Immunology 1995 **86** 212–218

Regional variation in the proliferative rate and lifespan of αβ TCR$^+$ and γδ TCR$^+$ intraepithelial lymphocytes in the murine small intestine

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SUMMARY

Using double staining for T-cell receptor (TCR) and 5-bromo-2′-deoxyuridine (BRdU) we have examined the proliferation rates and lifespan of murine intraepithelial lymphocytes (IEL's) *in vivo*. After a 24-hr pulse of BRdU the number of labelled αβ TCR$^+$ IEL was significantly higher in the ileum than the duodenum. In contrast, incorporation of BRdU into γδ TCR$^+$ IEL was significantly higher in the duodenum than the ileum. This regional variation was also seen after a 4-hr pulse of BRdU indicating that the differences probably reflect local rates of proliferation in the epithelium. Over a 6-day labelling period, the accumulation of labelled αβ TCR$^+$ and γδ TCR$^+$ IEL was linear, which allowed IEL lifespan to be calculated. There was considerable variation between groups of mice but the 50% population renewal time for αβ TCR$^+$ IEL was 12–36 days in the duodenum and 9–11 days in the ileum, and for γδ TCR$^+$ IEL was 12–21 days in the duodenum and 26–100 days in the ileum. The incorporation of BRdU into Vβ8$^+$ IEL showed the same regional variation as αβ TCR$^+$ IEL and the Vδ4 population behaved like the total γδ TCR$^+$ IEL population. In contrast Vβ11$^+$, potentially self-reactive IEL, showed a regional pattern of labelling like γδ TCR$^+$ IEL. Incorporation of BRdU into both αβ TCR$^+$ and γδ TCR$^+$ IEL in germ-free mice was very low and did not show marked regional variation. αβ TCR$^+$ and γδ TCR$^+$ IEL from both proximal and distal bowel were cytotoxic. Therefore αβ TCR$^+$ and γδ TCR$^+$ IEL show different rates of division in different sections of the gut, perhaps reflecting responses to different antigens. Both αβ TCR$^+$ and γδ TCR$^+$ IEL reside in the epithelium for weeks during which time the gut epithelial population will have been renewed many times.

INTRODUCTION

In mice intraepithelial lymphocytes (IEL) of the intestine comprise two main populations; a CD8αβ, αβ T-cell receptor (TCR)$^+$ population and a CD8αα, γδ TCR$^+$ population.[1] Initial studies in athymic mice supported the idea that the αβ TCR$^+$ population were mainly thymus derived whereas the γδ TCR$^+$ were thymus independent and differentiated locally in the gut from bone marrow precursors.[2–5] Studies in adult thymectomized, lethally irradiated, bone marrow reconstituted animals, however, showed that all IEL subsets could be thymus independent.[6] In contrast all IEL subpopulations can be reconstituted in neonatally thymectomized mice by thymus grafting.[7] While there is as yet no resolution to these different results from different systems, there is now evidence that the thymic stroma itself can regulate extrathymic development of IEL,[8] so that some IEL can be thymus dependent but not thymus derived. There is also data to suggest that at different times in the life of the mouse, IEL are derived from different sources. Thus early in ontogeny, most αβ TCR$^+$ and γδ TCR$^+$ are thymus derived, whereas later in ontogeny they develop extrathymically.[7,9]

One of the aspects of IEL immunobiology least understood is the lifespan of the different populations. This is especially important since the cells reside at a site where the surrounding epithelial cells are renewed every 48 hr.[10] Early studies using [^{3}H]thymidine labelling indicated that only a small fraction of the cells are dividing and that some IELs are long lived.[11,12] In contrast after a low dose of X-irradiation the number of IEL drops by about 25% but recovers within a few days, indicating that they may be rapidly renewed.[13] More recently, using parabiotic mice, the surprising observation was made that even after 5-weeks parabiosis, there was little evidence of cells from one parabiont migrating to the epithelium of the other.[14] This would indicate that either IELs are long lived and are slowly replaced, or are self-renewing.

In this study we have directly examined the proliferation rates of αβ and γδ TCR$^+$ IEL subsets within defined regions of the gut using pulse labelling with 5-bromo-2′-deoxyuridine (BRdU). We show major regional differences in the proliferation rates and lifespan of αβ TCR$^+$ and γδ TCR$^+$ IEL in different parts of the gut.

Received 2 April 1995; revised 19 June 1995; accepted 20 June 1995.

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MATERIALS AND METHODS

Mice

Female BALB/c mice were obtained from A Tuck and Sons Ltd (Battlesbridge, Essex, UK) and were used between 6 and 18 weeks of age.

Labelling with BRdU

5-bromo-2′-deoxyuridine (Sigma Chemical Co., Dorset, UK) was dissolved in phosphate-buffered saline (PBS) (50 mg/ml). 1 mg or 10 mg was given intraperitoneally on a daily basis. There were always three mice per group.

Antibodies

For cell-surface staining, H57.597 ($\alpha\beta$ TCR, ref. 15), GL3 ($\gamma\delta$ TCR$^+$, ref. 16) and KJ16 (Vβ8.1, 8.2, ref. 17) were obtained from culture supernatants. Antibodies RR3-15 (Vβ11, ref. 18) and GL2 (Vδ4, ref. 19) were obtained from Pharmingen (AMS Biotechnology UK Ltd, Whitney, Oxon, UK). Binding of hamster anti-mouse $\gamma\delta$, or hamster anti-mouse $\alpha\beta$ was visualized using biotinylated goat anti-hamster IgG (Vector Laboratories, Peterborough, UK) followed by avidin-fluorescein isothiocyanate (FITC) (Sigma). Binding of anti-Vβ8 and Vδ4 was visualized by FITC rabbit anti-mouse IgG (Dako Ltd, High Wycombe, Bucks, UK). Rat anti-BRdU (SeraLab, Crawley Down, Sussex, UK) followed by TRITC rabbit anti-rat IgG was used to visualize nuclear BUdR incorporation.

Surface labelling and BRdU detection by immunofluorescence

5-μm frozen sections were cut and double stained for TCR and BRdU based on the method of Penit.[18] Sections were air dried and fixed in acetone for 10–15 min, then washed with PBS. Primary antibodies to the TCR were added to sections and incubated for 1 hr at room temperature. Sections were washed in PBS and secondary FITC-conjugated antibodies were added (rabbit anti-rat or biotinylated goat anti-hamster followed by avidin-FITC) and incubated for 30 min at room temperature in the dark. Sections were again washed in PBS and then placed in 70% ethanol for 15 min followed by 15 min in 4 M HCl and then 0·1 M borax, pH 8·5, for 5 min. Sections were washed twice in PBS and anti-BRdU antibody was applied. Slides were incubated for 1 hr in the dark. TRITC rabbit anti-rat was then added after washing sections in PBS and incubated for 30 min. Sections were washed twice in PBS, mounted in glycerol, and examined using the ×50 oil objective of a Leitz–Dialux epifluorescence microscope (Leica UK Ltd, Milton Keynes, Bucks, UK). At least 100–200 TCR labelled IEL (green) were counted and the number of these showing red nuclear staining for BRdU noted. Unless stated otherwise, results are the average of three mice per point.

Isolation of IELs

This was carried out as previously described.[20] Briefly, Peyers patches, fat and mesentery were removed from the small intestine. The gut was then divided into the proximal 25% and distal 25%, opened longitudinally and cut into 5-mm pieces. The pieces were then washed in Hanks' balanced salt solution (HBSS) and incubated for 30 min, with stirring, at 37° in 100 ml HBSS + 1 mM ethylenediaminetetraacetic acid (EDTA). The supernatant, containing epithelial cells and IEL's, was collected and the incubation repeated twice more with HBSS alone. Cells were then put into a 43/70% discontinuous Percoll gradient and spun at 600 *g* for 20 min. Cells at 43/70% interface were collected and washed in RPMI-1640 + 10% fetal calf serum (FCS).

FACScan analysis of isolated IELs

Isolated IELs were stained with, anti-$\alpha\beta$ TCR (H57.597) and anti-$\gamma\delta$ TCR (GL3) monoclonal antibodies (mAb) for 1 hr on ice. Biotin-labelled anti-hamster IgG was then applied to the cells after thorough washing with PBS/azide and incubated for a further 30 min. Labelled cells were visualized with avidin-FITC. Cells were analysed on a Becton Dickinson FACScanner.

Immunohistochemistry

Frozen sections of duodenum or ileum were stained by the indirect immunoperoxidase technique with anti-$\alpha\beta$ TCR, anti-$\gamma\delta$ TCR$^+$, anti Vδ4, anti-Vβ8 and anti-Vβ11 exactly as described previously.[2] The numbers of labelled cells was determined by differential counts of labelled cells and enterocytes and are expressed as labelled cells per 100 enterocytes. At least 100 labelled cells and adjacent enterocytes were counted per section.

Redirected cytotoxicity assay

1×10^6 target P815 cells were washed with PBS and labelled with 100 μCi sodium chromate for 1 hr. After extensive washing, labelled P815 cells were plated with varying dilutions of isolated cells in 96-well plates with or without the addition of 50 μl of antibody supernatant from hybridoma lines H57.597 or GL3. Maximal lysis was obtained by lysing cells with triple lysis buffer whilst spontaneous release was obtained from P815 cells cultured alone. Plates were incubated for 37° for 5 hr, after which 100 μl of supernatant was taken from each well for gamma counting.

% lysis was calculated by:

$$\frac{\text{experimental} - \text{spontaneouse release}}{\text{maximum} - \text{spontaneous release}} \times 100.$$

Statistics

Where applicable results are shown as mean ± 1 standard deviation (SD). In the experiments where accumulation of label was followed over extended periods and three mice per point were used, the results shown are the average of the values from each mouse. BRdU incorporation of IEL subsets in different regions of the intestine was compared by paired Student's *t*-test. The linearity of the accumulation of BRdU-labelled IEL, and comparisons of the slopes of the lines were tested by regression analysis.

RESULTS

Labelling of IEL 24 hr after a single injection of BRdU

Mice received a single injection of 10 mg BRdU and were killed 24 hr later. Labelled IEL could be clearly distinguished from labelled enterocytes by their green membrane fluorescence and basal position in the epithelium. From the outset of these studies it was clear that there were major variations in

Table 1. Incorporation of BRdU (24 hr after injection of 10 mg) into $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in the duodenum and ileum of BALB/c mice

			% BRdU$^+$			
			$\alpha\beta$ TCR$^+$		$\gamma\delta$ TCR$^+$	
Experiment no.	Age (weeks)	Time in animal unit (days)	Duo.	Ileum	Duo.	Ileum
1	8	1	14·3	23·3	20·0	6.6
2	10	15	7·0	13·1	4·1	2·0
3	10	26	6·3	17·6	11·8	3·5
4	10	26	3·3	8·6	7·6	5·5
5	12	1	2·5	7·8	4·5	2·2
6	12	35	4·1	12·6	5·6	1·6
7	14	10	15·8	20·0	10·8	6·6
8	18	59	1·0	3·2	3·6	2·0
Mean			6·8	13·3	8·5	3·7

Three mice per experiment. $\alpha\beta$ TCR$^+$ IEL duodenum versus ileum $P < 0.0001$, paired t-test; $\gamma\delta$ TCR$^+$ IEL duodenum versus ileum $P < 0.001$, paired t-test.

incorporation of BRdU between different groups of mice. In eight separate experiments, differences in the percentage of labelled $\alpha\beta$ and $\gamma\delta$ TCR$^+$ IELs in the duodenum and terminal ileum were apparent. Although there was a wide range in the number of labelled cells between experiments, which was not attributable to the age of the mice or the time in the animal facility, there was always higher labelling of $\alpha\beta$ TCR$^+$ in the ileum compared to the duodenum, and for $\gamma\delta$ TCR$^+$ IEL, the situation was reversed (Table 1).

To determine if these differences were due to differences in division in the epithelium, mice were sacrificed 4 hr after BRdU treatment. Again the same pattern was seen (Table 2) with maximal labelling of $\alpha\beta$ TCR$^+$ IEL in the ileum and maximal labelling of $\gamma\delta$ TCR$^+$ IEL in the duodenum.

We next examined intestine from the distal jejunum, about half-way down the small intestine. Twenty-four hours after a single injection of BRdU, labelling of $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL was the same in this region (Fig. 1), indicating that the increased labelling of $\gamma\delta$ TCR$^+$ was confined to the duodenum and the increased regional labelling of the $\alpha\beta$ TCR$^+$ was confined to the ileum.

Table 2. Incorporation of BRdU (4 hr after injection of 10 mg) into $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in the duodenum and ileum of BALB/c mice

	% BRdU$^+$			
	$\alpha\beta$ TCR$^+$		$\gamma\delta$ TCR$^+$	
Mouse	Duo.	Ileum.	Duo.	Ileum
1	1·0	3·4	3·9	1·5
2	0·5	3·0	3·0	2·8
3	1·0	4·7	4·0	1·5

Renewal rates of $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in different regions of the gut

The accumulation of labelled IELs in the gut was measured by giving mice a single injection of BRdU daily for 6 days (Fig. 2). For $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in the duodenum and ileum, the accumulation of labelled cells was linear. In the experiment shown, after 6 days, over 20% of the $\alpha\beta$ TCR$^+$ IEL were labelled in the ileum and 7% were labelled in the duodenum; 17% of the $\gamma\delta$ TCR$^+$ IEL were labelled in the duodenum and only 3·5% in the ileum, confirming again the major differences in the rates of proliferation of these two subsets in different regions of the gut.

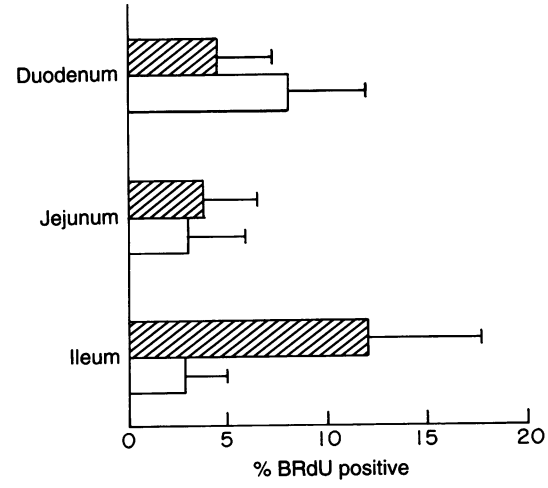

Figure 1. Incorporation of BRdU into $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in the duodenum, end of the jejunum, and ileum (24-hr pulse). $\alpha\beta$ TCR$^+$ IEL show significantly more labelling in the ileum than duodenum or jejunum ($P < 0.05$) and $\gamma\delta$ TCR$^+$ show significantly more incorporation of BRdU in the duodenum than the jejunum or ileum ($P < 0.01$). Data are mean ± 1 SD from six mice. (▨) α/β; (□) γ/δ.

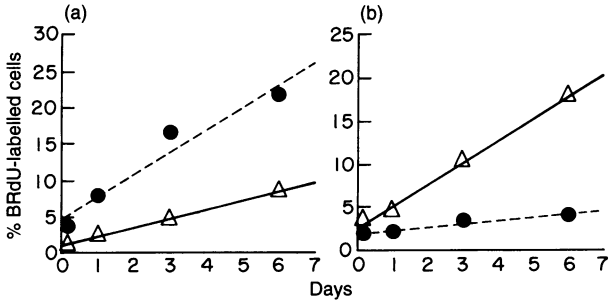

Figure 2. Accumulation of labelled $\alpha\beta$ TCR$^+$ (Fig. 2a) and $\gamma\delta$ TCR$^+$ IEL (Fig. 2b) in the duodenum and ileum from 4 hr to 6 days continuous labelling. The regression coefficients of all the lines were greater than 0·9. The rate of accumulation of BRdU labelled $\alpha\beta$ TCR$^+$ was significantly greater in the ileum than the duodenum ($P < 0{\cdot}001$) and the rate for $\gamma\delta$ TCR$^+$ was significantly greater in the duodenum than the ileum ($P < 0{\cdot}001$). Three mice per point. Mice received 1 mg BRdU per day. (△) α/β duo.; (○) α/β ileum; (△) γ/δ duo.; (●) γ/δ ileum.

The linear accumulation of labelled IEL indicates that IEL populations show one-in-one-out kinetics and therefore it is possible to determine population renewal rates. This is shown for two experiments in Table 3, where IEL were labelled for 6 days and the 50% renewal rates extrapolated from the slope of the line of the percentage of BRdU$^+$ cells with time. The rate of increase of BRdU-labelled IEL per day was taken as the daily renewal rate. It should be noted that there was considerable variation between the two experiments although the same pattern was seen in both. $\alpha\beta$ TCR$^+$ IEL are renewed faster in the ileum than the duodenum and the reverse in the case for $\gamma\delta$ TCR$^+$ IEL. Even with the variation it is possible to conclude that $\gamma\delta$ TCR$^+$ IEL are renewed rather slowly in the ileum (50% renewal approximately every 4–8 weeks) and that $\alpha\beta$ TCR$^+$ are also rather slowly renewed in the proximal intestine (around 2–5 weeks).

Persistence of labelled IEL

Mice were injected with BRdU daily for 9 days to achieve substantial labelling of the main IEL populations and the loss of labelled cells over the next 6 weeks was followed (Table 4). After 9 days labelling, as expected there were higher numbers of labelled $\alpha\beta$ TCR$^+$ IEL in the ileum than in the duodenum, and higher numbers of labelled $\gamma\delta$ TCR$^+$ IEL in the duodenum than in the ileum. Cell populations that are rapidly renewed will show a rapid decrease in labelling compared with those that are slowly renewed. Accordingly, labelled $\alpha\beta$ TCR$^+$ IEL were replaced at a rate of 0·4% per day in the duodenum but 1·3% per day in the ileum, whereas $\gamma\delta$ TCR$^+$ IEL were replaced faster (0·7% per day) in the duodenum than the ileum (0·4% per day).

Table 4. Persistence of BRdU-labelled IEL

	% labelled			
	$\alpha\beta$ TCR$^+$		$\gamma\delta$ TCR$^+$	
Days after cessation of labelling	Duo.	Ileum	Duo.	Ileum
0	20·3	33·6	61·0	16·0
3	20·2	21·3	39·7	13·9
11	10·9	5·9	16·4	1·0
40	3·0	0·3	0·6	0·3
Loss of labelled cells/day (%)	0·4	1·3	0·7	0·4

BRdU incorporation into $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL subpopulations

Twenty-four hours after a single injection of BRdU, labelling of Vβ8$^+$ IELs paralleled that of $\alpha\beta$ TCR$^+$ IELs, with higher labelling in the ileum than in the duodenum (Fig. 3). Likewise, for the Vδ4 subset of $\gamma\delta$ TCR$^+$ IEL, labelling was similar to $\gamma\delta$ TCR$^+$ IELs, being higher in the duodenum than the ileum. Potentially self-reactive (I-E^d) Vβ11$^+$ IELs were dividing and in addition showed a higher rate of proliferation in the duodenum than the ileum.

Immunohistochemistry was used to determine the density of IEL in the epithelium in the ileum and duodenum. In the duodenum there were 7·2 $\alpha\beta$ TCR$^+$ IEL per 100 enterocytes and 4·6 $\gamma\delta$ TCR$^+$ IEL per 100 enterocytes. In the ileum there were 6·3 $\alpha\beta$ TCR$^+$ IEL per 100 enterocytes and 3·3 $\gamma\delta$ TCR$^+$ per 100 enterocytes. IEL expressing Vβ8 comprised a major subpopulation (20–30%) of the $\alpha\beta$ TCR$^+$ IEL in the duodenum and ileum (2–3 Vβ8$^+$ IEL per 100 enterocytes), confirming earlier studies.[2] IEL expressing Vδ4 made up a

Table 3. Renewal rate per day and 50% population renewal rates of $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL in duodenum and ileum

	Renewal rate (% per day)				Population 50% renewal time (days)			
	$\alpha\beta$ TCR$^+$		$\gamma\delta$ TCR$^+$		$\alpha\beta$ TCR$^+$		$\gamma\delta$ TCR$^+$	
Experiment no.	Duo.	Ileum	Duo.	Ileum	Duo.	Ileum	Duo.	Ileum
1	1·4	4·5	2·4	0·5	36	11	21	100
2	4·1	5·6	4·1	1·9	12	9	12	26

Renewal rate per day was calculated as the slope of the line of the increase in BRdU-labelled cells with time. 50% population renewal time was extrapolated from the time it would take 50% of the $\alpha\beta$ TCR$^+$ or $\gamma\delta$ TCR$^+$ IEL to be labelled. Mice were injected with 1 mg BRdU per day.

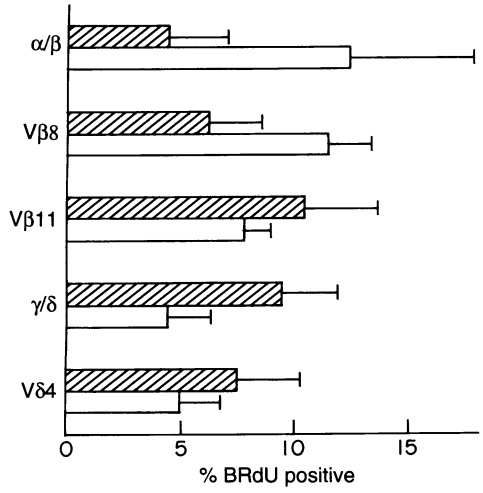

Figure 3. BRdU incorporation in IEL expressing Vβ8, Vβ11 and Vδ4. There were six mice per point (mean ± 1 SD) and the animals were killed 24 hr after administration of BRdU. IEL expressing Vβ8 showed significantly greater incorporation in the ileum than duodenum ($P < 0.003$), those expressing Vδ4 and Vβ11 showed significantly higher incorporation in the duodenum than the ileum ($P < 0.03$). (▨) duodenum; (□) ileum.

similar proportion of the γδ TCR$^+$ IEL (20–30%) in the duodenum and ileum. In contrast IEL expressing Vβ11 were uncommon (< 1 per 100 enterocytes) and it required multiple sections to count sufficient IEL for reliable measurements.

Functional analysis of αβ TCR$^+$ and γδ TCR$^+$ IEL in different regions of the intestine

Cytotoxic function was examined using redirected cytotoxicity assays with P815 target cells to determine if this also reflected regional variation. αβ TCR$^+$ IELs from both proximal and distal regions display similar degrees of cytotoxicity, 59·9% in proximal IELs and 48·7% in distal IELs at an effector : target ratio of 100 : 1 (Fig. 4a). γδ IELs however (Fig. 4b), display greater lytic activity in the proximal intestine (33·1%) than in the distal intestine (15·0%). However, when the proportion of γδ IELs in each region was analysed by FACScan it was found

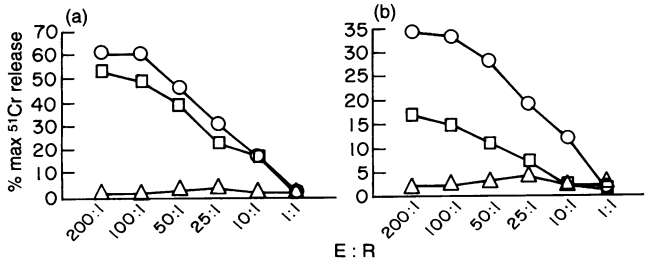

Figure 4. Redirected cytotoxicity of IEL isolated from the proximal 25% and distal 25% of mouse small intestine. Some jejunum had to be included as well as duodenum since the mouse duodenum is so short that it is impossible to isolate sufficient cells for analysis. Fig. 4(a) shows the cytotoxic activity of IEL using anti-αβ TCR$^+$ and Fig. 4(b) shows the cytotoxic activity with anti-γδ TCR$^+$. Cytotoxicity without (w/o) antibody was minor. Results are the average of three experiments using three mice each. (△) w/o antibody; (○) proximal IEL; (□) distal IEL.

Table 5. Incorporation of BRdU (24 hr after injection of 10 mg) into αβ TCR$^+$ and γδ TCR$^+$ IEL in the duodenum and ileum of germ-free BALB/c mice

	% BRdU$^+$			
	αβ TCR$^+$		γδ TCR$^+$	
Mouse	Duo.	Ileum.	Duo.	Ileum
1	0·5	0	0·5	2·6
2	0·8	0	0·9	2·9
3	1·9	1·8	2·1	0

that IEL isolated from proximal intestine contained a significantly higher percentage of γδ IELs, (24·9%, $n = 3$ experiments), compared with IEL isolated from distal intestine (17·4%, $n = 3$ experiments). Thus it is likely that the reduced cytotoxicity seen with γδ TCR$^+$ IEL from distal intestine reflects the relative paucity of these cells rather than any functional defect.

Incorporation of BRdU into IEL in germ-free mice

IEL were rare in the intestine of germ-free mice, especially those cells expressing the αβ TCR. However, 24 hr after a single injection of BRdU into three germ-free mice, only very low numbers of IEL were labelled (Table 5). Note that in two of the three mice no αβ TCR$^+$ IEL-incorporating BRdU were seen in the ileum.

DISCUSSION

In this paper we show that there are major differences in the rates of proliferation of αβ TCR$^+$ and γδ TCR$^+$ IEL in different regions of the intestine of young BALB/c mice. Using BRdU and double labelling for TCR it was shown that proliferation of αβ TCR$^+$ IEL is maximal in the ileum, whereas proliferation of γδ TCR$^+$ IEL is maximal in the duodenum and minimal in the ileum. These regional variations were seen in every mouse examined, although there were marked differences in the absolute numbers of dividing cells between experiments. We have no simple explanation for this variation between experiments although it is noteworthy that if incorporation of BRdU was low in αβ TCR$^+$ IEL, it was also low in γδ TCR$^+$ IEL. All mice were specific pathogen free and obtained from the same vendor. There was marked variation in different groups of mice analysed immediately after they arrived in the animal facility and in those that has been housed for prolonged periods. Within batches, however, labelling was consistent, allowing us to do time-course studies. We feel that these results are most likely due to bacterial or viral contamination of groups of mice during shipping and maintenance in our animal facility, which is not barrier maintained.

Despite variation between groups of mice it is clear that both αβ TCR$^+$ and γδ TCR$^+$ IEL populations are renewed at a much slower rate than the epithelial cells that surround them. The mouse epithelium is renewed every 48–72 hr[10] whereas the 50% population renewal rate for IEL takes from over 1 week to over 8 weeks, depending on the cell type and the region of the

bowel examined. These experiments cannot determine whether renewal of IEL is from an extra-intestinal precursor or is due to local self-renewal. However the failure to observe substantial mixing of IEL in parabionts of genetically defined mice[14] would argue that renewal is local and that the contribution from the periphery is minimal. Consistent with this, some IEL are labelled after a short pulse of BRdU indicating local proliferation in the epithelium.

As IEL can be resident in the epithelium for weeks then they must have a mechanism to remain in position while enterocytes flow past them. Human IEL express laminin/collagen receptors that might enable them to adhere to the basement membrane.[21] The basal position of IEL in the epithelium is also consistent with them adhering to the basement membrane.[22]

It is generally assumed that proliferation of T cells is an antigen-driven event and thus it seems reasonable to assume that the differences observed in this paper reflect regional variation in antigen exposure. The observation that proliferation of $\alpha\beta$ TCR$^+$ IEL was higher in the ileum than duodenum suggests that the stimulus for their proliferation might be the normal flora, which is much more abundant at this site.[23] There is evidence that $\alpha\beta$ TCR$^+$ IEL in mice are derived from Peyer's patch T blasts,[24,25] and once a cell has left the Peyer's patch and lodged in the ileal epithelium, there is probably a higher chance of it re-encountering antigens of the flora in the ileum than the duodenum. An alternative explanation for the results with $\alpha\beta$ TCR$^+$ is that there are regional differences in the ability of enterocytes to present lumenal antigens to $\alpha\beta$ TCR$^+$ IEL. In mice it has been reported that the expression of class II major histocompatibility complex (MHC) products on enterocytes is higher in the ileum than the duodenum.[26]

The unexpected observation of this paper is that proliferation of $\gamma\delta$ TCR$^+$ IEL was always higher in the duodenum than the ileum. Indeed turnover of ileal $\gamma\delta$ TCR$^+$ IEL was very slow. We consider that this reflects variation in the proliferation of $\gamma\delta$ TCR$^+$ cells in the epithelium rather than labelling of a precursor which then acquires the $\gamma\delta$ TCR$^+$ in the epithelium, because this effect was seen after a short pulse of BRdU. The specificity of $\gamma\delta$ TCR$^+$ IEL is still not known and they express relatively diverse TCRs[27] so, as yet, it is impossible to determine the stimulus for their proliferation. It is tempting to speculate that in the proximal bowel they may play a role in regulating oral tolerance to foods since $\gamma\delta$ TCR$^+$ lymphocytes from the gut and lung can break tolerance.[28,29] This however would involve them recognizing a fed nominal antigen, which has not yet been demonstrated.

Although it is now well established that $\alpha\beta$ TCR$^+$ IEL with potential self-reactivity are present in IEL,[14,30,31] this is the first study in which any functional analysis has been carried out *in vivo*. IEL expressing Vβ11, reactive with I-E^d which are usually deleted in the periphery, were present in IEL and were dividing. Since $\alpha\beta$ TCR$^+$ IEL have cytolytic function[16] it remains to be determined why the Vβ11$^+$ cells did not lyse adjacent epithelial cells which will express I-E. If the stimulus for proliferation of the Vβ11$^+$ IEL is via the TCR it would be unusual that this is not accompanied by functional activation. IEL isolated from mice transgenic for a $\gamma\delta$ TCR$^+$ which recognize a non-classical MHC class I gene product are unresponsive *in vitro*, suggesting that they are anergic *in vivo*.[32] However with time these cells disappear suggesting that they are not renewed.

Although we had the opportunity to examine only three germ-free mice, these experiments were revealing in that incorporation of BRdU into both $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL was low in the ileum and duodenum. This is readily explainable for the $\alpha\beta$ TCR$^+$, due to the lack of gut bacteria, but it also suggests that $\gamma\delta$ TCR$^+$ IEL are responding to something in the gut lumen that is present in normal mice, but absent in germ-free mice.

Both $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ IEL are constitutively cytotoxic.[14,33] We could find no meaningful difference in cytotoxic activity at different sites, which could not be explained by the relative number of the effector cells. Redirected cytotoxicity is, however, a relatively crude assay and perhaps more subtle analysis of cells at different sites might be more informative. The idea that there may be regional variation in IEL function in the intestine is, however, supported by studies that show major phenotypic and functional differences between IEL from the small and large intestine.[34]

ACKNOWLEDGMENTS

This work was supported by the BBSRC, the Wellcome Trust and the Joint Research Board of St Bartholomew's Hospital. The authors are grateful to Dr Phillip Carter who supplied the germ-free mice, supported by NIH DK34987 and State of North Carolina CVM204754.

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