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II Aspects of proliferation and migration of epithelial lymphocytes in the small intestine of mice

SUMMARY Mice were given either intraperitoneal tritiated thymidine ($^3\text{H-Tdr}$) or colchicine to study proliferation and migration of intestinal epithelial lymphocytes. Both labelled medium and large lymphocytes ('immunoblasts') were observed throughout the epithelium, crossing the basement membrane and within villous lymphatics for at least seven days after $^3\text{H-Tdr}$ administration. Epithelial lymphocytes are predominantly young cells, actively dividing at the rate of 1% per hour. They do not migrate along the villi, unlike epithelial cells, but circulate rapidly through the epithelium, returning to the lamina propria at the rate of approximately 3 epithelial lymphocytes/1000 epithelial cell nuclei/hour. The labelling pattern of epithelial lymphocytes and intralymphatic cells with time was very similar suggesting that epithelial lymphocytes therefore may directly enter adjacent lymphatics and hence gain access to thoracic duct lymph.

In addition to plasma cells and Peyer's patches, the small intestine contains a diffuse population of lymphocytes, of which epithelial lymphocytes form a well defined component. It has been established that small lymphocytes in thoracic duct lymph recirculate through Peyer's patches (Gowans and Knight, 1964), whereas a substantial number of the larger lymphocytes in thoracic duct lymph 'home' to the lamina propria (Gowans and Knight, 1964; Griscelli, Vassalli, and McCluskey, 1969). A large proportion of these latter cells become plasma cells (Hall and Smith, 1970; Hall, Parry, and Smith, 1972; Guy-Grand, Griscelli, and Vasalli, 1974), the remainder becoming medium-sized lymphocytes both within the lamina propria and interepithelial cell spaces (Guy-Grand *et al.*, 1974).

It has been suggested that most of the epithelial lymphocytes either degenerate or migrate through the intestinal epithelium into the lumen (Bunting and Huston, 1921; Wolf-Heidegger, 1939; Andrew and Andrew, 1945). This view is questionable since in part I (Marsh, 1975) it was shown that epithelial lymphocytes 'transform' and become immunoblasts within the small intestinal epithelium. These findings suggested that epithelial lymphocytes may be immunocompetent cells responding to local antigenic stimulation, and therefore were more likely to re-enter the lamina propria rather than be sloughed into the intestinal lumen.

The aim of this study was to gain further information about the proliferation and migration of epithelial lymphocytes in adult mouse jejunum: the results provide further insight into the behaviour and possible fate of epithelial lymphocytes.

Methods

Adult male mice were kept in metabolic cages to minimize coprophagy, fed on pellets and tap water, and allowed to adapt to this environment for seven days.

EXPERIMENT 1

The material used was described in a previous paper (Marsh and Trier, 1974b). Briefly, 24 mice weighing 20-30 g (Charles River Labs, N. Wilmington, Mass) were given six consecutive intraperitoneal injections of 25 μ Ci of (3 H-methyl)-thymidine (3 H-Tdr) of specific activity 6.7 Ci/mmol (New England Nuclear Corporation, Boston, Mass) at hourly intervals, and killed in pairs thereafter at six, 12, 24, 36, 48, 60, 72, 80, 96, 120, 144 and 168 h after the first injection. It was thus possible to observe labelled epithelial lymphocytes over several days, to allow for successive divisions of lymphocytes within this period, and thus to determine overall trends in the labelling pattern

of epithelial lymphocytes.

EXPERIMENT 2

Eight adult male mice weighing between 20 and 30 g (Charles River Labs, Kent) each received 250 μ g colcemid intraperitoneally. At hourly intervals thereafter, two mice were sacrificed and the remainder were given a further intraperitoneal injection of 250 μ g colcemid. By this method sustained levels of colchicine, sufficient to arrest all dividing cells, were maintained in each animal until sacrificed.

Segments of upper jejunum were fixed in ice-cold chrome-osmium (Dalton, 1955) containing 0.9 mmol CaCl_2 , dehydrated in an ascending ethanol series and embedded in Epon. Sections 1 μ m thick were cut perpendicular to the mucosal surface with glass knives on Sorvall MT2-B or Reichert OMU-3 ultramicrotomes.

EXPERIMENT 1

Six consecutive 1 μ m sections were mounted per slide: a 10 μ m step was discarded between each slide. Sections were dipped in Ilford K5 emulsion and exposed at 4° for four to six weeks in the presence of anhydrous calcium sulphate. Latent images were developed in D19, fixed and stained with toluidine blue (Trump, Smuckler, and Benditt, 1961).

EXPERIMENT 2

Three consecutive 1 μ m sections were mounted per slide and stained with toluidine blue: a 8 μ m step was discarded between successive slides.

All preparations were examined with an oil-immersion objective, only one section per slide being used for quantitation. A cell was considered labelled if three or more grains were observed over its nucleus.

MORPHOLOGICAL OBSERVATIONS

Criteria for the morphological identity of epithelial lymphocytes, including immunoblasts, in 1 μ m toluidine blue-stained Epon sections of jejunum are detailed in part I.

QUANTITATIVE MEASUREMENTS

(1) *Labelled and unlabelled epithelial lymphocytes per 1000 epithelial cell nuclei*

The number of labelled and unlabelled epithelial lymphocytes per 3000 villous epithelial cell nuclei was counted in each animal. The means for each pair of animals were expressed per 1000 epithelial cell nuclei. Epithelial lymphocyte labelling was classified as 'heavy' (> 16 grains/nucleus) or 'light' (< 15 grains/nucleus).

(2) *Total labelled epithelial lymphocytes/1000 epithelial cell nuclei per one-third segments of villi*

Villi were arbitrarily divided into proximal, middle and distal one-thirds: labelled epithelial lymphocytes, irrespective of grain density, were counted per 1000 epithelial cell nuclei in each villous segment. Means for each pair of animals were expressed as total labelled epithelial lymphocytes/1000 epithelial cell nuclei per one-third segment of villi.

(3) *Diameter and grain density of intralymphatic lymphocytes*

For each pair of animals, the diameter of 1200 lymphocytes identified within mucosal lymphatics was measured with a calibrated ocular graticule. They were graded as small ($< 7 \mu\text{m}$), medium ($7\text{--}10 \mu\text{m}$), and large lymphocytes ($> 10 \mu\text{m}$ in diameter). Grain density was classified as 'heavy' (> 16 grains/nucleus) or 'light' (< 15 grains/nucleus).

(4) *Epithelial lymphocyte mitotic arrests per hour*

The number of colchicine-induced mitoses among 2000 epithelial lymphocytes was counted per animal. The means for each animal pair were expressed as the number of mitotic arrests per 100 epithelial lymphocytes.

The metaphase arrests observed in this experiment were considered to be those of epithelial lymphocytes.

Some dividing eosinophil leucocytes were also arrested but, compared with epithelial lymphocytes, their mitotic figures were smaller and more densely stained; their cytoplasmic granules, showing green metachromasia with toluidine blue, were often evident in the surrounding cytoplasm. All other cells comprising the villous epithelium were confidently excluded, since mitotic division of columnar epithelial cells (Leblond and Messier, 1958), goblet cells (Merzel and Leblond, 1969) and argentaffin cells (Ferreira and Leblond, 1971), or their precursors, is restricted solely to the generative zones of the crypts and does not occur along the villi.

Results

Label was confined predominantly to medium-sized epithelial lymphocytes, although labelled immunoblasts were regularly observed throughout the seven-day observation period (fig 1). In addition, labelled lymphocytes were frequently observed in transit across the basement membrane in radioautographs of $1 \mu\text{m}$ Epon sections (fig 2). Virtually all were medium-sized lymphocytes (fig 2a), but very rarely immunoblasts were also observed in this position (fig 2b). Spontaneous mitotic figures in epithelial lymphocytes (fig 3), many of which were labelled, were observed at a frequency of approximately 1-2/1000 epithelial cell nuclei. The density

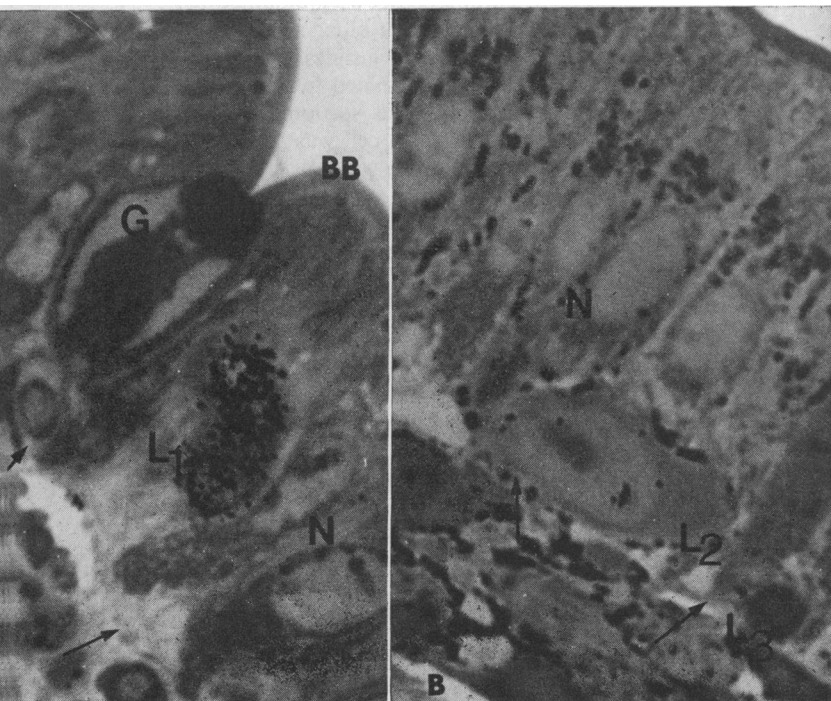


Fig 1 A Radioautograph of $1 \mu\text{m}$ toluidine blue-stained section of jejunum 12 hr after $^3\text{H-Tdr}$. Heavily labelled immunoblast (L_1) $16 \mu\text{m}$ in length, lies within epithelium, displacing nucleus (N) of adjacent epithelial cell. Epithelium extends from brush border (BB) to basement membrane (arrows). Goblet cell (G) ($\times 1900$).

B Radioautograph of mouse jejunum seven days after $^3\text{H-Tdr}$. A large, lightly labelled immunoblast (L_2) lies in epithelium, parallel to basement membrane (arrows). Compare size (length = $18.5 \mu\text{m}$) with adjacent small lymphocyte (L_3) (diameter = $5 \mu\text{m}$). Epithelial cell nucleus (N). Dark material in supranuclear Golgi zone, intercellular spaces and lamina propria is lipid ($\times 1900$).

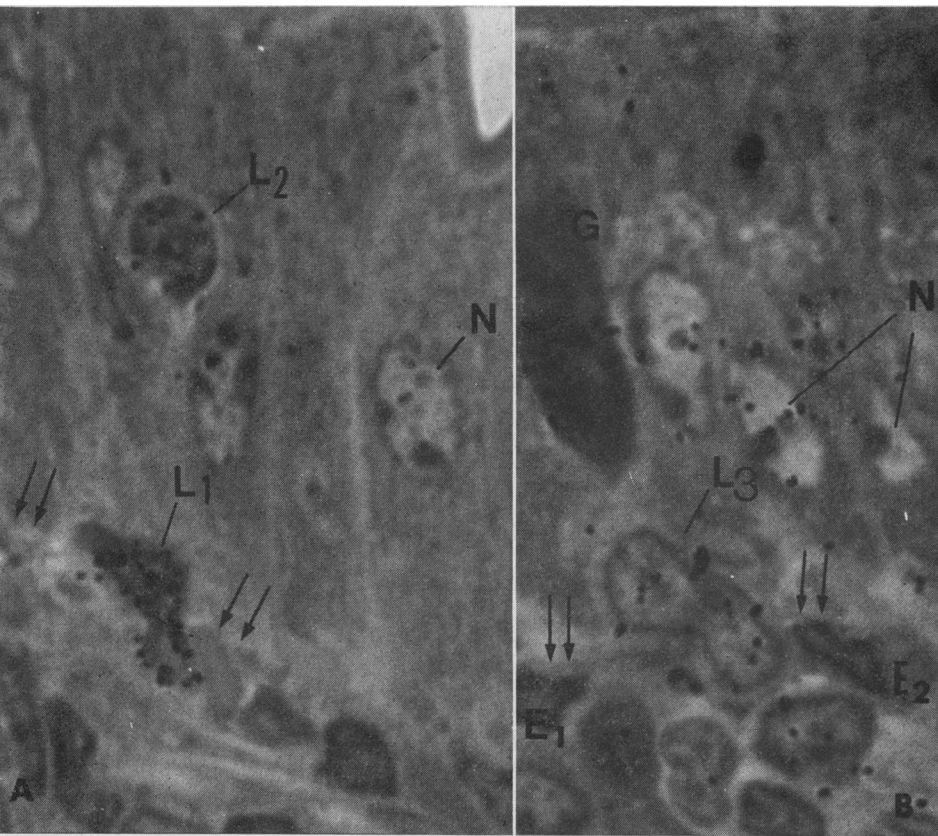


Fig 2 These 1 μ m radioautographs illustrate labelled epithelial lymphocytes migrating through the basement membrane (double arrows):

A A heavily labelled, medium sized lymphocyte (L_1) is entering epithelium (day 6 after $^3\text{H-Tdr}$). It has a typical 'hand-mirror' appearance, its narrow waist indicating perforation through basement membrane. A lightly labelled epithelial lymphocyte (L_2) lies within epithelium. Epithelial cell nucleus (N) ($\times 2400$).

B This 1 μ m section shows a large cell (L_3) across the basement membrane (double arrows) the direction of which is indicated by densely staining nuclei of endotheial cells (E_1 and E_2) comprising subepithelial capillary network of villus. Cell L_3 (approximately 15 μ m in length) is characteristic of an immunoblast, with large euchromatic nucleus: its cytoplasm, seen as a pale crescent adjacent to the lower pole of a goblet cell (G) trails behind the nucleus. This cell, therefore, is almost certainly leaving the epithelium (seven days after $^3\text{H-Tdr}$) ($\times 2400$).

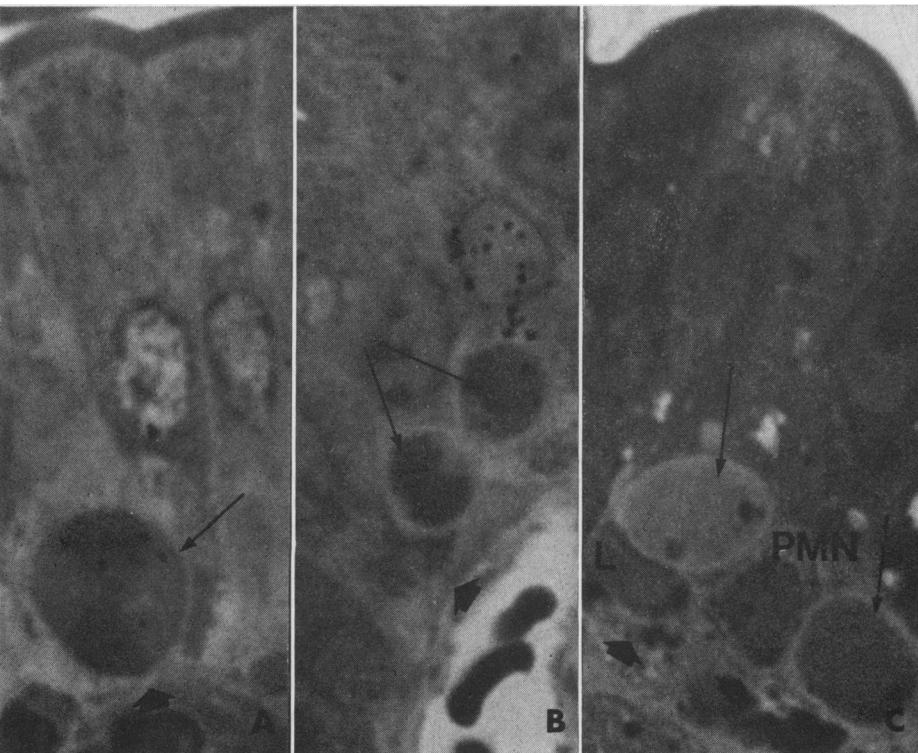


Fig 3 These 1 μ m radioautographs illustrate mitotic figures (long arrows) in dividing epithelial lymphocytes. Arrow heads indicate basement membrane:

A Probable anaphase, showing two distinct clumps of lightly labelled chromatin within dividing lymphocyte.

B Telophase, with almost complete separation of daughter epithelial lymphocytes.

C This micrograph shows two metaphase-arrested epithelial lymphocytes in jejunal epithelium of animal given four hourly injections of colchicine. Compare size with that of adjacent eosinophil leucocyte (PMN). A third medium-sized epithelial lymphocyte (L) comprises this group of cells ($\times 2400$).

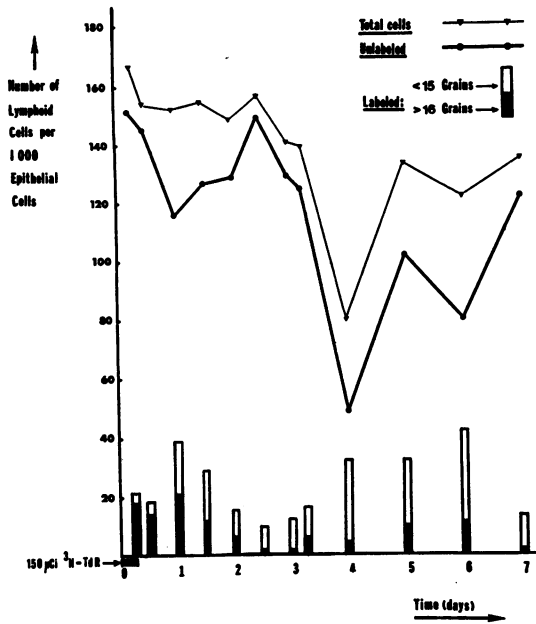


Fig 4 This medium-sized lymphocyte shows certain ultrastructural differences from that in figure 3. Nuclear membrane is irregular and deeply invaginated: there is some increase in euchromatin and a prominent nucleolus (Ne). Cytoplasm is more abundant, containing two well defined Golgi stacks (G), one of which is located adjacent to plasma membrane; a centriole (C), several mitochondria (M), and numerous lysosomes (L). This cell is clearly transitional between small epithelial lymphocytes and the medium-sized cells illustrated in figures 5 and 6 ($\times 18\ 200$).

of immunoblasts (labelled and unlabelled) was of the order of 3/1000 epithelial cell nuclei.

Counts of labelled epithelial lymphocytes in each pair of animals agreed very closely: the distribution of their means with time is shown in figure 4. Following a six-hr dose of $150\ \mu\text{Ci}\ ^3\text{H-Tdr}$, two peaks of labelled epithelial lymphocytes were observed between six and 60 hr and 72 and 168 hr, with maxima occurring at 24 hr (day 1) and 144 hr (day 6): the proportion of labelled epithelial lymphocytes at these times was approximately 25% and 35%, respectively. The lowest number occurred at 60 hr (2.5 days) when means of only two heavily labelled and seven lightly labelled epithelial lymphocytes per 1000 epithelial cell nuclei were counted.

Between 60 and 96 hr (day 2.5-4) after $^3\text{H-Tdr}$ administration, the total population of epithelial lymphocytes decreased rapidly, occasioned predominantly by an equally rapid drop in unlabelled epithelial lymphocytes from 154 to 42/100 epithelial cell nuclei/hour. This represented a rate of fall of 112/36, or approximately 3 epithelial lymphocytes/1000 epithelial cell nuclei/hour. The ultimate recovery of the total population of epithelial lymphocytes between days 4 and 7 (fig 4) was also accompanied by a rise in the number of labelled epithelial lymphocytes entering the epithelium during the same period.

The distribution of labelled epithelial lymphocytes with time in each one-third segment of the villi was symmetrical (fig 5), therefore clearly indicating that epithelial lymphocytes do not migrate along the villi, unlike epithelial cells. The sequential migration of the latter is indicated by the hatched bars (fig 5)

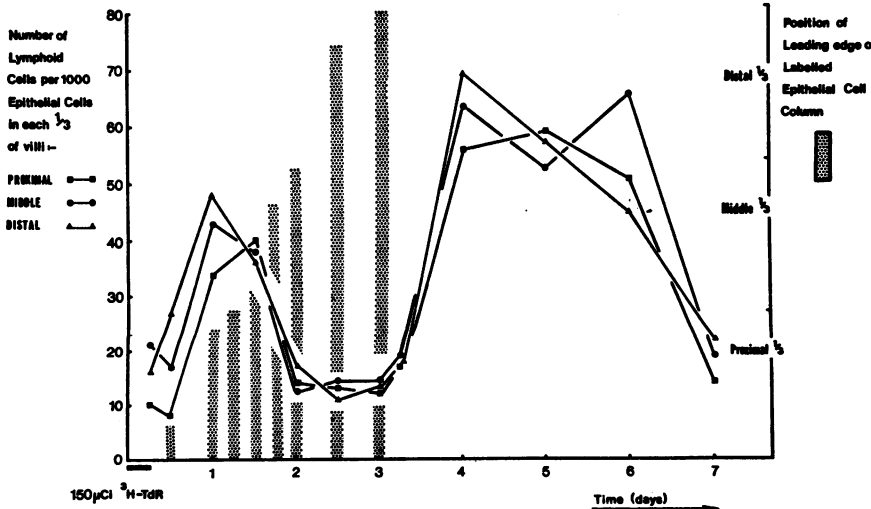


Fig 5 This diagram shows the number of all labelled epithelial lymphocytes (expressed as lymphoid cells/1000 epithelial cells) in the proximal middle and distal one-third segments of villi with time. The shaded bars represent progressive migration of epithelial cells with time. The proportion of labelled epithelial lymphocytes in each segment of the villi is identical at each time point, indicating that they do not migrate along the villi, unlike epithelial cells. Thus exchange of epithelial lymphocytes, into and out of epithelium, occurs at a similar rate throughout the entire villus.

which show that the leading edge of labelled epithelial cells entered the proximal one-third of the villi at 12 hr and reached their tips 48-60 hr later, ie, on days 2.5-3. The epithelial cells being shed into the intestinal lumen at this time were the same cells which had been exposed initially to peak levels of $^3\text{H-Tdr}$. Reutilization of $^3\text{H-Tdr}$ from sloughed epithelial cells is unlikely to have affected the labelling pattern of epithelial lymphocytes, however, since throughout the same period of observation, the grain density of adjacent proliferating crypt cells fell progressively.

Measurement of lymphocytes within mucosal lymphatics (table) showed that 74.4% were small lymphocytes ($< 7 \mu\text{m}$ in diameter), 19.3% medium-sized (7-10 μm in diameter) and 6.3% large lymphocytes ($> 10 \mu\text{m}$ in diameter). These figures parallel previous observations (Gesner and Gowans, 1962) indicating that mouse thoracic duct lymph contains 5% large lymphocytes. In contrast 25% medium sized lymphocytes and 38% large lymphocytes were labelled compared with only 3.5% small lymphocytes (fig 6). Their distribution with respect to grain

Lymphocyte Diameter	No. Cells Counted (% total count)	No. Cells Labelled (% labelled)	Grain Density	
			< 15	> 16
Small ($< 7 \mu\text{m}$ diameter)	893 (74.4)	31 (3.4)	26	5
Medium (7-10 μm diameter)	231 (19.3)	58 (25.1)	38	20
Large ($> 10 \mu\text{m}$ diameter)	76 (6.3)	28 (37.8)	19	9
Total	1200	117	83	34

Table Percentage distribution of labelled and unlabelled lymphoid cells within mucosal lymphatics of adult mouse jejunum

density is illustrated in figure 7. The fall in labelled lymphocytes between 48 and 80 hr after $^3\text{H-Tdr}$ injection closely reflected the biphasic pattern of epithelial lymphocyte labelling (fig 4).

Following hourly injections of intraperitoneal colchicine, epithelial lymphocyte mitoses (fig 3c) accumulated at the rate of approximately 1/100 epithelial lymphocytes/hour (fig 8). The mean

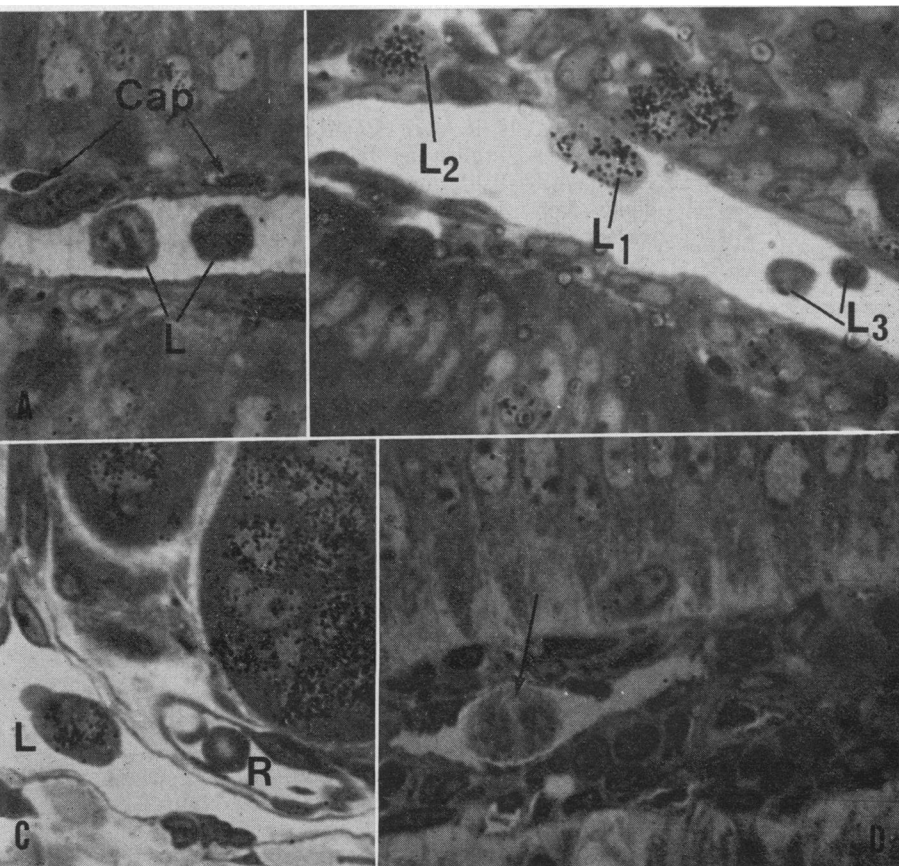


Fig 6 The following are radioautographs of intra-lymphatic lymphocytes in 1 μm sections of mouse jejunum: A This shows two labelled, medium-sized lymphocytes within central lacteal. Sub-epithelial capillary network (Cap).

B This villous lymphatic contains two unlabelled small lymphocytes (L_3) and a densely labelled lymphoblast (L_1). Another heavily labelled large cell (L_2) lying within a sub-epithelial capillary could be a 'blood-borne' immunoblast returning to the lamina propria

C This basal lymphatic contains a large, heavily labelled lymphocyte. Erythrocyte (R) in adjacent capillary provides size reference for all lymphocytes illustrated in this figure.

D A dividing lymphoblast within central villous lymphatic (all magnifications $\times 1200$).

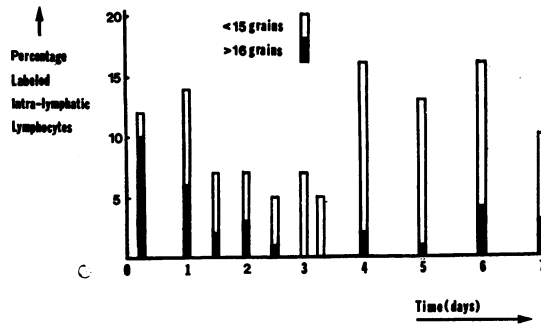


Fig 7 This diagram shows the percentage of labelled intralymphatic lymphocytes against time. Their grain density, at each time point, is shaded appropriately.

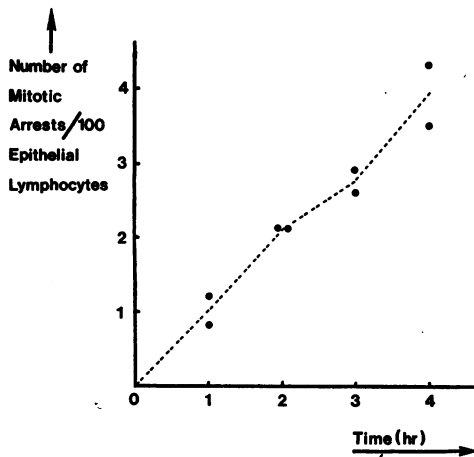


Fig 8 This graph shows the rate of accumulation of mitotic arrests in epithelial lymphocytes (expressed as a percentage of all epithelial lymphocytes counted) of mice given intraperitoneal colchicine at hourly intervals. Mean percentages for each pair of animals are shown by dotted line.

number of epithelial lymphocytes/1000 epithelial cell nuclei in this group of animals was 280, and since mouse intestinal villi are clothed by approximately 3000 epithelial cells (Hagemann, Sigdestad, and Leshner, 1970), the overall lymphoid cell population per villus was approximately 840 epithelial lymphocytes. Thus for each villus it followed that 1% or approximately nine epithelial lymphocytes divide hourly, ie, three epithelial lymphocytes/1000 epithelial cell nuclei/hour, and second, that epithelial lymphocytes must leave the epithelium at a similar rate, otherwise their density within the villous epithelium would rise progressively.

Discussion

The epithelial lymphocyte cell population of intestinal villi, although anatomically distinct, is not physiologically 'closed'. Analysis of cell turnover is, therefore, difficult and complicated by exchange of lymphocytes across the basement membrane, of mitosis within the epithelium and of further possible losses of epithelial lymphocytes into the lumen. Despite the importance of these considerations, the results of this study support the following conclusions: (1) that epithelial lymphocytes do not migrate along the villi, unlike epithelial cells; (2) that epithelial lymphocytes divide at the rate of 1/100 epithelial lymphocytes/hour; (3) that epithelial lymphocytes leave the epithelium at the rate of 3 epithelial lymphocytes/1000 epithelial cell nuclei/hour; (4) that epithelial lymphocytes are young cells which rapidly circulate through the villous epithelium; and (5) that some medium and large epithelial lymphocytes probably migrate from epithelium into adjacent mucosal lymphatics, thereby gaining access to thoracic duct lymph.

The result of colchicine administration showed that 1% epithelial lymphocytes divide hourly. Because prolonged colchicine administration severely affects intestinal morphology (Clark and Harland, 1963) these observations could not be pursued indefinitely. If it were supposed that epithelial lymphocytes proliferate linearly with time (as suggested by fig 8), then approximately 25% would divide daily, and the entire population could be replaced within three to four days, thus confirming previous observations that epithelial lymphocytes are young, actively dividing cells (Darlington and Roger, 1966; Fichtelius, 1968).

Second, the colchicine study provided an indirect estimate of the rate of loss of epithelial lymphocytes from the epithelium, assuming that the overall population remains constant (Fichtelius, Yunis, and Good, 1968). This estimate (3 epithelial lymphocytes/1000 epithelial cell nuclei/hour) was based on calculated cell populations for individual mouse villi (Hagemann *et al*, 1970) and approximated the rate of fall of unlabelled epithelial lymphocytes (3/1000 epithelial cell nuclei/hour) observed between 60 and 96 hr in experiment 1 (fig 4). This latter fall, which reflected mean epithelial lymphocyte counts in the jejunum of several animals, followed the reduction in the entry of new (heavily labelled) cells into the epithelium approximately three days after $^3\text{H-Tdr}$ injection. Subsequent recovery of the total population of epithelial lymphocytes appeared dependent both on the entry of new lymphocytes into the epithelium between days 4 and 7 (represented by a second peak of labelled epithelial lymphocytes in

fig 4) and presumably on mitotic expansion of labelled and unlabelled epithelial lymphocytes already present within the epithelium at this time. These observations suggest that epithelial lymphocytes leave the epithelium rapidly, and second that maintenance of the total population is dependent on a continuous supply of new lymphocytes into the villous epithelium.

The reduced rate of entry of labelled epithelial lymphocytes into the epithelium between day 2.5-3 (fig 4) was unexpected, and is difficult to explain. In another radioautographic study of mouse small intestine, an almost constant proportion of labelled epithelial lymphocytes was observed over 10 days (Darlington and Rogers, 1966). These latter workers used ^3H -Tdr of lower specific activity and in a dose one-fifth that employed here. The labelling pattern observed here (fig 4) thus may have resulted from radiation damage to precursors of epithelial lymphocytes. The second peak of labelled epithelial lymphocytes (day 4-7, fig 4) was not due to ^3H -Tdr re-utilization since the grain density of adjacent proliferating crypt cells showed a gradual and progressive fall throughout the entire study.

It is evident from fig 5, despite other claims (Fichtelius, 1968), that epithelial lymphocytes do not migrate along the villi or exfoliate from their tips, unlike columnar epithelial cells. Rather, the symmetry of labelling of epithelial lymphocytes in each one-third segment of the villi clearly showed that exchange of lymphocytes between epithelium and lamina propria occurs at all points along the epithelial basement membrane of the villi. This is in keeping with previous work showing that over 95% of epithelial lymphocytes are located basally within the epithelium (Darlington and Rogers, 1966; Shields, Touchon, and Dickson, 1969; Meader and Landers, 1967; Toner and Ferguson, 1971) and frequently penetrate the basement membrane (Meader and Landers, 1967; Toner and Ferguson, 1971; Trier, 1968; Toner, 1968) and subepithelial fibroblast sheath (Marsh and Trier, 1974a) of the villous epithelium. Furthermore, since a large proportion of epithelial lymphocytes undergo blast transformation within the epithelium (Marsh, 1975), such cells would be expected to re-enter the lamina propria rather than be sloughed into the lumen. The view that a physiologically significant loss of epithelial lymphocytes occurs into the lumen (Bunting and Huston, 1921; Wolf-Heidegger, 1939; Andrew and Andrew, 1945) is uncertain and lacks convincing quantitative support (Murray and Woods, 1964; Kotani, Yamashita, Rai, Seiki, and Horii, 1967). In this study, over 10^4 epithelial lymphocytes were counted in high-resolution radioautographs, yet none were seen abutting against, or

extending through, the tight junctions of adjacent columnar epithelial cells: on the other hand both labelled and unlabelled epithelial lymphocytes were frequently observed crossing the basement membrane (fig 2).

In deciding what happens to epithelial lymphocytes on re-entering the lamina propria, therefore, attention was paid to the distribution of labelled lymphoid cells within villous lymphatics. Their labelling pattern with time (fig 7) was remarkably similar to that of epithelial lymphocytes (fig 4) thus suggesting a close relationship between the flow of lymphocytes through the epithelium and lymphatics. Indeed, since medium and occasional large lymphocytes were observed throughout the epithelium, crossing the basement membrane and within mucosal lymphatics, it seemed likely that a proportion of epithelial lymphocytes might directly enter villous lymphatics and thus contribute to the larger lymphocytes circulating within thoracic duct lymph.

Morphologically, many epithelial lymphocytes resemble stimulated T cells (part I), and thus could contribute to the predominant T cell component (75%) of mouse thoracic duct lymph. However, about 33% of large lymphocytes in thoracic duct lymph are B cells (Guy-Grand *et al*, 1974) of which many originate from the intestine, as shown by their exclusive synthesis of IgM and IgA immunoglobulin (Mandel and Asofsky, 1968; Uhr and Vitetta, 1974). Although some of these presumably arise from Peyer's patches (Craig and Cebra, 1971) others might originate from B lymphocytes within epithelial lymphocytes (Ferguson and Parrott, 1972; Ferguson and Parrott, 1974). The mechanism by which IgA-secreting plasma cells are produced against specific intraluminal antigen (Crabbé, Nash, Bazin, Eyssen, and Heremans, 1969), and whether they derive either from Peyer's patch precursors or from activation of B epithelial lymphocytes is still unknown and requires further experimental study.

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