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Phenotypically distinct subpopulations of T cells in domes and M-cell pockets of rabbit gut-associated lymphoid tissues

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SUMMARY

Follicle epithelium and domes of gut-associated lymphoid tissue (GALT) contain populations of lymphocytes which first contact antigen taken up from the intestine. In order to study the association of lymphocytes with M cells in follicle epithelium, monoclonal antibodies (mAb) were generated by immunizing BALB/c mice with lymphocytes populating GALT domes from NZW rabbits, and their specificity was assessed by immunohistochemistry and flow cytometry. mAb 3C10 (IgM) and 3B6 (IgG3) recognized subpopulations of intraepithelial lymphocytes associated with M cells. mAb 3C10 also identified macrophage–lymphocyte clusters in domes and tingible body macrophages in germinal centres of GALT but did not react with cells in T-dependent areas (TDA) or B cells in follicles. mAb 3B6 recognized lymphocytes in domes and B cells in follicles but not T cells in TDA of GALT. The distribution of $3B6^+$ cells overlapped with, but was more restricted than, that of Ia^+ cells. Analysis of lymphocytes in follicle epithelium showed that >95% of lymphocytes associated with M cells were Ia^+. T cells represented ~95% of intraepithelial lymphocytes in the appendix and ~65% in Peyer's patches. A majority of intraepithelial lymphocytes was recognized by mAb 3B6, but mAb 3C10 identified only ~30%. Because neither 3C10 nor 3B6 recognized lymphocytes in TDA of GALT, these results indicate that most lymphocytes associated with M cells are a distinct phenotype of Ia^+ T cells.

INTRODUCTION

Gut-associated lymphoid tissues (GALT) are composed of numerous lymphoid follicles with a follicle epithelium containing specialized M cells (Faulk *et al.*, 1970; Waksman & Ozer, 1976). M cells exhibit distinctive structural features such as short microvilli and apical vesicles and enclose clusters of mononuclear leucocytes in the supranuclear region, creating a cytoplasmic pocket or central hollow (Owen & Jones, 1974; Bye, Allen & Trier, 1984). M cells lack the dimeric IgA receptor (Pappo & Owen, 1988) but express distinctive surface molecules which are not present on adjacent enterocytes (Pappo, 1989). M cells take up and transport protein antigens (Bockman & Cooper, 1973; Owen, 1977; Neutra *et al.*, 1987), viruses (Wolf *et al.*, 1983), bacteria (Owen *et al.*, 1986) and protozoa (Marcial & Madara, 1986) to lymphoid cells in the dome beneath the epithelium. Intraepithelial cells in the intestinal epithelium, which localize in the subnuclear region, include populations of cytotoxic cells and T-cell receptor (TcR) γ/δ^+ cells (Dobbins, 1986; Takagaki *et al.*, 1989), but little is known about the function of lymphocytes enclosed within M cell pockets in the follicle epithelium of GALT.

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The rabbit has provided a valuable model for studying the differentiation and transport functions of M cells. M cells in the rabbit constitute about 50% of the follicle epithelial cells; M-cell pockets contain aggregates of mononuclear cells (Pappo, Steger & Owen, 1988); and the transport efficiency of M cells in the rabbit is higher than in rodents (Pappo & Ermak, 1989). Several monoclonal antibodies (mAb) have been developed recognizing antigens on rabbit T- and B-cell surfaces (Jackson *et al.*, 1983; Lobel & Knight, 1984), but their specificity for lymphocytes populating domes and the follicle epithelium of GALT have not been examined. In this study, we describe two mAb which define distinct subpopulations of lymphocytes resident in domes and the follicle epithelium of rabbit GALT. Furthermore, we show that most lymphocytes in follicle domes and M-cell pockets are T cells which express class II molecules, and that these lymphocytes are phenotypically distinct from T cells in thymus-dependent areas.

MATERIALS AND METHODS

Animals

Female New Zealand white (NZW) rabbits, 2·5–3 kg in weight, were obtained from Animals West (Soquel, CA) and Rabbitek (Modesto, CA). Four-week-old female BALB/c mice were obtained from Simonsen Laboratories (Gilroy, CA).

Monoclonal antibodies

The hybridoma cell lines L11/135, which secretes an IgG1 mAb that recognizes rabbit T lymphocytes (Jackson *et al.*, 1983), and 2C4, which secretes an IgG2a mAb against rabbit Ia (Lobel & Knight, 1984), were obtained from the American Type Culture Collection (Rockville, MD). The hybridoma cell line 5D9 with specificity for rabbit M cells was grown as described previously (Pappo, 1989).

Preparation of cell populations for immunization and cell fusion

Rabbit dome cell populations from Peyer's patches and appendix were obtained as described elsewhere (Pappo *et al.*, 1988). Mice were immunized i.p. at Days 0 and 30 with 10^7 GALT cells in phosphate-buffered saline (PBS; 0·01 M phosphate, pH 7·4), emulsified in an equal volume of complete Freund's adjuvant. Seven days after the last injection, these mice were boosted i.v. with a similar number of GALT cells in PBS and somatic cell hybridization was carried out 3 days later (Köhler & Milstein, 1975) using 30% polyethylene glycol 1000 and the P3/NS1/1-AG4-1 cell line (ATCC), at a ratio of 8:1.

Screening of supernatants

Hybridoma culture supernatants were screened on GALT tissue sections by immunohistochemistry. Fresh frozen cryosections (7 μm thick) were fixed with acetone, and incubated with 100 μl of hybridoma supernatants, followed by a 1/200 dilution of biotinylated anti-mouse IgG and IgM, and then avidin conjugated to biotinylated horseradish peroxidase (ABC; Vector Laboratories, Burlingame, CA). Reagents were applied to tissue sections for 30 min, and the sections washed three times with PBS. Labelled sites were detected with 0·05% diaminobenzidine tetrahydrochloride (Organon Teknika, Durham, NC) and 0·01% H_2O_2 in 0·1 M Tris-HCl buffer, pH 7·3, and sections were counterstained with methyl green. Controls included substitution of the primary reagent with NS1 medium or with mAb of unrelated specificity, and omitting the hybridoma supernatant. Representative hybridomas producing antibodies which reacted with dome lymphocytes (3B6 and 3C10), follicle B cells (6D4 and 3E12), and all lymphocytes (4F3) were selected and cloned by limiting dilution. The isotype and light chain composition of antibodies in hybridoma supernatants was assessed by ELISA with specific reagents (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Immunohistochemical distribution of molecules recognized by mAb

Tissue sections of Peyer's patches, appendix, spleen, and thymus from four NZW rabbits were incubated with hybridoma culture supernatants and the reaction product was developed as described above. Lymphoid compartments were scored from + to +++ based upon the proportion of lymphocytes labelled (<33%, 33–66%, >66%). Negative controls included incubation of sections with the secondary reagents, and with isotype-matched mAb of unrelated specificity. Negative control slides did not exhibit labelling of any region, except for scattered cells with reaction product from endogenous peroxidase.

For quantification of lymphocyte numbers in the follicle epithelium, M cells were identified by labelling with mAb 5D9. Lymphocyte counts were made in 350 μm long segments of the follicle epithelium where 5D9$^+$ M cells were present in serial sections. Data were expressed as cell number ± SEM per 500 μm of follicle epithelium.

Fluorescence staining and flow cytometry

Dispersed lymphoid cell populations from each organ were incubated with culture supernatants (100 μl) for 20 min at 4° in V-bottomed microtitre plates (10^6 cells/well), washed three times with PBS containing 0·5% bovine serum albumin (BSA) and 0·1% NaN_3, and reincubated with 25 μg/ml of biotin-conjugated goat anti-mouse IgG or IgM (Vector Laboratories, Burlingame, CA). After being washed, the cells were incubated with the appropriate dilution of FITC-conjugated avidin (Becton-Dickinson, Mountain View, CA), washed, fixed with 1% paraformaldehyde in PBS (pH 7·4) and analysed with a FACS 440 (Becton-Dickinson). Control samples were incubated with secondary reagents, or with hybridoma medium.

Table 1. Reactivity of a panel of mAb with different lymphoid tissue compartments

	mAb						
	4F3	L11/135	3E12	6D4	3C10	3B6	2C4
GALT							
TDA	+++	+++	−*	−	−	−	++†
Corona	+++	+	+++	+++	+	+++	+++
Germinal centre	+++	+	+++	+++‡	+	+++§	+++
Dome	+++	++	++	++	++	+++	+++
Spleen							
PALS	+++	+++	−*	−	−	−	++†
Follicles	+++	+	+++	+++	−	+++	+++
Red Pulp	+++	++	++	++	++	+	+++
Thymus							
Cortex	+++	+++	−	−	−	−	++†
Medulla	+++	+++	−	−*	−*	++	+++

* A few, scattered positive cells.

† Reticular pattern.

‡ Reduced reactivity or negative in periphery of germinal centre.

§ Weak reactivity in appendix.

RESULTS

Lymphocyte compartmentalization in GALT

The reactivity of the lymphocyte-specific mAb with rabbit GALT is shown in Table 1. Lymphocytes in all compartments of GALT were labelled with mAb 4F3 (IgG1, κ), which accounted for >95% of the cells stained (Fig. 1).

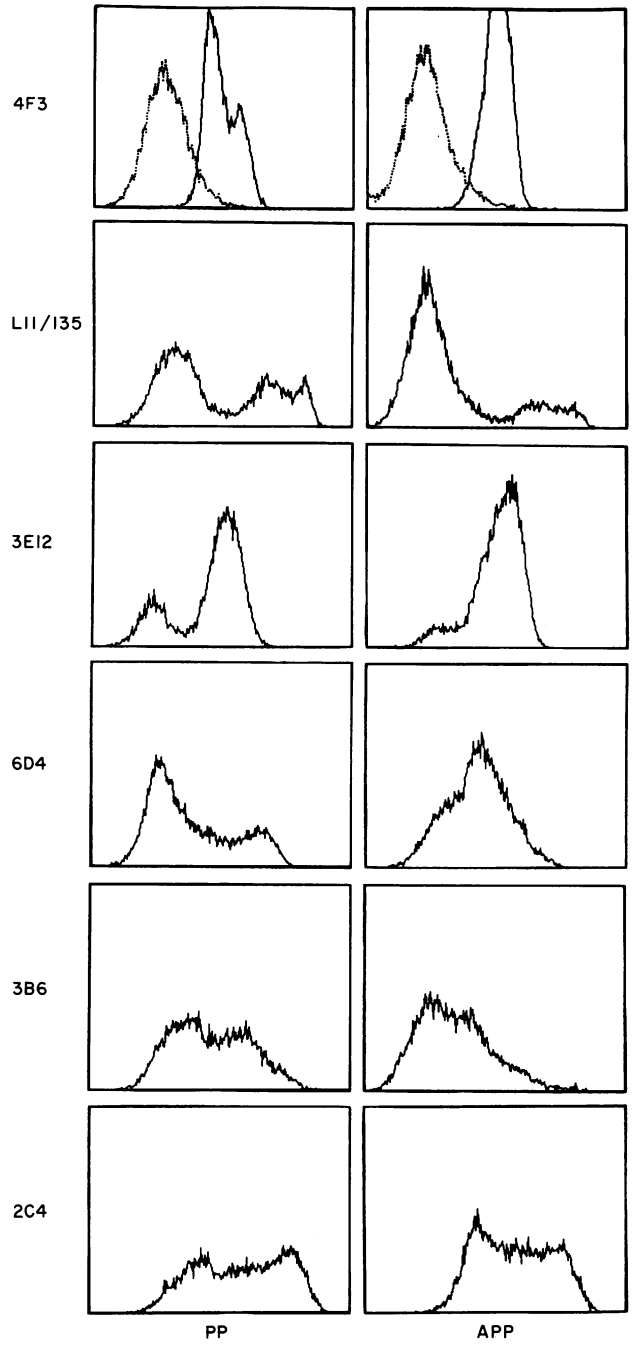

Figure 1. Flow cytometric analysis of Peyer's patch (PP) and appendix (APP) lymphocytes labelled with mAb 4F3, L11/135, 3E12, 6D4, 3B6, and 2C4. *x*-axis, fluorescence intensity. *y*-axis, relative number of cells. In the first panel, control cells (dotted line) incubated with hybridoma culture medium instead of mAb are shown.

T-cell areas were identified by labelling with mAb L11/135 (Table 1). L11/135 labelled all cells in the T-dependent areas (TDA), scattered cells in corona and germinal centre, and about half the cells in the dome of GALT (Fig. 2a). By flow cytometry, T cells represented about 40% of lymphoid cells in Peyer's patches and 10–20% in appendix (Fig. 1).

Two mAb produced by immunization with rabbit GALT cell populations reacted with B-cell compartments. mAb 3E12 (IgG1, κ) labelled all cells in coronas and germinal centres, about half the cells in the dome, and a few scattered cells in TDA (Table 1). By flow cytometry, 3E12$^+$ cells formed a single, distinct peak of intermediate intensity and accounted for about 75% of cells in Peyer's patches and 90% in the appendix (Fig. 1). mAb 6D4 (IgG1, κ) densely labelled follicle coronas, less densely labelled germinal centres, about half the cells in the dome, but did not label TDA (Table 1, Fig. 2b). By flow cytometry, about 35% of cells in Peyer's patches (mostly bright)

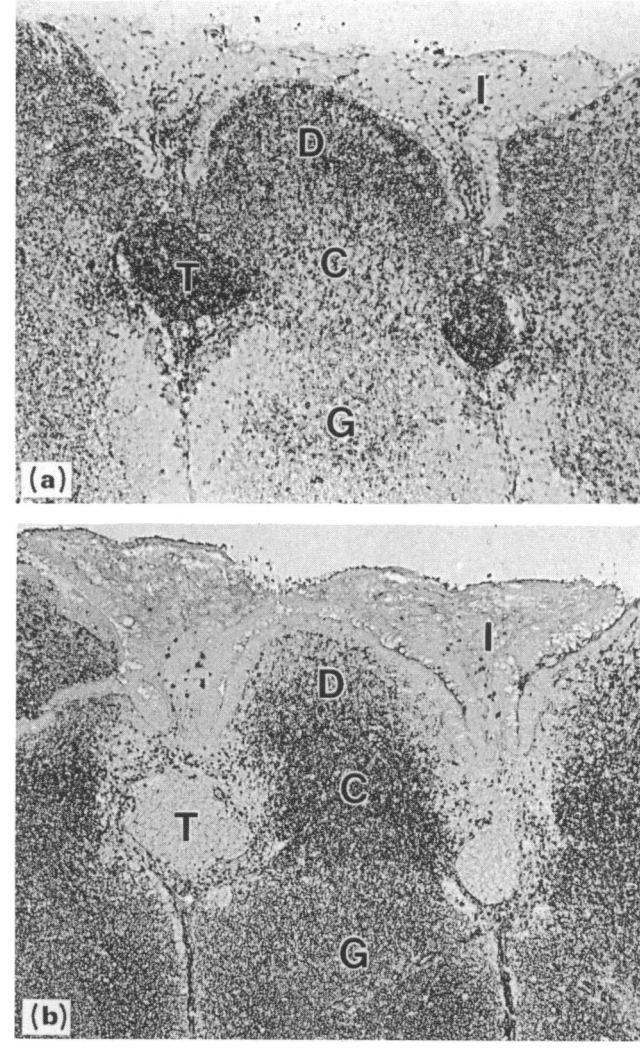

Figure 2. T-cell (a) and B-cell (b) compartments in GALT (appendix). (a) L11/135 reacts with TDA (T) and scattered cells in corona (C) and germinal centre (G). (b) 6D4 identifies B cells in corona and germinal centre. Both L11/135$^+$ and 6D4$^+$ cells populate the dome (D). Lumen located at top of figure. Interdomal areas (I) composed of colonic epithelium and lamina propria separate domes (× 50).

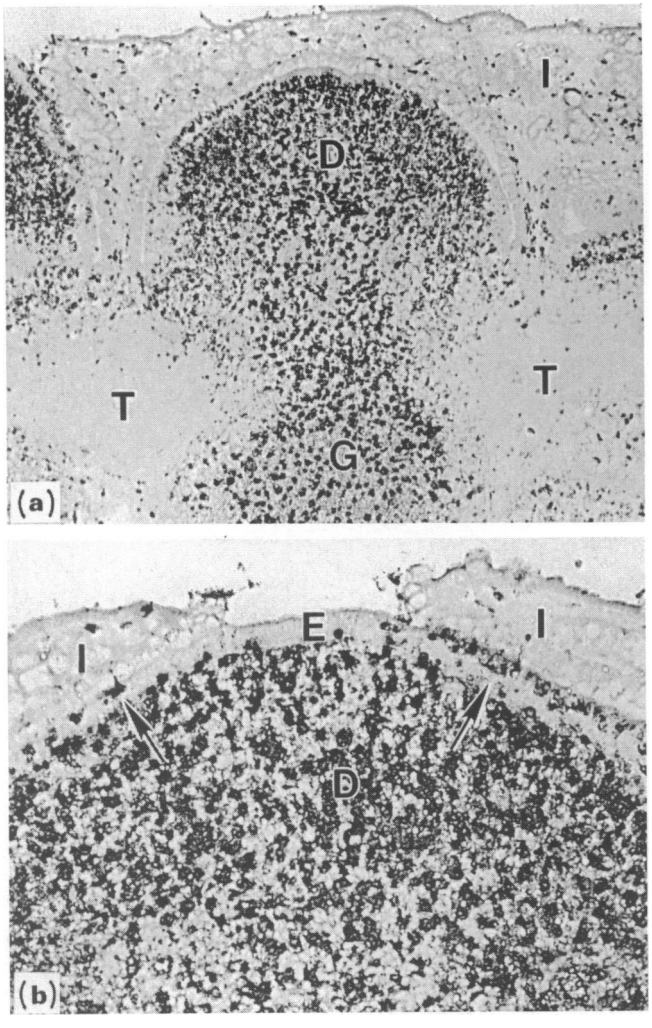

Figure 3. 3C10 reactivity in GALT (appendix). (a) 3C10 reacts with macrophage–lymphocyte clusters in the dome (D) and tingible body macrophages in the germinal centre (G). Lymphocytes in TDA (T) are unlabelled. (b) Higher magnification of dome showing $3C10^+$ cells (arrows) in the follicle epithelium (E). Apical surfaces of interdomal colonic epithelium and follicle epithelium are closely apposed to each other. I, interdomal area (a, × 50; b, × 120).

and about 70% of cells in appendix (mostly dull) were $6D4^+$ (Fig. 1). The proportions of bright and dull cells corresponded to the size of coronas and germinal centres seen by immunohistochemistry. Thus, in Peyer's patches, the large corona corresponded to a distinct bright peak, whereas in the appendix the large germinal centre corresponded to a large dull peak (Fig. 1).

Recognition of dome macrophage–lymphocyte clusters by mAb 3C10

mAb 3C10 (IgM, κ) labelled macrophage–lymphocyte clusters in the dome, tingible body macrophages in the germinal centre, and a subpopulation of intraepithelial lymphocytes in the follicle epithelium of GALT (Table 1; Fig. 3). It was unreactive with lymphocytes defined by L11/135 in TDA or by 6D4 or 3E12 in coronas and germinal centres. The cytoplasm of large mononuclear cells in the dome and germinal centre was labelled, and lymphocytes in the dome and follicle epithelium displayed

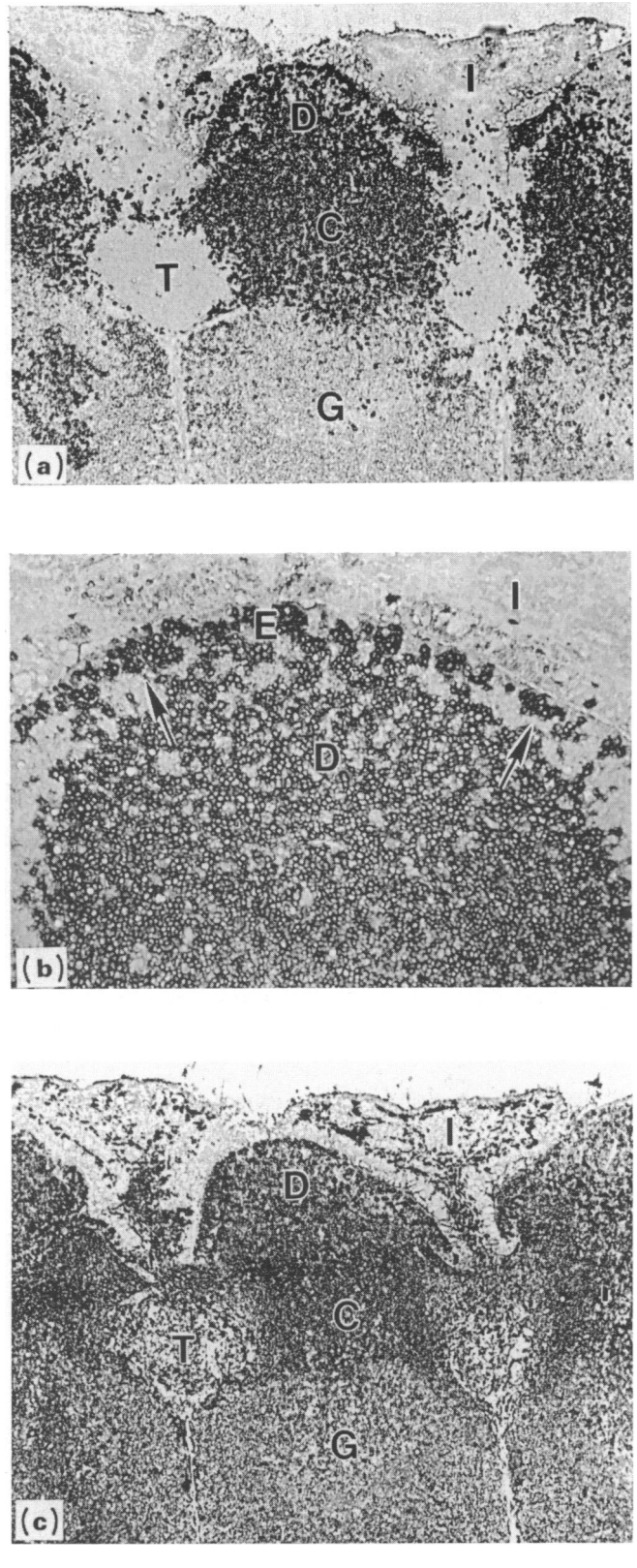

Figure 4. 3B6 reactivity in GALT (appendix) (a, b) compared to class II reactivity (2C4) (c). (a) 3B6 is reactive with lymphocytes in dome (D) and corona (C). In TDA (T), cells are unreactive with 3B6 (a), but 2C4 detects cells with a reticular pattern (c). (b) Higher magnification of dome showing $3B6^+$ cells (arrows) in follicle epithelium (E). G, germinal centre; I, interdomal area (a, c, × 50; b, × 120).

Table 2. Lymphocyte counts in M-cell pockets defined by a panel of mAb

	mAb					
	4F3	L11/135	6D4	3C10	3B6	2C4
Appendix	112±2* (100)†	102±12 (95)	6±3 (5)	34±9 (31)	81±12 (75)	112±14 (100)
Peyer's patches	89±12 (94)	61±15 (65)	33±10 (35)	35±8 (37)	91±12 (96)	100±8 (100)

* Cell counts ± SEM per 500 μm segment of follicle epithelium.
† (Percentage) relative to the sum of L11/135$^+$ and 6D4$^+$ cells.
Average length of appendix epithelium = 1·48 ± ·16 mm.
Average length of Peyer's patch epithelium = 1·59 ± ·31 mm.

granular perinuclear staining. In the dome, 3C10$^+$ cells were present in areas defined by both L11/135 and 6D4. By flow cytometry, mAb 3C10 failed to identify a distinct positive cell population.

mAb 3B6 reacts with a subpopulation of Ia$^+$ cells

mAb 3B6 (IgG3, κ) reacted with lymphocytes in the dome and follicle epithelium but not cells defined by L11/135 in TDA of GALT (Table 1, Fig. 4a, b). The expression of 3B6 antigen overlapped with but was more restricted than that of Ia$^+$ cells identified by mAb 2C4. Both 3B6 and 2C4 labelled lymphocytes in domes and B-cell populations (Table 1). In TDA, however, 3B6 did not label cells (Fig. 4a), whereas 2C4 labelled cells in a reticular pattern (Fig. 4c). In follicles, the reactivity of 3B6 with germinal centres in appendix was weak in comparison to the dome (Fig. 4a). By flow cytometry, heterogeneous cell subpopulations with low, intermediate, and high levels of fluorescence intensity were seen with both mAb (Fig. 1).

3C10 and 3B6 lymphocytes in dome epithelium are primarily Ia$^+$ T cells

In the follicle epithelium, the proportion of lymphocytes was determined in M-cell pockets (Table 2). Greater than 95% of lymphocytes in the epithelium reacted with mAb 4F3 and 2C4. T cells represented about 95% of lymphocytes in the appendix

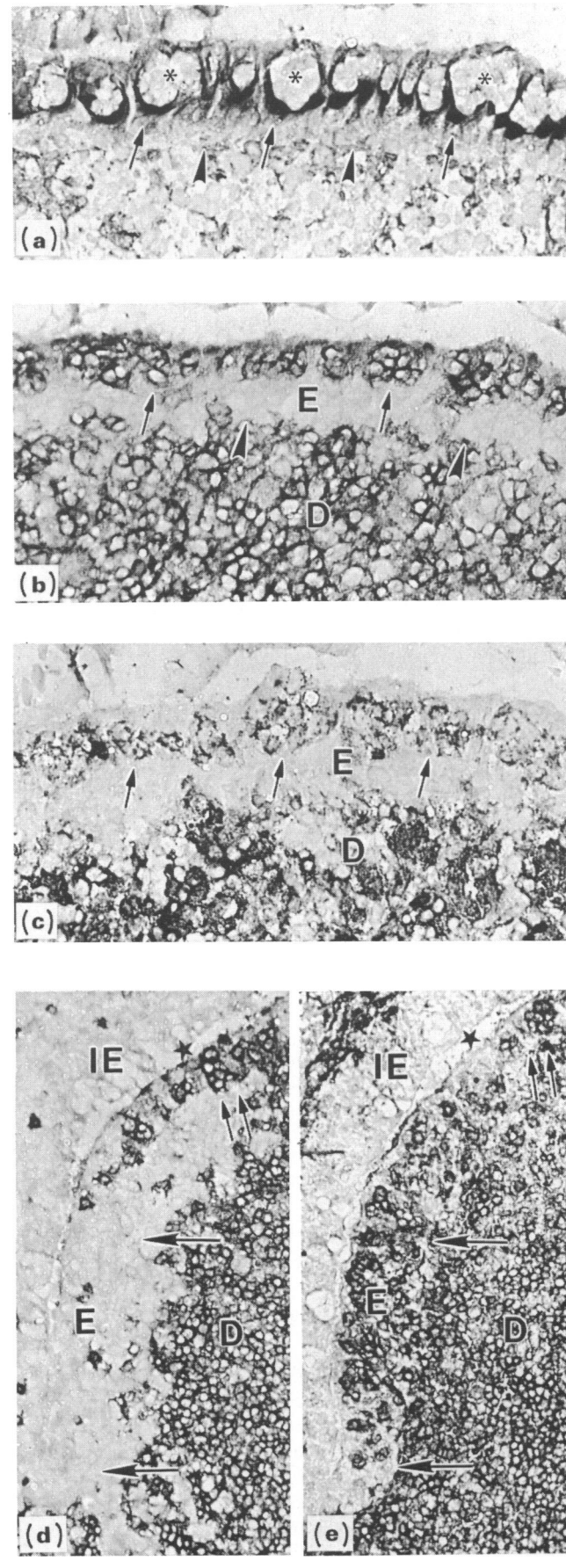

Figure 5. Distribution of lymphocyte subsets in follicle epithelium. (a) M cells (arrows) defined by mAb 5D9. Reaction product is concentrated above the level of epithelial cell nuclei enclosing clusters of lymphocytes in M-cell pockets (asterisks). Basal lamina of follicle epithelium denoted by arrowheads. (b) L11/135 reacts with most lymphocytes (arrows) in M-cell pockets. (c) 3C10 gives a perinuclear granular reaction product in intraepithelial lymphocytes (arrows). (d, e) Differential expression of 3B6 by lymphocytes in follicle epithelium of appendix. Most lymphocytes in follicle epithelium (E) at base of dome (area between single arrows) are unreactive with mAb 3B6 (d) but reactive with mAb 2C4 (e). Further up the dome, lymphocytes reactive with both 3B6 and 2C4 are found (double arrows). Ia-bearing cells labelled by mAb 2C4. A star denotes the lumen. D, dome; IE, interdomal colonic epithelium. (a–c, × 300; d,e, × 190).

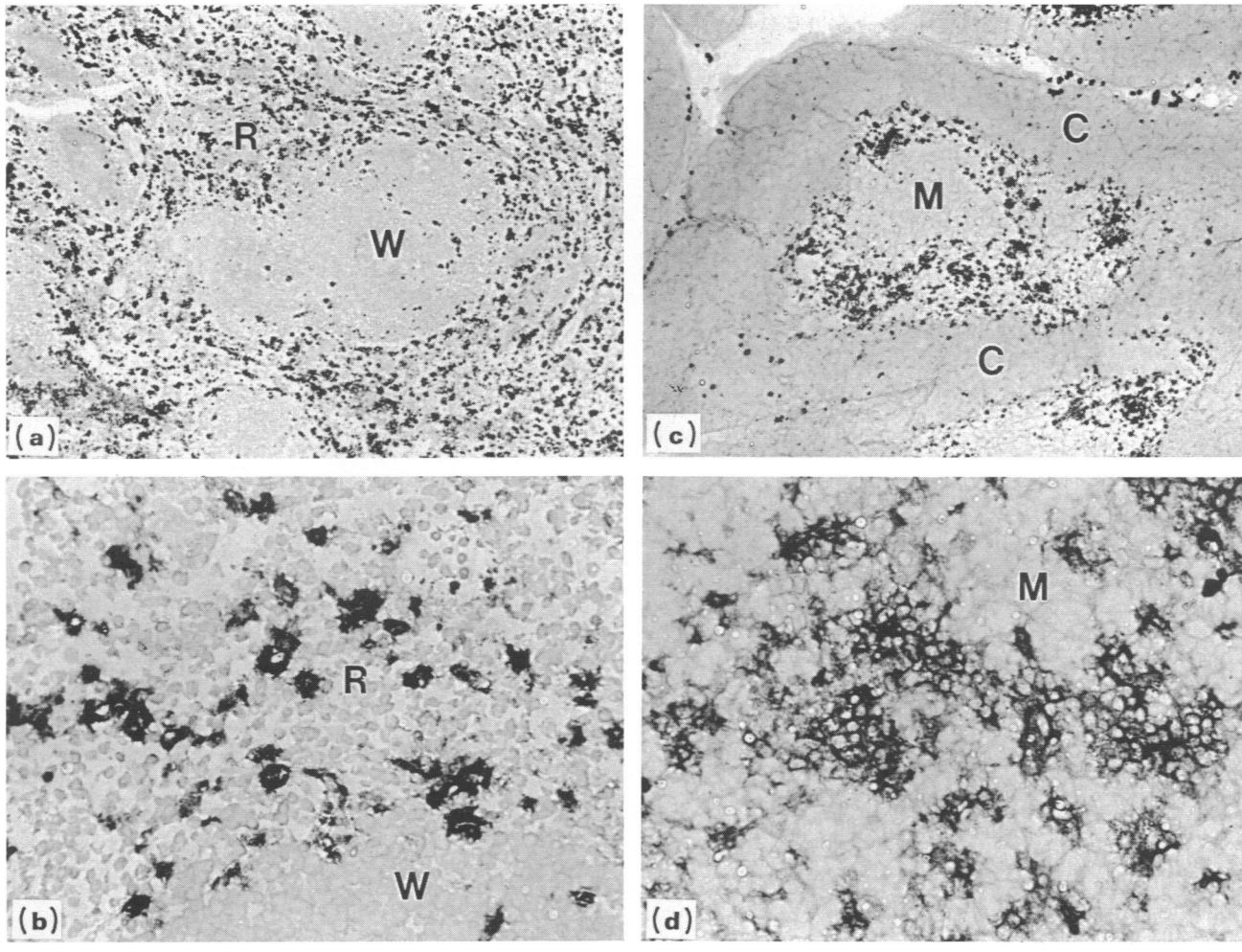

Figure 6. Distribution of 3C10$^+$ and 3B6$^+$ cells in non-GALT lymphoid compartments. (a, b) 3C10 reactivity in spleen. 3C10 labels primarily macrophages in red pulp (R) but not white pulp (W). (c, d) 3B6 reactivity in the thymus. 3B6 labels clusters of thymocytes in the outer medulla (M). C, cortex (a, c, × 50; b, d, × 300).

and 65% in Peyer's patches, with B cells accounting for the remaining lymphocytes. 3C10$^+$ cells accounted for about 30% of lymphocytes in M-cell pockets (Fig. 5a–c). 3B6$^+$ cells accounted for about 75% of lymphocytes in appendix and greater than 95% in Peyer's patches (Table 2).

In the appendix, the phenotype of lymphocytes correlated with their position on the sides of domes (Fig. 5d,e). Within and directly beneath the epithelium at the base of the dome, lymphocytes were reactive with L11/135 and 2C4 but not 3C10 nor 3B6. At sites distant from crypts, lymphocytes were reactive with L11/135, 2C4, 3C10, and 3B6.

3C10 and 3B6 reactivity in spleen and thymus

The reactivity of the panel of mAb was examined in spleen and thymus (Table 1). 3C10 recognized solitary mononuclear cells in the red pulp but not the white pulp of the spleen (Fig. 6a, b). In the thymus, it was unreactive with cells in cortex and rarely reactive with mononuclear cells in the medulla (Table 1).

3B6 labelled follicles and red pulp but not periarteriolar lymphoid sheaths (PALS) in the spleen (Table 1). In the thymus, it reacted with clusters of thymocytes in the outer medulla but was unreactive with cortical thymocytes (Fig. 6c, d). Comparison with serial sections showed that all medullary thymocytes were reactive with 2C4 but, in the cortex, 2C4$^+$ cells formed a reticular meshwork surrounding unreactive thymocytes (Table 1).

DISCUSSION

This study characterized the tissue distribution of two mAb of novel specificity for GALT dome and M-cell pocket lymphocytes. The distribution of T cells and B cells in this study was similar to distributions reported in rabbits (Sell, Raffel & Scott, 1980; Loar, Dennison & Sell, 1986), rodents (Mayrhofer, Pugh & Barclay, 1983; Witmer & Steinman, 1984; Ermak & Owen, 1986) or ungulates (Parsons *et al.*, 1989). T cells identified by mAb L11/135 (Jackson *et al.*, 1983) were found in TDA and scattered in follicle coronas and germinal centres. Because CD4$^+$ cells localize in coronas and germinal centres of rodents (Ermak & Owen, 1986) it is reasonable to assume that the L11/135$^+$ cells in rabbit coronas and germinal centres represent a population of CD4$^+$ T cells. The mAb 6D4 and 3E12, which reacted with B-cell regions, had a tissue distribution similar to

that of sIgM$^+$ cells (Sell *et al.*, 1980). 6D4 was expressed at higher density on corona B cells than germinal centre B cells. Coronal B cells have been shown to express surface IgD, which is absent on germinal centre cells; however, they do not bind the lectin peanut agglutinin (PNA) which binds to germinal centre cells (Butcher *et al.*, 1982).

The distribution of cells reactive with mAb 3C10 in GALT indicated that this mAb labelled dome lymphocytes and dome and germinal centre macrophages. Dome and germinal centre macrophages have been shown to be distinct subpopulations in GALT based upon expression of cell-surface molecules, ultrastructure, and histochemical staining patterns (LeFevre, Hammer & Joel, 1979; Lause & Bockman, 1981; Ermak & Owen, 1986). The antigen labelled by 3C10 was distributed in the cytoplasm of positive cells. The virtual absence of 3C10$^+$ cells in the thymus indicated that this antigen did not appear on T cells during intrathymic development but may have become expressed on T cells in the GALT microenvironment. 3C10 was also reactive with macrophages in splenic red pulp, but only in GALT were populations of lymphocytes recognized. The presence of 3C10$^+$ lymphocytes in GALT may be related to their specificity for mucosal tissues. Alternatively their presence may reflect different immunological states between GALT, which has large secondary follicles with germinal centres, and spleen, which only has a few, small germinal centres. The reactivity of 3C10 on both cell populations raises the possibility that this molecule is expressed on lymphocytes during interactions with macrophages.

The distribution pattern of mAb 3B6 in GALT, spleen, and thymus suggested that it reacted with a subpopulation of Ia-bearing cells. Ia molecules have been shown to be present on activated T cells but not most T cells in TDA, medullary thymocytes, B cells and dendritic cells in thymus, spleen and GALT (Mayrhofer, Pugh & Barclay, 1983; Dijkstra & Döpp, 1983; Ewijk, 1984). In contrast, 3B6 was reactive with dome T cells but not TDA lymphocytes, a subpopulation of medullary thymocytes, and B cells. Unlike the mAb 2C4 (Lobel & Knight, 1984), 3B6 did not label dendritic cells in thymic cortex or in TDA of GALT. The distribution pattern suggested that 3B6 on T cells may be expressed during T-cell development in the thymus. Although it has been shown that rabbit thymocytes migrate to GALT domes (Barg & Draper, 1975), it is not known whether 3B6-bearing medullary thymocytes migrate to GALT domes. The shared expression of 3B6 by a subpopulation of medullary thymocytes and dome T cells suggests that 3B6$^+$ cells in thymus may migrate to GALT domes and populate M-cell pockets.

Staining of serial sections of GALT domes indicated that most lymphocytes located in M-cell pockets in the rabbit are Ia-bearing T cells, including lymphocytes defined by mAb 3B6 and 3C10. In the mouse, both CD4$^+$ and CD8$^+$ cells were identified in the follicle epithelium (Ermak & Owen, 1986). Lymphocytes in follicle domes represent a population of migrating lymphocytes which localize to the follicle epithelium (Bhalla & Owen, 1983). The basal lamina beneath the follicle epithelium contains numerous lymphocyte-sized pores (McCluggage, Low & Zimny, 1986; Pappo *et al.*, 1988), suggesting that the lymphocytes in M-cell pockets frequently migrate into and out of the epithelium. Follicle epithelial cells migrate along the basal lamina from the base to the tip of each dome and are renewed every 3–4 days (Bhalla & Owen, 1982). The increase in proportion of 3C10$^+$ and 3B6$^+$ lymphocytes in appendiceal follicle epithelium at areas distant from crypts suggests that lymphocytes in M-cell pockets may acquire 3C10 or 3B6 reactivity during contacts with M cells. Alternatively, 3B6$^+$ cells may preferentially associate with mature M cells on the sides of appendiceal domes. The observation that most lymphocytes in the follicle epithelium are Ia-bearing T cells which express molecules not found in mature T-cell areas of GALT, spleen or most of the thymic medulla indicates that these are a phenotypically distinct subset of T cells.

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