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T-cell recirculation in the sheep: migratory properties of cells from lymph nodes

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Summary. T lymphocytes derived from different sources in sheep were compared for their ability to recirculate from blood to lymph. Nylon wool columns were used to prepare T-cell-enriched populations from efferent intestinal lymph, efferent prescapular lymph and from cell suspensions of mesenteric lymph nodes and prescapular lymph nodes. With each animal, T cells from two of the above sources were labelled *in vitro*, one population with fluorescein isothiocyanate the other with rhodamine isothiocyanate; both populations were returned to the animal at the same time by intravenous injection. The intestinal lymph and prescapular lymph were continuously monitored to compare the recirculating properties of the two populations of T cells. This technique led to confirmation of the earlier reports in sheep of a preferential recovery of intestinal lymph T cells and of prescapular lymph T cells in the lymph from which the cells were originally collected. This phenomenon was much less evident with T cells from mesenteric nodes and prescapular nodes and in a number of experiments a random pattern of recirculation occurred. It is concluded that there are differences in the composition of the T-cell population in a node compared with that of the lymph draining the node. The advantages of using fluorescently-labelled cells to study lymphocyte migration are discussed.

Abbreviations: BSS, Hanks's balanced salt solution; FITC, fluorescein isothiocyanate; Int. lymph, intestinal lymph; i.v., intravenous(ly); MLN, mesenteric lymph node(s); PBS, phosphate-buffered saline; PsLN, prescapular lymph node(s); Ps lymph, prescapular lymph; TRITC, rhodamine isothiocyanate.

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INTRODUCTION

It has been well established that a large proportion of the small lymphocytes in lymph nodes and the spleen as well as in the lymph are migratory cells recirculating between the blood and the lymph. Studies of this phenomenon in small laboratory rodents have usually involved the intravenous injection of radiolabelled cells with subsequent measurements of radioactivity in the lymphoid tissues and in the thoracic duct lymph (Sprent, 1973; Freitas, Rose & Parrott, 1977; Freitas, Rose & Rocha, 1980). In sheep, the chronic collection of efferent lymph from lymph nodes has enabled a more precise examination of lymphocyte migration through individual organs and different lymphoid tissues (Cahill, Poskitt, Frost & Trnka, 1977; Chin & Hay, 1980; Hall, 1980).

It was shown with these types of experiment that there are differences in the migratory properties of lymphocytes depending on the nature of the cells and the site from which they were collected. For example, lymphoblasts from the intestinal lymph of sheep or from mesenteric lymph nodes of rats or mice preferentially migrate to the tissues associated with the gut

(Griscelli, Vassalli & McClusky, 1969; Hopkins & Hall, 1976). In contrast, lymphoblasts from sites distant from the gut show no preference for the gut-associated tissues (Griscelli *et al.*, 1969; Hopkins & Hall, 1976). In addition, the migratory properties of small T lymphocytes have been examined in sheep and it has been confirmed in a number of studies that the T cells in intestinal lymph are more likely to reappear preferentially in intestinal lymph after intravenous injection. Likewise, T cells in the lymph from other regions, such as the head, forelimbs or hindlimbs, preferentially reappear in the lymph from these sites and not in the lymph from the intestine (Cahill *et al.*, 1977; Chin & Hay, 1980). This asymmetry in lymphocyte recirculation was taken to indicate the existence of two distinct pools of recirculating T cells in sheep.

Other studies failed to demonstrate any dichotomy in the migratory properties of small T lymphocytes in rats or mice (Freitas *et al.*, 1977, 1980). However, the experimental protocols used in these experiments were, of necessity, different from those used in the sheep studies, because it is not feasible to collect lymph chronically from different sites in rats or mice.

The present work was undertaken to determine whether or not the differences in the migratory properties of small T lymphocytes observed in the sheep, as opposed to the mouse, were due to specific properties of the cell populations used in the experiments or to species differences. A further extension of the previous experiments in the sheep was to label the two populations of T lymphocytes by *in vitro* incubation of one population with rhodamine isothiocyanate and the other population with fluorescein isothiocyanate. With this approach, two populations could be reinjected i.v. at the same time into the donor sheep and the migratory properties studied simultaneously. This made it possible to interpret the conflicting results from small laboratory rodents and sheep.

MATERIALS AND METHODS

Animals

The animals used were of either sex from commercial flocks of White Alpine sheep and Black Face sheep aged between 8 months and 1 year. They were housed in metabolism cages and given free access to hay, water and salt lick.

Surgical procedures

The sheep was prepared for surgery and the efferent lymphatic duct draining the prescapular node was cannulated (Heitmann, 1970). The contralateral prescapular lymph node was excised to make a cell suspension. A cannula was secured in the jugular vein for intravenous infusions. A long paracostal incision was then made in the right flank of the sheep and part of the intestine was withdrawn to expose the ileocaecal junction and the mesenteric lymph node chain associated with the distal jejunum and the ileum. A small mesenteric node, weighing about 2 g, associated with the jejunum, was selected for excision. The node was carefully freed of overlying mesentery and the blood vessels under the node were exposed by blunt dissection and then ligated. The node was removed to prepare a cell suspension and the incision in the mesentery was closed with interrupted sutures.

At the ileal end of the mesenteric lymph node chain in sheep there is usually a large kidney-shaped node which receives lymph from most of the ileum. The large efferent lymphatic from this node was cannulated by using standard techniques. The intestines were repositioned in the abdomen, the abdominal wall sutured closed, and the animal allowed to recover from anaesthesia.

Lymph collection and cell counting

Lymph was collected at varying intervals at room temperature in plastic bottles containing a 1 ml solution of sterile normal saline with 500 international units of preservative-free heparin (Liquemin; Hoffmann-La Roche, Basel, Switzerland), 500 international units of penicillin and 500 μg streptomycin (Flow Laboratories, Inc., Rockville, Md, U.S.A.). The viability of collected lymphocytes, assessed by trypan blue dye exclusion, was always over 95%. The cell concentration in the lymph was estimated by diluting samples with Isoton II, and analysing with a Coulter counter model Fn equipped with a 100 μm aperture (Coulter Electronics Inc., Hialeah, Fl., U.S.A.).

Preparation of cell suspensions

Efferent lymph was centrifuged and the cells washed three times in Hanks's balanced salt solution (HBSS) and then adjusted to the required cell concentration. A prescapular lymph node and mesenteric lymph node were removed from the anaesthetised animal as described previously. Extracapsular fat was removed, the capsule stripped away and the lymph node teased apart in HBSS. The cells were washed three times in HBSS; the procedure included a 1 g sedimentation step before each wash to eliminate cell clumps and debris.

Cells were resuspended in HBSS at a concentration of 5×10^7 viable mononuclear cells/ml. Suspensions of T-cell-enriched lymphocytes were prepared by nylon wool fractionation as described elsewhere (Cahill, Poskitt, Frost, Julius & Trnka, 1978).

Labelling of lymphocytes in vitro

The method described by Butcher & Weissman (1980) for labelling mouse cells with fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (TRITC) was used.

Stock solutions of 1 mg/ml FITC (Nordic Laboratories, Tilburg, The Netherlands) and TRITC (Nordic Laboratories, Tilburg, The Netherlands) in phosphate-buffered saline, pH 7·4 (PBS) were prepared and undissolved material was removed by centrifugation. Aliquots of this stock solution were stored at −20°.

Cells were labelled by the addition of either 10 μl FITC per ml cell suspension (1×10^8 cells/ml) or 5 μl TRITC per ml. After 15 min at 37° the cells were washed twice in HBSS, counted, and the intensity of labelling was checked with the fluorescence microscope (Leitz Orthoplan fitted with Ploemopak 2 for epifluorescence). Cells were reinfused i.v. into the sheep in numbers from 1×10^8–1×10^9 in volumes of 10–20 ml. Immunoglobulin on the surface of lymphocytes was detected with a standard immunofluorescence assay (Cahill *et al.*, 1978).

Counting of FITC- and TRITC-labelled cells in efferent lymph

The white cell concentration of the sample was determined in a haemocytometer or with the Coulter counter. Cell suspensions were then adjusted to contain a defined cell concentration (up to 2×10^8/ml). The chambers of a Cunningham slide, modified to hold 20 μl, were filled with the lymph samples. The chamber was scanned under the fluorescence microscope and the total number of fluorescent cells of one colour was determined. Filters were switched and the number of fluorescent cells of the other colour in the same chamber was counted. In this way the total number of labelled cells in a given collection of lymph was determined. During the first days after injection the fluorescent cells in efferent lymph could easily be seen with × 40 microscope objectives, whereas at later times, when the cells had lost some of their fluorescence intensity, × 50 or × 100 objectives were needed.

RESULTS

Sheep were prepared by cannulating the efferent lymphatics from both a prescapular lymph node and a mesenteric (ileocaecal) lymph node. In addition, with some of these sheep, a prescapular lymph node and a mesenteric (jejunal) lymph node were excised and cell suspensions prepared. T cells from these four different sources were compared for their ability to recirculate. The cell populations were passed through nylon wool columns, which reduced the proportion of cells with surface immunoglobulin to less than 5% of the total. This population, enriched in T cells, will be subsequently referred to as T cells. One population of T cells was labelled with fluorescein and the other population with rhodamine. Both populations were injected intravenously at the same time and their reappearance in the efferent lymph from a mesenteric node and a prescapular node were compared for up to 240 hr after injection.

T cells from lymph

One sheep was used to compare the recirculation pattern of the T cells from the two sources of efferent lymph i.e. draining from a mesenteric node and a prescapular node. The aim was to see if fluorescently-labelled lymphocytes from lymph would show a bias in their recirculation similar to that shown by the isotopically-labelled lymphocytes used in earlier studies (Scollay, Hopkins & Hall, 1976; Cahill *et al.*, 1977).

Figure 1 shows the number of labelled cells per 10^4 lymphocytes collected in the two sources of lymph as a function of the time after injection. A clear-cut asymmetrical recirculation was observed with preferential reappearance of prescapular lymph T cells in the prescapular lymph and the reappearance of intestinal lymph T cells in lymph from a mesenteric node. Labelled cells appeared in the lymph after a few hours and increased rapidly in abundance during the first day. These results confirm and extend those reported elsewhere with isotopically-labelled cells (Scollay *et al.*, 1976; Cahill *et al.*, 1977; Chin & Hay, 1980). With fluorescent cells, however, the increase in number continued beyond the first day and reached a maximum approximately 2 days after injection and the subsequent decline occurred at a much slower rate than in the previous studies with isotopically-labelled cells (Cahill *et al.*, 1977). During the 210 hr after injection 17·4% of injected intestinal lymph T cells and 21·8% of the injected prescapular lymph T cells were recovered in the lymph collections. The vast majority of the labelled lymphocytes recovered in the lymph

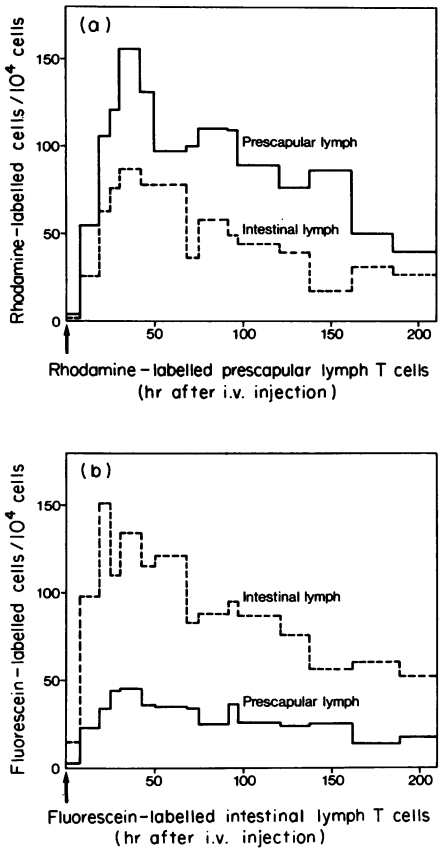

Figure 1. Changes in the concentration of fluorescent cells in the prescapular lymph and in the intestinal lymph after i.v. injection of T cells derived from lymph. A bias in the recirculation pattern is obvious. (a) Rhodamine-labelled prescapular lymph T cells ($1{\cdot}5 \times 10^9$) injected i.v. (b) Fluorescein-labelled intestinal lymph T cells ($7{\cdot}3 \times 10^8$) injected i.v.

were small to medium in size and less than 5% were large lymphocytes. Examination of cell suspensions stained for surface immunoglobulin showed that virtually all the fluorescent cells which appeared in lymph had no surface immunoglobulin.

T cells from lymph nodes

Three sheep were reinjected with their own lymphocytes prepared from cell suspensions of both prescapular lymph nodes and mesenteric lymph nodes. The recovery of both T cell populations was monitored in prescapular lymph and intestinal lymph. The values for each animal are given in Table 1 and the recovery curves for two of these animals are shown in Figs 2 and 3. It was found with one sheep that the T cells derived from lymph nodes exhibited a non-random recirculation pattern. Specifically, mesenteric lymph node T cells preferred to migrate into intestinal lymph rather than into prescapular lymph. Conversely, prescapular lymph node T cells preferred to migrate into prescapular lymph (Fig. 2 and Table 1; sheep 668). However, with the T cells purified from the nodes the asymmetry in recirculation was less obvious than with the T cells purified from lymph. In the second sheep no bias was obvious in the recirculation of T cells purified from either lymph node. In this animal the recovery of both populations was the same in the lymph from the mesenteric and from the prescapular nodes (Fig. 3 and Table 1; sheep 653). In the third sheep the recirculation of mesenteric lymph node T cells showed no bias, but the prescapular lymph node T cells showed a slight preference to migrate into the prescapular lymph (Table 1; sheep 655).

T cells purified from lymph nodes and from lymph

In an additional two sheep the migratory potential of T cells derived from lymph nodes and of T cells derived from lymph draining a related region were compared simultaneously in the same animal. The cells from lymph were labelled with one fluorochrome, whereas those from the corresponding lymph node were labelled with the alternative fluorochrome. A comparison of the recoveries in lymph of these two cell populations (Table 2) confirms the trends reported in the previous two sections. Both cell populations showed a biased recirculation but the differences were more evident for the T cells derived from lymph. The absolute recoveries of labelled cells in the lymph up to 80 hr were 5%–13% of the number injected, rising to 11%–28% after 200 hr.

DISCUSSION

The results of these experiments confirm the existence in sheep of at least two classes of T lymphocytes based on differences in recirculation properties. There is a clear distinction between the T cells in lymph from prescapular lymph nodes and the T cells in lymph from mesenteric lymph nodes. Each of these two cell populations showed a clear preference to leave the bloodstream and return to the lymph from which they were originally collected.

The results were obtained with the aid of a cell-

Table 1. Recirculation of lymph node T cells

Sheep no.	Injected cells (i.v.) Source	Label	Number	Relative recovery ratio* Int. lymph:Ps lymph	Absolute recovery (%)‡ 80 Hr	200 Hr
653	PsLN	FITC	$3{\cdot}4 \times 10^8$	0·91	6·9	13·1
	MLN	TRITC	$7{\cdot}3 \times 10^8$	0·94	6·4	12·1
668	PsLN	TRITC	$4{\cdot}2 \times 10^8$	0·75†	13·5	23·8
	MLN	FITC	$1{\cdot}1 \times 10^9$	1·21†	14·7	24·7
655	PsLN	FITC	$4{\cdot}2 \times 10^8$	0·64†	15·3	25·2
	MLN	TRITC	$4{\cdot}9 \times 10^8$	0·99	10·7	17·5

* For each population the value was calculated for the percentage of the injected dose recovered in the intestinal lymph and in the prescapular lymph. The ratio of the relative recovery of each population in the 2 sources of lymph gives an indication of whether or not there is a bias in recirculation.

† The ratios which indicate that preferential recirculation occurred.

‡ Cumulative recovery of injected dose in both lymphatics.

PsLN, prescapular lymph node cells; MLN, mesenteric lymph node cells; Ps lymph, prescapular lymph; Int. lymph, intestinal lymph.

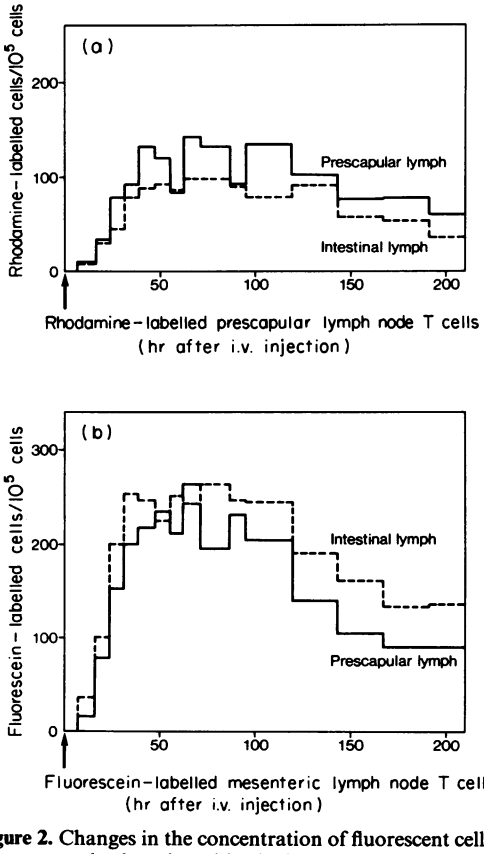

Figure 2. Changes in the concentration of fluorescent cells in the prescapular lymph and in the intestinal lymph after i.v. injection of T cells derived from lymph nodes (Sheep 668). A small bias in the recirculation pattern is obvious. (a) Rhodamine-labelled prescapular lymph node T cells ($4{\cdot}2 \times 10^8$) injected i.v. (b) Fluorescein-labelled mesenteric lymph node T cells ($1{\cdot}1 \times 10^9$) injected i.v.

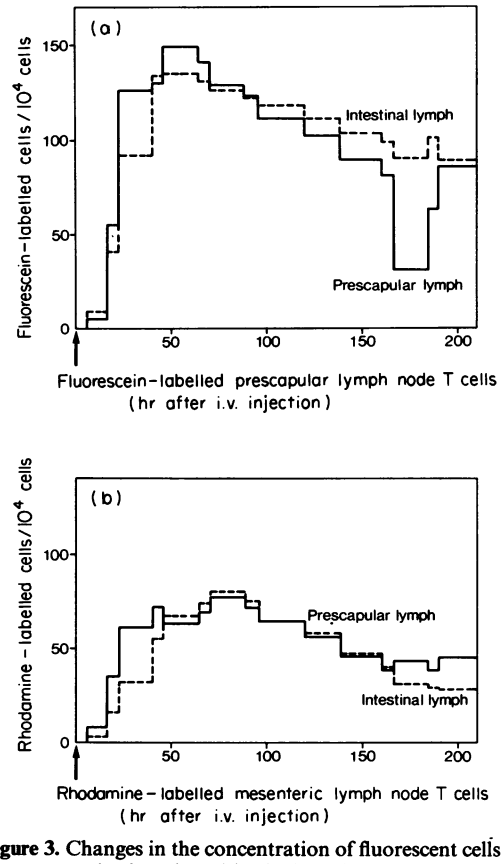

Figure 3. Changes in the concentration of fluorescent cells in the prescapular lymph and in the intestinal lymph after i.v. injection of T cells derived from lymph nodes (Sheep 653). There is no bias in the pattern of recirculation. (a) Fluorescein-labelled prescapular lymph node T cells ($3{\cdot}4 \times 10^8$) injected i.v. (b) Rhodamine-labelled mesenteric lymph node T cells ($7{\cdot}3 \times 10^8$) injected i.v.

Table 2. Comparison of recirculation of T cells from lymph node and efferent lymph

Sheep no.	Injected cells (i.v.) Source	Label	Number	Relative recovery ratio* Int. lymph:Ps lymph	Absolute recovery (%)‡ 80 Hr	200 Hr
667	MLN	TRITC	$5{\cdot}9 \times 10^8$	1·45	10·2	19·9
	Int. lymph	FITC	$2{\cdot}1 \times 10^9$	1·75	12·6	28·4
657	Ps lymph	FITC	$7{\cdot}0 \times 10^8$	0·57	5·5	11·0
	PsLN	TRITC	$2{\cdot}2 \times 10^8$	0·65	11·6	28·2

*‡ See legend to Table 1 for details and abbreviations.

labelling technique that has not previously been used in sheep. Two different fluorescent dyes were used to label the T cells *in vitro* so that after the cells had been injected i.v. it was possible to discriminate simultaneously between the recirculation properties of cells from two different sources. As a result of the direct observation of many labelled cells recovered in lymph it became obvious that most of the cells which contributed to the biased recirculation pattern were small cells and not large transforming lymphocytes. It therefore seems unlikely that the phenomenon is due to blast cell recirculation (Freitas *et al.*, 1980). In addition the recirculation differences can be observed for many days after i.v. injection, but the reappearance of the labelled blast cells in lymph is a transient event which almost ceases 1 day after injection (Griscelli *et al.*, 1969; Reynolds, 1976).

Studies in rats and mice of the migration pattern of T cells from mesenteric lymph nodes, subcutaneous lymph nodes, and thoracic duct lymph (Freitas *et al.*, 1977, 1980) failed to detect any differences similar to those reported in this and earlier studies in sheep. One reason for the conflicting results might relate to differences in the source of the T cells used, i.e. the lymph nodes from rodents and the efferent lymph from sheep. The recirculation data presented here suggest that the T-cell population isolated from a lymph node is not equivalent to the T-cell population in the efferent lymph from a node, at least in sheep. T cells isolated from lymph nodes did not consistently show the same degree of preferential recirculation that can invariably be demonstrated with T cells from lymph. Thus, in three out of eight experiments, the recirculation of both mesenteric lymph node T cells and of prescapular lymph node T cells appeared to be completely random and comparable with the results of the studies done in rats and mice. Also, with the experiments in which the recirculation of lymph node T cells and of efferent lymph T cells were followed simultaneously, the extent of the bias in cell recirculation was less obvious with the lymph node T cells than with the lymph T cells. We take these results to be evidence that the lymph node, when compared with efferent lymph, contains a smaller fraction of the total T-cell population that show a biased recirculation. Thus, in sheep, the lymph node cannot be considered to be the best source of cells for studies of lymphocyte recirculation.

The use of fluorescent dyes as a means of labelling living cells for use in migration studies needs some comment. The technique was introduced by Butcher & Weissman (1980) who showed by syngenic cell transfers in mice that fluorescent labelling had no effect on the viability or on the migratory properties of lymphocytes (Butcher, Scollay & Weissman, 1980). We have confirmed these observations in our studies with sheep. Also a comparison of our results with those from other studies in sheep which used ^{51}Cr- or ^{111}In-labelled lymph cells (Çahill *et al.*, 1977; Chin & Hay, 1980) suggest that fluorescent labelling gives a more accurate picture of lymphocyte recirculation. For example, the recovery of the i.v. injected dose in the lymph is several times higher with fluorescent labelling than with radiolabelling (see Cahill *et al.*, 1977; Chin & Hay, 1980). One possible reason for this difference is the progressive loss of isotopes from radiolabelled cells. Fluorescent dye is also lost from cells but the quantification is done by visual counting, and not by determining the total amount of fluorescence. Therefore, labelled cells containing fluorochrome can be reliably detected for many days, or even weeks, after i.v. injection. The resultant greater accuracy may be the reason why the recovery curves in lymph have a peak 1 day later and a much slower decline when the T cells are labelled with fluorochrome instead of with isotopes (Cahill *et al.*, 1977; Chin &

Hay, 1980). Another advantage of fluorochrome labelling is that some of the variations between experiments are reduced by simultaneously following the migration of separate cell populations labelled with different fluorescent dyes. This approach provides no difficulty in discriminating between the two cell populations.

Finally this technique allows the analysis of other features such as the size and the surface markers of cells known to have recirculated. This will thus enable studies of the migratory pathways of various lymphocyte subclasses to be made, without having to devise a method of first purifying the population in question.

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