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A new antigenic determinant on intra-epithelial lymphocytes and its association with CD45

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SUMMARY

Rat monoclonal antibodies were prepared against intra-epithelial lymphocytes (IEL) isolated from the gut of Balb/c mice and screened for selective reactivity with mucosal lymphocytes. One antibody, M371, identified a new surface antigen on 30–40% of IEL. It bound to very few, if any, lymphocytes within the lamina propria and to none in other lymphoid tissues; neither did it stain lymph node lymphocytes that had been stimulated in culture with mitogens or alloantigens. The data suggest that M371 identifies a sessile population of IEL and that expression of the antigen is induced locally in the epithelium. In addition to IEL, M371 bound to some goblet cells in the mid and distal small gut but not in the proximal region. Double-staining experiments showed that M371 was highly specific for IEL with the phenotype Lyt-2^+, Lyt-3^-, Thy-1^-, $CD3^+$ and stained a majority of cells in this subpopulation. M371 precipitated a surface molecule approximately 275,000 MW in size, which was also precipitated by antibodies to CD45. Treatment of fixed IEL with sodium periodate prevented staining by M371, suggesting involvement of carbohydrate in the epitope. The specificity of M371 was shown to differ from that of the antibodies CT1 and CT2, which identify a carbohydrate determinant of CD45 expressed on cytotoxic lymphocytes and IEL. The possibility that the gut epithelium provides an environment for the functional differentiation of thymus-independent mucosal T cells is discussed.

INTRODUCTION

It has always been attractive to speculate that intra-epithelial lymphocytes (IEL) in the gut may have an important role in mucosal immunity. Their abundance and close proximity to the external environment has provided reasonable justification for this view. IEL are heterogeneous with respect to size, cytoplasmic granularity and surface phenotype but most display T-cell characteristics (Dobbins, 1986, Ernst, Befus & Bienenstock, 1985). Despite a substantial research effort in recent years their true function and pathway of differentiation is still quite obscure. Recently, these cells have attracted renewed and widespread attention because of reports that most of them express T-cell receptors composed of γ and δ chains (Goodman & Lefrancois, 1988; Bucy *et al.*, 1988; Bonneville *et al.*, 1988) in contrast to the $\alpha\beta$ complex which, in man and the mouse, is overwhelmingly predominant among T cells in other lymphoid compartments. Such reports have encouraged the idea that IEL may be the product of a novel T-cell lineage.

We have prepared a large panel of monoclonal antibodies against mouse intra-epithelial lymphocytes and screened them for selective reactivity against mucosal lymphoid tissue. The purpose was to identify unusual surface features that could provide insights into the function or differentiation of these cells, in particular the selective 'homing' of thoracic duct T-blasts to mucosal surfaces (Husband & Dunkley, 1988) and putative functional interactions between lymphocytes and gut epithelial cells. Two antibodies were selected. The first, M290, stained a majority of T lymphocytes in the intestinal villus and precipitated novel surface structures of approximate molecular size 135,000 and 175,000 MW (Kilshaw & Baker, 1988). A similar antigen on mucosal lymphocytes of rat and man has recently been described by Cerf-Bensussan *et al.* (1986, 1987). The second antibody, M371, has a more restricted distribution among lymphocyte subsets and is the subject of the present communication.

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MATERIALS AND METHODS

Animals

Specific pathogen-free BALB/c male mice, approximately 8–10 weeks old, were obtained from Harlan Olac Ltd (Bicester, Oxon) and retained within local animal accommodation for up to 4 weeks.

Preparation of monoclonal antibodies to intra-epithelial lymphocytes (IEL)

IEL were isolated from BALB/c mice by the method of Davies & Parrott (1981). The purity of preparations was routinely assessed by staining with monoclonal antibodies to CD45; cells that were not of haemopoietic origin consistently comprised

15% or less of the preparation. The contaminants were large spherical granulated cells thought to be derived from the crypt epithelium (Lefrancois, 1987).

IEL ($1{\cdot}5 \times 10^7$) were injected intravenously into Lou/c rats weekly for 4 weeks. Rat spleen cells were then fused with the mouse cell partner P3X63Ag8, 653 by conventional techniques. Supernatants were screened by immunohistochemical staining of BALB/c duodenum and selected for ability to stain IEL but not lymph node cells. Hybridomas M290 and M371 satisfied this selection criterion.

Other antibodies

The following monoclonal and polyclonal antibodies were used in this study: FITC-conjugated rat anti-Lyt-2 (53.6.72; Becton-Dickinson); rat anti-Lyt-3 (53.5; Ledbetter *et al.*, 1981); rat anti-CD45, leucocyte common antigen (YBM42.2.2; Watt *et al.* 1983; and M1/9.3; Springer *et al.*, 1978); hamster anti-CD3 (145-2C11; Leo *et al.*, 1987); mouse anti-Thy-1.2 (5a-8; Cedar-Lane Laboratories); mouse anti-TcR $V_\beta 8{\cdot}1$–$8{\cdot}3$ (F23.1; Staerz *et al.*, 1985); rat anti-TcR $V_\beta 8{\cdot}1$–$8{\cdot}2$ (KJ16; Haskins *et al.*, 1984); mouse anti-rat-kappa chain (RG7/9.1 and RG7/7.6); phycoerythrin-labelled sheep anti-rat Ig (Serotec) that had been absorbed with mouse IgG to render it species specific; biotinylated sheep anti-rat Ig (Amersham International); FITC-labelled sheep anti-mouse Ig (Amersham International). The rat IgG2a antibody 19.34 (anti-aflatoxin) and the mouse IgG1 antibody NOA-1, (anti-ovalbumin) were used as negative controls for surface staining. In addition, GY11/217 rat IgG2c anti-rat class I MHC was used as a control in immunoprecipitation experiments.

Immunohistochemistry and lymphocyte surface staining

Frozen sections (6μ) of mouse gut were fixed in acetone (5 min) and treated with the following sequence of reagents, applying each for 25 min at room temperature; M371, biotinylated anti-rat Ig, streptavidin-biotin-horseradish peroxidase complex and, finally, diaminobenzidine (DAB) with hydrogen peroxide. Endogenous peroxidase activity was eliminated by incorporating sodium azide (0·5%) in the DAB solution. Sections were counterstained with haematoxylin and eosin.

For lymphocyte surface staining, IEL (3×10^6) in 100 μl of Eagle's minimum essential medium (MEM) containing 5% fetal bovine serum and 0·1% sodium azide were treated at 0° with monoclonal antibodies used singly or as pairs of rat and mouse antibodies. Antibody binding was detected using FITC-labelled anti-mouse Ig, phycoerythrin-labelled anti-rat Ig or the two reagents used in sequence. Fluorescence was assessed using a FACS 420 (Becton-Dickinson) equipped with a data lister and Consort 30 software system. Fluorescence intensity is expressed on an arbitary scale spanning approximately two decades. FITC was examined using a 540 nm short pass filter and phycoerythrin with a 560 nm long pass filter. Fluorochromes were excited with a 488 nm, 400 milliwatt argon laser. Alternatively, fluorescence was assessed by microscopy; for each preparation approximately 400 cells were counted.

Surface iodination and immunoprecipitation of IEL surface antigens

Freshly isolated IEL (2×10^7) were iodinated using 2 mCi Na [^{125}I] and 10 μg iodogen (Pierce, UK Ltd). They were then lysed in 0·6 ml 1·0% Nonidet P-40, 2 mM phenyl methyl sulphonyl fluoride, 2 mM EDTA, 10 μM pepstatin, 10 mM iodoacetamide in phosphate-buffered saline (PBS). After centrifuging the lysate at 100,000 *g* for 30 min at 4° the supernatant was 'precleared' by treatment for 1 hr at 4° with 10 μl protein A sepharose CL-4B (Pharmacia, Uppsala, Sweden). Surface antigens were precipitated by addition of 10 μl protein A–Sepharose that had been precoated with M371 (0·3 ml culture supernatant containing 20 μg IgG2c) or control antibody. For precipitation with anti-CD45 rat IgG2a antibodies (YBM 42.2.2 and M1/9.3), protein A–Sepharose beads were first coated with monoclonal mouse IgG2a anti-rat Kappa chain (40 μg RG7/9.1 per 10 μl beads) followed by 20 μg anti-CD45 antibody. Coated beads were mixed with IEL lysates for 1 hr at 4° and then washed. In experiments involving sequential precipitation, lysates were pretreated twice with 20–40 μl antibody-coated beads, each treatment lasting 45 min. Precipitated antigens were reduced with 5% 2-mercaptoethanol, separated by SDS–PAGE gradient gel (5–8%) and detected by autoradiography using Kodak X-AR5 film and enhancing screens.

Periodate oxidation of surface antigens

Isolated IEL were fixed for 30 min at room temperature with 2% paraformaldehyde and then treated for 10 min with 0·1 M glycine in phosphate-buffered saline(PBS). They were then incubated for 20 min at 37° in 0·05 M sodium acetate (pH 5·5) with or without 2 mM sodium m-periodate. The cells were then washed in MEM containing 5% fetal bovine serum and stained by immunofluorescence for FACS analysis.

ELISA with Tamm–Horsfall glycoproteins (THG)

Sd (a^+) and Sd (a^-) THG (Dr W. M. Watkins, CRC, Harrow, Middex) were dissolved in PBS at 10 μg/ml and coated onto microtitre plates overnight. Subsequently PBS containing 0·05% Tween 20 was used to wash plates and dilute reagents. Serial dilutions of M371 were applied to plates for 1 hr at room temperature. Rat Ig was then detected using biotinylated anti-rat Ig followed by streptavidin-biotin-peroxidase complex and finally o-phenylenediamine. For inhibition studies M371 was applied to the plate for 1 hr and the antibody CT-1 was then added for another hour. Binding of mouse Ig was detected by a similar peroxidase method.

RESULTS

Tissue distribution of M371-positive cells

Frozen sections of BALB/c small intestine prepared from samples taken at a range of sites along the gut were stained with M371 by the immunoperoxidase technique. In the proximal third of the gut, M371 stained only lymphocytes within the epithelium (Fig. 1a, b). In the mid and distal gut $M371^+$ IEL were present at a lower frequency and a variable proportion of goblet cells were also stained, both on the villi and in the crypts (Fig. 1c). In athymic BALB/c nude mice, the majority of IEL were $M371^+$.

Stained cells were not seen in the lamina propria at any level of the gut. Frozen sections and isolated lymphocytes were prepared from other lymphoid organs and tested with M371, (isolated cells being tested both for surface staining of viable cells and cytoplasmic staining of acetone-fixed cytosmears). $M371^+$ cells were not detected at all in bone marrow, spleen,

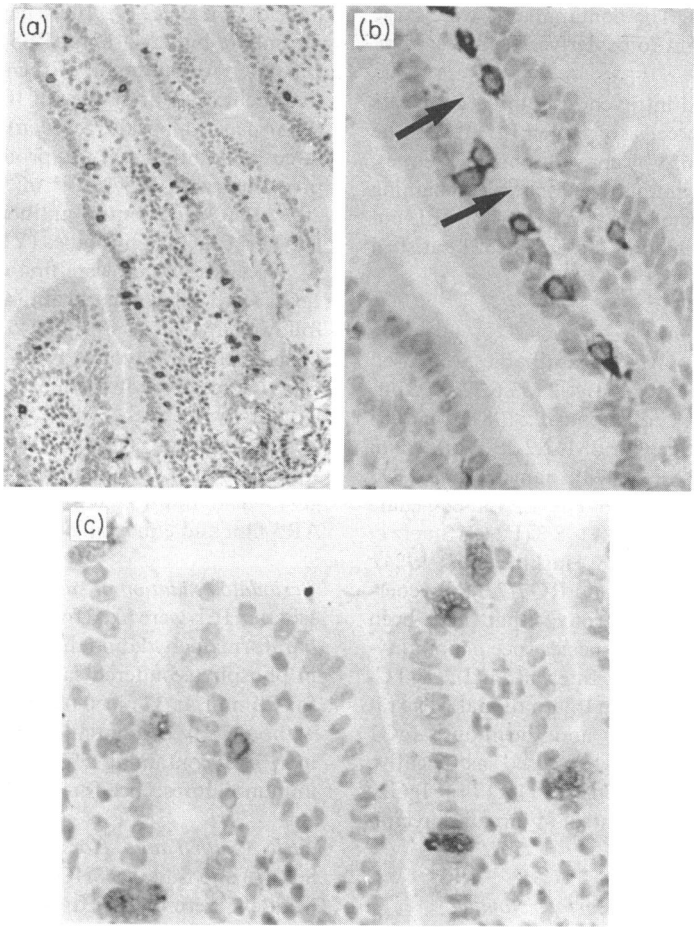

Figure 1. Immunoperoxidase staining with M371, (a) and (b) IEL in duodenal villi. The arrows in (b) mark the line of the basement membrane under the left hand row of epithelial cells. (c) stained goblet cells in mid-gut.

Table 1. Surface staining of BALB/c IEL

Antibody or marker	% positive ± SD
M371	35 ± 5
M290	84 ± 2
Lyt-2	78 ± 6
Thy-1	39 ± 8
CD3	83 ± 3
TcR V_β8·1–8·3	21 ± 7
TcR V_β8·1 8·2	19 ± 1
CD45	92 ± 3

peripheral and mesenteric lymph nodes, thymus and Peyer's patches. Neither did IEL in the bronchiolar epithelium show positive staining. Lymph node cells that had been stimulated in culture with concanavalin (Con A) also gave negative results.

Surface phenotype of IEL

Table 1 shows results obtained by staining the surface of IEL with M371 and antibodies to other markers. The percentage values are shown to establish the characteristics and purity of our IEL preparations in comparison with those of other laboratories. The percentage of cells expressing TcR V_β8 is discussed more fully elsewhere (Viney, Macdonald & Kilshaw 1989). Antibody M290 identifies a new surface marker found mainly on epithelial lymphocytes (Kilshaw & Baker 1988).

Figure 2 shows a series of FACS profiles defining the IEL subset stained by M371. It shows that M371 identifies, almost exclusively, Thy-1$^-$, Lyt-2+ cells (Fig. 2a, b) and that few of these express $\alpha\beta$ TcR V_β8 (Fig. 2c). Because 90% of the IEL population as a whole stains for CD3, discounting the small proportion of non-lymphoid contaminants (Fig. 2d and Table 1), it follows that most M371$^+$ cells must be CD3$^+$. The Thy-1$^-$ Lyt-2$^+$ subset encompasses about 50% of IEL (Fig. 2e, Table 1) and M371 stains most of these but probably not all. Detection of Lyt-3 only on Thy-1$^+$ IEL (Fig. 2f) implies that the M371$^+$ population probably does not express the normal Lyt-2/3 heterodimer.

Table 2 shows numerical data pooled from FACS analysis and fluorescence microscopy on the co-expression of surface markers. In summary, M371$^+$ IEL display the following phenotype: CD3$^+$ Thy-1$^-$, Lyt-2$^+$ Lyt-3$^-$, and TcR V_β8 is sparsely represented.

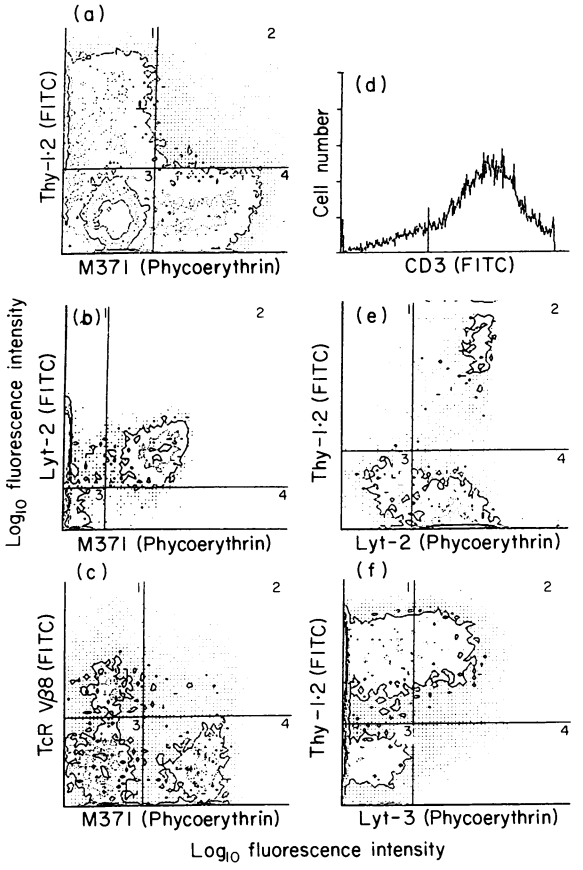

Figure 2. Two-colour FACS contour plots showing co-expression of IEL surface markers. TcR $V_\beta 8$ was stained with antibody F23.1. Staining for CD3 is shown only as a simple histogram.

Table 2. Co-expression of IEL surface markers

Subset	Distribution of markers within subset % positive ±SD	
M371	Thy-1	1·1±0·8
	Lyt-2	99·2±1·2
	TcRV_β8·1-8·3	8·0±1·65
Lyt-3	Thy-1·2	91·7±0·7
Lyt-2	Thy-1·2	31·5
TcR V_β8·1-8·2	Thy-1·2	89·3±3·3

Immunoprecipitation studies

Autoradiographs of a series of immunoprecipitates separated by SDS-PAGE are shown in Fig. 3. M371 precipitated from the surface of IEL, an antigen with an approximate molecular size of 275,000 MW (tracks a, e). A gradation of material of lower molecular weight, down to 200,000 MW, was consistently present. Pre-absorption of the IEL lysate with the monoclonal antibody YBM42.2.2, specific for an invariant determinant of the leucocyte common antigen CD45, almost completely removed the antigen (track c) and precipitated, in addition bands at 180,000 and 200,000 MW (track d). The reciprocal absorption experiment was performed with a second antibody, M1/9·3, specific for a monomorphic determinate of CD45. This gave a precipitation pattern similar to that of YBM42.2.2 (track f) and pre-absorption of the lysate with M371 removed the 275,000 MW component (track g). It is noticeable that the 180,000 and 200,000 MW bands were less prominent after this pre-absorption but the significance of the observation has not been investigated. The two antibodies to CD45 gave identical precipitation patterns with lysates from lymph node cells. Prominent bands were seen at 180,000, 200,000, and 215,000 MW and faint bands at 235,000 and 275,000 MW (tracks h, i, k, l). Smaller components were also present and were probably degradation products. M371 did not precipitate any antigens from lymph node cell lysates (track j).

Treatment of surface antigens with sodium periodate

IEL that had been fixed with paraformaldehyde were incubated with sodium m-periodate or with acetate buffer and then surface-stained with M371 (Fig. 4). Treatment with 2 mM periodate largely prevented staining (compare Fig. 4a, b). Residual low level fluorescence was largely due to an increase in background caused by periodate and was seen in controls. Staining with YBM42.2.2, anti-CD45, was unaffected by periodate treatment (Fig. 4c, d).

Relationship with CT antibodies

Our data suggests that M371 detects an IEL surface antigen that is in some way associated with CD45. The effect of periodate indicates that carbohydrate may be important for the integrity of the epitope. Superficially there are similarities between M371 and the antibodies CT-1 and CT-2, which detect carbohydrate epitopes on CD45 that are expressed on IEL and cytotoxic T-cell lines. (Lefrancois & Bevan, 1985; Lefrancois, 1987). It is notable too that CT antibodies stain some epithelial cells in the villi and crypts (Lefrancois, 1987), but it is unclear whether or not goblet cells give positive results.

The relationship between the antibodies was investigated in more detail. CT-1 and CT-2, though differing in fine specificity, both recognize an o-linked oligosaccharide with the following structure: GalNAcβ1, 4[SAα2, 3]-gal (Conzelmann & Lefrancois, 1988). This is found on the human blood group Sd (a^+) Tamm–Horsfall glycoprotein and on erythrocytes of the rare Cad blood group (Donald *et al.*, 1983). Therefore, M371 was tested by ELISA for binding to Sd(a^+) Tamm–Horsfall glycoprotein and for inhibitory activity preventing CT-1 binding to this antigen. Figure 5 shows that M371 was inactive by both criteria.

In further studies M371 was tested for binding to cytotoxic lymphocytes that had been generated in mixed lymphocyte culture by three successive stimulations (C57BL/6 anti-DBA/2) in the presence of supernatant from ConA-stimulated rat spleen cells. Results were entirely negative. In contrast, 6% of these cells stained brightly with CT-1.

DISCUSSION

The specificity of M371 is remarkable. Our data show that it is selective for IEL with the phenotype Thy-1^-, Lyt-2^+ and stains

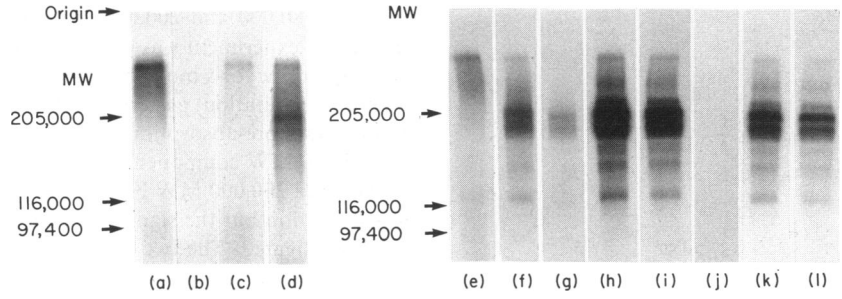

Figure 3. Autoradiographs of ^{125}I-labelled lymphocyte surface antigens separated by SDS–PAGE. IEL antigens were precipitated by: (a) M371; (b) GY11/217, IgG2c negative control; (c) M371, after pre-absorption of the cell lysate with YBM42.2.2 anti-CD45; (d) YBM42.2.2; (e) M371 (separate experiment); (f) M1/9.3 anti-CD45; (g) Ml/9·3 after pre-absorption of the lysate with M371. Lymph node cell surface antigens were precipitated by: (h, k) YBM42.2.2; (i, l) M1/9.3; (j) M371. In tracks a–d, e–j, and k, l the film was exposed for 4 days, 3 days and 1 day, respectively.

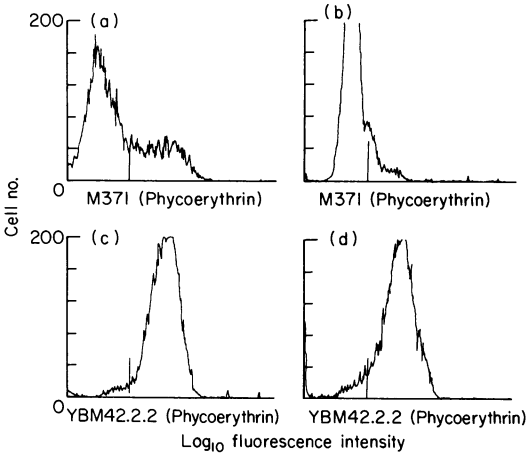

Figure 4. IEL were fixed with paraformaldhyde and treated with sodium periodate (b,d) or buffer only (a,c) and then stained using M371 (a, b) or YBM 42.2.2. anti-CD45 (c, d) and analysed by flow cytometry.

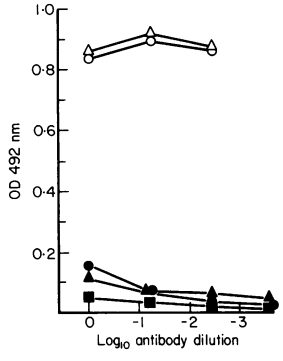

Figure 5. M371 was tested by ELISA for binding to Sd(a$^+$) and Sd(a$^-$) Tamm–Horsfall glycoproteins (THG) and for inhibitory activity on the binding of CT-1. (●) M371 with Sd(a$^+$)THG; (▲)M371 with Sd(a$^-$)THG; (■)control antibody 19·34; (○) CT-1 in the presence of M371; (△) CT-1 in the presence of control antibody 19.34. Serial dilutions of M371 started with neat culture supernatant; CT-1 ascitic fluid was used at 1:400.

the majority but probably not all of these. The lineage and function of cells in this subpopulation are not properly understood but they are thymus independent and are present in the gut epithelium of athymic nude mice in nearly normal numbers and represent the predominant IEL phenotype in such animals.

The observation that M371 identifies an antigen in some goblet cells could suggest that surface staining of IEL merely reflects non-specific uptake of intestinal mucins by lymphocytes that are close at hand. The very precise subset distribution of M371 reactivity and the abundant presence of stained IEL in the proximal small gut where stained goblet cells were absent or sparse denies this explanation. In contrast, it is likely that both cell types synthesize independently a similar carbohydrate epitope. An analogous situation exists for the CT antibodies which react with both IEL and epithelial cells.

It is particularly interesting that M371$^+$ cells were almost completely absent from the lamina propria and, indeed, were undetected in all other lymphoid compartments investigated. This invites the conclusion that this major subpopulation is unable to leave the epithelium and recirculate. The same argument implies that expression of the antigen is induced after lymphocytes have arrived in the epithelium and perhaps represents one facet of a differentiation process taking place in that environment.

Lyt-3 was expressed only on Thy-1$^+$ IEL. Thus, the CD8 complex on M371$^+$ lymphocytes appears to involve only the Lyt-2 chain. This is consistent with observations from another laboratory (D. Guy-Grand, unpublished data) that a substantial proportion of Lyt-2$^+$ IEL fails to be stained by the antibody H35-17-2, which recognizes the Lyt-2/3 heterodimer (Goldstein *et al.*, 1982). In view of the selective retention of Lyt-3 on Thy-1$^+$ cells, it seems unlikely that the result could be explained by the greater susceptibility of the Lyt-3 chain to proteolysis during cell isolation (Ledbetter *et al.*, 1981). The genes coding for the two chains are located on chromosome 6 and are separated by only 36 kb (Gorman, 1988). They are in the same transcriptional orientation and, because the two chains are nearly always expressed together, the genes have been considered to be under common regulatory control. The function of the Lyt-3 chain is unknown and transfection experiments have established that the Lyt-2 homodimer is sufficient for functional recognition of MHC class I (Gabert *et al.*, 1987). The situation in IEL is unusual and requires investigation at the transcription level.

Immunoprecipitation studies showed that M371 identifies an antigen with a molecular size of approximately 275,000 MW. The substantial diminution of antibody binding after treatment of cell surfaces with periodate suggests that carbohydrate is probably involved in the structure of the epitope. This view is supported by the observation that heating paraformaldehyde-fixed IEL at 100° did not affect the ability of M371 to stain the cell surface (data not shown). The rather smeared appearance of the antigen on SDS–PAGE may be attributable to differential glycosylation. The pattern was unchanged by treatment of the solubilized antigen with endoglycosidase F (data not shown) but the possibility of O-linked glycosylation has yet to be investigated.

Experiments with two different monoclonal antibodies to monomorphic determinants of CD45 established that the antigen was either an integral feature of a CD45 variant that has an unusually high molecular weight, or was closely associated with CD45 in the cell membrane and co-precipitated with it. Further experiments are required to resolve this point. A preliminary observation that some IEL express a 260,000 MW form of CD45 (Thomas & Lefrancois, 1988) lends support to the former explanation. On the other hand, it has been observed that Ly5 (CD45) binds with high affinity to sulphated carbohydrates (Parish, Hogarth & McKenzie 1988). Surprisingly, both antibodies to CD45 precipitated a similar, but rather faint, 275,000 MW band from lymph node cells. This component did not bind to M371 and its significance is unclear. The other CD45 bands seen with lymph node cells (180, 200, 215, 235,000 MW agree with the results of others (Lefrancois & Goodman, 1987). Notwithstanding the rather smeared pattern of high molecular weight components in the CD45 precipitations of IEL, bands at 220,000 and 235,000 MW (typical of peripheral Lyt-2$^+$ T cells) seem to be absent. Neither is the IEL pattern similar to that of cytotoxic T-cell clones nor T cells stimulated in mixed lymphocyte culture (Lefrancois & Bevan, 1984). Further studies with antibodies to restricted epitopes of CD45 would be instructive.

The failure of M371 to bind to Sd(a$^+$) Tamm–Horsfall urinary glycoproteins or to inhibit binding of CT-1 unequivocally establishes a distinction between these antibodies. This is reinforced by the observation that M371 did not stain cytotoxic T cells.

Recently it has been claimed that IEL express T-cell receptors composed largely of γ and δ chains (Goodman & Lefrancois, 1988; Bonneville *et al.*, 1988; Bucy *et al.*, 1988). On the other hand, data presented here and elsewhere (Viney *et al.*, 1989) shows that about 20% of IEL express the Vβ8 variant of $\alpha\beta$ TcRs but that expression is biased strongly toward the Thy-1$^+$ subpopulation. Thus, it is likely that M371$^+$ IEL express $\gamma\delta$ chains. The thymic independence of M371$^+$ IEL and the expression of CD3 on most IEL in nude mice (Viney *et al.*, 1989) encourage speculation that a primitive pathway of lymphocyte differentiation, possibly involving induction of $\gamma\delta$ expression may occur under the influence of the gut epithelium. The very restricted Vγ gene usage (Bonneville *et al.*, 1988) suggests a rather narrow receptor repertoire. In the rat it has been demonstrated that all morphological types of IEL can arise directly from cells of bone marrow origin (Mayrhofer & Whateley, 1983) and it is conceivable that the gut epithelium to some degree parallels the thymus in providing an environment for TcR gene rearrangement and maturation. Developement of $\gamma\delta$ TcRs on lymphocytes in lymph nodes and spleen has been shown to be largely thymus dependent (Pardoll *et al.*, 1988). This finding would not necessarily contradict our suggestion because M371$^+$ IEL are probably sessile and would not be expected to populate other lymphoid organs.

The function of the new antigen is unclear and the data so far provided little basis for speculation except that it could be involved in selective retention of IEL in the epithelium. M371 should serve as a valuable tool to investigate differentiation and function in a population of lymphocytes that is currently attracting widespread attention.

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