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Classification of newly formed and migrating splenic lymphocytes

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SUMMARY

Newly formed lymphoid cells in the spleens of normal young pigs were selectively labelled with ^{3}H-TdR, using an extracorporeal perfusion system. One day later splenic and blood lymphocytes were separated by rosetting with sheep red blood cells for classical T cells and by a direct anti-globulin rosetting reaction for B cells. The proportion of newly formed lymphocytes in these subsets was determined by autoradiography. In this study we show that while both B and T lymphocytes are produced, B cells show a much higher incidence of newly formed cells. Labelled populations of spleen derived T and B lymphocytes were also found in the blood, but the B cells showed a lower labelling incidence than in the spleen.

INTRODUCTION

In recent years the heterogeneity of lymphocytes has been demonstrated using an ever increasing number of surface or functional markers. However, the migration and proliferation kinetics of most lymphocyte subsets have not been studied. The role of a single lymphoid organ in lymphocytopoiesis and lymphocyte recirculation can be examined by selective labelling of that organ under physiological conditions. The amount of lymphocyte production (Pabst, Munz & Trepel, 1977) and the fate of the newly formed splenic lymphocytes (Pabst & Nowara, 1982) have been studied previously by use of an extracorporeal perfusion system (Pabst, Reilmann & Neuhaus, 1980). In recent experiments it was demonstrated that blood E rosette forming T and B lymphocytes recirculate within the pig spleen, while the large number of null cells does not (Binns, Pabst & Licence, 1981). The aims of the present study were to classify the lymphocytes which are produced within the pig spleen and to characterize the newly formed lymphocytes which emigrate to other organs. For this purpose the established lymphocyte marker techniques for pig T and B lymphocytes (Binns *et al.*, 1977, 1979), whose interrelationship and biological properties have been studied in some detail (Binns, 1982), were combined with the perfusion technique for selective labelling of the spleen (Pabst *et al.*, 1980). Thus the proliferating lymphocytes were labelled in their normal microenvironment.

MATERIALS AND METHODS

Animals. Five normal young pigs of the Large White Babraham herd (mean age 10·5 weeks,

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mean weight 24 kg) were starved 1 day before the operation but were given free access to food and water after the operation.

Labelling the spleen. The abdomen was opened through a midline incision under halothane general anaesthesia. After cannulating the artery and vein running to the mid-portion of the spleen, the main splenic vessels were clamped and the spleen connected to a simple closed circuit system for extracorporeal normothermic perfusion (Pabst *et al.*, 1980; Pabst & Binns, 1981). The perfusate, consisting of the medium RPMI 1640 supplemented with 3·5g% dextran (mol. wt = ~70,000 daltons) and 2,500 units of heparin, was oxygenated and pumped through the spleen at an arterial pressure of about 140 mm Hg and a flow rate of approximately 130 ml/min. This closed perfusion circuit had a volume of 300 ml. For 15 min 100 μCi tritiated thymidine (^{3}H-TdR, spec. act. 5 μCi/mM, Radiochemical Centre, Amersham, England) was included in the perfusate as a DNA precursor label. After this labelling period, 450–650 ml of fresh, non-radioactive perfusate was pumped through the spleen to wash out the unincorporated label. Finally 0·3 mg adrenaline was introduced intra-arterially with the last of the perfusate to cause the spleen to contract. In this way a spillover of the label into the general circulation was practically avoided, as in previous experiments (Pabst & Nowara, 1982). The clamps on the main splenic vessels were then opened and the normal blood circulation through the spleen was re-established.

Cell preparation. One day later the pigs were anaesthetized, 50–100 ml blood was taken from the splenic vein and defibrinated and the spleen excised. The spleens were flushed with approximately 250 ml phosphate-buffered saline (PBS) to wash out the intravascular leucocytes. A cell suspension was prepared from the whole spleen, the red cells were removed by flash lysis and the suspension washed with PBS. The lymphocytes from the splenic venous blood were prepared as described previously (Binns *et al.*, 1981). In brief, the defibrinated blood was treated with carbonyl iron to remove phagocytic cells, dextran was used to enhance red cell sedimentation and the leucocyte rich supernatant was centrifuged on Ficoll-Triosil. Classical T lymphocytes were detected by their receptors for sheep red blood cells revealed in the presence of dextran (termed DS$^+$ rosette forming cells; Binns *et al.*, 1977) and surface immunoglobulin positive B lymphocytes identified by using the direct anti-globulin rosetting reaction (termed DARR$^+$ cells; Binns *et al.*, 1979). Rosettes were formed in 1·5 ml plastic microcentrifuge tubes by centrifuging 0·5 ml lymphocytes at 10^7/ml either with 1 ml 2% sheep RBC in 6% dextran (150 K daltons mol. wt) or with 0·5 ml 2·5% in PBS donkey RBC coupled with sheep IgG anti-pig IgG by chromic chloride treatment. After overnight incubation the rosettes were gently resuspended and allowed to sediment for 30 min in conical 50 ml Falcon tubes (2070F) at 1 ***g*** and room temperature.

The supernatant (~4·5 ml) contained unrosetted red cells and lymphocytes with few rosettes: these lymphocyte preparations were named DS$^-$ and DARR$^-$ respectively. The pellets (~0·5 ml), comprising mainly rosette forming cells, were termed the DS$^+$ and DARR$^+$ preparations. After resuspension in 50% fetal calf serum, several cytocentrifuge preparations were made of these fractions and smears were made of the cell suspension of blood and spleen lymphocytes. Thus, for splenic blood lymphocytes and for splenic tissue lymphocytes there were five preparations each: the starting cell suspension and the DS$^+$, DS$^-$, DARR$^+$ and DARR$^-$ fractions. The slides were fixed in absolute methanol for 20 min and then processed for routine autoradiography using Kodak AR 10 stripping film. After an exposure time of 100–140 days the slides were developed and stained with Giemsa.

Evaluation. Lymphocytes with more than five grains were counted as labelled, contrasting with a negligible maximum background labelling of two grains per cell. Most lymphocytes had more than 30 grains. In the DS$^+$ and DARR$^+$ preparations only well spread rosettes without red cells lying on top of the lymphocytes were evaluated. Rosette forming cells were defined as having more than five red cells. In the DS$^-$ and DARR$^-$ preparations, only lymphocytes with no red cells in contact were counted. On average more than 2,500 rosette forming lymphocytes or non-rosetting cells were evaluated per preparation.

RESULTS

The histological structure of the perfused spleen was well preserved and no signs of damage to the

endothelium were seen (Fig. 1), as in previous experiments (Pabst & Nowara, 1982; Binns *et al.*, 1981). In preparing and evaluating the rosette preparations care was taken to count only well spread rosettes (Fig. 2), since the intervention of overlying red cells between the lymphocyte and the film can prevent its exposure, because of the very short track length of the emission from ^3H.

In the splenic lymphocyte population which was used to set up the rosetting reaction, a mean of $5{\cdot}9 \pm 3{\cdot}2\%$ was labelled. One animal showed an exceptionally high labelling index of 11·4%. A histological examination of this spleen revealed many lymphoid blast cells and mitotic figures in the

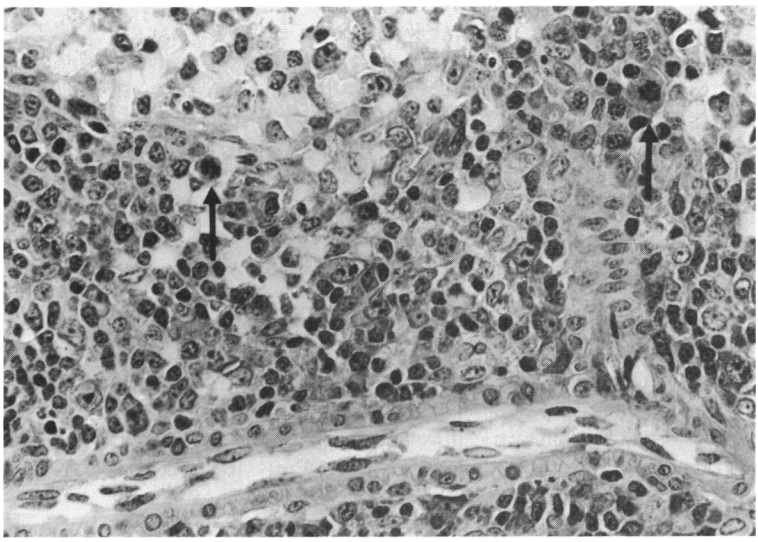

Fig. 1. Histological section of a pig spleen 1 day after selective perfusion with ^3H-TdR containing medium. The splenic structure is well preserved. Two mitoses are indicated by arrows. Methacrylat embedded, 2 μm, Giemsa stain, magnification × 350.

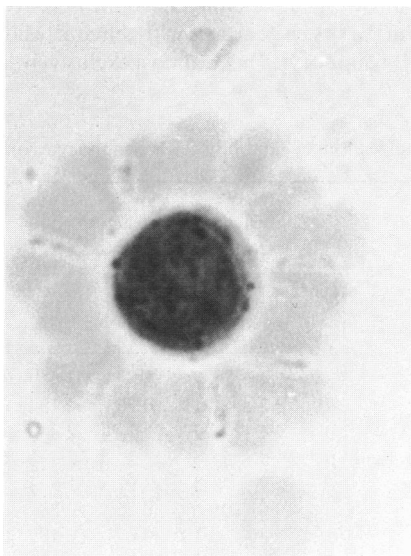

Fig. 2. The radioactively labelled splenic lymphocyte forming a rosette with sheep red blood cells demonstrates that this is a T lymphocyte and was produced in the spleen 1 day previously. Giemsa, autoradiography 140 day exposure time, magnification × 1,100.

white pulp (Fig. 1). The labelling indices for the T and B lymphocyte fractions in the spleen and in the blood are shown in Table 1. While the DS^+ fraction in the spleen had a mean labelling index of only a sixth of that of all spleen cells, the $DARR^+$ lymphocytes on average had a labelling index over two times higher. Thus the cell turnover rate is considerably higher among splenic B cells than T cells. The percentages of labelled lymphocytes in the DS^- and $DARR^-$ fractions demonstrate that some newly formed splenic lymphocytes had neither T nor B cell surface markers. The mean labelling index for spleen derived lymphocytes in the blood was 0·99%. Although the labelling index for $DARR^+$ cells was higher (120%), the difference was far less pronounced than in the spleen. In the splenic cell preparations the B cells had on average a labelling index 15 times higher than the T cells but in the blood only 4·3 times higher. Thus, there appear to be marked differences in the rate of production and release from the spleen of T and B lymphocytes. However, precise comparisons of the B:T labelling index ratios in blood and spleen are difficult in this dynamic *in vivo* situation, since these would have to take into account not only differing rates of cell division but also rates of splenic emigration to the blood and of entry to the recirculating and sessile lymphoreticular tissue pool, as well as cell life span.

DISCUSSION

Previous experiments have shown that only lymphoid cells in the perfused spleen incorporate the radioactive precursor during the labelling period (Pabst & Nowara, 1982). In two experiments, the amount of ^{3}H-TdR which remained in the animal was measured by scintillation counting and found to be only 15–20 μCi, confirming that spillover of radioactivity into other lymphoid organs was avoided by this selective labelling procedure and that all labelled lymphocytes found 1 day after perfusion must have been produced within the spleen. Press, Rosse & Clagett (1977) using mice, demonstrated that the rapidly renewed splenic lymphocytes were roughly divided into 70% B, 20% T and 6% null lymphocytes. In that study the mice were labelled by systemic injection of ^{3}H-TdR. Due to the high rate of migration of newly formed cells from one organ to another the data cannot be interpreted as a classification of lymphocytes produced in the spleen. It was surprising that so many newly formed splenic lymphocytes expressed receptors for sheep red blood cells and had surface immunoglobulin as early as 1 day after their birth. In mouse bone marrow the maturation of newly formed lymphocytes obviously takes longer (for review see Osmond, 1980). The local production of B lymphocytes in the spleen contrasts with the conclusion of Rosse *et al.* (1978) that in guinea-pigs rapidly renewed B lymphocytes have almost exclusively been recently generated in the bone marrow.

Table 1. Labelling indices of whole spleen (S) and blood (B) lymphocytes and their fractions from five pigs

	% labelled cells in fraction from pig number					
Cell fraction	R 15	R 18	R 21	R 25	R 26	
S	3·6	11·4	3·7	5·7	5·0	5·9±3·2
DS^+	1·7	2·2	0·13	0·58	0·18	0·96±0·94
DS^-	7·0	2·1	0·7	3·1	2·4	3·0±2·4
$DARR^+$	14·0	14·0	18·8	13·3	12·5	14·5±2·5
$DARR^-$	1·4	2·9	1·3	3·9	1·3	2·2±1·2
B	0·5	3·0	0·6	0·56	0·28	0·99±1·1
DS^+	0·19	0·76	0·18	0·07	0·18	0·28±0·28
DS^-	0·3	0·80	0·36	0·62	0·23	0·46±0·24
$DARR^+$	0·7	3·4	0·47	1·44	0·05	1·2±1·3
$DARR^-$	0·16	1·5	0·37	0·7	0·035	0·55±0·59

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