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THE MIGRATION OF RAT THORACIC DUCT LYMPHOCYTES THROUGH SPLEEN *IN VIVO*

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Summary.—Rat thoracic duct lymphocytes have been labelled *in vitro* using [^{3}H]5-uridine. After injection intravenously into syngeneic recipients their fate has been followed by scintillation counting and autoradiography. During the first 24 hours the distribution of radioactivity between different organs changed greatly with time. Lungs and liver contained about 50% of the injected radioactivity within 0·5 hours and lost most of this within 4 hours. In the spleen the radioactivity increased for about 4–6 hours and then decreased, whereas that in mesenteric and peripheral lymph nodes increased almost linearly for 24 hours. Free [^{3}H]uridine and heat-killed cells failed to accumulate in lymphoid organs. When labelled lymphocytes were injected into splenectomized animals their lymph nodes accumulated more radioactivity earlier than the controls and remained more radioactive for at least 24 hours. It seems that one population of lymphocytes which accumulates in the rat spleen is released to accumulate subsequently in lymph nodes.

WHEN rat small lymphocytes are labelled and injected into syngeneic recipients they may be detected subsequently in peripheral lymphoid tissues. This technique has been used (1) to investigate the route of recirculation of lymphocytes in rats (Gowans and Knight, 1964; Griscelli, Vassalli and McCluskey, 1969), (2) to delineate thymus-dependent areas in mouse lymphoid tissues (Parrott and de Sousa, 1971), (3) to demonstrate B-lymphocyte recirculation in rats (Howard, Hunt and Gowans, 1972; Howard, 1972), (4) to differentiate between different populations of small lymphocytes in mice (Zatz and Lance, 1970), (5) to demonstrate the immune elimination of allogeneic lymphocytes in lymphoid tissues of mice (Bainbridge and Gowland, 1971) and (6) to follow the fate of injected large lymphocytes in rats (Hall, Parry and Smith, 1972).

This paper reports a method for following the rate of tritium labelled rat thoracic duct lymphocytes after their intravenous injection into syngeneic recipients, by scintillation counting and autoradiography. The results show that during the 24 hours after their injection the distribution of lymphocytes between different tissues changes rapidly with time. In particular, the time course of migration of the injected cells through the spleen *in vivo* is very similar to that through an isolated perfused rat spleen (Ford, 1969*a*, *b*) and one population of injected cells can home to either spleen or lymph nodes. A preliminary report was published some time ago (Born and Bradfield, 1968).

MATERIALS AND METHODS

The animals were adult male inbred Wistars weighing between 250 and 350 g.

Collection of rat thoracic duct lymphocytes.—Thoracic ducts were cannulated under ether anaesthesia as described by Bollman, Cain and Grindley (1948*b*) with the modification of Gowans (1959). The rats in restraining cages (Bollman *et al.*, 1948*a*) were continuously infused intravenously with Krebs–Ringer solution containing penicillin 100 μg/ml, streptomycin 100 μg/ml and heparin 2 u/ml, flowing at 2 ml/hour. Lymph was collected at 4° into sterile tissue culture medium (TC 199, Glaxo Ltd) containing either heparin, 20 u/ml, or sodium citrate, 3·8% w/v. Collections were made between 4 and 16 hours after cannulation.

Labelling of lymphocytes.—Lymph in TC 199 was centrifuged at 100 *g* for 10 min and the sedimented lymphocytes were resuspended in TC 199, containing 20% of either rabbit serum or autologous rat serum, to a final cell concentration of $1{\cdot}0 \times 10^8$ per ml. [^{3}H]5-uridine (from the Radiochemical Centre, Amersham; specific activity 15–20 Ci/mmol) was added to a final concentration of 10 μCi/ml. The culture was incubated at 37° for 1 hour. The cells were then washed 3 times and finally resuspended in TC 199 for injection. Thoracic duct lymphocytes prepared in this way contained about $1{\cdot}5 \times 10^4$ d/min per million cells.

The viability of lymphocytes was assessed by examining cells in culture at 37° under phase contrast microscopy after incubation for 2 hours in Mackaness chambers (Mackaness, 1952). Characteristic motility was seen in more than 80% of the cells. In addition, one preparation of labelled cells was injected intravenously into a restrained rat which had a thoracic duct fistula and the labelled cells were subsequently recovered from the draining lymph.

Injection of cells.—Between $0{\cdot}5 \times 10^8$ and 1×10^8 labelled lymphocytes in 1·0 ml were injected into the tail veins of restrained unanaesthetized recipients, which were then released. The recipients were killed between 0·5 and 24 hours after injection.

Measurement of radioactivity in tissues.—Tissues were removed and weighed when appropriate. Pieces were cut up, weighed on filter paper discs and incubated in scintillation counting vials with 2 ml of hyamine hydroxide (Hyamine-10x, Packard Instruments Co. Ltd) at 55° for 36 hours. After cooling, 0·3 ml hydrogen peroxide was added to each vial. After a further 24 hours, to allow all oxygen to escape, 15 ml of scintillant, containing 2-butoxyethanol as secondary solvent, was added. Radioactivity was measured using a Packard Tricarb scintillation counter and corrections made for quenching using an automatic external standard.

Autoradiography.—Tissues were fixed in 10% buffered formol saline; 4 μm sections were covered by Kodak AR 10 stripping film and exposed for 4 weeks before staining through the emulsion with methyl-green pyronin or haematoxylin and eosin.

RESULTS

Fate of labelled lymphocytes after intravenous injection

Rat thoracic duct lymphocytes were labelled with [^{3}H]uridine and injected intravenously into unanaesthetized syngeneic recipients, which were then released. The recipients were killed between 0·5 and 24 hours after injection and their tissues prepared for scintillation counting and autoradiography.

Liver and *lungs* each accumulated between 20 and 30% of the injected dose of radioactivity within 30 min although, because of its greater weight, the concentration of radioactivity in liver was only about one-tenth of that in the lungs (Fig. 1). Autoradiography showed that the labelled lymphocytes in the liver were in the sinusoids but had not been phagocytosed by Küpffer cells; those in the lungs were in the alveolar walls, presumably in the lumen of capillaries. The radioactivity in both liver and lungs declined rapidly so that, after 8 hours, they contained 5 and 2% of the injected dose respectively and these amounts did not change significantly during the next 16 hours.

In the spleen, radioactivity accumulated for 4 hours after injection and then

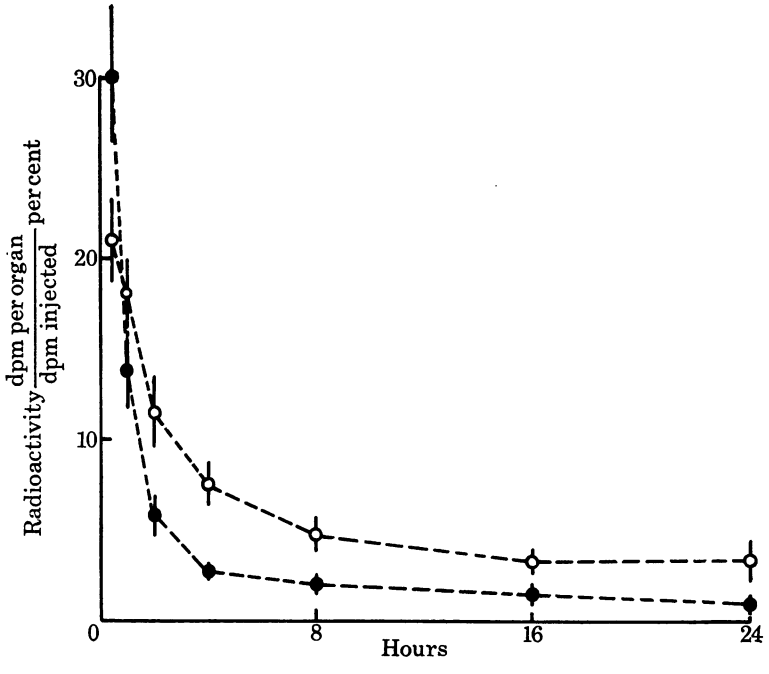


FIG. 1.—Accumulation of radioactivity in liver and lungs of rats injected intravenously with viable syngeneic thoracic duct lymphocytes labelled *in vitro* with [^{3}H]uridine. Abscissa: time (hours) after injection of cells. Ordinate: radioactivity (d/min) in whole organ expressed as a percentage of the injected dose of radioactivity. Each point represents the mean (± s.e. mean) of groups of 5 rats killed between 0·5 and 4 hours and groups of 3 killed between 8 and 24 hours after injection.
○– – –○, liver; ●– – –●, lung.

declined from a maximum of 30% of the injected dose to about 7% 20 hours later (Fig. 2). Autoradiographs showed labelled lymphocytes in the marginal zone at the periphery of the white pulp of the periarteriolar sheaths 30 minutes after injection. After 1 hour labelled cells were seen distributed throughout the white pulp (Fig. 3), whereas 3 hours later they were seen clustered around the central arteriole (Fig. 4).

Lymph nodes.—The time course of increase in concentration of radioactivity was similar in both mesenteric and peripheral lymph nodes. By making an assumption for the total weight of lymph node tissue (Andreasen, 1943) the accumulation of radioactivity in the lymph nodes could be expressed as total radioactivity per lymph node tissue (Fig. 2). After a rapid initial increase there was little further increase for about 4 hours; thereafter the lymph nodes accumulated radioactivity linearly. Autoradiographs showed labelled lymphocytes, initially in association with the specialized endothelium of the post capillary venules and thereafter throughout the paracortical area.

Other tissues.—The time course for the uptake of radioactivity by Peyer's patches was very similar to that for lymph node. In some experiments samples were taken from kidney, muscle (diaphragm), thymus and small gut (excluding Peyer's patches). In one experiment the kidneys accumulated about 1·5% of

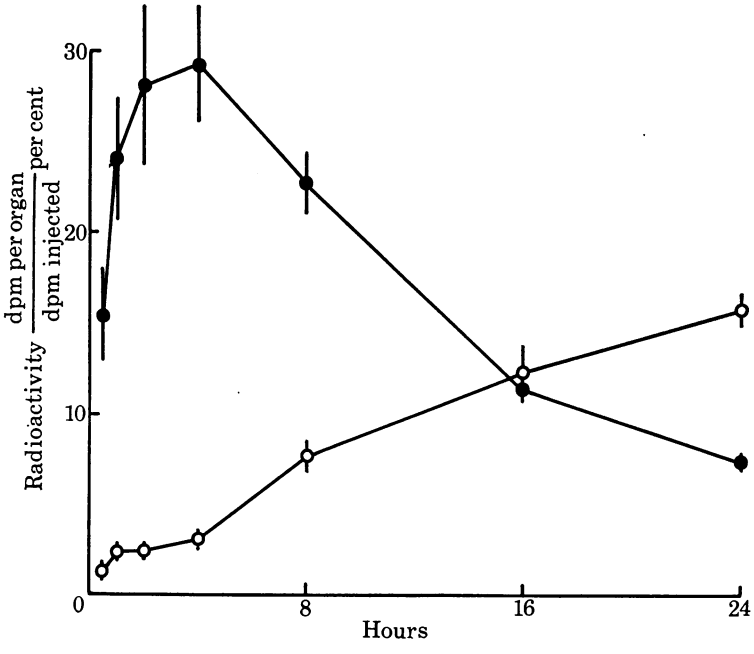


FIG. 2.—Accumulation of radioactivity in spleen and pooled lymph nodes (see text) of rats injected intravenously with viable syngeneic thoracic duct lymphocytes labelled *in vitro* with [^{3}H]uridine. Abscissa: time (hours) after injection of cells. Ordinate: radioactivity (d/min) in whole organs expressed as a percentage of the injected dose of radioactivity. Each point represents the mean (± s.e. mean) of groups of 5–7 rats except for a group of 3 killed 16 hours after injection.

●——●, spleen; ○——○, pooled lymph nodes.

the injected dose of radioactivity after 30 min; apart from this the concentrations were below 1% of the injected dose per g of tissue and usually not significantly above background. Measureable amounts of radioactivity were later recovered in the urine so that its transient appearance in the kidneys may have been associated with the excretion of soluble tritiated uridine.

Injection of [^{3}H]uridine alone

[^{3}H]uridine was injected intravenously into 8 rats in a dose 10 times greater than that normally associated with the injected labelled lymphocytes. Individual rats were killed between 0·5 and 24 hours after injection. Although all tissues accumulated measurable levels of radioactivity none contained more than 2%

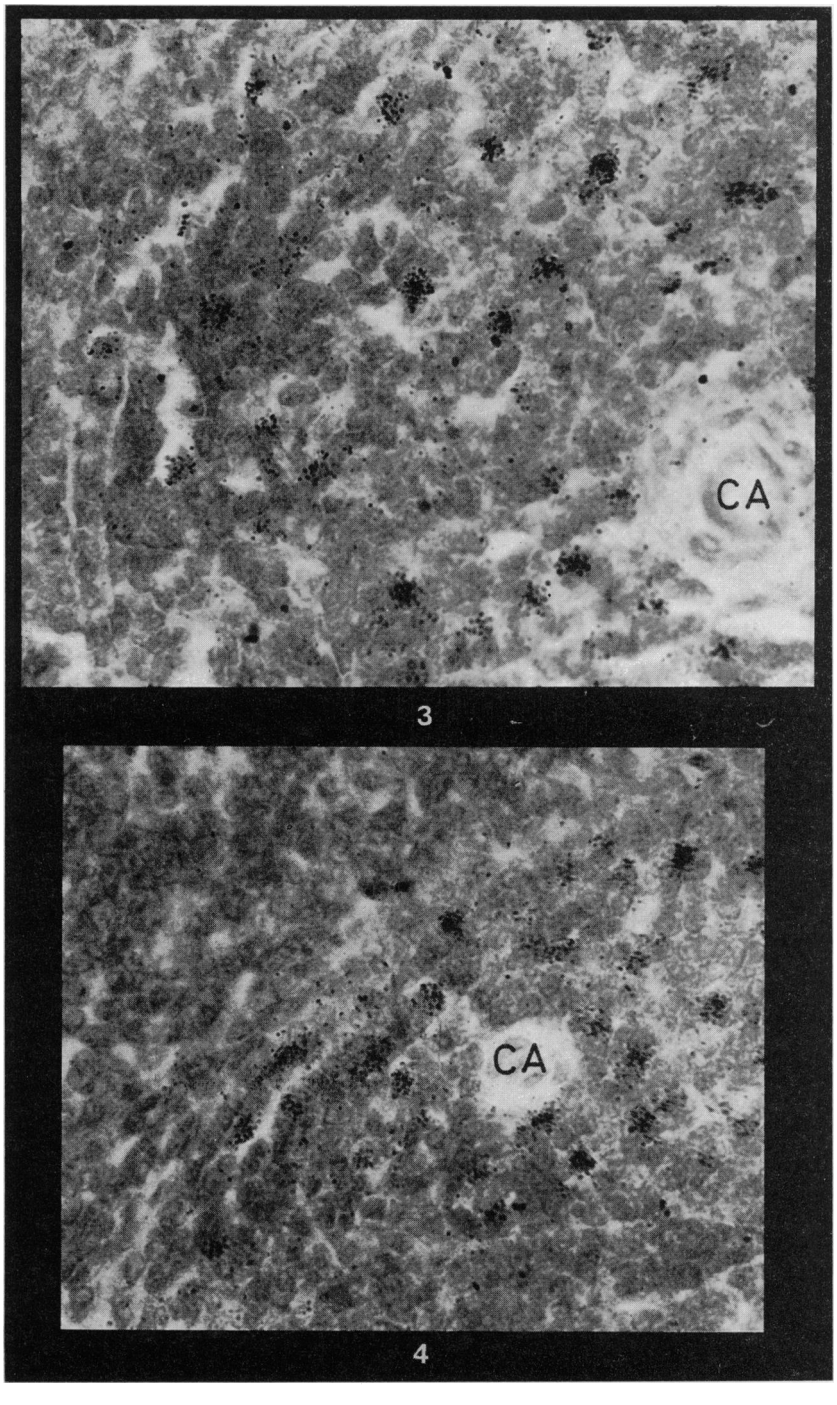
EXPLANATION OF PLATE

Autoradiographs to show the white pulp of spleen of rats injected intravenously with viable syngeneic thoracic duct lymphocytes labelled *in vitro* with [^{3}H]uridine.

C.A. = central arteriole. (Methyl green pyronin × 600.)

FIG. 3.—Spleen from rat killed 1 hour after injection. Labelled cells are distributed throughout the white pulp.

FIG. 4.—Spleen from rat killed 4 hours after injection. Labelled cells are clustered around the central arteriole.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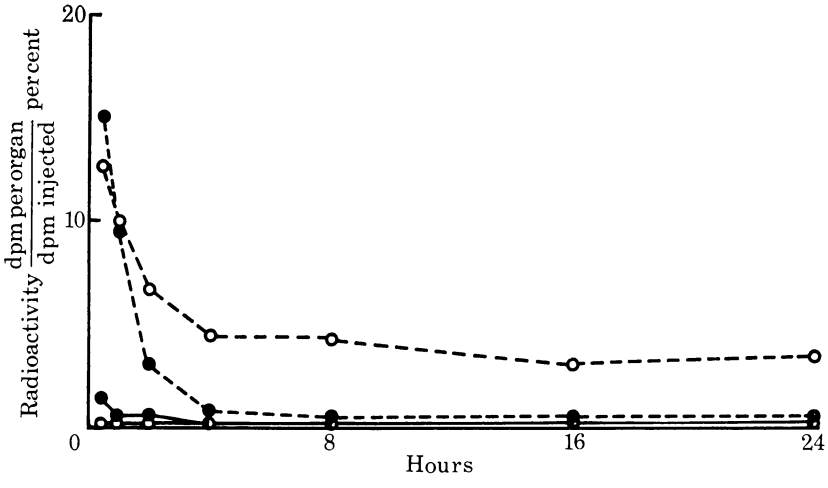

FIG. 5.—Accumulation of radioactivity in tissues of rats injected intravenously with heat killed syngeneic thoracic duct lymphocytes labelled *in vitro* with [^{3}H]uridine. Abscissa: time (hours) after injection of cells. Ordinate: radioactivity (d/min) in whole organ expressed as a percentage of the injected dose of radioactivity.
○- - -○, liver; ●- - -●, lung; ●——●, spleen; ○——○, pooled lymph nodes.

of the injected dose of radioactivity. From this result, and from that of the previous experiment, it was concluded that after the injection of labelled cells the accumulation of radioactivity by any tissue represented the accumulation of labelled lymphocytes in that tissue.

Injection of heat-killed labelled lymphocytes

Thoracic duct lymphocytes were labelled, washed and then heated to 48° for 20 min when all were dead, as indicated by their fixed appearance and lack of motility under phase contrast microscopy. Most cells were morphologically intact and retained 68% of the radioactivity found in cells not so heated. After injection of heat-killed cells into 8 rats, radioactivity accumulated transiently in liver and lungs but there was no subsequent accumulation in spleen, lymph nodes or Peyer's patches (Fig. 5). From this result it was concluded that the accumulation of radioactivity in lymphoid organs after the injection of labelled lymphocytes represented the accumulation of viable cells.

Effect of previous splenectomy on the uptake of viable labelled lymphocytes by lymph nodes

Rats were splenectomized 10 days before receiving an intravenous injection of labelled thoracic duct lymphocytes; control rats underwent no operation; sham operated rats underwent an abdominal operation without splenectomy. The recipients were killed 8 or 24 hours after injection of the cells and their tissues were prepared for scintillation counting. The results are shown in Fig. 6. Eight hours after injection the lymph nodes had accumulated about twice the number of injected lymphocytes in the absence of a spleen than in its presence ($P < 0{\cdot}001$). After 24 hours this ratio had fallen to 1·3 : 1 ($0{\cdot}1 > P > 0{\cdot}05$).

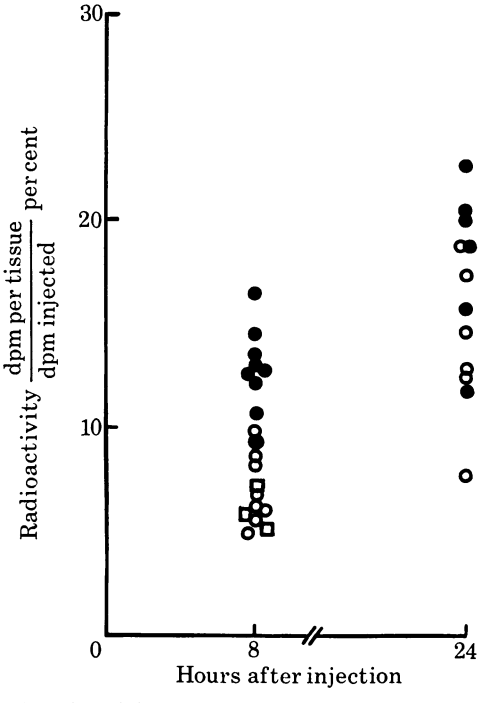

FIG. 6.—Accumulation of radioactivity in lymph nodes of rats with and without spleens injected intravenously with viable syngeneic thoracic duct lymphocytes labelled *in vitro* with [^{3}H]uridine, measured 8 and 24 hours after injection. Radioactivity (d/min) in pooled lymph node tissue is expressed as a percentage of the injected dose of radioactivity. Each point represents the mean of duplicate from one animal.
○, control rats; □, sham operated rats; ● splenectomized.

This indicated that, when present, the spleen reduced the availability of injected lymphocytes to lymph nodes by itself taking them up at a fast rate but, as might be predicted from Fig. 2, this effect was smaller after 24 hours than after 8 hours.

DISCUSSION

When [^{3}H]uridine was used to label rat thoracic duct lymphocytes *in vitro* the pattern of labelling was similar to that found by others (Goldschneider and McGregor, 1968*b*; Ford, 1969b; Kay and Cooper, 1969). This tritium label has made possible concomitant quantitation and localization of injected cells in the organs of recipients. Since B-lymphocytes in rat thoracic duct lymph label comparatively lightly with [^{3}H]uridine (Howard *et al.*, 1972) the techniques used in our experiments probably detected mainly T-lymphocytes.

After intravenous injection labelled lymphocytes were held up transiently in liver and lungs. This phenomenon has been described previously (Weisburger *et al.*, 1951; Gowans and Knight, 1964; Goldschneider and McGregor, 1968*b*; Hall *et al.*, 1972) and probably results from changes in surface properties of the cells due to handling *in vitro*. It does, however, have the implication that the subsequent availability of the cells to lymphoid tissues depends, at least

in part, on the time course of their trapping in liver and lungs. This complicates the interpretation of experiments designed to demonstrate altered ability of injected lymphocytes to home to lymphoid tissues (Gesner and Ginsberg, 1964; Woodruff and Gesner, 1969; Gesner, Woodruff and McCluskey, 1969) because any agent which prevents or delays the uptake of injected lymphocytes into lymphoid tissues can do so either directly by acting on the mechanism of migration or indirectly by preventing or delaying the release of the lymphocytes from liver and lungs.

After the injection of [^{3}H]uridine by itself, or of labelled dead lymphocytes, no radioactivity accumulated in the spleen. Therefore, when radioactivity accumulated there after the injection of living cells, it was concluded to represent accumulation of viable lymphocytes. The uptake of such cells rose to a maximum 4–6 hours after injection and then the radioactivity diminished. Autoradiographs showed that during this time labelled cells accumulated first at the periphery of the periarteriolar sheath, migrated towards the central arteriole and then disappeared. No histological evidence was seen for an exit pathway from white to red pulp (Mitchell, 1973). These observations on the time course of uptake and release of lymphocytes and on their route of migration are similar to the findings of others *in vivo* (Goldschneider and McGregor, 1968*a*, *b*) and in the isolated perfused rat spleen (Ford, 1969b). The results do not indicate whether lymphocytes released from the spleen can re-enter the spleen without first traversing other lymphoid tissues.

During 24 hours after their intravenous injection viable lymphocytes accumulated in lymph nodes at an almost constant rate. The fact that there was no delay in the time course of uptake of injected cells into mesenteric lymph nodes compared with peripheral lymph nodes showed that the cells were presenting themselves to each of these tissues in the same way, and disproved the possibility that cells present to mesenteric lymph nodes in afferent lymph only after traversing the lamina propria of the gut or the Peyer's patches.

More lymphocytes entered lymph nodes in the first 8 hours after injection in the absence of the spleen than in its presence. This showed that many of the injected lymphocytes can enter either of these lymphoid tissues but that when the spleen is present it reduces the supply of injected lymphocytes to the lymph nodes. After 24 hours, however, the absence of a spleen makes little difference to uptake by lymph nodes because, in control animals, many of these lymphocytes have by this time traversed the spleen and entered lymph nodes.

Spleen seeking and lymph node seeking populations of lymphocytes have been distinguished in mice (Zatz and Lance, 1970). Our observations, however, add to the evidence in rats that one population of recirculating lymphocytes can traverse spleen and lymph nodes (Ford, 1969*a*; Roser and Ford, 1972) and conform to the idea that this population migrates through spleen more rapidly than through lymph nodes (Ford and Gowans, 1969).

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