Immunology 1987 **62** 273–277

Lymphocyte migration into the lamina propria of the gut is mediated by specialized HEV-like blood vessels

S. H. M. JEURISSEN, A. M. DUIJVESTIJN,* Y. SONTAG & G. KRAAL *Department of Histology, Medical Faculty, Free University, Amsterdam, The Netherlands*

Accepted for publication 2 June 1987

SUMMARY

Migration of lymphocytes into the lamina propria of the small intestines was studied in mice using short-term *in vivo* migration experiments in combination with immunocytochemistry and autoradiography. The results show that, shortly after intravenous injection, most of the lymphocytes present in the lamina propria are actually located within the capillary network of the villi. Furthermore, it was shown that lymphocytes leave the blood stream and enter the lamina propria via small blood vessels at the base of the villi. These blood vessels can be discriminated by their positive staining with MECA-325, a monoclonal antibody that is specific for high endothelial venules (HEV) in lymphoid organs. From the results it is concluded that the gut contains specialized venules at specific sites, involved in the emigration of lymphocytes, comparable to HEV in lymphoid organs. The flatness of the endothelium of these MECA-325-positive intestinal blood vessels, which is in contrast to the situation in lymphoid organs, could not be changed by inducing an intestinal inflammation. This flatness may be directly correlated to the less efficient transmigration of lymphocytes, as demonstrated in our experiments.

INTRODUCTION

An important characteristic of lymphocytes is their ability to migrate continuously into and from lymphoid organs. The immigration of lymphocytes into lymphoid organs via the afferent lymphatics or blood vessels (Gowans & Knight, 1964; Guy-Grand, Griscelli & Vassalli, 1974; Cahill *et al.*, 1977) and their subsequent emigration via the efferent lymphatics have been studied extensively (Scollay, Butcher & Weissman, 1980; Jeurissen, Sminia & Kraal, 1984). It has been shown that immigration into lymphoid organs from the blood is mediated by specialized venules characterized by high endothelium, and therefore called high endothelial venules (HEV; Gowans & Knight, 1964; Stamper & Woodruff, 1976; Kraal, Duijvestijn & Hendriks, 1987). These HEV have been described in lymph nodes, Peyer's patches, bronchus-associated lymphoid tissue and granuloma, but not in the spleen (Gowans & Knight, 1964; Van der Brugge-Gamelkoorn & Kraal, 1985; Freemont & Ford, 1985). The existence of a specific selection mechanism based on the occurrence of determinants and related receptors on endothelial cells and lymphocyte subsets, respectively, was demonstrated using *in vitro* HEV adherence assays (Butcher, Scollay & Weissman, 1979, 1980; Kraal, Weissman & Butcher, 1983). Based on these experiments, a model was outlined in which two different determinant-receptor sets exist, one specific for Peyer's patches (PP) and one for peripheral lymph nodes (PLN), which was confirmed by the development of specific monoclonal antibodies (Gallatin, Weissman & Butcher, 1983; Rasmussen *et al.*, 1985; Chin *et al.*, 1986).

Little attention has been paid to the migration of lymphocytes into non-lymphoid tissues. In the lamina propria (LP) of the gut many lymphocytes can be seen, whereas lymphocytes are also abundantly present inbetween epithelial cells of the intestines. It is unknown how lymphocytes enter the lamina propria since no blood vessels with the morphological criteria of HEV can be found. From several studies using labelled lymphoblasts it has been concluded that lymphocytes are able to leave the blood throughout the entire villus (Marsh, 1975; Hall & Smith, 1970). With the development of MECA-325, a monoclonal antibody specific for HEV in lymphoid organs (Duijvestijn *et al.*, 1987), we wished to re-examine the migration of lymphocytes into the lamina propria. It was found that MECA-325 specifically reacts with venous blood vessels in between the

* Present address: Dept. of Histology, Medical Faculty, State University, Groningen, The Netherlands.

Abbreviations: DNCB, dinitrochlorobenzene; FITC, fluorescein isothiocyanate; HEV, high endothelial venule(s); LP, lamina propria; PLN, peripheral lymph node(s); PP, Peyer's patch(es).

Correspondence: Dr S. H. M. Jeurissen, Dept. of Virology, Central Veterinary Institute, PO Box 365, 8200 AJ Lelystad, The Netherlands.

crypts and, combining autoradiography and immunohistochemistry, we were able to demonstrate that lymphocytes cross the endothelium of these vessels.

MATERIALS AND METHODS

Animals

Female $(C_3D_2)F_1$ mice were obtained from G. O. Bomholtgard Ltd, Rye, Denmark, and were kept under routine laboratory conditions. In some experiments 200 μg dinitrochlorobenzene (DNCB) in 0·1 ml vegetable oil were given orally for 4 days.

Cell labelling and transfer

Pooled lymph nodes (usually mesenteric, popliteal, inguinal, axillary, bronchial and cervical lymph nodes) were suspended in Earle's medium containing 1% new-born calf serum and 10 μmol HEPES.

For quantitative experiments, cells were labelled with fluorescein isothiocyanate (FITC; Isomer I, BBL Microbiology Systems) in a concentration of 30 μg/30 × 10^6 cells/ml for 30 min at 37° (viability was ≥90%).

For autoradiography, cells were radiolabelled with [^{3}H]leucine (Amersham International, Amersham, Bucks, U.K.; 10 μCi/10^7 cells/ml) for 60 min at 37°.

After labelling, cells were spun through serum and injected intravenously into syngeneic recipients (30–40 × 10^6 cells per animal).

Quantification of intravenously injected FITC-labelled cells in the gut

At various time-points after injection of the cells (15 and 30 min, 1, 3, 7 and 24 hr) the mice were killed. Pieces of small intestine were removed at random throughout the intestines and snap-frozen in liquid nitrogen. Using a fluorescence microscope the numbers of FITC-labelled cells in the gut were determined per whole section in three different pieces of gut, of which every tenth section (thickness 8 μm) was collected to a total of 10 sections. In addition, the percentage of FITC-labelled donor cells in each recipient's blood was determined. Peripheral blood was obtained via cardiac puncture and decoagulated with heparin. Erythrocytes were removed by NH_4Cl shock. The percentage of labelled donor cells was determined by counting at least 500 cells per sample. The data of donor cells per gut section and blood were determined for each mouse individually.

In vitro *assay of lymphocyte binding*

This technique has been described previously (Butcher *et al.*, 1979). Briefly, rhodaminated mesenteric and peripheral lymph node cells were incubated at 4° on unfixed frozen sections of small intestine with or without Peyer's patches. The slides were gently agitated throughout incubation. After 30 min the sections were fixed and examined under dark-field illumination and UV epi-illumination. In order to remove excessive amounts of mucus, in some experiments gut segments were rinsed with 0·1% dithiothreitol in saline.

Immunohistochemistry

Rat anti-mouse monoclonal antibody, MECA-325, was applied on cryostat sections, followed by incubation with a highly specific rabbit anti-rat Ig coupled to peroxidase (Dako, Glostrup, Denmark). Peroxidase activity was detected by incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, MO) in 0·5 mg/ml Tris-HCl buffer, pH 7·6, containing 0·01% H_2O_2.

Autoradiography

The combination of autoradiography and immunohistochemistry has been described previously (Van Rooijen & Streefkerk, 1976). Thirty minutes, 2 hr and 7 hr after injection mice were killed and pieces of small intestine, with or without Peyer's patches, mesenteric lymph nodes and spleen were removed and snap-frozen in liquid nitrogen. After immunohistochemical staining the sections were covered with stripping film (Eastman Kodak Co., Rochester, NY) and exposed for 3 weeks in the dark. After development the sections were counterstained with haematoxylin.

RESULTS

Quantification of intravenously injected FITC-labelled lymphocytes in the gut

These experiments were performed to be able to correlate the rate of entry of cells into the intestinal tissues and the presence of

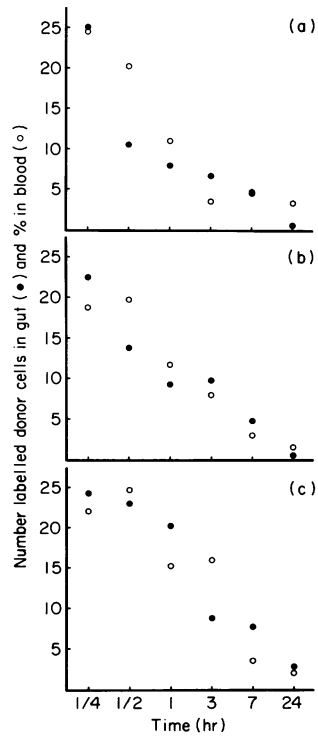

Figure 1. The effect of time on the localization of FITC-labelled donor lymphocytes in the gut. Lymphocytes (30–40 × 10^6) were injected i.v., and segments of small intestine were removed at time-points as indicated. The mean numbers of FITC-labelled lymphocytes per cross-section of the gut were determined (solid symbols). The percentages of FITC-labelled cells in peripheral blood of the recipients at these time-points were determined (open symbols). The symbols at a given time-point represent the percentage of labelled donor cells in blood and gut sections of the same mouse. Within an experiment all animals received the same dose of donor cells. Three consecutive experiments are represented in (a), (b) and (c).

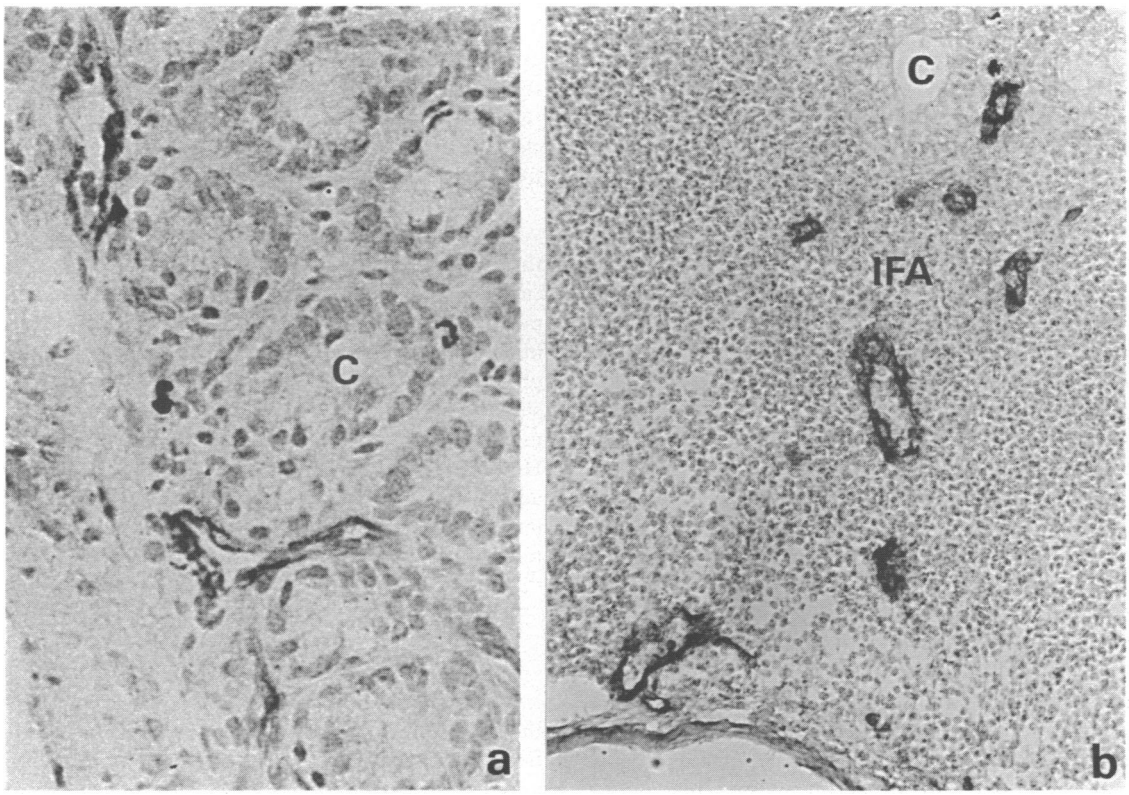

Figure 2. MECA-325 antibodies specific for high endothelial venules (HEV) in lymphoid organs applied on cryostat sections of gut tissue (a) and Peyer's patches (b). (a) In between the muscularis externa and the crypts, venous blood vessels stain with MECA-325. The villi are completely devoid of positive staining. These MECA-325-positive blood vessels appear to have flat endothelium (magnification × 400). (b) In Peyer's patches, MECA-325 antibodies specifically stain HEV, which are predominantly located in the interfollicular area (magnification × 200; C: crypts; IFA: interfollicular area).

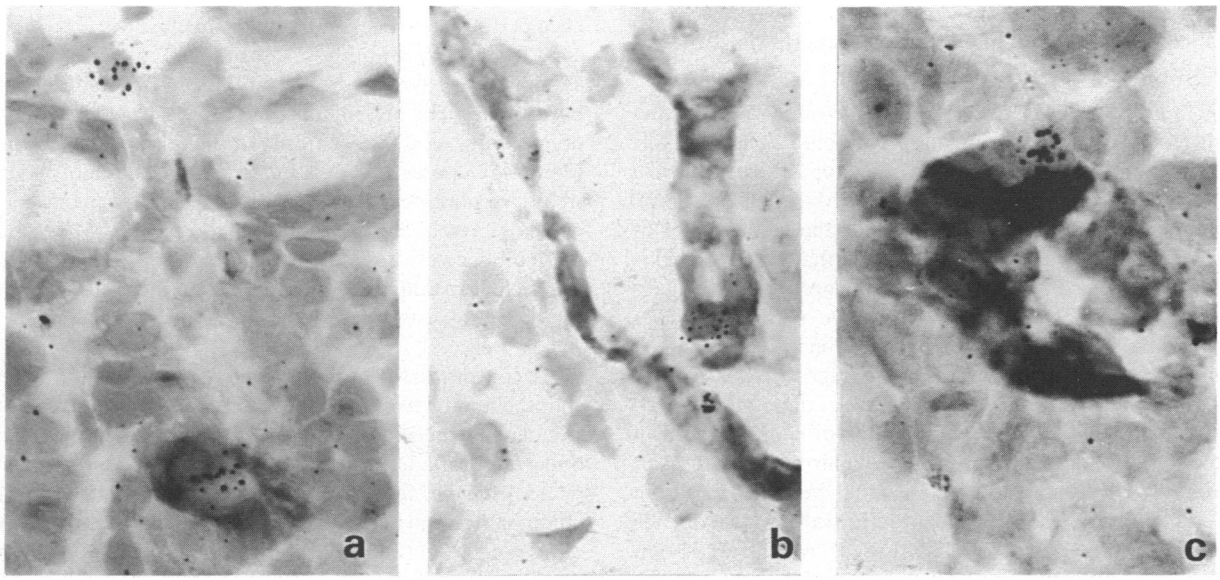

Figure 3. Lymphocytes migrate from the blood into the lamina propria of the gut via specialized venules. A combination of autoradiography and immunoperoxidase staining with MECA-325 was used to detect the exit of lymphocytes from the blood. Thirty minutes after the intravenous injection of [^{3}H]leucine-labelled lymphocytes, cells were found crossing MECA-325-positive blood vessels. (a) Labelled cell inside MECA-325-positive blood vessel, and one cell already in the lamina propria (magnification × 900). (b) Labelled cell passing through the endothelium (magnification × 900). (c) Labelled cell just outside the vessel (magnification × 1350). Due to the differences in plane of focus between the grains in the exposed film and the immunoperoxidase staining in these microphotographs, only the grains are in focus.

cells in the blood stream. Recipient mice were injected i.v. with FITC-labelled lymph node cells, and at several time-points after injection the number of donor cells per cross-section of the small intestines was determined and correlated with the number of injected cells present in peripheral blood. The results are shown in Fig. 1. Fifteen minutes after intravenous injection an average of 20–25 FITC-labelled cells could be counted per cross-section of the small intestines. At this time-point one-fifth of the peripheral blood leucocytes consisted of FITC-labelled donor lymphocytes. One hour after cell transfer, the values of FITC-labelled cells in gut sections and peripheral blood had declined to half of the values at 15 min. At 3 hr and 7 hr the numbers of FITC-labelled cells in both gut sections and peripheral blood had further decreased. At the time-point of 24 hr very few FITC-labelled lymphocytes were present in the gut in correlation with very small percentages of FITC-labelled cells in the blood. In contrast to the decreasing number of labelled cells in both intestinal tissue and blood at later time-points, a constant increase of labelled cells was seen in sections of the Peyer's patch. Especially during the first 3 hr after injection, a rapid increase was observed, starting around the HEV in these organs. The abundance with which labelled cells would concentrate in and around HEV was never observed in any blood vessels in the intestines outside the Peyer's patches. In order to gain more insight into the precise location at which lymphocytes leave the blood stream into the lamina propria an *in vitro* adherence assay was performed as described for HEV in lymphoid organs. However, we were not able to detect specific binding to lymphocytes of any type of blood vessel outside PP. This was mainly due to the excessive high background of binding that is observed on sections of the intestines. Attempts to bring this background down by removing the mucus, which may be especially responsible for the non-specific binding, by incubating the intestinal tissues with dithiothreitol also failed.

Migration of lymphocytes into the lamina propria via MECA-325-positive venules

Although no classical HEV can be demonstrated in the lamina propria by morphology or using the *in vitro* binding assay, it was found that small venules in between the crypts adjacent to the muscularis externa stained positively with MECA-325 (Fig. 2). This monoclonal antibody is specific for the endothelium of HEV in lymphoid organs, but the epitope that is recognized is not directly involved in the adhesion of lymphocytes (Duijvestijn *et al.*, 1987). The MECA-325-positive vessels are never observed in the villi, and their endothelium is quite flat. A combination of autoradiography and immunoperoxidase staining was used to investigate the possible role of these MECA-325-positive blood vessels in mediating lymphocyte migration from the blood into the lamina propria. Thirty minutes after intravenous injection, labelled cells could be detected within MECA-325 blood vessels, as well as in the wall and just outside these vessels in the lamina propria (Fig. 3). At this time-point radiolabelled cells could also be detected in the lamina propria, but whether these cells were located inside the abundant vascular system of the lamina propria or had left the blood stream was impossible to determine. The number of radiolabelled cells in and around these MECA-325-positive vessels shortly after injection was always very low in contrast to the many radiolabelled cells that could be detected in and around the Peyer's patch HEV at the same time. At later time-points, 3 hr and 7 hr after cell transfer, very few cells were seen associated with the MECA-325-positive vessels, whereas at these time-points labelled cells could occasionally be detected in the lamina propria and inbetween epithelial cells. These results clearly suggest a role of these vessels in the exit of lymphocytes from blood into the lamina propria.

The flatness of the endothelium of the MECA-325-positive vessels in the gut could be a reflection of a quiescent state of these vessels. It is known that venules at sites of inflammation can attain the plump morphology of HEV in combination with positive staining for MECA-325. Factors produced at such inflammatory sites may be involved in the induction of these phenomena, and we therefore wished to determine whether a state of inflammation, induced in the gut, could alter the morphology of the MECA-325 vessels in the lamina propria. However, an acute inflammation induced in the gut by oral administration of DNCB, leading to extensive granulocyte influx and oedema in the lamina propria, did not alter the morphology of these MECA-325 vessels.

DISCUSSION

In this study the migration of lymphocytes from the blood into the lamina propria of the gut was investigated using a combination of autoradiography and immunohistochemistry. It appeared that lymphocytes are able to enter the lamina propria via small MECA-325-positive venules at the base of the villi. In analogy to Peyer's patches and lymph nodes, where MECA-325 antibodies specifically recognize high endothelial venules (HEV), we assume that MECA-325-positive vessels represent specific sites in the gut where lymphocytes can leave the blood by passing through the endothelial wall.

Most of the studies on lymphocyte migration in the gut have been performed using lymphoblasts labelled with DNA precursors (Hall, Parry & Smith, 1972). In this study using [^{3}H]leucine as marker, all lymphocytes were labelled, including lymphoblasts that were present in low numbers in the lymph node preparations used. The majority of the cells associated with the MECA-325-positive vessels in the gut had the morphology of small lymphocytes and therefore we feel confident that the results represent extravasation phenomena of small recirculating lymphocytes. Based on the observations of other authors (Griscelli, Vassalli & McCluskey, 1969), it cannot be ruled out that lymphoblasts especially may use other sites of extravasation as well.

In contrast to MECA-325-positive HEV in lymph nodes, the endothelium of the intestinal vessels was flat and could not be stimulated to become higher after the induction of an inflammation with oral administrations of DNCB. The latter could be related to the fact that a chronic rather than an acute inflammation will induce cuboidal endothelium as found at sites of inflammation (Smith, McIntosh & Morris, 1970). From the small number of cells that was found to be associated with the intestinal vessels, it was concluded that the rate at which lymphocytes leave these vessels is very low in comparison with that observed with HEV in Peyer's patches. It is unclear whether this is directly related to the flatness of the endothelium lining these MECA-325-positive venules. In ruminant species no cuboidal endothelium can be demonstrated in lymph nodes and

Peyer's patches, in spite of active recirculation of lymphocytes in these species.

A low transmigration rate was also apparent from our experiments in which the fate of FITC-labelled cells was followed after transfer. Although many transferred cells were found in the villi initially, their number dropped rapidly, concomitant with that in the blood, clearly demonstrating that the vast majority of labelled lymphocytes found in the gut are within the extensive vascular system of the lamina propria. The decrease of labelled cells in the villi and blood must be due to redistribution of these cells in other parts of the body, e.g. spleen and liver, and is not caused by a loss of label from these cells. Coinciding with the decrease in blood and villi, increasing numbers of labelled cells can be found in Peyer's patches associated with the HEV.

Lymphocyte migration via HEV is regulated and influenced by the presence of receptors on the endothelial surface that combine with determinants on the lymphocyte membrane (Jalkanen *et al.*, 1986; Kraal *et al.*, 1986). It has now been well established that these receptors differ on HEV from lymph nodes and Peyer's patches (Gallatin *et al.*, 1983; Rasmussen *et al.*, 1985; Chin *et al.*, 1986). Furthermore, selective mechanisms of extravasation in the gut itself have been described using mesenteric IgA lymphoblasts (Tseng, 1981; Weisz-Carrington *et al.*, 1979). MECA-325-positive venules in the gut resemble HEV in antibody staining and function. It would be of great interest to see whether similar or different receptor systems as found on regular HEV are involved in the transmigration of lymphocytes through these specific vessels.

REFERENCES

Butcher E.C., Scollay R.G. & Weissman I.L. (1979) Lymphocyte adherence to high endothelial venules: characterization of a modified *in vitro* assay, and examination of the binding of syngeneic and allogeneic lymphocyte populations. *J. Immunol.* **123,** 1996.

Butcher E.C., Scollay R.G. & Weissman I.L. (1980) Organ specificity of lymphocyte migration: mediation by highly selective lymphocyte interaction with organ-specific determinants on high endothelial venules. *Eur. J. Immunol.* **10,** 556.

Cahill R.N.P., Pashitt C.D., Frost H. & Trnka Z. (1977) Two distinct pools of recirculating T lymphocytes. Migratory characteristics of nodal and intestinal T lymphocytes. *J. exp. Med.* **145,** 420.

Chin Y.-H., Rasmussen R.A., Woodruff J.J. & Easton T.G. (1986) A monoclonal anti-HEBF PP antibody with specificity for lymphocyte surface molecules mediating adhesion to Peyer's patch high endothelium of the rat. *J. Immunol.* **136,** 2556.

Duijvestijn A.M., Kerkhove M., Bargatze R. & Butcher E.C. (1987) Lymphoid tissue- and inflammation-specific endothelial cell differentiation defined by monoclonal antibodies. *J. Immunol.* **138,** 713.

Freemont A.J. & Ford W.L. (1985) Functional and morphological changes in post-capillary venules in relation to lymphocytic infiltration into BCG-induced granulomata in rat skin. *J. Pathol.* **147,** 1.

Gallatin W.M., Weissman I.L. & Butcher E.C. (1983) A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature (Lond.)*, **304,** 30.

Gowans J.L. & Knight E.J. (1964) The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. London, Ser. B.* **159,** 257.

Griscelli C., Vassali P. & McCluskey R.T. (1969) Distribution of large dividing lymph node cells in syngeneic recipient rats after intravenous injection. *J. exp. Med.* **130,** 1427.

Guy-Grand D., Griscelli C. & Vassalli P. (1974) Gut-associated lymphoid system—nature and properties of large dividing cells. *Eur. J. Immunol.* **4,** 435.

Hall J.G., Parry D.M. & Smith M.E. (1972) Distribution and differentiation of lymph-borne immunoblasts after intravenous injection into syngeneic recipients. *Cell Tiss. Res.* **5,** 269.

Hall J.G. & Smith M.E. (1970) Homing of lymph-borne immunoblasts to gut. *Nature (Lond.)*, **226,** 262.

Jalkanen S., Reichert R.A., Gallatin W.M., Bargatze R.F., Weissman I.L. & Butcher E.C. (1986) Homing receptors and the control of lymphocyte migration. *Immunol. Rev.* **91,** 39.

Jeurissen S.H.M., Sminia T. & Kraal G. (1984) Selective emigration of suppressor T cells from Peyer's patches. *Cell. Immunol.* **85,** 264.

Kraal G., Duijvestijn A.M. & Hendriks H.R. (1987) The endothelium of the high endothelial venule. A specialized endothelium with unique properties. *Exp. Cell Biol.* **55,** 1.

Kraal G., Twisk A., Tan B. & Scheper R.J. (1986) A surface molecule on guinea pig lymphocytes involved in adhesion and homing. *Eur. J. Immunol.* **12,** 1515.

Kraal G., Weissman I.L. & Butcher E.C. (1983) Differences in *in vivo* distribution and homing of T cell subsets to mucosal *vs* non-mucosal lymphoid organs. *J. Immunol.* **130,** 1087.

Marsh M.N. (1975) Electron-microscopic evidence of blast transformation in epithelial lymphocytes of mouse small intestinal mucosa. *Gut*, **16,** 665.

Rasmussen R.A., Chin Y.-H., Woodruff J.J. & Easton T.G. (1985) Lymphocyte recognition of lymph node high endothelium. VII. Cell surface proteins involved in adhesion defined by monoclonal anti-HEBFLN (A.11) antibody. *J. Immunol.* **135,** 19.

Scollay R.G., Butcher E.C. & Weissman I.L. (1980) Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* **10,** 210.

Smith J.B., McIntosh G.H. & Morris B. (1970) Migration of cells through chronically inflamed tissues. *J. Pathol.* **100,** 21.

Stamper H.B. & Woodruff J.J. (1976) Lymphocyte homing into lymph nodes: *in vitro* demonstration of the selective affinity of recirculating lymphocytes for high endothelial venules. *J. exp. Med.* **144,** 828.

Tseng J. (1981) Transfer of lymphocytes of Peyer's patches between immunoglobulin allotype congeneic mice: repopulation of the IgA plasma cells in the gut lamina propria. *J. Immunol.* **127,** 2039.

Van der Brugge-Gamelkoorn G.J. & Kraal G. (1985) The specificity of the high endothelial venule in bronchus-associated lymphoid tissue (BALT). *J. Immunol.* **134,** 3746.

Van Rooijen N. & Streefkerk J.G. (1976) Autoradiography and immunohistoperoxidase techniques applied to the same tissue section. *J. immunol. Meth.* **10,** 379.

Weisz-Carrington P., Roux M.E., McWilliams M., Phillips-Quagliata J.M. & Lamm M.E. (1979) Organ and isotype distribution of plasma cells producing specific antibody after oral immunization: evidence for a generalized secretory immune system. *J. Immunol.* **123,** 1705.