Immunology 1985 **56** 301

Surface markers on lymphocytes leaving pig lymph nodes

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Accepted for publication 6 June 1985

Summary. Mesenteric lymph nodes of normal young pigs were perfused *in vitro* at physiological temperature. Cell-free perfusion medium was pumped into the artery for more than 2·5 hr, and lymphocytes were continuously released into the venous effluent. Recirculating lymphocytes emigrate from pig lymph nodes by entering the blood vasculature directly and not via efferent lymphatics. The presence of lymphocytes in paracortical venular walls after 2 hr of perfusion with new medium suggests that these are the sites of emigration. The rate of emigration of lymphocytes from mesenteric lymph nodes was estimated to be 6×10^7/hr. Study of the lymphocyte populations emerging from the perfused lymph nodes showed that B lymphocytes and E-rosette forming T lymphocytes, but almost no Null lymphocytes, are involved. While the proportion of B lymphocytes remained constant during the perfusion period, E-rosette forming lymphocytes increased significantly. Lymphocyte subpopulations differ profoundly in their capacity to migrate through lymph nodes.

INTRODUCTION

It is generally accepted that lymphocyte recirculation is an essential phenomenon in the immune system, disseminating virgin, effector and memory cells throughout the lymphoid organs and facilitating the co-operation of different subsets of lymphocytes with each other and antigen-presenting cells (for reviews see Ford, 1975; Hopkins & McConnell, 1984). There are at least two levels at which lymphocyte recirculation is controlled: (i) the interaction of lymphocytes with high-walled endothelium in post-capillary venules (high endothelial venules, HEV) in lymph nodes which regulates their entry into lymphatic tissues, and (ii) factors regulating the exit from the lymphoid organ. The migration of lymphocytes across HEV and through the lymph nodes has been the main topic of interest in *in vivo* studies of lymphocyte recirculation (Nieuwenhuis & Ford, 1976; Fossum, Smith & Ford, 1983), and now a monoclonal antibody (MEL14) has been described which seems to be directed against a 'homing' receptor on mouse lymphocytes which enables their adherence to HEV in peripheral lymph nodes (Gallatin, Weissman & Butcher, 1983). The second level of control of lymphocyte recirculation, the exit, has attracted much less interest. Using different techniques of local labelling of lymph nodes, we have shown that lymphocytes leave lymph nodes preferentially via lymphatics in sheep but predominantly via the blood in pigs (Binns, Pabst & Licence, 1985). In this respect, pigs differ from all species studied to date, and sheep are apparently conventional (Ford, 1975). The aims of the present study were to quantify the emigration and to classify the emigrating lymphocytes by surface markers in experiments in which pig mesenteric lymph nodes were perfused *in vitro*, using a modified version of a perfusion system

Abbreviations: DARR, direct antiglobulin rosetting reaction; DS rosettes, rosettes with sheep red blood cells formed in the presence of dextran; HEV, high endothelial venules; SRBC, sheep red blood cells.

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which had been used to demonstrate and quantify lymphocyte release from the pig spleen (Pabst & Trepel, 1975).

MATERIALS AND METHODS

Animals and general surgical procedures

Eight normal young pigs of the German Landrace and Large White breeds were used. Seven animals had a mean weight of 21·7±0·9 (SE) kg and one larger pig weighed ~90 kg. Under general anaesthesia, the abdomen was opened, the mesentery of the terminal ileum between the mesenteric lymph nodes and intestine was carefully ligated near the lymph nodes, the gut was disssected away, the superior mesenteric artery and vein cannulated and the now isolated, cannulated nodes removed from the pig. Thus, only ~20–30% of the mesenteric lymph nodes and the remaining distally ligated mesentery was attached to the cannulas. The lymph nodes were flushed with about 50 ml perfusate at room temperature to wash out most of the red cells, prevent clotting and cool the nodes until perfusion was started.

Perfusion system

The perfusion system was comparable to that used previously for selective perfusion of the spleen (Pabst & Trepel, 1975; Pabst & Binns, 1981; Binns, Pabst & Licence, 1981). In brief, it consisted of a pump with adjustable flow, a membrane oxygenator, a perspex organ chamber and a device to measure the perfusion pressure. A reservoir for perfusion medium, most of the system's silastic tubing and the organ chamber were kept in a 39° water-bath to ensure perfusion of the tissue at body temperature. The perfusate consisted of RPMI-1640 with 35 g/l each of two dextrans of molecular weights 70,000 and 150,000, the latter included to increase further the intravascular colloidal osmotic pressure. An open perfusion system was used in these experiments, so that all venous effluent was collected in tubes. For at least 2·5 hr, fresh perfusate (containing no cells) was pumped into the artery and the cell number in the collected venous determined in 50-ml timed aliquots. Then, the venous return effluent samples were pooled into ~half-hour collections and the lymphocytes characterized by surface markers. At the end of the perfusion, samples of lymph nodes were fixed for histology.

Surface markers

Lymphocytes isolated from the peripheral blood and from the perfusate were used, after washing four times, to identify recirculating subpopulations. Standard techniques were used for sheep red blood cell rosettes (S rosettes) in saline or in the presence of dextran (DS rosettes), revealing the two subsets of rosette-forming T cells (Binns, 1978) which show some differences in behaviour *in vivo* (Binns, 1982). B lymphocytes were identified by the direct antiglobulin rosetting reaction

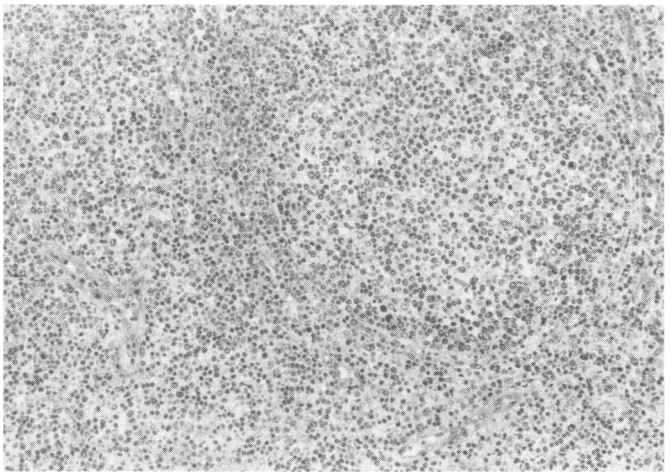

Figure 1. Histological section of a mesenteric lymph node after *in vitro* perfusion for 2·5 hr. A normal germinal centre can be seen. The paracortex with HEVs is less densely populated than normal. (Glycolmethacrylate, 2 μm, Giemsa stain, magnification ×88.)

Table 1. Weight of perfused lymph nodes and absolute number of leucocytes released per minute from the nodes.

Exp. no.	Weight of perfused lymph nodes (g)	Leucocytes released	
		$\times 10^3$/min	$\times 10^3$ per g lymph node/min
1	6·5	90	13·8
2	3·8	110	28·9
3	6·0	70	11·7
4	10·9	700	64·2
5	13·4	580	43·3
6	13·2	250	18·9
7	11·6	160	13·8
Mean ± SE	9·3 ± 1·5	280 ± 96	27·8 ± 7·4

(DARR) (Binns *et al.*, 1979). The rosettes were counted after incubation of the pelleted lymphocytes and indicator cells at 4° overnight, and the proportions of B, T and Null cells calculated in the latter from the formula:

$$\% \text{ Null} = 100 - (\% \text{ DARR} + \% \text{ DS rosettes}).$$

RESULTS

Preservation of the perfused lymph nodes

During the 2·5 hr period of normothermic perfusion, the vascular resistance proved very stable, with perfusion pressures of 80–120 mm Hg. The PO_2, PCO_2 and pH were measured five times in the arterial and venous perfusate. The mean arteriovenous difference for oxygen was 125 ± 2·9 (SE) mm Hg, and the veno-arterial difference for CO_2 was 10·9 ± 1·5 and for the pH 0·24 ± 0·01. The venous PO_2 was always higher than 100 mm Hg. These data document the oxygen consumption and production of carbon dioxide and were used as criteria of viability and normal physiological function. At the end of the perfusion period, the mesentery was edematous but the lymph nodes seemed to be of normal size. In Fig. 1, a histological section of a perfused lymph node is shown, demonstrating the well-preserved structure of all compartments of the node.

Closed-circuit perfusion

In an initial experiment, the lymph nodes were perfused in a closed-circuit system (in contrast to all subsequent 'straight-through' perfusions) and the venous return was not collected. Instead, the lymphocyte numbers in the perfusate were determined by sampling at regular intervals. At 40 min after starting the perfusion, 14×10^3 lymphocytes per ml were found in the perfusate, and by 90 and 120 min the concentration had reached $16{\cdot}5 \times 10^3$/ml and $17{\cdot}8 \times 10^3$/ml, respectively. Thus, from about 1 hr onwards, roughly the same number of lymphocytes were homing into the lymph nodes as were released, demonstrating that these emigrating lymphocytes are recirculating cells. Moreover, these recirculating lymphocytes in the perfusate could only have left the lymph nodes via the vein and not via lymphatics, since only the artery and vein were cannulated.

Number of lymphocytes released

Table 1 shows the absolute number of lymphocytes released from the lymph nodes throughout the perfusion period. A mean of $27{\cdot}8 \pm 7{\cdot}4 \times 10^3$ lymphocytes per g of lymph node emigrated per 1 min over a 2·5 hr period. When these data are used to estimate lymphocyte release per hour for all mesenteric lymph nodes, they give a total of about 6×10^7 lymphocytes. The estimated lymphocyte release in our previous *in vivo* experiments (Binns *et al.*, 1985) was $\sim 10^8$ lymphocytes per hour.

Markers on emigrating lymphocytes

The lymphocyte subpopulations in the peripheral blood and in half-hour samples of the venous effluent are shown in Fig. 2. There are four noteworthy aspects. (i) During collection, the proportion of B cells

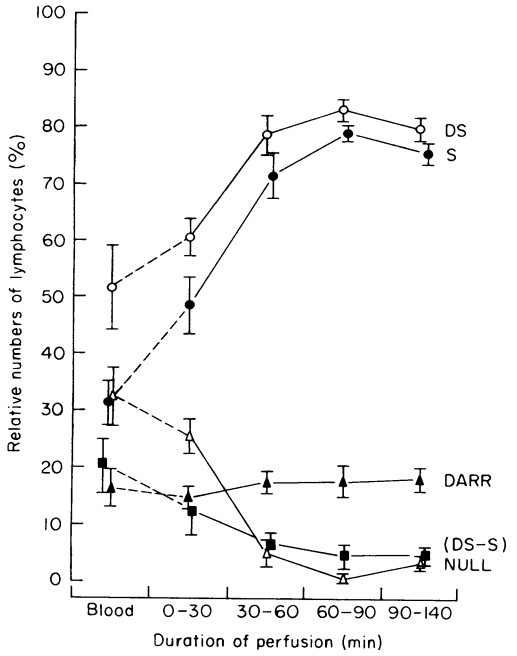

Figure 2. Relative numbers of lymphocyte subpopulations in the blood and in pooled venous samples released from isolated perfused mesenteric lymph nodes (mean ± SE).

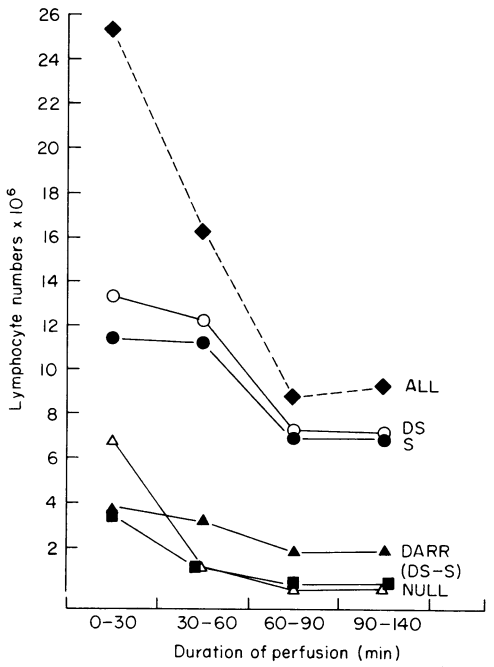

Figure 3. Comparison of the absolute number of all lymphocytes and subpopulations of lymphocytes released via the vein during normothermic perfusion of mesenteric lymph nodes (mean ± SE).

among the emigrating, recirculating lymphocytes remains at a constant level. (ii) In contrast, the proportion of T lymphocytes forming rosettes with SRBC increases dramatically and this is obvious for the subsets revealed by rosette formation both in saline and in dextran. (iii) However, the ratio of strong to total E-rosette forming cells (i.e. S/DS) was 0·62 ± 0·05 in the blood and increased in the half-hour samples as follows: 0–30 min: 0·79 ± 0·07; 30–60 min: 0·91 ± 0·02; 60–90 min: 0·95 ± 0·02; 90–140 min: 0·95 ± 0·02. These figures indicate that from ~30 min onwards, the great majority of weakly rosetting lymphocytes have disappeared and there is hardly any difference between S and DS rosettes. (iv) A striking observation was the rapid disappearance of Null lymphocytes from the population of released lymphocytes. Thus, from 30 min, nearly all lymphocytes are either B or strongly rosette-forming T lymphocytes. Moreover, since the vast majority of lymphocytes in the first half-hour collection were released within the first few minutes, the Null lymphocytes probably emerged during a much shorter period than 30 min.

In Fig. 3, the absolute numbers of all lymphocytes and of the subpopulations are plotted against time of perfusion. There is an obvious drop in the cell output from the first to the second sample, which is almost entirely caused by the rapid drop of the Null and the weakly E-rosette forming (DS-S) lymphocytes. Otherwise, the data are comparable to the proportions shown in Fig. 2.

Histological evidence of the lymphocyte emigration site in the lymph node

Figure 4 shows that lymphocytes were present in the walls of postcapillary venules in the paracortex of lymph nodes even after 2·5 hr of perfusion with clean lymphocyte-free medium, suggesting that these vessels are sites of lymphocyte emigration for the lymph node.

DISCUSSION

Pig lymph nodes have a peculiar structure (for review, see Binns, 1982), but the entry of recirculating lymphocytes is via HEV as in other species and the time

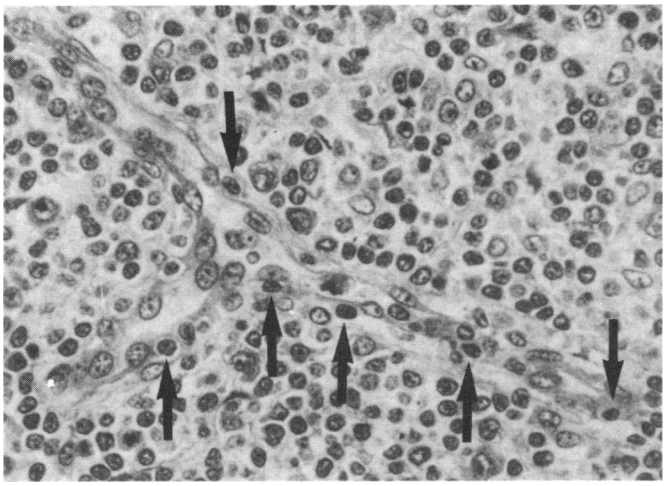

Figure 4. Histological section of a mesenteric lymph node after *in vitro* perfusion for 2·5 hr. The postcapillary venules in the paracortex show lymphocytes (indicated by arrows) in or under the endothelium of their walls. (Glycolmethacrylate, 2 μm, Giemsa stain, magnification $\times$288.)

needed to pass through the HEV of pig lymph nodes (Pabst & Geisler, 1981) is comparable to that for rat lymph nodes (Fossum *et al.*, 1983).

At least two interesting aspects emerged from the present study. Firstly, the experiments demonstrate once more that, in pigs, lymphocytes emigrate from lymph nodes by a direct vascular entry and not via efferent lymphatics, as we have recently shown by other techniques (Binns *et al.*, 1985), and that these are recirculating cells since they can rapidly re-enter into lymph nodes as shown by the closed-circuit experiment. Although cell-free perfusate was pumped into the arterial side of the vascular bed for more than 2 hr, lymphocytes continued to be released into the vein for the whole time of perfusion, in a way reminiscent of similar experiments using pig spleens (Pabst & Trepel, 1975). In contrast, in experiments on isolated perfused mesenteric lymph nodes of the rat, no entry into the venous side was found (Sedgley & Ford, 1976).

The site of direct vascular emigration of recirculating lymphocytes from the pig lymph nodes has not been clear. However, the presence of several lymphocytes in the walls of postcapillary venules in the paracortex after over 2 hr of perfusion with lymphocyte-free perfusate suggests that these vessels are a site of emigration. Thus, labelled lymphocytes introduced intravenously, systemically or locally, cross the PCV wall rapidly and migrate away into the lymphoid tissue in less than 1 hr (Pabst & Geisler, 1981), suggesting that the lymphocytes which were entering the lymph node when it was taken for perfusion should have long since left the vicinity of PCVs. In contrast, those continuing to leave the lymph node during perfusion should continue to cross the endothelium at their site of exit and be visible there. This observation of the apparent site of vascular emigration is consistent with the previous less direct evidence revealed by (i) the presence of lymphocyte plugs in venules in stimulated lymph nodes and (ii) the finding of occasional labelled lymphocytes in venules after infusion of [^{3}H]adenosine-labelled cells in the afferent lymphatic (McFarlin & Binns, 1973).

The second interesting observation concerns the changes in lymphocyte populations released during the perfusion. Pig lymphocytes are well characterized by several surface markers (for review, see Binns, 1982). An interesting population is the lymphocytes bearing neither T- nor B-cell characteristics, the Null cells. These lymphocytes disappear after thymectomy, are non-adherent to nylon wool, and do not seem to be involved in *in vitro* mitogen, antibody or cytotoxic responses (Binns, 1982). The present experiments show that, whereas B and strongly rosetting T lymphocytes are involved in lymphocyte recirculation, Null lymphocytes do not recirculate. The presence of Null cells in the first half-hour collection, most of which emerged in the first 10 min, may be explained by the presence of some residual blood in the vascular bed

of the lymph nodes, or possibly by an intravenously marginal pool of lymphocytes which can be rapidly mobilized. In previous emigration studies on isolated perfused pig spleens, and on lymph nodes and spleens labelled *in vitro*, B and T lymphocytes were also involved but no Null cells were found (Binns *et al.*, 1981, 1985). When human spleens were perfused, the subpopulations released also differed from blood lymphocytes, showing changes in the B:T and T_8:T_4 ratio towards those of the spleen (Reinecke & Pabst, 1983). All these data support the view that recirculation is selective. The shift in the ratio of strong: total E-rosette forming lymphocytes could mean that lymphocytes with a weak affinity for SRBC, which need the presence of dextran in the assay to demonstrate rosette formation, are either immature cells which do not enter lymph nodes, or else they do home to lymph nodes and after a certain residence time they re-emerge as strong rosette-forming cells.

Thus, these studies of the pig lymph node, isolated and perfused *in vitro* at physiological temperature, both confirm the peculiar route of emigration of recirculating lymphocytes in pig lymph nodes and the marked differences in the capacity of different lymphocyte subpopulations to recirculate, and promise to provide a valuable tool for future study of the factors affecting the recirculation behaviour of functional lymphocyte subsets in health and disease.

ACKNOWLEDGMENTS

R. Pabst was supported by the Deutsche Forschungsgemeinschaft (Pa240/3), and R. M. Binns was supported by the European Economic Community Agricultural Division (0172).

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