodamine isothiocyanate (TRITC) labelled rabbit anti-L1¹⁰ or unlabelled rabbit anti-S-100 protein whose red signal was achieved in the last incubation step by means of TRITC labelled anti-rabbit IgG.⁹ For paired staining of CD68 and HLA-DR the first marker was detected by biotinylated goat anti-mouse IgG1 and Streptavidin Texas Red, and the second by FITC conjugated goat anti-mouse IgG2a (Table I). The gut epithelium could in this combination be delineated by costaining for cytokeratin with a primary rabbit antiserum followed by 7-amino-4-methylcoumarin-3-acetic acid (AMCA) conjugated goat anti-rabbit IgG (Table I).¹¹

To discriminate clearly between various L1 containing cell types in the same section, five step three colour staining was performed as follows: (a) monoclonal antibody to either eosinophil cationic protein or neutrophil elastase

TABLE I Primary and secondary antibody reagents used for two colour immunohistochemistry

Reagent	Working concentration and incubation time	Source
Rabbit antiserum to human L1, both chains (IgG, 0.5 g/l)	1:100 20 hours	Authors' laboratory ^a
TRITC-labelled rabbit anti-human L1, both chains (IgG, 0.5 g/l)	1:100 20 hours	Authors' laboratory ^a
Mouse anti-human L1 (Mac 387), both chains (ascites, IgG1)	1:1000 20 hours	DAKO A/S ^b
Mouse anti-CD68, clone KP1 (supernatant, IgG1)	1:10 20 hours	Pulford K A F and Mason D Y, Oxford ^c
Rabbit antiserum to S-100 protein	1:336 20 hours	DAKO Corporation ^d
Mouse anti-human HLA-DR, clone L243 (purified, IgG1)	1:20 20 hours	Becton Dickinson ^e
Mouse anti-human HLA-DR, clone BMA 022 (purified, IgG2a)	1:200 20 hours	Behring ^f
Mouse anti-human eosinophil cationic protein (ECP), clone EG2 (supernatant, IgG1)	1:20 20 hours	Spry C J F, London ^g
Mouse anti-human neutrophil elastase, clone NP57, (supernatant IgG)	1:200 20 hours	DAKO A/S ^b
Rabbit antiserum to human epidermal keratin	1:100 20 hours	Authors' laboratory ^h
FITC labelled goat anti-mouse IgG2a	1:20 1.5 hours	Southern Biotech Assoc ⁱ
Biotinylated goat anti-mouse IgG1	1:50 1.5 hours	Southern Biotech Assoc ⁱ
TRITC-labelled swine anti-rabbit Ig	1:16 30 minutes	Authors' laboratory ^j
Biotinylated horse anti-mouse IgG	1:80 3 hours	Vector laboratories ^k
AMCA labelled goat anti-rabbit IgG	1:20 30 minutes	Vector laboratories ^k
FITC labelled Avidin B	1:200 30 minutes	Vector laboratories ^k
Texas Red labelled Streptavidin	1:400 30 minutes	BRL ^l
FITC labelled Streptavidin	1:50 30 minutes	Boehringer ^m

^aDale I, *et al*, *Am J Clin Pathol* 1987; 87: 681–99; ^bGlostrup, Denmark; ^cPulford, *et al*, *J Clin Pathol* 1989; 42: 414–21; ^dSanta Barbara, CA, USA; ^eSunnyvale, CA, USA; ^fMarburg, Germany; ^gSt George's Hospital and Medical School, London; ^hHuitfeldt H, Brandtzaeg P. *Histochemistry* 1985; 83: 381–9; Birmingham, AL, USA; ⁱBrandtzaeg P, Rognum TO. *Histochem J* 1983; 15: 655–99; ^jBurlingame, CA, USA; ^kGaithersburg, MD, USA; ^lMannheim, Germany.

TABLE II Combinations of cell markers used in two colour immunofluorescence staining after different tissue preparation

Flourescein label*	Rhodamine label*	Tissue fixation
1 CD68 (KP1)	L1	Formalin
2 HLA-DR	L1	Ethanol
3 ECP (EG2)	L1	Ethanol
4 HLA-DR (L243)	ECP (EG2)	Ethanol
5 CD68 (KP1)	S-100 protein	Formalin
6 L1	S-100 protein	Formalin
7 HLA-DR (BMA 022)	S-100 protein	Formalin
8 CD68 (KP1)	HLA-DR (L243)	Ethanol

*See Table I.

or a mix of both was first applied; (b) biotinylated horse anti-mouse IgG; (c) mix of FITC-labelled Streptavidin, monoclonal antibody to CD68, and unconjugated antiserum to L1; (d) biotinylated horse anti-mouse IgG; and (e) AMCA-conjugated goat anti-rabbit IgG mixed with Streptavidin Texas Red. Eosinophils (EG2⁺) or neutrophils (elastase⁺) or both (EG2⁺ and elastase⁺) stained green, macrophages (CD68⁺) red, and L1⁺ cells blue (see Fig 3 below). Expression of L1 could therefore be related to each phenotype, allowing in particular CD68⁺ macrophages to be distinguished from neutrophils expressing this marker.¹²

Reagents were appropriately diluted (Table I) in phosphate buffered solution containing bovine serum albumin at 125 g/l, and all incubations of the primary antibodies took place for 20 hours at room temperature. Biotinylated anti-mouse IgG and Streptavidin were applied for three hours or 30 minutes, respectively, whereas the subclass specific goat anti-mouse IgG conjugates were applied for 1½ hours. Negative controls were obtained by omitting or exchanging the primary antibodies. To ensure that conjugates were not non-specifically bound to eosinophils, control sections were treated with 1% chromotrope 2R (Sigma, St Louis, USA).⁷

After a final wash in phosphate buffered saline (10–15 minutes), all sections were briefly rinsed in deionised water, air dried, and mounted in buffered (pH 8) polyvinyl alcohol.

IMMUNOFLUORESCENCE COMBINED WITH CYTOCHEMISTRY

Monoclonal anti-CD68 or anti-L1 was applied for 20 hours on ethanol fixed sections, and both markers were visualised as described above with FITC fluorescence. For costaining of acid phosphatase the sections were thereafter incubated for one hour at 37°C in the following substrate solution: 2 ml Naphthol AS-MX Phosphate acid (Sigma 388-B), 2 ml 2.5 M acetate buffer (pH 5.2), 46 ml distilled water, and Fast Garnet GBC, 15 mg per 50 ml (Sigma F-6504).

FLUORESCENCE MICROSCOPY AND HISTOMORPHOMETRY

The tissue sections were examined in a Leitz Orthoplan fluorescence microscope equipped with a Ploem-type epi-illuminator, an ×25 Leitz immersion objective, and an ×10 ocular. The filter system facilitated repeated study of individual cells with regard to fluorescein (green), rhodamine or acid phosphatase (red), and AMCA (blue) emission. The spatial relation between the two or three colours was further evaluated in double or triple exposed colour slides taken on Kodak Ektachrome 800 ASA daylight film (see Figs 1–3).

An ocular grid (Leitz code No. 519902), to count the fluorescent gut cells, was moved systematically along the villous epithelium and the typical flattened goblet cell depleted follicle associated epithelium of Peyer's patch domes. By permitting one side of the frame to touch the basement membrane, the subepithelial zone was divided into areas of 0.008 mm², which usually

included the whole lamina propria of the villi and the so called 'mixed cell zone' of the Peyer's patch domes. Cells expressing L1 within each grid and in the corresponding epithelial layer were counted in relation to the CD68 marker. In such enumerations, CD68 negative eosinophils¹² as well as CD68 positive macrophages could generally be distinguished from neutrophils (which showed much brighter staining for L1 than the macrophages). Preliminary counts suggested that only about 5% of the L1 positive cells in the lamina propria were neutrophils, a finding supported by staining for chloroacetate esterase in the same sections.¹³

Results

CD68⁺ macrophages in the ileal lamina propria were deemed to coexpress L1 quite rarely (about 0.5%). Most L1⁺ cells at this site were thus taken to represent granulocytes, which was supported by the fact that the CD68⁺L1[−] cells showed a much stronger reaction for acid phosphatase than those expressing L1 in all ethanol fixed specimens examined. Acid phosphatase was also expressed apically on the epithelium, which served as an inherent control.

CD68⁺ macrophages were on the whole slightly more numerous than L1⁺ granulocytes in formalin fixed tissue (Table III). In preliminary counts on ethanol fixed sections (n=8) the values were somewhat reversed (56% v 44%), probably because of L1 leaching when cross linking fixative was omitted.⁵ The L1⁺ granulocytes were often quite numerous at the base of the villi and in the crypt area (Fig 1A), while CD68⁺ macrophages were relatively predominant near the tips of the villi (Fig 2A). The few positive intraepithelial cells seen in the villus epithelium after costaining for CD68 and L1 were usually granulocytes; less than 10% were CD68⁺L1[−] macrophages (six of a total of 64 evaluated cells).

Relatively few L1⁺ cells were seen in the dome area of Peyer's patches (defined as the zone beneath the typical flattened follicle associated epithelium containing few or no goblet cells); and they were totally absent in the germinal centres of Peyer's patches (Fig 1A), as previously seen in other lymphoid tissues.³ Conversely, CD68⁺L1[−] macrophages abounded in the dome area (Fig 2B and Table IV), and such large cells were also frequently seen in the germinal centres where they apparently represented tingible body macrophages.

In the lamina propria about 90% of the L1⁺

TABLE III Median percentage (range) of cells in normal ileal lamina propria recognised by two colour immunofluorescence staining in formalin fixed mucosal specimens (n=8)

Antibody combination*	
Anti-L1	Anti-CD68
48 (30–60)	52 (40–60)

*Only six cells out of 2302 (mean cell count per specimen, 259) were stained by both antibodies.

TABLE IV Median number (range) of subepithelial cells per grid (0.008 mm²) recognised by two colour immunofluorescence staining in formalin fixed normal ileal mucosal specimens (n=8)

Location*	Antibody combination	
	Anti-L1	Anti-CD68
Villus	5.5 (1.8–8.2)	6.2 (3.7–8.5)
PP dome†	0.8 (0.3–1.5)	3.9 (1.0–5.7)

*Median number of grids examined per specimen in villus and Peyer's patch (PP) dome was 26 and 14, respectively; †Beneath goblet cell depleted parts of follicle associated epithelium.

Figure 1: Formalin fixed mucosal sections subjected to two colour immunofluorescence staining of (A) Peyer's patch for L1 (green) and S-100 protein (red), and (B) villus mucosa for L1 (green) and EG2 (red). Note abundant S-100 protein⁺, probably interdigitating dendritic cells in dome and also some close to the villous epithelium on the right, and that L1⁺ cells are mainly located in lamina propria outside the Peyer's patch (A). All EG2⁺ eosinophils contain L1 (B). VE=villus epithelium; FAE=follicle associated epithelium; FC=follicle centre (original magnification (A) $\times 160$; (B) $\times 410$).

cells taken to be granulocytes were by the specific marker eosinophil cationic protein shown to be eosinophilic granulocytes (median number of EG2⁺ cells examined per specimen: 120, range 9–225; $n=7$). In fact, no L1⁺ eosinophils were seen in the lamina propria (Fig 1B) by contrast with lymph nodes where some such cells apparently lacked this protein (see below). The density of both L1⁺ cells (Table IV) and EG2⁺ cells (Table V) was on average seven times higher in the ileal villi than in the Peyer's patch domes.

Also most of the CD68⁺ macrophages occurring in the fairly normal peripheral lymph nodes lacked L1. In some of the mesenteric lymph node specimens, however, many macrophages were CD68⁺L1⁺, perhaps because of increased traffic of mononuclear cells as suggested by comparatively pronounced sinus histiocytosis;

Figure 2: Ethanol fixed sections of normal ileal villus (A) and Peyer's patch (B) subjected to two colour immunofluorescence staining with KP1 for CD68 (red) and HLA-DR (green). Note abundant CD68⁺DR⁺ macrophages (yellow) in subepithelial area. The endothelium of small vessel expresses only DR (arrowed) and two granulocytes are stained only for CD68, one indicated by arrow (A). Note double expression of CD68 and DR apically on epithelium (A). There are numerous double expressing macrophages (yellow) in the dome (B). Small CD68⁺DR⁺ cells represent mainly B lymphocytes, whereas larger such cells are probably of interdigitating dendritic variety. FAE=follicle associated epithelium; FC=follicle centre (original magnification $\times 410$).

on average, 50% of all L1⁺ cells without granulocyte markers expressed CD68 in these nodes (Fig 3A, Table VI). Also, CD68⁺ smaller round cells considered to be monocytes often occurred intravascularly in ileal lamina propria and lymph node pulpa as well as in marginal sinuses. By contrast with the normal situation, most CD68⁺ macrophages (70–90%) infiltrating areas of lymph nodes occupied by malignant B cells expressed L1 (Fig 3B). These tumour areas contained only scattered L1⁺ granulocytes and some of the eosinophils (0–20%) seemed to lack L1.

The abundant subepithelial and intrafollicular CD68⁺ macrophages seen in Peyer's patches

Figure 3: Ethanol fixed sections of normal mesenteric lymph node (A) and peripheral lymph node with B-cell lymphoma (B) subjected to three colour staining with KP1 for CD68 (red), eosinophil cationic protein and neutrophil elastase (green), and L1 (blue). Green emission omitted in (A) because of absence of granulocytes. Note CD68⁺L1⁺ and CD68⁺L1⁻ macrophages in both (A) and (B). A granulocyte (heavy arrow) is triple stained in (B) (original magnification $\times 410$).

were all HLA-DR positive (Fig 2B), as were the S-100 protein⁺ interdigitating dendritic cells sometimes found beneath the follicle associated epithelium (Fig 1A). Conversely, most L1⁺ cells both in Peyer's patches and lamina propria did not express HLA-DR; only 1% (range 0–8.9%) of these cells were DR⁺ (median number of L1⁺ cells evaluated per ethanol fixed ileal specimen: 115, range 75–201; n=8). This implied that eosinophils in normal gut mucosa generally do not express class II molecules, which was directly confirmed by paired staining for eosinophil cationic protein (EG2) and HLA-DR (median number of EG2⁺ cells examined: 101, range 64–280, n=4) in which only four double positive cells were found.

Most S-100 protein⁺ interdigitating dendritic cells were clustered in the interfollicular zones of Peyer's patches. Also at this site they were CD68⁻L1⁻ but expressed strongly HLA-DR. Only four S-100 protein⁺ cells in eight double stained formalin fixed sections seemed to express CD68 and two L1 (median 65, respectively 73, S-100 protein⁺ cells were examined). A few S-100 protein⁺, comparatively large elements (deemed not to be nerve fibres) were seen scattered or as small clusters in the villous lamina propria (Fig 1A).

Follicle associated epithelium and villous

TABLE V Median number (range) of subepithelial eosinophils recognised by EG2 immunofluorescence in ethanol fixed normal ileal mucosal specimens (n=7)

Location*	Cells/grid (0.008 mm ²)
Villus	2.8 (1.4–3.3)
PP dome†	0.4 (0–0.7)

*Median value of grids examined per specimen in villus and Peyer's patch (PP) dome was 25 and 16, respectively; †Beneath goblet cell depleted parts of follicle associated epithelium.

TABLE VI Median percentage (range) of CD68 positive mononuclear phagocytes detected by three colour immunofluorescence staining among L1 positive cells in ethanol fixed lymph nodes*

Peripheral nodes (n=7)	Mesenteric nodes (n=8)
0 (0–3.5)	51 (0–94.6)

*Median cell count (range) per specimen in peripheral and mesenteric lymph nodes was 203 (83–250) and 120 (41–227), respectively.

epithelium showed apical expression of CD68, apparently related to the brush border in a granular pattern completely congruent with that of HLA-DR (Fig 2A). Because the CD68 antigen is derived from lysosomes,⁶ this finding is in agreement with the fact that lysosomal proteins may be subjected to apical transport, thus reaching the microvilli of polarised cells.⁵

Discussion

The M_r approximately 36.5 K myelomonocytic L1 protein complex belongs to the S-100 protein family and may play a part in the regulation of cellular proliferation and differentiation.⁵ Its two chains (L1_L and L1_H) are regrettably referred to by a bewildering collection of names, various authors having different preferences (MRP-8 and MRP-14; p8, p14; CFA or calgranulin A and B).⁵ The most recent proposal is calprotectin because of L1's calcium binding properties and its anti-microbial effects shown *in vitro*.⁵ In this study we examined expression of L1 by macrophage subpopulations in the normal distal ileum using CD68 and S-100 protein as cell markers. Similar examinations were performed in reactive lymph nodes for comparison, because previous studies have shown striking expression of L1 by macrophages infiltrating pathological processes.⁵ In the normal ileum, however, we found that virtually all CD68⁺ macrophages and (not unexpectedly)⁵ S-100 protein⁺ dendritic cells lacked this protein.

Lamina propria macrophages thus show remarkably downregulated L1 production in the normal state. The usual course for mononuclear cells after extravasation is in fact to lose functional capacity; resident macrophages in normal tissues are therefore in some aspects less differentiated than monocytes, but they may nevertheless be more active in other instances.² Macrophage heterogeneity is indeed quite common, even within a single lymphoid organ.¹⁴ Monoclonal antibodies have greatly helped to dissect such subpopulations, but the results are often inconclusive because of overlapping reactivity and lack of related functional information. Available information about the distribution of various macrophage subsets in the human gut (Table VII) has mostly been based on single immunohistochemical staining in serial tissue sections, rendering evaluation of multiple marker expression difficult or impossible. The monoclonal antibodies 25F9 and RFD7 bind to mature human tissue macrophages¹⁵ whereas RFD1 identifies interdigitating dendritic cells. Interestingly, in normal colonic lamina propria most cells (87%) recognised by these reagents

TABLE VII Labelling of cells of the monocyte/macrophage family with monoclonal antibodies previously reported for normal human ileal mucosa including Peyer's patches

Antibody code and reference	Reactivity	Peyer's patches			Mucosal lamina propria
		Dome area	Follicle centre	Interfollicular area	
RFD1 ³⁵	Dendritic cells (some B cells)	+	+	+	+
RFD7 ³⁵	Tissue macrophages	—	—	—	+
RFD9 ³⁶	Epitheloid cells and tingible body macrophages	—	+	—	—
Y1/82 ³⁷	Monocytes and macrophages	+	+	—	+
EBM11 ³⁸	Tissue macrophages	+	+	—	+
UCHM1 ³⁹	Monocytes	—	+	—	—
25F9 ¹⁵	Mature macrophages	+	+	—	+

seem to be positive for all three markers² and can apparently function both as phagocytes and antigen presenting cells.^{2,16} By contrast, antibody RFD7 does not react with macrophages in the dome region or germinal centres of Peyer's patches,¹⁷ while antibodies EBM11 and Y1/82 directed against CD68,⁶ have been found to detect most intestinal macrophages. This also includes the two latter tissue sites, which is in keeping with our findings based on antibody KP1.

It is quite possible that cooperation between various macrophage subsets takes place in Peyer's patches. Wright *et al*¹⁸ reported that particulate antigens, after initial phagocytosis, may be reprocessed for presentation to T cells, and Guidos *et al*¹⁹ proposed a functional complementation between mononuclear phagocytes and interdigitating dendritic cells. The first subpopulation may thus provide processed antigens in a form more suitable for presentation by dendritic cells lacking acid phosphatase,^{2,20} whereas the second (with strong constitutive class II expression) may present very efficiently antigenic peptides to CD4⁺ T cells. Perhaps such a dichotomy exists between the CD68⁺ and S-100 protein⁺ cell populations in Peyer's patches. A particular difference between murine class II-expressing macrophages and interdigitating dendritic cells seems to be that only the second subset is capable of triggering resting T cells,²¹ whereas the first needs primed T cells to induce immune responses.²² Formation of clusters with unprimed T cells has thus been shown to be a functional feature of dendritic cells²³; this may explain the striking appearance of S-100 protein⁺ cells in Peyer's patches, which are considered important sites for induction of primary immune responses.²⁴

The exact activation state of normal lamina propria macrophages is currently unknown, but the lack of intercellular adhesion molecule-1 (CD54)²⁵ may restrict their function in antigen presentation especially to unprimed CD4⁺ T cells despite being pan-class II-positive.²⁶ With regard to their lack of L1, this feature is shared with resident macrophages in many other normal tissues such as tonsils, peripheral lymph nodes, thymus, spleen, and liver.⁵ We have recently found that in inflammatory bowel disease, large numbers of CD68⁺L1⁺ mononuclear cells occur in the affected gut mucosa. They most likely represent recently emigrated monocytes and may thus turn out to be highly responsive young 'inflammatory macrophages'.²⁷ It is currently unknown, however, whether this subset belongs to the same functional category as the

CD68⁺L1⁺ mononuclear cells infiltrating malignant lymph node processes and often present in normal (reactive) mesenteric lymph nodes. Further studies are also required to evaluate any disparity in their expression of the two L1 chains. These peptides are complexed in both neutrophils and monocytes,^{28,29} but recently extravasated mononuclear cells have been claimed to express only the L_H chain in acute inflammation – contrasting with chronic inflammatory conditions in which macrophages often are found to express both chains.^{5,27} One hypothesis is thus that lack of L1_L downregulation might reflect failure of monocyte differentiation to macrophages in chronic inflammation.²⁹ More work is needed, however, to clarify such putative differential regulation of the two L1 chains; possible immunohistochemical artifacts cannot be discounted because quite contrasting results have been reported with regard to expression of the two chains in immune granulomas.^{5,27}

Our results show that eosinophilic granulocytes are the main cellular source of L1 in the normal gut. Circulating eosinophils have been reported to be negative for L1¹⁰ and they apparently show only variable positivity in inflamed tissue.⁵ It is remarkable, therefore, that in normal ileal mucosa these cells all express L1. As well as its anti-microbial activities,⁵ an anti-proliferative function has recently been shown for L1 and tentatively ascribed to its inhibition of casein kinase II.⁵ Such a putative immunosuppressive effect of L1 might accord with the intestinal distribution seen of the L1⁺ eosinophils: they are localised mainly in the lamina propria where a downregulating effect on lymphocyte proliferation would be beneficial to dampen immune responses to food antigens, but they are virtually absent beneath the follicle associated epithelium of Peyer's patches where adequate immunostimulation to potentially pathogenic antigens is desirable. Interestingly, L1 may share an immunoregulatory function with another eosinophil product, eosinophil cationic protein, which has been shown to inhibit the proliferation of human T and B cells and also the immunoglobulin production of plasma cell lines *in vitro*.^{30,31} This inhibition can be reversed by IL-6,³⁰ a cytokine which has been implicated as a positive factor for mucosal IgA production.³²

In vitro experiments have suggested that secretory IgA is a very potent activator of eosinophils as measured by degranulation, being two to three times more potent than monomeric IgA.³³ Locally produced polymeric IgA antibodies may therefore be the principal immune stimulator of eosinophil effector functions at mucosal surfaces, both in protective and tissue destructive situations.³⁴ Much more work is required, however, to clarify possible immunoregulatory and immunopathological interactions between eosinophils and the mucosal IgA system in the human gut.

Supported by the Norwegian Research Council for Science and the Humanities, the Norwegian Cancer Society, and Rakel and Otto Kr Bruun's Legacy. Hege E Svendsen is thanked for excellent secretarial assistance.

1 Tew JG, Thorbecke J, Steinmann RM. Dendritic cells in the immune response: characteristics and recommended nomenclature (a report from the Reticuloendothelial Society Committee on Nomenclature) *Reticuloendothel* 1982; 31: 372–80.

- 2 Adams DO. Macrophage activation. In: Roitt IM, Delves PJ, eds. *Encyclopedia of immunology*. London: Academic Press, 1992: 1020-6.
- 3 Verspaget H, Beeken W. Mononuclear phagocytes in the gastrointestinal tract. *Acta Chir Scand (suppl)* 1985; 525: 113-26.
- 4 King PD, Katz DR. Mechanisms of dendritic cell function. *Immunol Today* 1990; 11: 206-11.
- 5 Brandtzaeg P, Dale I, Gabrielsen TØ. The leucocyte protein L1 (calprotectin): usefulness as an immunohistochemical marker antigen and putative biological function. *Histopathology* 1992; 21: 191-6.
- 6 Pulford KAF, Rigney EM, Micklem KJ, Jones M, Stross WP, Gatter KC, et al. KP1: a new monoclonal antibody that detects a monocyte/macrophage associated antigen in routinely processed tissue sections. *J Clin Pathol* 1989; 42: 414-21.
- 7 Tai PC, Spry CJF, Peterson C, Venge P, Olsson I. Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein. *Nature* 1984; 309: 182-4.
- 8 Brandtzaeg P. Mucosal and glandular distribution of immunoglobulin components. Immunohistochemistry with a cold-ethanol fixation technique. *Immunology* 1974; 26: 1101-14.
- 9 Brandtzaeg P, Rognum TO. Evaluation of tissue preparation methods and paired immunofluorescence staining for immunocytochemistry of lymphomas. *Histochem J* 1983; 15: 655-99.
- 10 Dale I, Brandtzaeg P, Fagerhol MK, Scott J. Distribution of a new myelomonocytic antigen (L1) in human peripheral blood leucocytes. Immunofluorescence and immunoperoxidase staining features in comparison with lysozym and lactoferrin. *Am J Clin Pathol* 1985; 84: 24-34.
- 11 Halstensen TS, Scott H, Brandtzaeg P. Intraepithelial T cells of the TcR $\gamma\delta^+$ CD8 $^-$ and V δ 1/J δ 1 $^+$ phenotypes are increased in coeliac disease. *Scand J Immunol* 1989; 30: 665-72.
- 12 Pulford KAF, Sipos A, Cordell JL, Stross WP, Mason DY. Distribution of the CD68 macrophage/myeloid associated antigen. *Int Immunol* 1990; 2: 973-80.
- 13 Leder LD. Über die selektive fermentcytochemische Darstellung von neutrophilen myeloischen Zellen und Gewebsmastzellen im Paraffin-schnitt. *Klin Wochenschr* 1964; 42: 553-5.
- 14 Dijkstra CD, Döpp EA, Joling P, Kraal G. The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 1985; 54: 589-99.
- 15 Hume DA, Allan W, Hogan PG, Doe WF. Immunohistochemical characterisation of macrophages in human liver and gastrointestinal tract: expression of CD4, HLA-DR, OKM1, and the mature macrophage marker 25F9 in normal and diseased tissue. *J Leucoc Biol* 1987; 42: 474-84.
- 16 Mahida YR, Jewell DP. Respiratory burst capacity of human intestinal macrophage subpopulations. In: MacDonald TT, Challacombe SJ, Bland PW, Stockes CR, Heatley RV, Mowat AM, eds. *Advances in mucosal immunology*. London: Kluwer Academic, 1990: 701-6.
- 17 Mahida YR, Patel S, Jewell DP. Mononuclear phagocyte system of human Peyer's patches: an immunohistochemical study using monoclonal antibodies. *Clin Exp Immunol* 1989; 75: 82-6.
- 18 Wright MD, Wood PR, Coia G, Cheers C. Particulate antigens may be reprocessed after initial phagocytosis for presentation to T cells in vivo. *Immunol Cell Biol* 1987; 65: 505-10.
- 19 Guidos C, Sinha AA, Lee K-C. Functional differences and complementation between dendritic cells and macrophages in T-cell activation. *Immunology* 1987; 61: 269-76.
- 20 Seldenrijk CA, Drexhage HA, Meuwissen SGM, Pals ST, Meijer CLJM. Dendritic cells and scavenger macrophages in chronic inflammatory bowel disease. *Gut* 1989; 30: 486-91.
- 21 Inaba K, Steinman RM. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. *J Exp Med* 1984; 160: 1717-35.
- 22 Austin JM. Lymphoid dendritic cells. *Immunology* 1987; 62: 161-70.
- 23 Inaba K, Steinman RM. Accessory cell T lymphocyte interactions. Antigen-dependent and -independent clustering. *J Exp Med* 1986; 163: 247-61.
- 24 Pappo J, Owen RL. The lymphoid system and immunologic defense of the digestive tract. In: Motta PM, Fujita H, eds. *Ultrastructure of the digestive tract cells*. Boston: Martinus Nijhoff, 1981: 181-99.
- 25 Malizia G, Calabrese A, Cottone M, Raimondo M, Trejdosiewicz LK, Smart CJ, et al. Expression of leukocyte adhesion molecules by mucosal mononuclear phagocytes in inflammatory bowel disease. *Gastroenterology* 1991; 100: 150-9.
- 26 Unanue ER. Antigen presentation. In: Roitt IM, Delves PJ, eds. *Encyclopedia of Immunology*. London: Academic Press, 1992: 111-4.
- 27 Zwadlo G, Brügger J, Gerhards G, Schlegel R, Sorg C. Two calcium-binding proteins associated with specific stages of myeloid cell differentiation are expressed by subsets of macrophages in inflammatory tissues. *Clin Exp Immunol* 1988; 72: 510-5.
- 28 Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hagar G, Karast M, Sorg C. Calcium-dependent complex assembly of the myeloid differentiation proteins MRP-8 and MRP-14. *J Biol Chem* 1991; 266: 13462-7.
- 29 Edgeworth J, Gorman M, Bennett R, Freemont P, Hogg N. Identification of p8,14 as a highly abundant heterodimeric calcium binding protein complex of myeloid cells. *J Biol Chem* 1991; 266: 7706-13.
- 30 Kimata H, Yoshida A, Ishioka C, Iiang Y, Mikawa H. Eosinophil cationic protein inhibits immunoglobulin production and proliferation in vitro in human plasma cells. *Cell Immunol* 1992; 141: 422-32.
- 31 Peterson CGB, Skoog V, Venge P. Human eosinophil cationic proteins (ECP and EPX) and their suppressive effects on lymphocyte proliferation. *Immunobiology* 1986; 171: 1-13.
- 32 McGhee JR, Mestecky J, Elson CO, Kiyono H. Regulation of IgA synthesis and immune response by T cells and interleukins. *J Clin Immunol* 1989; 9: 175-99.
- 33 Abu-Ghazaleh RI, Fujisawa T, Mestecky J, Kyle RA, Gleich GJ. IgA-induced eosinophil degranulation. *J Immunol* 1989; 142: 2393-400.
- 34 Silverstein DS. Eosinophils. In: Roitt IM, Delves PJ, eds. *Encyclopedia of immunology*. London: Academic Press, 1992: 512-4.
- 35 Poulter LW, Campbell DA, Munro C, Janossy G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scand J Immunol* 1986; 24: 351-7.
- 36 Janossy G, Bofill M, Poulter LW, Rawlings E, Burford GD, Navarrete C, et al. Separate ontogeny of two macrophage-like accessory cell populations in the human fetus. *J Immunol* 1986; 136: 4354-61.
- 37 Davey FR, Cordell JL, Erber WN, Pulford KA, Gatter KC, Mason DY. Monoclonal antibody (Y1/82A) with specificity towards peripheral blood monocytes and tissue macrophages. *J Clin Pathol* 1988; 41: 753-8.
- 38 Franklin WA, Mason DY, Pulford K, Falimi B, Bliss E, Gatter KC. Immunohistological analysis of human mononuclear phagocytes and dendritic cells by using monoclonal antibodies. *Lab Invest* 1986; 54: 322-35.
- 39 Hogg N, MacDonald S, Slusarenko M, Beverley PCL. Monoclonal antibodies specific for human monocytes, granulocytes and endothelium. *Immunology* 1984; 53: 753-67.