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Lymphocyte migration in the spleen: the effect of macrophage elimination

G. KRAAL, H. RODRIGUES, K. HOEBEN & N. VAN ROOIJEN *Department of Cell Biology and Immunology, Free University, Amsterdam, The Netherlands*

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SUMMARY

To study the influence of macrophages on the migration and distribution of lymphocytes in the spleen, macrophages were eliminated from the spleen of mice by injection of liposomes in which DMDP was encapsulated. This leads to an elimination of macrophages in both the red pulp and marginal zone of the spleen within 1–2 days. In these animals the distribution of lymphocytes was determined by transfer of either syngeneic fluoresceinated or Ly 5 congeneic cells. It was found that after elimination of the macrophages the number of lymphocytes immigrating into the spleen had decreased, although a comparable mode of compartimentalization was found with an initial localization in the marginal zone and a subsequent distribution into the white pulp. After this elimination spleen macrophage subsets return with different kinetics, and in this way the influence of the red pulp macrophages, the marginal zone macrophages and the marginal metallophilic macrophages on lymphocyte immigration and redistribution could be investigated. A quantitative decrease of immigration was still found when red pulp and marginal metallophilic macrophages had repopulated their compartments, but was only fully restored when the last population to repopulate the spleen after treatment with DMDP-liposomes, the marginal zone macrophages, had returned. Experiments with isolated T and B cells showed that the elimination of macrophages had a profound effect on the localization of B cells in the white pulp, whereas it hardly affected T cells.

INTRODUCTION

The spleen functions as an extensively branched arterio-venous filter positioned in the bloodstream and as a consequence a very large number of blood-borne lymphocytes will continuously pass this organ per day. Calculations using radiolabelled cells showed that in the rat the exchange rate of small lymphocytes from the blood into the spleen is in the order of 8×10^7 lymphocytes per hour (Ford, 1968).

Lymphocytes that enter the spleen will leave the bloodstream in the marginal zone where small arterioles end in sinuses. The majority of the lymphocytes will be carried along by the bloodstream into the red pulp and will eventually leave the spleen by the venous blood stream. Yet another part of the cells will actively migrate from the marginal zone into the compartments of the white pulp. Calculations on the transit time and the percentage of cells redistributing between red pulp and white pulp showed that 90% of incoming cells will end up in the red pulp, with a short transit time of 5 min, and 10% of the cells will migrate into the white pulp, with an average stay of 4–5 hr (Ford, 1968, 1969; Hammond, 1975). However, it is not known what mechanisms make the lymphocytes move one way or the other. From the multiple data on lymphocyte migration in the spleen it is concluded that all recirculating lymphocytes redistribute in the spleen in similar fashions, irrespective of their source (Zatz & Lance, 1970; Smith & Ford, 1983). The differential distribution of blood-borne lymphocytes in the spleen may therefore be the result of differences in motility or may be based on the accidental position a cell will occupy after leaving the arterial bloodstream into the marginal sinus with a more slowly flowing bloodstream. Studies on the localization of lymphocytes in the spleen have mainly focused on the white pulp (Pabst & Geisler, 1981), and it is not known whether the lymphocytes that are present in the red pulp are merely passing through or if specific retention can occur in the splenic cords. The routes lymphocytes follow from the marginal zone into their respective white pulp compartments have been demonstrated in various studies using autoradiography after transfer of radiolabelled cells (Ford, 1968; Nieuwenhuis & Ford, 1976; Pabst, 1988). Both B and T lymphocytes were found accumulating in the marginal zone as early as 15 min after transfer. Using enriched T-cell fractions a discrimination could even be made between localization in the outer and inner part of the marginal zone (Brelinska & Pilgrim, 1983). After this initial localization B cells were found to concentrate in the outer parts of the periarteriolar lymphocyte sheath (PALS), moved towards the bases of the coronas and were located in the coronas by 18 hr after cell transfer (Nieuwenhuis & Ford, 1976; Van Ewijk & Nieuwenhuis, 1985). T cells, on the other hand, rapidly moved from the

Abbreviations: DMDP, dichlorodimethylene-diphosphonate; PALS, periarteriolar lymphocyte sheath.

Correspondence: Dr G. Kraal, Dept. of Histology, Free University, P.O. Box 7161, 1007MC Amsterdam, The Netherlands.

marginal zone into the central region of the PALS and could be detected there within 1 hr. The accumulation of lymphocytes in the marginal zone after cell transfer and the presence of many macrophages in this area have prompted several investigators to suggest a role of these macrophages in directing the migration of lymphocytes (Freitas & De Sousa, 1976; Brelinska, Pilgrim & Reisert, 1984).

Recently a method has been described to eliminate macrophages in the spleen by a single injection of liposomes in which dichlorodimethylene-diphosphonate (DMDP) is encapsulated (Van Rooijen & Van Nieuwmegen, 1984). After elimination, the various macrophage subpopulations show striking differences in their mode of reappearance (Van Rooijen, Kors & Kraal, 1989). At given times spleens can be found that contain no macrophages (except for those in the white pulp), red pulp macrophages only, or a mixture of red pulp macrophages and marginal metallophilic macrophages. Eventually, the marginal zone macrophages also reappear. Using this model we studied the kinetics of lymphocyte migration in the spleen to see whether the presence of macrophages and macrophage subsets in particular would influence the retention of lymphocytes and the migration into the white pulp.

MATERIALS AND METHODS

Animals

C57Bl/6(Ly 5.2) mice were purchased from Bomholtgard (Ry, Denmark). C57Bl/6(Ly 5.1) mice were a gift of Dr E.A. Boyse (Sloan Kettering Cancer Center, New York, NY) and were bred and maintained in our facilities.

Preparation of liposomes with entrapped DMDP

Multilamellar liposomes were prepared as described earlier (Van Rooijen & Claassen, 1988). In brief, 75 mg phosphatidylcholine and 11 mg cholesterol (Sigma Chemical Co., St Louis, MO) were dissolved in chloroform in a round-bottomed flask. The thin film that formed on the interior of the flask after low vacuum rotary evaporation at 37° was dispersed by gentle rotation for 10 min in 10 ml phosphate-buffered saline (PBS; 0·15 M NaCl: 10 mM phosphate buffer, pH 7·4). For DMDP-containing liposomes, 1·89 g DMDP were added to 10 ml PBS. More DMDP could not be dissolved in the buffer. DMDP was a kind gift of Procter and Gamble, U.S.A. In order to wash the liposomes, these were centrifuged twice in PBS at 100,000 *g* for 30 min to remove free non-entrapped DMDP. The amount of liposome-entrapped DMDP was determined as described earlier (Claassen & Van Rooijen, 1986). After washing of the liposomes, these were resuspended in 4 ml PBS and 0·2 ml (containing about 2 mg of liposome entrapped DMDP) was injected intravenously in mice at indicated time-points.

Immunohistochemistry of macrophages and lymphoid cells in the spleen

Blocks of fresh splenic tissue were frozen in liquid nitrogen and stored at −20°. Cryostat sections of 8–10 μm thickness were fixed in acetone for 10 min and air-dried for at least 30 min. After washing in 0·01 M PBS (pH 7·4) the sections were incubated with culture supernatants of various monoclonal rat anti-mouse antibodies at saturating concentrations in PBS containing 0·1% bovine serum albumin (BSA; Poviet, Tilburg) for 30 min. After washing thoroughly in PBS, the slides were incubated with a 1:100 dilution of peroxidase-conjugated rabbit anti-rat IgG (Dako, Glostrup, Denmark) in PBS/BSA containing 1% normal mouse serum for 30 min. After washing in PBS again, the peroxidase activity was visualized with 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Sigma) in 0·5 mg/ml Tris-HCl buffer (pH 7·6), containing 0·01% H_2O_2. The sections were stained for 10–15 min at room temperature. Control slides were incubated in the same way, omitting the first step.

Acid phosphatase activity in the sections of spleen tissue was demonstrated as described by Eikelenboom (1978).

Quantification of immigrating cells

C57Bl/6(Ly 5.2) mice were injected with C57Bl/6(Ly 5.1) spleen cells (25×10^6 per animal) and at given time-points the animals were killed and their spleens were frozen in liquid nitrogen. Using a cryostat, approximately every tenth section was picked up and stained for the presence of donor allotype cells. Using a MOP-Videoplan (Kontron) with integrated XY tablet, the surface area of either white pulp areas (periarteriolar lymphocyte sheath and follicles) or marginal zone was determined, as well as the number of donor lymphocytes in that given area. This was done for at least 10 representative areas per spleen per animal. Thereafter the number of donor cells per unit area white pulp or marginal zone of each section was determined.

Monoclonal antibodies

The following monoclonal antibodies were used: MOMA-2 (Kraal, Rep & Janse, 1987) was used to stain all macrophages in the spleen. Marginal metallophilic macrophages were specifically stained by the monoclonal antibody MOMA-1 (Kraal & Janse, 1986). Marginal zone macrophages were specifically stained by the monoclonal antibody ERTR9 (Dijkstra *et al.*, 1985). Anti-Ly 5.1 (clone A-20) and anti-Ly 5.2 (clone 104-2) antibodies were a kind gift of Dr E. A. Boyse, Memorial Sloan Kettering Cancer Institute, New York, NY. They were biotinylated as described previously (Kraal *et al.*, 1986) and used in combination with peroxidase-conjugated avidin.

RESULTS

To find out whether the presence of macrophages would influence the passage of lymphocytes through the spleen, animals were treated with DMDP-liposomes and 48 hr later, when virtually all macrophages in the spleens of these mice have been eliminated, syngeneic FITC-labelled lymphocytes were injected intravenously. Determination of the percentage of labelled cells by FACS-analysis showed that at the various time-points after injection of the cells, fewer labelled donor cells could be demonstrated in the spleens of DMDP-treated animals than in those of control animals (Fig. 1). That these differences were not induced by variation in injected cell numbers was shown by determining the percentage of donor lymphocytes in the lymph nodes of these animals. Elimination of splenic macrophages by DMDP liposomes does not affect the macrophages in lymph nodes because the intravenously injected liposomes are not able to pass endothelial cells. The number of cells entering the lymph nodes after transfer was therefore used as an internal control to measure the variation of injected lymphocytes per animal, and from Fig. 1 it can be seen that this variation is very small.

From these results it could not be deduced whether the depletion of the macrophages from the spleen had an effect on

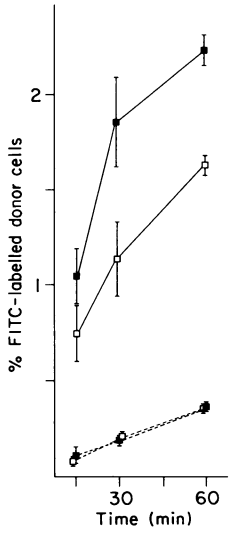

Figure 1. Animals were treated with DMDP-liposomes and 48 hr later they were injected with syngeneic FITC-labelled spleen cells. After 15, 30 and 60 min spleens and lymph nodes were removed and suspended and the percentage of labelled cells in the organs was determined by FACS analysis (FACSstar, Becton-Dickinson) on 10,000 cells. The results are expressed as the mean (±SD) of four animals. Closed squares represent control animals and open squares are animals that were treated with DMDP liposomes. The solid lines represent the percentages of cells found in the spleens of these animals whereas the broken lines represent the data found in the lymph nodes.

the localization of lymphocytes in red pulp or white pulp or both. To be able to determine the precise localization of lymphocytes during their passage through or into the various splenic compartments, we used the Ly 5 congenic mouse strain combination. This way by using immunohistochemistry, donor lymphocytes could be located on the base of their specific staining and their numbers in the various compartments of the spleen were determined. At 15, 30 and 60 min after cell transfer the numbers of donor cells in marginal zone and white pulp were determined. This was performed on various days after elimination of macrophages by DMDP-liposomes to be able to study the influence of the differential return of the macrophage subsets. The depletion of the macrophages, as well as the return of the subsets in time, was checked in each animal using staining for acid phosphatase in combination with specific monoclonal antibodies. In concordance with data from the literature an initial localization of transferred cells was found in the marginal zone, whereafter the number of cells in the white pulp area gradually increased (Fig. 2). In these determinations no discrimination was made between localization in follicles or PALS and all cells that had clearly left the marginal zone were scored as present in the white pulp. Comparing the DMDP-animals with intact control animals it was evident that, as already observed in the overall quantification of immigrant cells by FACS analysis, the total number of cells was lower in the animals that had been treated with liposomes and this was clear for both compartments investigated (Figs 3 & 4). Only at 84 days after treatment, when the macrophage populations in the spleen are histologically normal and even the marginal zone macrophages have fully regained their positions, were no differences observed between control and DMDP-liposome-treated animals.

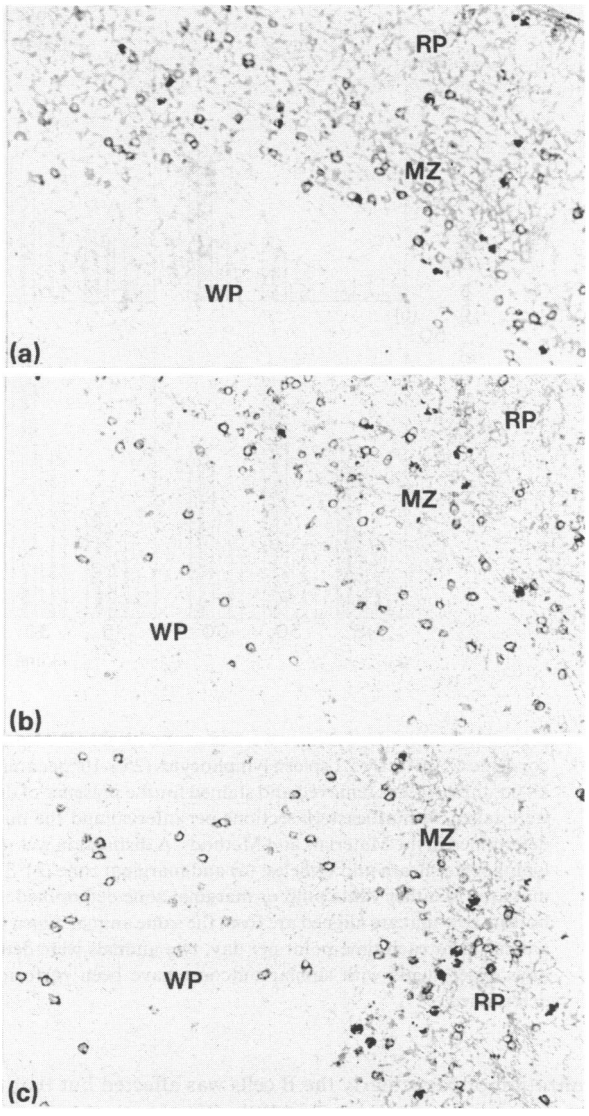

Figure 2. C57Bl/6.Ly 5.2 mice were treated with DMDP-liposomes and at 48 hr after treatment the animals were injected with congenic C57Bl/6.Ly 5.1 spleen cells. At 15, 30 and 60 min after cell transfer spleen sections were stained for the presence of donor type lymphocytes. (a) A section of the spleen is shown at 15 min after transfer. The lymphocytes are predominantly found in the marginal zone and start to appear in the red pulp. (b) At 30 min after transfer, cells start to leave the marginal zone for the outer PALS area of the white pulp, and (c) 60 min after transfer numerous cells can be found in the PALS of the spleen. RP, red pulp; MZ, marginal zone; WP, white pulp; Magnification 14× objective.

Since in these studies transfers were performed with unseparated splenic lymphocyte suspensions, we wished to determine whether macrophage elimination would equally affect the localization and retention of T and B cells. Therefore, Ly 5.2 spleen suspensions were separated in either T cells by nylon-wool purification, or B cells by complement lysis of T cells, and these enriched populations were injected into Ly 5.1 control or liposome-treated animals. When the number of allogeneic donor cells was determined in these animals it was clear that the

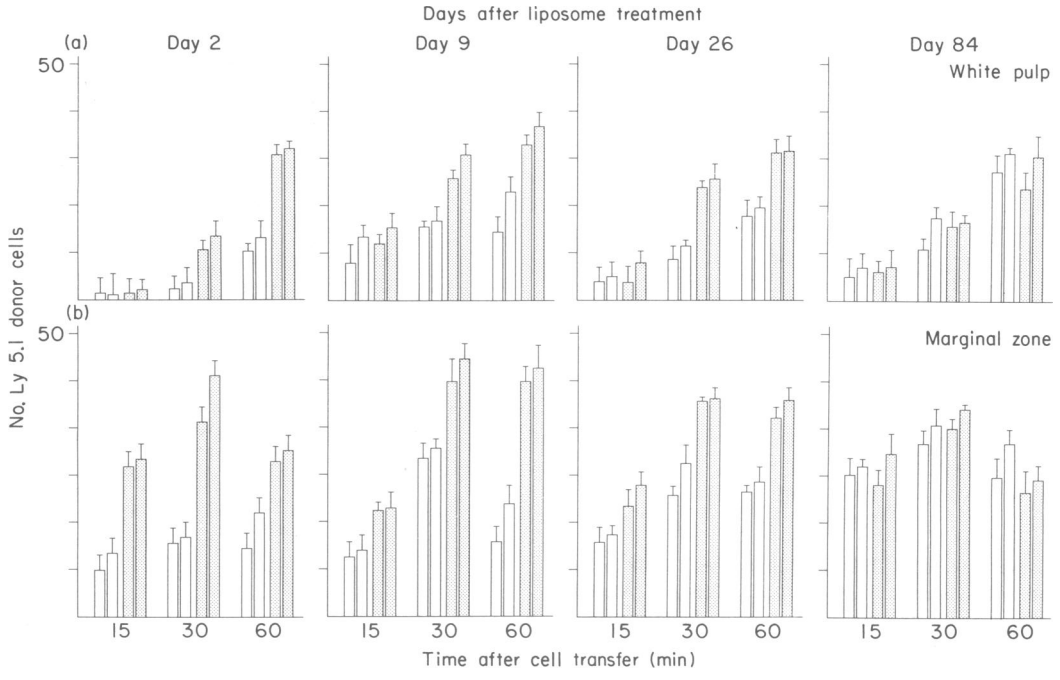

Figure 3. C57Bl/6.Ly 5.2 mice were treated with DMDP-liposomes and at various days after treatment the animals were injected with congenic C57B16.Ly 5.1 spleen lymphocytes (25×10^6 per animal). At short intervals after injection (15, 30 and 60 min) the animals were killed, their spleens removed and stained for the presence of donor type lymphocytes. From each animal sections throughout the spleen were taken with at least 10 sections per interval and the number of lymphocytes present in a representative area of the spleen was determined in the Materials and Methods. A distinction was made between the presence of lymphocytes in the white pulp (periarteriolar lymphocyte sheath and follicles; (a) and marginal zone (b). The results are represented as the mean ($\pm$SD) number of donor cells per unit area of either white pulp or marginal zone determined in at least 10 sections of an individual animal. The bars in the upper and bottom row that are aligned are from the same animal. Open bars represent DMDP-liposomes-treated animals, dotted bars are control animals. For each time-point per day, two animals were determined. The data are from representative experiments. At intermediate days experiments with similar outcome have been performed but were omitted from the graph because fewer time-points were determined.

localization of particularly the B cells was affected but that the elimination of macrophages had little effect on the localization of T cells in the marginal zone and white pulp (Fig. 4).

DISCUSSION

One of the effects of the elimination of the macrophages in the spleen after treatment with DMDP-liposomes could be a change in blood flow in this organ, especially due to the relatively empty venous sinuses in the red pulp. This may directly influence the retention of the cells and can therefore explain the differences in number of lymphocytes found in treated versus control animals, in particular in the short intervals after transfer (15–60 min) as tested in our experiments. Also at longer intervals, such as 2 and 24 hr after cell transfer, differences between the two groups were evident (because these time-points were not determined at all days after DMDP treatment, the results are not included in the graphs). However, 9 days after liposome treatment the majority of macrophages, namely all those in the red pulp, have returned to their positions. From this time-point on, it is hard to assume that the bloodflow will still be very different and yet the kinetics of the transferred cells in treated animals are still different from those of the control group. In fact, the patterns show similar differences up until the last time-point of 84 days, where both groups are comparable. Furthermore, the fact that B-cell localization in particular is affected and T cells enter the white pulp in comparable fashions also points to a specific macrophage–lymphocyte interaction rather than a haemodynamic phenomenon. It cannot be ruled out that B cells, more than T cells, are affected by free DMDP released from liposomes or dying macrophages, influencing their recirculatory behaviour. So far we have found no evidence that free DMDP affects lymphocytes but if this would be the case it could only occur early after injection, because the amount of liposomes administered is titered to be optimal and is no overdose. All liposomes are therefore cleared very rapidly from the bloodstream, as can also be deduced from the repopulation of the red pulp macrophages seen after 8–10 days. Furthermore, it has been demonstrated that free DMDP has a very short half-life in serum of only several minutes (Fleisch, 1988). We therefore assume that the presence of macrophages in the marginal zone is in some way involved in the migration of B lymphocytes into white pulp. Two likely candidates are to be found: (i) the marginal zone macrophages, dispersed throughout the marginal zone, are large cells with long extensions showing intimate contacts with surrounding B cells and associated with the immune response against T-independent type 2 antigens (Humphrey & Grennan, 1981; Dijkstra *et al.*, 1985), and (ii) the

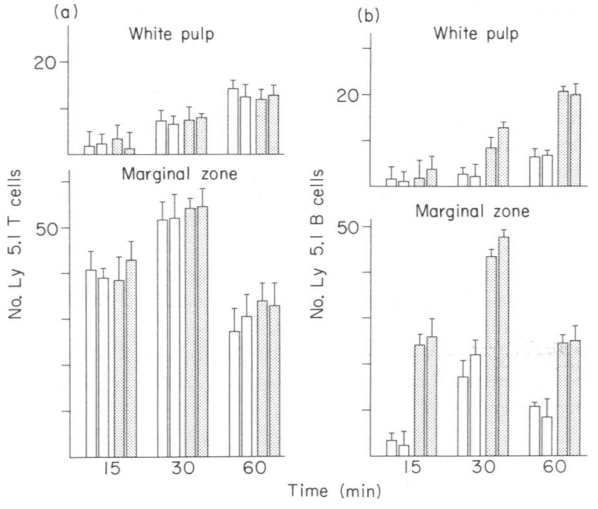

Figure 4. C57Bl/6.Ly 5.2 mice were treated with DMDP-liposomes and at 48 hr after treatment they were injected with either purified T cells (a) or purified B cells (b) from C57Bl/6.Ly 5.1 mice at 25×10^6 lymphocytes per animal. At 15, 30 and 60 min after cell transfer the animals were killed and the number of donor type cells in the three compartments of the spleen was determined as described in Fig. 3. Open bars represent DMDP-liposome-treated animals, dotted bars are control animals. T cells were purified from spleen cells suspensions by nylon-wool adherence or double-passage through a Sephadex G-25 column. Their purity in the experiments was over 95%. B cells were purified from spleen cells suspensions by a double-step complement lysis in which cells were first incubated with cytotoxic anti-Thy-1 monoclonal antibody (clone 30-H-12), washed and thereafter incubated at 37° in 10% guinea-pig serum. After washing twice through serum the purity of the B cells was found to be over 90%.

marginal metallophilic macrophages, at the border of marginal zone and white pulp, with extensions suggestively directed into the white pulp. Although their function is still largely speculative (Snook, 1964; Eikelenboom, 1978), these macrophages may be involved in antigen presentation (Kraal, Janse & Claassen, 1988).

Both types of macrophages are completely eliminated after a single injection of DMDP-liposomes in the mouse (Van Rooijen *et al.*, 1989). At the end of the second week the marginal metallophilic macrophages start to repopulate their specific location as judged by their expression of MOMA-1 antigen and non-specific esterase content. Therefore, in our experiments animals at Day 26 after elimination have normal populations of this subset of macrophages but still show impaired retention and migration of lymphocytes, as determined in the cell transfer system, indicating that this cell type is not directly involved in the trafficking of lymphocytes. Isolated T- and B-cell populations have not been tested at these later time-points, but we feel that since at the early time-point, B-cell localization in particular was affected and the spleen cell suspensions used for these transfers predominantly consist of B cells (at least 65%), the findings at the later time-points reflect the behaviour of B cells in particular.

The marginal zone macrophages are still completely absent at 26 days, and these cells will only start to reappear around 4–5 weeks after elimination. Their complete recovery is a very slow process, but at 84 days as tested in our experiments the spleens are again normal with respect to their macrophage subsets, and at this time-point the migration-kinetics of transferred cells are also comparable to control animals. This is a strong indication that the marginal zone macrophages form an important microenvironment for B cells, in accordance with the close associations described between these macrophages and B cells (Humphrey & Grennan, 1981) and the presence of particular B cells (Kumamaratne, Bazin & MacLennan, 1981) and B-memory cells (Liu, Oldfield & MacLennan, 1988) in the marginal zone area. Although we have previously demonstrated a role for C3b receptors on B cells in migration (Kraal *et al.*, 1985) the precise mode of interaction of B cells and macrophages in this area is still unclear and awaits further investigation.

REFERENCES

Brelinska R. & Pilgrim C. (1983) Macrophages and interdigitating cells; their relationship to migrating lymphocytes in the white pulp of rat spleen. *Cell Tissue Res.* **233,** 671.

Brelinska R., Pilgrim C. & Reisert I. (1984) Pathways of lymphocyte migration within the periarterial lymphoid sheath of rat spleen. *Cell Tissue Res.* **236,** 661.

Claassen E. & Van Rooijen N. (1986) Preparation and characteristics of dichloromethylene diphosphonate-containing liposomes. *J. Microencapsulation*, **3,** 109.

Dijkstra C.D., Van Vliet E., Dopp E.A., Van der Lely A.A. & Kraal G. (1985) Marginal zone macrophages identified by a monoclonal antibody: characterization of immuno- and enzyme-histochemical properties and functional capacities. *Immunology*, **55,** 23.

Eikelenboom P. (1978) Characterization of non-lymphoid cells in the white pulp of the mouse spleen: an *in vivo* and *in vitro* study. *Cell Tissue Res.* **195,** 445.

Fleisch H. (1988) Biphosphonates: a new class of drugs in diseases of bone and calcium metabolism. *Exp. Pharmacol.* V **83,** 441.

Ford W.L. (1968) The mechanism of lymphopenia produced by chronic irradiation of the rat spleen. *Br. J. Exp. Pathol.* **49,** 502.

Ford W.L. (1969) The kinetics of lymphocyte recirculation within the rat spleen. *Cell Tissue Kinet.* **2,** 171.

Freitas A.A. & De Sousa M. (1976) The role of cell interactions in control of lymphocyte traffic. *Cell. Immunol.* **22,** 345.

Hammond B.J. (1975) A compartmental analysis of circulatory lymphocytes in the spleen. *Cell Tissue Kinet.* **8,** 153.

Humphrey J.H. & Grennan D.G. (1981) Different macrophage populations distinguished by means of fluorescent polysaccharides. Recognition and properties of marginal zone macrophages. *Eur. J. Immunol.* **11,** 221.

Kraal G., Hardy R.R., Gallatin W.M., Weissman I.L. & Butcher E.C. (1986) Antigen induced changes in B cell subsets in lymph nodes: analysis by dual fluorescence cytofluorometry *Eur. J. Immunol.* **16,** 829.

Kraal G. & Janse M. (1986) Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. *Immunology*, **58,** 665.

Kraal G., Janse M. & Claassen E. (1988) Marginal metallophilic macrophages in the mouse spleen: effects of neonatal injections of MOMA-1 antibody on the humoral immune response. *Immunol. Lett.* **17,** 139.

Kraal G., Rep M. & Janse M. (1987) Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2: an immunohistochemical study. *Scand. J. Immunol.* **26,** 653.

Kraal G., Van Hoogstaten I., Klerx J.P.A.M. & van Dijk H. (1985) The selective localization of B lymphocytes in the spleen and the role of complement receptors *Eur. J. Immunol.* **15,** 681.

Kumamaratne D.S., Bazin H. & MacLennan I.C.M. (1981) Marginal zones, the major B cell compartment of rat spleens *Eur. J. Immunol.* **11,** 858.

LIU Y.-J., OLDFIELD S. & MACLENNAN I.C.M. (1988) Memory B cells in T cell-dependent antibody responses colonize the splenic marginal zone *Eur. J. Immunol.* **18,** 355.

NIEUWENHUIS P. & FORD W.L. (1976) Comparative migration of B and T lymphocytes in the rat spleen and lymph nodes. *Cell. Immunol.* **23,** 254.

PABST R. (1988) The spleen in lymphocyte migration. *Immunol. Today*, **9,** 43.

PABST R. & GEISLER R. (1981) The route of migration of lymphocytes from blood to spleen and mesenteric lymph nodes in the pig. *Cell Tissue Res.* **221,** 361.

SMITH M.E. & FORD W.L. (1983) The recirculating lymphocyte pool of the rat: a systemic description of the migratory behaviour of recirculating lymphocytes. *Immunology*, **49,** 83.

SNOOK T. (1964) Studies on the perifollicular region of the rat's spleen. *Anat. Rec.* **148,** 149.

VAN EWIJK W. & NIEUWENHUIS P. (1985) Compartments, domains and migration pathways of lymphoid cells in the splenic pulp. *Experientia*, **41,** 199.

VAN ROOIJEN N. & CLAASSEN E. (1988) *In vivo* elimination of macrophages in spleen and liver, using liposome-encapsulated drugs: methods and applications. In: *Liposomes as Drug Carriers* (ed. G. Gregoriadis), p. 131. John Wiley and Sons, Chichester, U.K.

VAN ROOIJEN N., KORS N. & KRAAL G. (1989) Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination *J. Leukocyte Biol.* **45,** 97.

VAN ROOIJEN N. & VAN NIEUWMEGEN R. (1984) Elimination of phagocytic cells in the spleen after intravenous injection of liposome-encapsulated dichloromethylene diphosphonate. An enzyme-histochemical study. *Cell Tissue Res.* **238,** 355.

ZATZ M.M. & LANCE E.M. (1970) The distribution of chromium-51 labeled lymphoid cells in the mouse. A survey of anatomical compartments. *Cell. Immunol.* **1,** 3.