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Numbers and phenotype of lymphocytes emigrating from sheep bone marrow after *in situ* labelling with fluorescein isothiocyanate*

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SUMMARY

In normal young lambs the bone marrow was selectively labelled with fluorescein isothiocyanate by a temporary perfusion of one hind-leg. One day later, the incidence of bone marrow emigrants in different lymph nodes, spleen, Peyer's patches, thymus, non-perfused bone marrow and blood was determined. The emigrants were also phenotyped by the use of monoclonal antibodies and classified into monocytes or lymphocyte subsets. Large numbers of lymphocytes left the bone marrow of the perfused leg during 1 day. Considerable numbers of cells migrated to other bone marrow compartments. Varying numbers of mononuclear emigrants were found in peripheral lymphoid organs, with labelling indices ranging from 1·06% in the blood to 0·004% in the thymus. In the spleen, comparable numbers of B- and T-lymphocyte emigrants from the bone marrow were found, whereas in the blood, lymph nodes and jejunal Peyer's patches many more emigrants were T lymphocytes than B lymphocytes. In the prescapular lymph nodes, for instance, 90·4% of emigrants were T cells but only 9·6% were B cells. Based on the large numbers of lymphocytes emigrating from the bone marrow, their phenotypes and their entry into other bone marrow compartments, it can be concluded that the bone marrow of young lambs is an integral part of the migratory route of lymphocytes.

INTRODUCTION

The proportion of lymphocytes among nucleated cells in haemopoietic bone marrow varies greatly between species. In rodents and young children up to 30% of the nucleated cells are lymphoid (Rosse, 1980), whereas in the sheep, pig and human adult only 5–10% are lymphoid. The bone marrow has been demonstrated to be the main site of B-lymphocyte production in rodents (Rosse, 1980; Osmond, 1980) but this does not appear to be the case in sheep (Reynolds, 1981). In the guinea-pig and mouse the bone marrow contains not only rapidly proliferating lymphocytes but also slowly renewed T and B lymphocytes (Rosse, 1972; Röpke, Hougen & Everett, 1975). These T lymphocytes seem to create problems such as graft versus host disease in bone marrow transplantation (Storb & Thomas, 1982). The bone marrow has attracted much less interest as a site of lymphocyte migration than as an organ of lymphocyte production (Tavasolli & Yoffey, 1983), although Gowans & Knight (1964) noticed that some *in vitro* RNA-labelled thoracic duct lymphocytes migrated to the bone marrow of rats. In man, lymphocyte migration to the bone marrow has been shown after corticosteroid administration, resulting in lymphopenia (Fauci, 1975).

Lymphocyte migration studies are often hampered by unknown effects of the *in vitro* labelling procedure and injection techniques on the *in vivo* migration behaviour (Hall, 1985). By using optimal collecting conditions for thoracic duct lymphocytes in rats, the proportion of i.v. injected lymphocytes localizing in the bone marrow was 2·4% (Smith & Ford, 1983) in contrast to 25% given before for thoracic duct lymphocytes in this species by using different collection techniques (Rannie & Donald, 1977).

Using a technique of perfusing lymphoid organs via the blood vessels, which enables lymphocytes to be labelled in their normal microenvironment (Pabst, Reilmann & Neuhaus, 1980; Pabst & Binns, 1981; Pabst, Kaatz & Westermann, 1983; Reynolds & Pabst, 1984), we studied lymphocyte emigration from the sheep bone marrow. The main aims of the present study were to determine: (i) the organs to which bone marrow lymphocytes emigrate, (ii) which lymphocyte subsets are found among the emigrants, (iii) the extrapolated total number of emigrating lymphocytes, and (iv) whether non-lymphoid mononuclear cells are found among the emigrants. A fluorescent dye, fluorescein isothiocyanate (FITC), which binds to any proteins, was used as a marker in order to label all lymphoid cells in the bone marrow, irrespective of whether they are long-lived or

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short-lived, locally produced or previous immigrants. By using monoclonal antibodies against surface markers on sheep lymphoid cells (Beya *et al.*, 1986; Miyasaka *et al.*, 1983, 1985), the emigrating cells could be classified.

MATERIALS AND METHODS

Selective perfusion of one hind-leg

Seven White Alpine and Black Jura lambs of both sexes were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland (mean weight 9·6±3·8 kg, mean age 4·1±2·0 weeks). The perfusion technique was comparable to that described for young pigs (Pabst *et al.*, 1983). In brief, under barbiturate anaesthesia the lambs were intubated and anaesthesia continued with halothan. The femoral artery and vein of the right leg were cannulated. Thick silicone tubes were pulled through a tunnel underneath the skin of the groin and fixed by tissue clamps. The tubes were used as a tourniquet to occlude other blood vessels to the hind-leg during perfusion. The skin blood flow was stopped by long-branched clamps. The prefemoral lymph node was excluded from the perfused area. By this technique the blood flow was prevented in all collateral branches from the obturatorial, gluteal and pudendal arteries and the leg was totally dependent on the oxygen and nutrients supplied by the extracorporeal perfusion system. The cannulae were connected to the perfusion system (Pabst *et al.*, 1980) which consisted of a roller pump, an oxygenator and a reservoir for the perfusate in a waterbath (Fig. 1). The priming volume was 400 ml. The perfusate consisted of medium RPMI-1640, pH 7·3, supplemented with 35 g/l of dextran (MW ~ 70,000) to provide a normal colloidal osmotic pressure. The hind-leg was perfused at a pressure of up to 130 mm Hg and a flow rate of up to 100 ml/min at 37°. The remaining red cells from the perfused leg resulted in a packed cell volume of 2·4±1·9% in the perfusate. FITC was added to give a final concentration of 35–100 μg/ml (FITC Isomer I, Sigma 7250). After 15 min of close circuit perfusion, up to 1 l of fresh perfusate without FITC was pumped through the leg under physiological pressure to wash out the remaining intravascular label so that there might be minimal spillover of the label onto the general circulation (Fig. 1). Therefore, all the peripheral blood cells that had been labelled were removed and all cells that could migrate out of the system were cells residing in the bone marrow. The tourniquet and clamps were opened and the blood circulation was re-established immediately. The cannulae were removed and the incision closed. Since the popliteal lymph node was included in the perfused area, this node was always excised immediately after the perfusion. Before, during and 10 min after the end of the perfusion, blood samples were drawn from the general circulation to check for the presence of labelled lymphocytes as a control for the selectivity of the labelling technique. About 30 min after re-establishing the blood flow to the leg, a prescapular lymph node was excised and a cell suspension prepared. In some experiments a blood sample was taken at 6–7 hr after perfusion.

Preparation of cell suspensions

One day after the perfusion a blood sample was taken, the animals were killed and the following tissues were excised: mesenteric and prescapular lymph node, spleen, thymus, jejunal and ileal Peyer's patches, contralateral non-perfused tibia, perfused tibia and distal parts of the perfused femur. These were examined for the presence of fluoresceinated cells. Cell suspensions were prepared and mononuclear cells isolated by Percoll

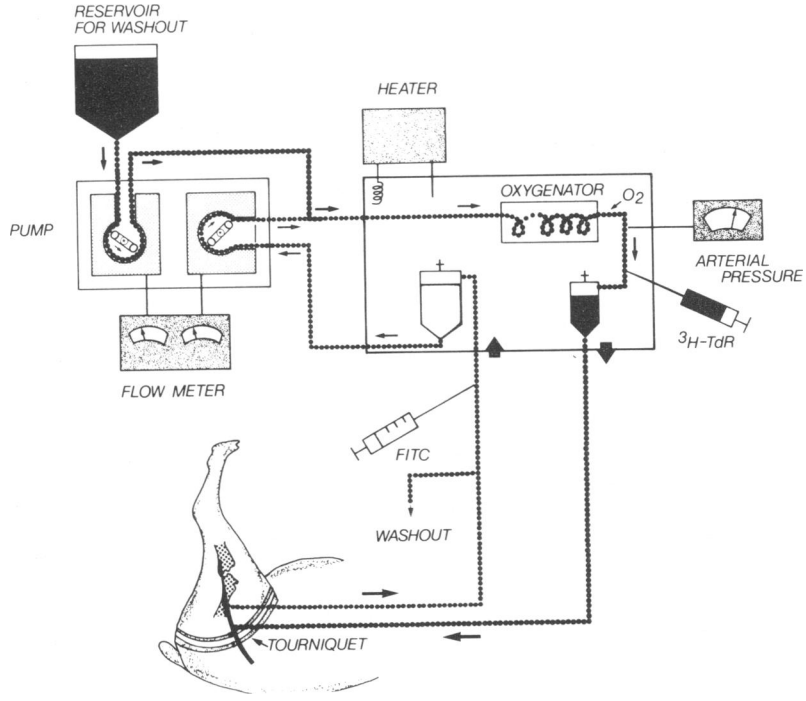

Figure 1. Schematic drawing of the perfusion circuit to label selectively one hind-leg of a lamb. A pump transports the perfusate through an oxygenator via a cannula into the femoral artery. The perfusate returns from the femoral vein to the reservoir. The second system is used to wash out the unincorporated label. A tourniquet occludes all other vessels to that leg during the perfusion.

centrifugation as described previously (Miyasaka & Trnka, 1985). B lymphocytes were identified by incubation with $F(ab')_2$ rabbit anti-sheep Ig. ST-1, a monoclonal antibody, recognizes all T cells in sheep (Beya *et al.*, 1986); T-80 monoclonal identifies a subpopulation of T lymphocytes (Miyasaka *et al.*, 1983) that are T-helper type cells for inducing cytotoxic lymphocytes (Ezaki *et al.*, 1985). Monoclonal antibody 175 labels all cells in the myeloid and erythroid series but not lymphoid cells (Miyasaka *et al.*, 1985). The staining technique has been described before (Miyasaka & Trnka, 1985). The cell suspensions were evaluated for FITC-positive cells using a Leitz microscope fitted with epifluorescence. Unlabelled cells were counted by phase-contrast optics. Depending on the labelling index, 5000–50,000 cells were counted in each cell suspension. For identifying lymphocyte subsets, at least 50 FITC-positive cells were examined in each test by the use of monoclonal antibodies described above.

RESULTS

The hind-leg was perfused with almost constant vascular resistance at normothermia. The skin, muscle and connective tissue all became yellowish. One day after perfusion no sign of tissue damage could be seen macroscopically, and the perfused bone marrow was well preserved histologically (Fig. 2).

Selectivity of labelling

The intravascular mononuclear cells of the perfused leg had been washed out by the flushing procedure before the restoration of the marrow circulation. In the perfusate all cells were brightly labelled. In the peripheral blood samples obtained during the perfusion, no labelled lymphocytes could be found. Ten to 15 min after re-establishing the blood supply to the leg, a mean of $0{\cdot}12 \pm 0{\cdot}06\%$ of the mononuclear cells in the circulation were as brightly labelled with FITC as those in the perfusate, and there was no background labelling as could be expected if a measurable spillover of FITC had happened. In each experiment one prescapular lymph node was excised about 30 min after perfusion of the leg. In these nodes a mean of 0·005% of the lymphocytes were stained. Since no leakage of FITC could be demonstrated during the perfusion, we concluded that all labelled cells found 1 day after selective labelling in different organs came from the bone marrow of the perfused hind-leg, and that they could not have picked up the label anywhere else.

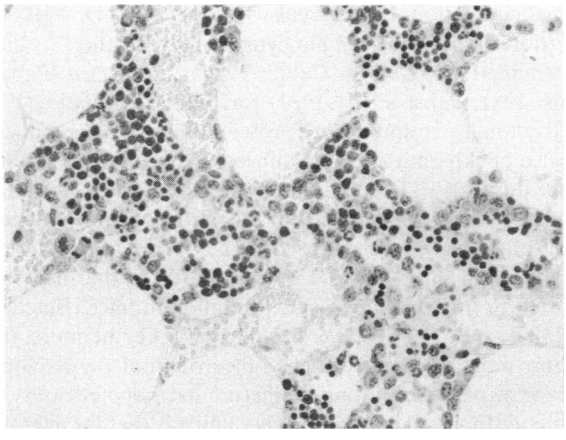

Figure 2. Histological section of tibial bone marrow 1 day after selective perfusion, demonstrating a normal histological structure (methacrylate embedded, 2 μm, Giemsa, magnification $\times 280$).

Organ distribution of bone marrow emigrants

The frequency of FITC-labelled mononuclear cells is shown in Fig. 3. The suspension from the perfused tibia had the highest mean labelling index of 71·5%. The lower and more variable labelling index in the perfused femur was due to the blood supply to the distal femoral epiphysis, which is only partly supported by the branches of the artery cannulated by this technique. No lymphoid organ had a labelling index as high as the blood with $1{\cdot}06 \pm 0{\cdot}69\%$. Six to seven hours after the perfusion the labelling index in the blood was already $0{\cdot}52 \pm 0{\cdot}25\%$. There was no obvious difference in the indices for mesenteric and prescapular lymph node. The spleen always showed a higher index than the lymph nodes. The scattered jejunal Peyer's patches had a labelling index of only 0·09%, which was, however, still much higher than that found in the continuous long Peyer's patch of the terminal ileum (0·01%). A consistently low index was seen in the thymus. Surprisingly, high labelling indices were found in distant marrow such as the contralateral non-perfused tibia. The majority of all emigrated labelled cells were of small to medium size. In the marrow cell suspensions, transitional cells and small lymphocytes could not easily be differentiated from cells of the erythroid series and/or myeloid series precursors by phase-contrast microscopy.

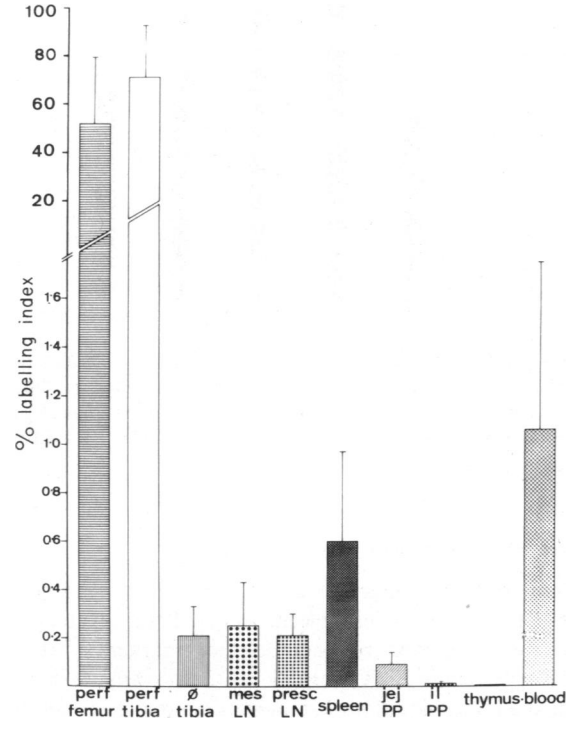

Figure 3. Labelling index (%) of mononuclear cells in bone marrow, blood and different lymphoid organs 1 day after selective labelling of the bone marrow of one hind-leg. The mean of seven experiments $\pm$ SD are shown (perf = perfused, ø = not perfused, mes and presc LN = mesenteric and prescapular lymph node, jej and il PP = jejunal and ileal Peyer's patches).

Table 1. Frequency of B and T lymphocytes and monocytes among emigrant mononuclear leucocytes found in different organs 1 day after selective labelling of one hind-leg

Organ	Surface phenotype of bone marrow emigrants (%)		
	sIg$^+$ (B lymphocytes)	ST-1$^+$ (T lymphocytes)	175$^+$ (erythroid and myeloid cells)
Peripheral blood	14·7±6·7	38·8±17·2	52·5±7·0
Mesenteric lymph node	12·7±6·2	55·7±10·7	22·5±3·8
Prescapular lymph node	7·3±3·4	68·7±11·4	23·6±9·7
Jejunal Peyer's patches	17·8±6·5	51·9±5·3	15·5±8·0
Spleen	15·0±5·9	30·5±6·7	48·5±6·5

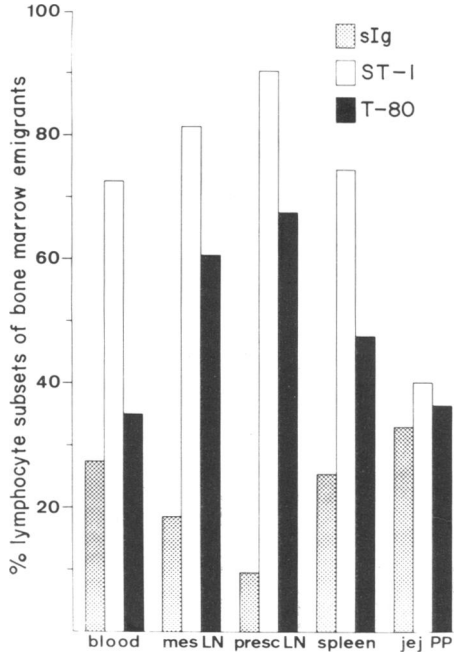

Figure 4. Relative frequency of B (sIg$^+$), T (ST-1) or T-cell subsets (T-80) among bone marrow emigrant lymphocytes found in different organs 1 day after selective perfusion of the bone marrow of one leg (mes and presc LN = mesenteric and prescapular lymph node, jej PP = Peyer's patches in the jejunum).

Surface markers on bone marrow emigrant cells

The emigrant mononuclear cells found in mesenteric and prescapular lymph nodes, spleen, jejunal Peyer's patches and blood were classified by their surface markers (Table 1). Surprisingly, many FITC-positive cells, especially those in the spleen and in the blood, were stained by a monoclonal antibody that identifies monocytes and cells of the erythroid lineage. These cells were on average larger than sIg$^+$ cells or T cells that are recognized by the ST-1 monoclonal antibody. From the data in Table 1, the relative frequencies of B, T and T-subset lymphocytes were calculated and are shown in Fig. 4. In the spleen there were comparable numbers of B- and T-cell emigrants from the bone marrow. In lymph nodes, however, up to nine times more T cells than B cells were found. The subset of T lymphocytes identified by the T80 antibody in relation to all T-lymphocyte emigrants from the bone marrow showed obvious differences between lymphoid organs (Fig. 4). In peripheral and mesenteric lymph nodes about three out of four of all T lymphocytes, in jejunal Peyer's patches about two out of three, and in the spleen and blood only about one in two were T-80-positive.

DISCUSSION

In this study we used an extracorporeal perfusion system (Pabst *et al.*, 1980; Pabst & Binns, 1981; Pabst *et al.*, 1983; Reynolds & Pabst, 1984) to label bone marrow cells *in vivo* under physiological conditions, and subsequently studied their migration. The advantage of the perfusion technique for labelling cells *in situ* to study their migratory behaviour is clear. By this method cells are labelled in their normal microenvironment by perfusion via the regional blood vessels at a constant temperature, flow rate, O_2 tension and pH without destroying the tissue architecture. Therefore, there is minimal alteration of the physiology of the labelled cells and tissues, and the cells and tissues are expected to perform their normal functions upon the restoration of the normal circulation. Fluorescent dyes such as FITC have been effectively used to label lymphocytes *in vitro* (Butcher, Scollay & Weissman, 1980; Chin & Cahill, 1984) and *in vivo* (Pabst & Binns, 1981; Pabst *et al.*, 1983) for migration studies. FITC labels cytoplasmic and surface proteins not only of lymphocytes but also of skin, muscle and connective tissue cells. Therefore, higher doses of FITC than those used for *in vitro* labelling of lymphocytes have been added to the perfusion system depending on the mass of the perfused tissue. Nevertheless, doses of FITC used in this study did not exceed those that have been reported to impede lymphocyte migration in mice (Butcher *et al.*, 1980) and sheep (Chin & Cahill, 1984). Furthermore, there was no morphological evidence of structural or cytological damage or of release of any immature haemopoietic cells into the blood stream. It is therefore very unlikely that the migratory behaviour of the lymphocytes was altered in the present study by labelling lymphocytes in their normal microenvironment with FITC, and we conclude that migration of cells from the bone

Table 2. The proportions and estimated absolute numbers of lymphocytes that emigrated from one hind-leg during 1 day after selective labelling with FITC

Organ	Total number of lymphocytes $\times 10^{10}$	Labelling index (%)		Total number of emigrated lymphocytes
		All cells	Lymphocytes ST-1$^+$ + sIg$^+$	
Blood	0·27	1·06	0·57	$0·154 \times 10^8$
Lymph nodes	5	mLN* 0·25 pLN† 0·21	0·17	$0·85 \times 10^8$
Peyer's patches	17 < 2 < 15	jej‡ 0·09 il§ 0·01	0·06 0·01	$0·12 \times 10^8$ $0·15 \times 10^8$
Thymus	18	0·004	0·004	$0·072 \times 10^8$
Spleen	1·6	0·6	0·27	$0·432 \times 10^8$
Total				$1·78 \times 10^8$

* mLN, mesenteric lymph node.
† pLN, prescapular lymph node.
‡ jej, jejunal Peyer's patches.
§ il, ileal Peyer's patches.

marrow took place at a normal rate and at a normal stage of differentiation. Only one time-point has been studied so far, but it is likely that an equilibrium of the emigrated lymphocytes was already obtained 1 day after the perfusion (Smith & Ford, 1983).

The present study reveals that a substantial proportion of the emigrant lymphocytes from the bone marrow are T cells, which emigrate to a different extent into different lymphoid organs. Different subpopulations of T cells may show differential emigration. We have examined only one subpopulation of T cells in this study. T-80 monoclonal antibody identified a subset of T lymphocytes in the blood. Based on observations that nearly all T cells in efferent lymph and in typical cell-traffic areas in lymphoid tissues such as the deep cortex of lymph nodes and periarteriolar lymphoid sheath of the spleen are T-80$^+$ and also that these T-80$^+$ cells have much higher actin activity than other T-cell subpopulations (Mély-Goubert, Dudler & Miyasaka, 1984), it has been suggested that T-80 mAb recognizes recirculating T cells (Miyasaka *et al.*, 1983, 1985). In this context, it is interesting that high proportions of emigrant T cells from the bone marrow were T-80$^+$. This tendency was particularly evident in jejunal Peyer's patches and in lymph nodes, and this may reflect the higher proportion of recirculating T cells among total T cells in these organs than in the spleen and blood. The presence of labelled cells in the contralateral tibia was notable and this was very similar to that observed in our previous studies in pigs (Pabst *et al.*, 1983). In addition, when we obtained the bone marrow from a perfused leg immediately after the completion of perfusion procedures, it was found that the majority of non-labelled cells in the labelled marrow were lymphoid and also T-80$^+$ (data not shown). Collectively, these results indicate that lymphocytes continuously enter and are released from the bone marrow.

It is not known in which compartments in lymph nodes and spleen these bone marrow emigrants enter. Detection of fluoresceinated cells on frozen sections has as yet been unsuccessful in our hands. The use of a fluorochrome such as rhodamine or the Hoechst dye 33342 (Brenan & Parish, 1984), which are more resistant to fading, might circumvent this problem and would possibly allow a simultaneous detection and *in situ* phenotyping of emigrant cells on tissue sections by a concurrent use of monoclonal antibodies and appropriate secondary reagents.

Since the labelling was restricted to the perfused tissue, and intravascular cells that were also labelled during perfusion were flushed out from the perfused area prior to restoration of the blood circulation, it was concluded that FITC-positive cells found in the periphery were all derived from the perfused marrow. Therefore, the extent of cell emigration from the marrow could be quantified. The total number of lymphocytes emigrated from the perfused marrow was calculated and is shown in Table 2. The distal femur and tibia contain only about 5% of all haemopoietic marrow in young lambs (M. Miyasaka and L. Dudler, unpublished observations), and therefore the number of lymphocytes emigrating from the total bone marrow is approximately 20 times the number given here, and is estimated to be $3·5 \times 10^9$ cells per day. Our preliminary results on the estimation of the total number of marrow lymphocytes suggest that about $1·5 \times 10^{10}$ lymphocytes exist in the marrow of young lambs such as those animals used in this study (M. Miyasaka, unpublished observations). The high incidence of bone marrow emigrants with a surface marker of non-lymphoid cells observed in this study indicates that we have probably overestimated the emigration of lymphocytes in our previous studies in pigs, because we made no attempt to differentiate lymphoid from non-lymphoid mononuclear cells (Pabst *et al.*, 1983).

A very low but discernible labelling index in the thymus could not solely be explained by the presence of labelled cells in the thymic vasculature. In previous experiments involving the labelling of bone marrow of young pigs (Pabst *et al.*, 1983), the blood vessels of these animals were flushed with large volumes of saline before the organ samples were excised for examination, and despite this procedure a comparable organ distribution could be found. Therefore, we interpret this meagre labelling index in the thymus as the reflection of the very small but significant extent of cell traffic through the thymus.

The obvious difference of the labelling index in jejunal and the continuous ileal Peyer's patches supports our earlier conten-

tion that sheep jejunal Peyer's patches are more like typical peripheral lymphoid organs, and ileal Peyer's patches are more like a primary lymphoid organ with almost no traffic area (Reynolds, Pabst & Bordmann, 1985).

Obviously more work is needed to elucidate the role of emigrating lymphocytes from the bone marrow. Phenotyping by using monoclonal antibodies that recognize functional subsets of T lymphocytes such as helper and cytotoxic/suppressor subsets will be very useful, and these antibodies are also becoming available in sheep (Ezaki *et al.*, 1985; Maddox, Mackay & Brandon, 1985). In addition, the role of the bone marrow in lymphocyte migration in sheep of other age-groups must be studied. The technique of selectively labelling one leg cannot, however, be used in adult sheep, because the haemopoietic marrow in the femur and tibia is largely replaced by fat.

The labelling of bone marrow cells in their normal microenvironment with FITC by a selective perfusion has obvious advantages, not only for lymphocyte studies but also for studies of granulocyte and erythrocyte kinetics.

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