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The appearance of fluorescein-labelled lymphocytes in lymph following *in vitro* or *in vivo* labelling: the route of lymphocyte recirculation through mesenteric lymph nodes

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Summary. Fluorescein isothiocyanate (FITC) has been used to study lymphocyte migration in sheep. After being labelled *in vitro* with FITC, lymphocytes migrated from blood into lymph at the same rate and with the same recovery as lymphocytes labelled with the radioisotope ^{51}chromium. The *in vivo* labelling of mesenteric lymph nodes (MLN) with FITC resulted in high numbers of labelled lymphocytes appearing in prescapular lymph. However, the appearance of the FITC-labelled lymphocytes in the prescapular lymph could be prevented by cannulating the main intestinal lymph duct prior to the *in vivo* labelling procedure. It was concluded that lymphocytes labelled *in vivo* within the MLN required an intact lymphatic system to reach the blood circulation and did not enter the venous circulation directly from the MLN.

INTRODUCTION

Radioisotopes were used to demonstate that lymphocytes recirculated from blood into tissues and back to the blood via the lymphatic system (Gowans & Knight, 1964) and they have been used to study lymphocyte traffic since then. The technique of labelling lymphocytes with the fluorochrome, fluorescein isothiocyanate (FITC) offered an additional way to study lymphocyte migration with a label which was not biohazardous, and enabled the labelled cells to be identified while still viable (Butcher & Weissman, 1980). FITC has been used for lymphocyte migration studies in mice (Butcher, Scollay & Weissman, 1980), pigs (Binns, Blakely & License, 1981) and in sheep (Reynolds *et al.*, 1982). The FITC-labelled lymphocytes in sheep (Reynolds *et al.*, 1982) appeared in lymph with different kinetics than previously shown for lymphocytes labelled with radioisotopes (Cahill *et al.*, 1977; Chin & Hay, 1980; Issekutz, Chin & Hay, 1980a, b).

FITC has also been used to label cell populations *in vivo*. Using this technique, thymocyte migration has been studied in mice (Scollay *et al.*, 1978; Scollay, Butcher & Weissman, 1980) and the route of lymphocyte recirculation has been studied in the pig (Pabst & Binns, 1981) where it was shown that a considerable number of lymphocytes labelled *in vivo* within the mesenteric lymph nodes (MLN) returned directly to the venous circulation in the MLN and few returned to the blood via the efferent intestinal lymph. This unique pathway of lymphocyte migration has been well established for the pig (Binns & Hall, 1966; Bennell &

Abbreviations: FITC, fluorescein isothiocyanate; MLN, mesenteric lymph nodes; HBSS, Hanks's balanced salt solution; FCS, foetal calf serum; c.p.m., counts per minute; PLN, peripheral lymph node.

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Husband, 1981a, b; Pabst & Geisler, 1981). In rats, perfused isolated MLN released few, if any lymphocytes into the venous efflux (Sedgley & Ford, 1976). The route of lymphocytes returning to the blood from the MLN of sheep has not been reported. However, lymphocytes in a popliteal lymph node returned to the blood via the efferent lymph, since cannulation of the efferent lymphatic draining a popliteal lymph node undergoing an immune response prevented the dissemination of the immune response throughout the rest of the sheep (Smith *et al.*, 1970).

The two aspects of the present study were (i) to investigate the migratory properties of sheep lymphocytes labelled *in vitro* with FITC, and to determine whether lymphocytes labelled with FITC or with a radioisotope exhibited any differences in the kinetics of lymphocyte migration and recoveries in lymph after infusion into the blood, and (ii) to elucidate the route of lymphocytes returning to the blood from the MLN by using the technique of labelling the MLN of sheep *in vivo* with FITC.

MATERIALS AND METHODS

Animals

Merino ewes, 1–2 years of age, were used in all experiments.

Surgery and lymph collection

The technique of cannulating the efferent lymph duct from single lymph nodes (Hall & Morris, 1962) was used to collect lymph from the prefemoral (Hall, 1967) or prescapular (Heitmann, 1970) lymph nodes. In some animals, the main intestinal lymph duct was also cannulated (Lascelles & Morris, 1961). At the time of surgery, a cannula was positioned in the jugular vein and secured in position.

Lymph was continuously collected into sterile polyethylene bottles which contained 1 ml of normal saline supplemented with 500 U preservative-free heparin (Commonwealth Serum Laboratories, Melbourne, Victoria), 500 IU penicillin and 500 μg streptomycin (Flow Laboratories, South Yarra, Victoria). The lymph collection bottles were changed at various times throughout the day. The cell concentration in each collection was determined using a model FN Coulter Counter fitted with a 100 μM aperture tube (Coulter Electronics Ltd., Dunstable, Bedfordshire, U.K.).

In vitro *cell labelling*

A stock solution of FITC (Research Organics Inc., Cleveland, OH, U.S.A.) was prepared in 0·4 M phosphate-buffered saline, pH 7·4 as described previously (Butcher & Weissman, 1980). The final concentration of FITC was 780 μg/ml.

Efferent lymph was collected for 3–4 hr. The cells were washed once in Hanks's balanced salt solution (HBSS) containing 1% foetal calf serum (FCS) and then resuspended at 1×10^8 cells/ml in HBSS containing 1% FCS. For FITC labelling, either 10 μg or 50 μg FITC was added to the cell suspension. For ^{51}chromium labelling, 50 μCi $Na_2[^{51}Cr]O_4$ (Amersham Australia Pty. Ltd., Sydney, N.S.W.) was added to the cell suspension. The cells were incubated with label for 30 min at 37°. Lymph plasma was added to stop the labelling reaction and the cell suspension centrifuged (5 min, 400 ***g***, 21°). The cells were washed twice in lymph plasma and then resuspended in 20 ml of lymph plasma. The labelled cells were returned to the sheep via the jugular vein cannula.

In vivo *cell labelling*

At the time of surgery, 5 ml of FITC solution was injected into the MLN. Multiple injections of 50–100 μl were made with a 27 gauge needle fitted to a 1 ml tuberculin syringe.

Counting of FITC-labelled lymphocytes

Lymphocytes collected in lymph were resuspended at a concentration of 10^8 cells/ml. A 10 μl aliquot of this cell suspension was placed on a microscope slide and a coverslip, 22 mm square, was placed on top. The edges of the coverslip were sealed with nail polish to form a counting chamber.

The proportion of FITC-labelled lymphocytes was determined as described previously (Scollay *et al.*, 1980). Using a grid of known size, the total number of cells in three random grids in one row of the chamber was counted. Using a simple conversion factor, the total number of cells in that row could be calculated. The number of FITC-labelled lymphocytes in that row were counted by scanning the entire row of the chamber under u.v. light. The number of rows of the chamber counted in this manner was determined by the proportion of FITC-labelled lymphocytes present. When very few FITC-labelled lymphocytes were present, the entire chamber was scanned.

A Zeiss microscope fitted with a IV F1 epifluorescence condenser and a HBO 50 W super-pressure mercury lamp was used for counting FITC-labelled

cells (Carl Zeiss Pty. Ltd., Carnegie, Victoria). A planneofluar 16 objective was used throughout to count cells.

Measurement of radioactivity

After aliquots of lymph were taken for determining the cell concentration and for counting FITC-labelled lymphocytes, the rest of the cells in each lymph collection were pelleted by centrifugation (5 min, 400 *g*, 21°). At the end of each experiment, all of the samples were placed in a Packard Auto-Gamma 500C gamma spectrometer (Packard Instruments, Downers Grove, IL, U.S.A.) and counted for 10 min.

Statistics

Statistical significance was determined using the *t*-statistic.

RESULTS

In vitro cell labelling

Lymphocytes labelled *in vitro* with FITC were returned to the venous circulation of the sheep. Over the following 72 hr, the proportion of FITC-labelled cells in lymph was determined.

When lymphocytes were incubated with 10 μg FITC/10^8 cells, more than 99% of all cells were labelled. The intensity of labelling varied, but about 5% of the cells were labelled very brightly. After being returned to the venous circulation, the concentration (FITC-labelled cells/10^4 cells) of labelled lymphocytes appearing in lymph increased over the first day and then declined over the next 2 days. This pattern was observed in six out of six sheep. A representative result is shown (Fig. 1b).

When lymphocytes were incubated with 50 μg FITC/10^8 cells, more than 99% of all cells were labelled very brightly. After being returned to the venous circulation, the concentration of labelled lymphocytes appearing in lymph increased over the first day and then declined over the next 2 days. This pattern was observed in six out of six sheep. A representative result is shown (Fig. 1b).

In the 72 h after intravenous injection, the number of FITC-labelled lymphocytes recovered in the efferent lymph of a single lymph node ranged from 1% to 8% of the number of labelled cells injected (Table 1). The number of labelled cells recovered in lymph depended on the cell output of the lymph node. Thus, more labelled lymphocytes were recovered in the

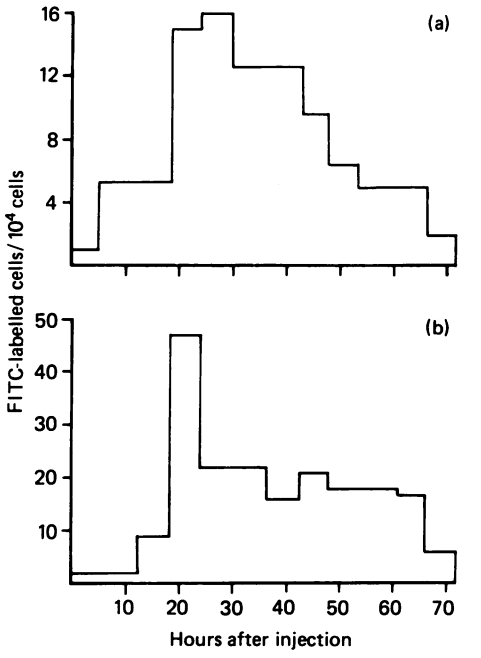

Figure 1. Appearance of FITC-labelled lymphocytes in efferent prescapular lymph following the injection of 2×10^8 lymphocytes labelled with (a) 10 μg FITC/10^8 cells or (b) 50 μg FITC/10^8 cells.

Table 1. Recovery of lymphocytes labelled *in vitro* with FITC in the efferent lymph of prescapular or prefemoral lymph nodes (72 hr after intravenous infusion)

Dose of FITC used to label cells (μg/10^8 cells)	% recovery	% recovery/10^9 cells
10	8·44	0·76
	6·36	0·40
	2·50	0·24
	1·71	0·42
	2·81	0·28
	3·94	0·45
		0·43 ± 0.07*
50	1·03	0·49
	3·73	0·42
	7·12	0·89
	3·77	0·92
	3·73	0·49
	1·22	0·43
		0·61 ± 0·10*

* Mean ± SE.

lymph of a lymph node with a high cell output compared with a lymph node with a low cell output. To normalize the recovery of labelled cells from lymph nodes with different cell outputs, the percentage recovery was divided by the total number of cells collected over the same period and expressed as % recovery/10^9 cells. Over the 72 hr after intravenous infusion, the mean recovery of lymphocytes labelled with 10 μg FITC/10^8 cells was 0·43%/10^9 cells. This was not significantly different from the mean recovery of lymphocytes labelled with 50 μg FITC/10^8 cells which was 0·61%/10^9 cells.

To compare the migratory patterns of FITC-labelled lymphocytes and ^{51}Cr-labelled lymphocytes, 2×10^8 lymphocytes were labelled with FITC and another 2×10^8 lymphocytes from the same lymph collection were labelled with ^{51}chromium. Both labelled cell populations were returned simultaneously to the venous circulation of the sheep. The kinetics of the appearance of labelled cells in lymph were not noticeably different for FITC-labelled and ^{51}Cr-labelled lymphocytes. The concentration of labelled cells (FITC-labelled cells/10^4 cells or c.p.m./10^7 cells) increased over the first day and then declined over the next 2 days. This pattern was observed in five out of five sheep. A representative result is shown (Fig. 2). The recoveries per unit cell number were not significantly different for FITC-labelled and ^{51}Cr-labelled lymphocytes, when using paired data analysis (Table 2).

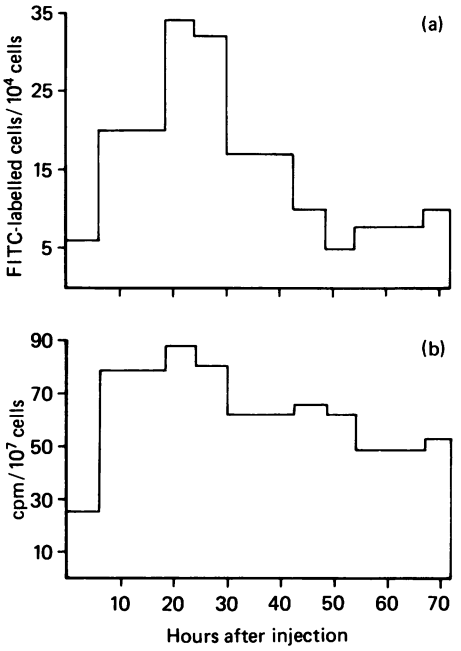

Figure 2. Appearance of labelled lymphocytes in efferent prescapular lymph. From the same lymph collection, 2×10^8 lymphocytes were labelled with (a) 50 μg FITC/10^8 cells or (b) 50 μCi ^{51}Cr/10^8 cells.

Table 2. Recovery of lymphocytes labelled *in vitro* with FITC or with ^{51}Cr in the efferent lymph of prescapular lymph nodes (72 hr after intravenous infusion)

Dose of FITC used to label cells (μg/10^8 cells)	% recovery/10^9 cells	
	FITC	^{51}Cr*
10	0·28	0·20
10	0·45	0·30
50	0·89	0·88
50	0·49	0·55
50	0·43	0·68

* ^{51}Cr was used to label lymphocytes at a dose of 50 μCi/10^8 cells.

In vivo cell labelling

In 3 sheep, 5 ml of FITC solution was injected into the MLN. The appearance of FITC-labelled lymphocytes was monitored in the efferent lymph of prescapular or prefemoral lymph nodes over the following 72 hr.

The concentration of FITC-labelled lymphocytes remained low during the first day and then rose rapidly to reach a maximum about 2 days after the injection of FITC. A representative result is shown in Fig. 3. The maximum concentrations of FITC-labelled lymphocytes in the prescapular or prefemoral lymph of the three sheep were 235, 438 and 143 FITC-labelled cells/10^4 cells. The corresponding lymph collections were made at 28–41·5 hr, 48–52 hr and 53–57 hr after the injection of FITC into the MLN.

To determine whether the FITC-labelled lymphocytes observed in lymph were labelled in the MLN or whether they were labelled in the systemic circulation by free FITC which had escaped the MLN, 5 ml of FITC was infused into the cranial mesenteric vein of a sheep. Only two FITC-labelled lymphocytes were observed in the prescapular lymph collection in the 72 hr after infusion of the FITC. This indicated that no significant numbers of lymphocytes were labelled by free FITC in the circulation.

In two sheep, 5 ml of FITC solution was injected

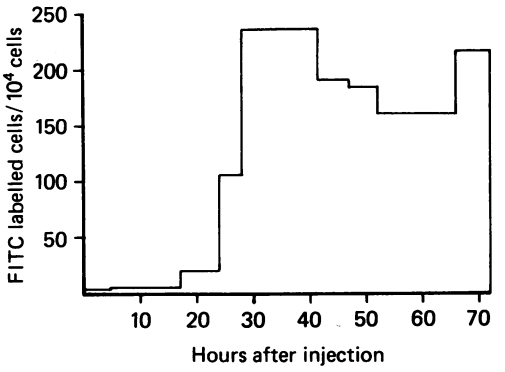

Figure 3. Appearance of FITC-labelled lymphocytes in efferent prefemoral lymph following the injection of FITC into MLN.

into the MLN after the main intestinal lymph duct had been cannulated. The presence of FITC-labelled lymphocytes was monitored for the following 72 hr in intestinal lymph and in prescapular lymph. In the intestinal lymph of one sheep, a maximum concentration of 25% FITC-labelled lymphocytes occurred in the first 4 hr after the injection of the FITC and fell to a level of 5% FITC-labelled cells between 32 and 72 hr after injection of the FITC (Fig. 4). The pattern of FITC-labelled cells appearing in the intestinal lymph of the second sheep was very similar. In contrast to the intestinal lymph, there was a striking paucity of FITC-labelled lymphocytes in the prescapular lymph of these sheep. In one sheep, only one FITC-labelled lymphocyte was observed in the lymph collected in the 72 hr after injection of FITC into the MLN. In the second sheep, three FITC-labelled lymphocytes were

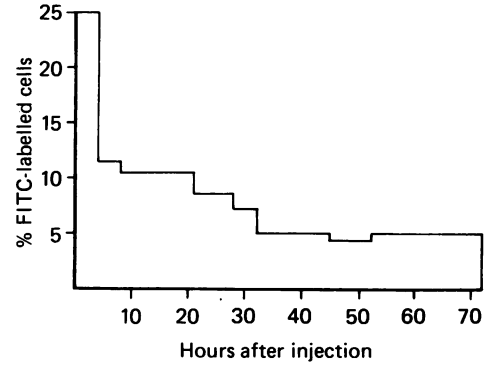

Figure 4. Appearance of FITC-labelled lymphocytes in the main intestinal lymph duct following the injection of FITC into MLN.

observed in the prescapular lymph during the 72 hr after injection of the FITC into the MLN.

DISCUSSION

In the present study, there was a difference between using FITC or radioisotope to follow lymphocyte migration. The detection of FITC-labelled lymphocytes relied on the visual identification of a cell labelled with FITC and was not concerned with how much FITC was present in that cell. The detection of a ^{51}Cr-labelled lymphocyte depended on the amount of radioisotope present in the labelled cells and also on the number of cells labelled. Despite this difference, both ways of tracing lymphocyte migration were remarkably similar.

When lymphocytes were labelled *in vitro* with 10 μg FITC/10^8 cells or 50 μg FITC/10^8 cells and then returned to the venous circulation, the appearance of labelled cells in lymph exhibited the same kinetics and recoveries for both labelling doses of FITC. By these criteria, the higher labelling dose of FITC was not adversely affecting the recirculatory capacity of the lymphocytes. Comparable labelling doses of FITC have been shown not to alter lymphocyte migration patterns in mice (Butcher *et al.*, 1980). With the higher dose of FITC, lymphocytes were labelled very brightly, which made identification of labelled cells in lymph much easier.

The migratory kinetics of lymphocytes labelled with FITC presented in this study were different from those previously reported (Reynolds *et al.*, 1982). When lymphocytes from the same lymph collection were labelled with FITC or with ^{51}chromium, both of the labelled cell populations appeared in lymph with the same kinetics. These migration patterns, with the maximum concentration of labelled cells in lymph 1 day after intravenous injection, were consistent with previous reports using radioisotopes to label lymphocytes (Cahill *et al.*, 1977; Chin & Hay, 1980; Issekutz *et al.*, 1980a, b). The recoveries of the lymphocytes labelled with FITC or with ^{51}chromium were not significantly different in this study and were consistent with the recoveries in previous studies using radioisotopes to label lymphocytes (Issekutz *et al.*, 1980a, b). The experiments presented here demonstrated quite clearly that the migratory properties of lymphocytes labelled with FITC and with ^{51}chromium were not different.

Following the *in vivo* labelling of MLN with FITC, a

very high concentration of FITC-labelled lymphocytes appeared in the prescapular lymph. The labelled cells which appeared in lymph were labelled in the MLN since the intravenous infusion of FITC did not label significant numbers of lymphocytes in the systemic circulation. When the FITC was injected into the MLN, any free FITC which escaped the MLN could not have labelled enough lymphocytes in the systemic circulation to make a substantial contribution to the number of labelled cells in lymph.

The main intestinal lymph duct collected all the lymph from the intestines (Lascelles & Morris, 1961). If this lymphatic was cannulated prior to the *in vivo* labelling of the MLN with FITC, labelled lymphocytes which appeared in prescapular lymph would have had to enter the venous circulation within the parenchyma of the MLN, because labelled cells returning to the blood via the efferent lymph would be diverted from the sheep into the lymph collection bottle.

When the main intestinal lymph duct was cannulated, there was a very high proportion of FITC-labelled lymphocytes in the intestinal lymph following injection of FITC into the MLN. It was unlikely that many of the FITC-labelled lymphocytes were labelled after entering the lymph collection bottle, since the room temperature of the collection bottle and the protein content of lymph would act together to limit the labelling of lymphocytes with FITC (Butcher & Weissman, 1980).

There was a striking paucity of FITC-labelled lymphocytes appearing in the prescapular lymph when the main intestinal lymph duct was cannulated. The few FITC-labelled lymphocytes which were observed in prescapular lymph were not significantly greater than those observed in the control experiment where FITC was infused intravenously. The *in vivo* labelling of MLN with FITC demonstrated that lymphocytes labelled in the MLN depended on an intact lymphatic system for returning to the venous blood.

The origin of the lymphocytes in efferent intestinal lymph is still unclear. About 90% of the cells in the efferent lymph of a peripheral lymph node (PLN) are recirculating lymphocytes derived from the blood within the lymph node (Hall & Morris, 1964, 1965). It is estimated that one in four blood lymphocytes will leave the blood to enter a PLN (Hay & Hobbs, 1977). On a per gram basis, the blood flow to a PLN and a MLN are the same (Hay & Hobbs, unpublished observations; Chin, 1982). However, the proportion of blood lymphocytes leaving the blood to enter the MLN is not known, although previous studies with radiolabelled lymphocytes suggest that substantial numbers of lymphocytes are entering the MLN from the blood (Cahill *et al.*, 1977). The extraction ratio in MLN may be very different from that in PLN for the following reason. The intestinal lymph cell output in a sheep with all of its MLN removed is not significantly decreased from the cell output of intestinal lymph in a sheep with its MLN intact (Reynolds, 1976; Hall, 1980). Since the cells leaving the bowel wall in afferent lymph could account for most of the cells in the intestinal lymph, very few lymphocytes in the blood are required to enter the MLN. If the MLN did extract the same proportion of lymphocytes from the blood as the PLN did, the total number of cells entering the MLN derived from the blood plus the cells entering the MLN from the bowel wall in afferent lymph would be twice as much as the cell output from the MLN in efferent lymph. The excess number of cells could not return to the blood from the parenchyma of the MLN because the present study demonstrates that lymphocytes in the MLN must return to the blood via the efferent lymph. There appears to be an imbalance in the number of lymphocytes migrating through the MLN which is difficult to resolve with current data.

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