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STATE OF THE ART

THE RECIRCULATION OF LYMPHOCYTES FROM BLOOD TO LYMPH: PHYSIOLOGICAL CONSIDERATIONS AND MOLECULAR MECHANISMS

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Only a few years ago, a review on the subject of lymphocyte recirculation consisted, essentially, of a summary of data derived from observations made on the migratory properties of subpopulations of lymphoid cells *in vivo* and on the architecture of the fixed lymphoid tissues through which these cells migrate. Thus, a typical 'state of the art' review addressed issues such as the non-randomness of lymphoblast and small lymphocyte migration patterns, the morphological and biochemical properties of the specialized endothelial cells which mediate this cellular traffic, the effect of antigen on lymphoid cell migration, and the relationship of lymphocyte traffic to the immune response. Since, at the time, very little was known about the molecular mechanisms involved in controlling or directing this traffic, this particular aspect was dealt with in a more speculative manner. The recent applications of hybridoma and molecular biological technologies to the study of lymphocyte migration have radically changed this emphasis. Today, with rare exceptions, a review on lymphocyte migration consists, almost exclusively, of a summary of descriptions of lymphocyte and endothelial cell-surface molecules thought to be involved in the initial adhesive event between circulating lymphocytes and specialized vascular endothelium (1,2). The cloning of several putative lymphocyte "homing receptors" and the ensuing discovery of structural relationships between these and other lymphoid and neural cell adhesion molecules, within and across species barriers, has prompted a considerable degree of excitement not only in the field of lymphocyte migration, in particular, but in a number of scientific disciplines, such as immunology, cell biology, and neurobiology, in general. In addition, it has become apparent that soluble mediators, including inflammatory cytokines, such as tumor necrosis factor species and the interferons, may influence lymphocyte traffic through particular tissues and, in so doing, may modulate local immune responses. It is quite possible that some or all of these molecules may be involved in lymphocyte migration during normal and pathological situations *in vivo.* However, the literature of the 25 years following Gowans' discovery of the recirculation of lymphocytes from blood to lymph (3,4) clearly indicates that this process cannot be adequately explained by just a few molecules.

PIONEERING DEVELOPMENTS: ANIMAL MODELS

The description of a technique for collecting lymph from the thoracic duct of the rat on a chronic basis (5) contributed significantly to the experiments which ultimately led to the discovery of the recirculation of small lymphocytes from blood to lymph. At a time when the lymphocyte was still quite enigmatic, Mann and Higgins (6) found that chronic drainage of the thoracic duct in dogs resulted in a progressive depletion of lymphocytes from the lymph. This observation seemed to perpetuate the long-held notion, which had been based on histological observations of intense mitotic activity in lymph nodes, that lymphocytes were short-lived cells. When the experiments of Mann and Higgins were repeated by Gowans in the rat (3), a startling discovery was made. The progressive fall in the output of lymphocytes in thoracic duct lymph which occurred during chronic drainage could be reversed by returning the lymph-borne cells to the blood. This observation provided indirect experimental support for the idea, which had been proposed over 50 years earlier, that lymphocytes continually recirculate from blood to lymph. Direct experimental support for this hypothesis came from experiments in which Gowans demonstrated that radiolabeled small lymphocytes appeared in thoracic duct lymph after intravenous injection (4). In a subsequent study, which involved tracing the fate of intravenously infused cells by autoradiography, Gowans and Knight (7) demonstrated that lymph nodes were the major sites of the exchange of lymphocytes between the blood and lymph. The details of the tempo of the process of lymphocyte migration were later examined in the rat in a highly systematic manner by Ford and his colleagues (8-10).

The landmark observations made by Gowans in the late 1950's were extended in significant ways by Morris and his colleagues in Canberra, who described techniques for the collection of lymph from groups of lymph nodes (11) or single lymph nodes (12) situated in various regions of the body of the sheep under physiological conditions. It was immediately shown that the cell output in the efferent lymph of the popliteal lymph node could not be diminished by irradiating the lymph node (13), thereby corroborating the view (4) of an extensive recirculation of lymphocytes from blood to lymph through lymph nodes. The opportunity to collect lymph from the regional drainage area of the popliteal lymph node, combined with the use of ^{3}H-thymidine to label actively dividing cells, enabled Hall and Morris (14) to establish conclusively that over 90% of the cells in the efferent lymph of a single subcutaneous lymph node were neither borne in afferent lymph nor recently divided cells, but were cells which recirculated from blood to lymph through the substance of the lymph node. The kinetics of this recirculation were examined at a later date (15).

The versatility of the sheep model was illustrated further in experiments in which lymph was chronically and quantitatively collected from a variety of non-lymphoid tissues (16). Small lymphocytes could be detected in lymph draining every non-lymphoid tissue examined but, between different tissues, there appeared to be no relationship between the protein concentration and lymphocyte output in the lymph (16). Later, however, the protein concentration in efferent lymph was found to be influenced by the process of lymphocyte extravasation within the lymph node (17,18).

By using techniques for the collection of lymph from the thoracic duct of the sheep fetus *in utero* (19), it was possible to demonstrate the establishment of a large-scale recirculation of lymphocytes from blood to lymph in the agammaglobulinemic, antigen-free, ovine fetus (20). Later studies, aided by descriptions of techniques for collecting lymph from single lymph nodes in the fetal lamb (21), described the kinetics and magnitude of lymphocyte recirculation through these organs (22-26). These studies on the development of lymphocyte recirculation in the ovine fetus, as well as

others on the kinetics and magnitude of lymphocyte traffic through dermal granulomas (27), hydronephrotic kidneys (27), and renal allografts (28-30), remain virtually unparalleled in any species system, not only in terms of their originality and elegance, but also with respect to their contributions to our present understanding of the embryogenesis of the immune system and the cellular dynamics of transplantation phenomena.

TISSUE-SPECIFIC PATTERNS OF LYMPHOCYTE MIGRATION

Lymphoblasts

In their original description of the localization of intravenously infused thoracic duct lymph cells in the rat, Gowans and Knight (7) observed that the small proportion of lymphoblasts present in the transfused population localized almost exclusively in the small intestine and not at all in peripheral lymph nodes. This important observation went seemingly unnoticed for several years until Griscelli et al (31) showed that a similar localization pattern could be produced by using lymphoblasts prepared from disaggregated mesenteric, but not peripheral, lymph nodes. Their findings were soon confirmed by Hall and his colleagues (32,33). The idea that antigen was directly involved in directing the migration of lymphoblasts to the gut was a popular one at the time but this notion was abandoned in favor of alternative hypotheses when it was shown that lymphoblasts migrated into grafts of syngeneic fetal gut (34) and into the gut of unsuckled, neonatal rats (35) at least as well as they migrated into the intestine of adult animals. The demonstration of immunoglobulin A (IgA) on the surface, as well as inside the cytoplasm, of a substantial proportion of gut-homing lymphoblasts (36) suggested that surface IgA (sIgA) might be involved in the selective migration of lymphoblasts to the gut. However, later studies cast considerable doubt on this possibility (37,38). More recent studies on the migratory properties of memory cells (see later section) may be relevant to experiments conducted using blast cells or, at least, recently divided cells.

Small Lymphocytes

The observation of the non-random migration of lymphoblasts to the small intestine suggested that the recirculation of small lymphocytes from blood to lymph might also be non-random. This issue was addressed independently by two groups who were working with the Hall and Morris (12) sheep model. Scollay, Hopkins and Hall (39), using unseparated efferent lymph cells, found that lymphocytes obtained from lymph draining a subcutaneous lymph node recirculated preferentially into lymph draining subcutaneous lymph nodes, whereas lymphocytes obtained from lymph draining intestinal lymph nodes recirculated preferentially through intestinal lymph nodes. Based on previous observations of non-random migration of B blasts in rodents, they concluded that the observed patterns of non-random recirculation reflected the migratory preferences of small B cells, not T cells, and that surface Ig mediated this process. The experiments of Cahill and his colleagues in Basel (40) clearly indicated that this was not the case. The tissue-specific recirculation patterns described by Scollay et al (39) could be reproduced by transfusing populations of lymph cells depleted of B lymphocytes by nylon wool separation. In addition, substantial amounts of radioactivity could be detected in the small intestine three hours after transfusion of lymphocytes obtained from lymph draining intestinal, but not subcutaneous, lymph nodes (40). This led to the conclusion that there are two subpopulations of T lymphocytes demarcated by their migratory specificities. One of these subpopulations was held to recirculate exclusively through the small intestine into intestinal lymph; the other was proposed to recirculate randomly through peripheral and mesenteric lymph nodes and not at all through the small intestine. In this

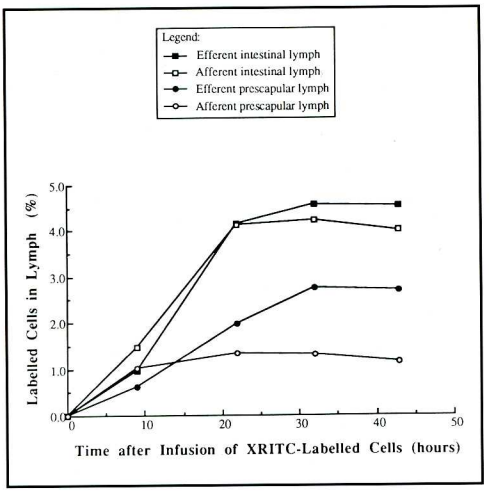

Fig. 1. Non-random recirculation of XRITC-labeled efferent intestinal lymph cells into efferent intestinal, afferent intestinal, efferent prescapular and afferent prescapular lymph. (Ref. 109, reprinted by permission of Blackwell Scientific Publications, Ltd.).

way, lymph draining intestinal lymph nodes would contain both randomly migrating and 'gut-seeking' small lymphocytes, whereas lymph draining subcutaneous lymph nodes would contain only randomly migrating lymphocytes. The apparent non-random migration of cells from lymph draining peripheral lymph nodes into lymph draining mesenteric lymph nodes would then be explained by a dilution effect (40). Although the experimental findings have been amply confirmed in this species (41,42) and evidence in favor of tissue-specific lymphocyte-endothelial cell recognition continues to mount (see below), the model, itself, was shown to be somewhat unsatisfactory, since the cells borne in afferent intestinal lymph display migratory properties which are not different from those of cells borne in efferent intestinal lymph (43,44).

At this point, it is worth noting that non-random migration of small lymphocytes could not be demonstrated in rodents by using thoracic duct lymph cells or cells obtained from disaggregated mesenteric and peripheral lymph nodes (45,46). The possibility that species differences were responsible for the observed discrepancy was not supported by two lines of evidence. First, ovine lymph-borne immunoblasts were found to display migratory properties which paralleled those seen in rodents. Thus, lymphoblasts borne in efferent intestinal lymph were found to migrate almost exclusively to the intestine, whereas lymphoblasts borne in lymph draining peripheral lymph nodes were observed to localize preferentially in peripheral lymph nodes (47). These patterns were also observed in the ovine fetus (48), which develops in an environment of extrinsic antigen. The second line of evidence emerged from another study in sheep. Small lymph-borne cells were found to recirculate non-randomly, thereby confirming the previous reports in this species (39-41). *See Fig. 1.* However, tissue-specific recirculation could not normally be demonstrated when the transfused small lymphocytes were obtained from mechanically disaggregated lymph nodes (42).

Eventually, the tissue-specific migration of small lymphocytes was demonstrated in mice by Butcher and his colleagues (49). Two types of experiments were performed. In the first, the interaction of Peyer's patch- and peripheral lymph node-derived lymphocytes with the high endothelial venules (HEV) of different lymphoid tissues was studied by using a modification (50) of a previously described *in vitro* assay of lymphocyte-endothelial cell recognition (51,52; the *in vitro* lymphocyte-binding assay will be described in detail at a later stage). Lymphocytes teased from Peyer's patches were found to bind preferentially to the HEV of Peyer's patches, whereas lymphocytes teased from lymph nodes were found to bind preferentially to the HEV of peripheral lymph nodes. Both types of cells were found to bind to the HEV of mesenteric lymph nodes to an intermediate extent. In the second type of experiment, the localization of these cell types in lymphoid tissues after intravenous infusion was examined. It was observed that the *in vivo* migratory properties of these cells could be

predicted by their *in vitro* HEV-binding patterns. Thus, Peyer's patch-derived lymphocytes migrated preferentially to Peyer's patches, whereas cells teased from peripheral lymph nodes localized more efficiently in peripheral lymph nodes. On the one hand, these patterns supported the model of Cahill et al (40); on the other hand, this model was unsatisfactory for explaining tissue-specific lymphocyte recirculation patterns in the sheep (43,44). While it is still possible that species differences exist, the experiments of Reynolds et al (42) stress the need for caution in interpreting the results of experiments, *in vitro* or *in vivo*, involving the use of lymphocytes obtained from fixed lymphoid tissues. It is likely that many small lymphocytes present within a fixed, secondary lymphoid tissue will be in a recirculatory 'mode'; however, it is equally likely that many cells will not. Since the migratory preferences which have been shown to date are relative rather than absolute, this point needs to be continually borne in mind.

Based on the experimental studies outlined above, it is now generally accepted that there exist subpopulations of small lymphocytes which display tissue-specific migratory preferences. However, the issue of whether the recirculatory preference displayed by a particular lymphocyte is an immutable property of that cell, or alternatively, is continually changing in response to immunological and/or non-immunological factors is still far from being resolved. In an effort to test the hypothesis that intestinal lymph contains a subpopulation of cells which *continually* recirculates through the intestinal lymphoid tissues in preference to other tissues, Abernethy et al developed a methodology by which the migratory pattern displayed by a single subpopulation of cells could be monitored on *two* consecutive transits from blood to lymph in the same sheep (53). Small lymphocytes borne in efferent intestinal lymph were collected, labeled *in vitro* with the green fluorochrome, fluorescein isothiocyanate (FITC), returned to the blood of the same sheep, and their recirculation into both intestinal and subcutaneous efferent lymph monitored. One day later, FITC-labeled lymphocytes which had recirculated into efferent intestinal lymph (putative 'gut-seeking' cells) were labeled *in vitro* with a red-shifted fluorochrome, substituted rhodamine isothiocyanate (XRITC). These double-labeled lymphocytes were then returned to the blood of the same sheep and their recirculation into both lymph compartments monitored. The migratory properties of the double-labeled cells were then compared with those of the single-labeled cells. If the migratory preference displayed by a particular subpopulation of cells was immutable, it was expected that the double-labeled subpopulation would be enriched for cells displaying the 'gut-seeking' phenotype and, consequently, would show a higher degree of selectivity for intestinal tissues than would the single-labeled population. This is exactly what was observed: in 12 out of 13 sheep, the intestinal:subcutaneous specific activity ratios were significantly higher for the double-labeled subpopulation (54). These results were therefore consistent with the hypothesis that intestinal lymph contained a subpopulation of cells which *continually* recirculated through the intestinal lymphoid tissues in preference to other tissues. The logical extension of this conclusion was that there existed individual cells whose migratory phenotype was possibly immutable.

Finally, there is evidence, from experiments in a number of species, that small lymphocytes show tissue-specificity in their recirculation through tissues other than intestinal lymph nodes and other gut-associated lymphoid tissues and peripheral lymph nodes. Studies in sheep demonstrate non-random recirculation of lymphocytes borne in afferent lymph draining dermal granulomas (41,55). The experimental observations cannot be explained by differential retention phenomena (56), so it has been proposed that there exists a population of small lymphocytes which selectively recirculates through the skin. It has subsequently been shown that the cell

responsible for the non-random recirculation is a T cell, not a B cell (57). This is not particularly surprising, since earlier studies (58,59), since confirmed by Miyasaka and his colleagues (60) and Cahill et al (61,62), have shown that B lymphocytes account for only 5-10% of lymphocytes in afferent subcutaneous lymph in the sheep, whereas they account for 20-35% of lymphocytes in blood and 20-30% of lymphocytes in lymph draining subcutaneous lymph nodes.

In another study in sheep, Spencer and Hall (63) observed that small lymphocytes obtained from lymph draining the caudal mediastinal lymph node recirculated equally well through the caudal lymph node and a peripheral lymph node, but less well through intestinal lymph nodes. Independently, Joel and Chanana (64) observed that small lymphocytes in efferent caudal mediastinal lymph showed a greater tendency to recirculate through the caudal lymph node than did cells from thoracic duct lymph. These data, as well as the results of *in vitro* studies of lymphocyte interactions with the HEV of gut- and bronchus-associated lymphoid tissues in rats and guinea pigs (65) and data obtained from perfusion studies by Pabst and his co-workers (66), have suggested that there is a separate pathway of lymphocyte migration through the lung. This view is clearly at odds with the previously held notion of a common mucosal immunologic system (67).

Recirculation of Small Lymphocytes in the Fetus

Any discussion of the tissue-specific migratory properties displayed by small lymphocytes would be incomplete without making reference to studies of lymphocyte recirculation in the fetus. As described earlier, there is an extensive recirculation of lymphocytes from blood to lymph in the fetal lamb (20) and in the fetal calf (68) which is established prior to the second half, and perhaps as early as the first third, of gestation. The magnitude of this recirculation increases with the vigorous lymphopoietic activity occurring during gestation. The rate of addition of small lymphocytes to the recirculating lymphocyte pool was calculated to be 3.6×10^6 cells/hr and 3.4×10^7 cells/hr for fetal lambs of 100 and 300 days gestational age (term=150 days), respectively (20). In the fetal calf, it was calculated that lymphocytes are added to the recirculating lymphocyte pool at a rate of 5×10^6 cells/hr and 1×10^9 cells/hr in fetuses of 120 and 188 days gestational age (term+280 days), respectively (68). Since the placenta in these species is epitheliochorial, the developing fetus is agammaglobulinemic and has no contact with maternal or any other antigen. Therefore, the idea that small, recirculating lymphocytes are cells which carry immunological memory is likely incorrect (see later section on Memory Cells). The studies of Morris and his co-workers in the sheep (20) demonstrate that this exchange of lymphocytes occurs within the substance of lymph nodes. Cahill et al (21) observed that the cells in lymph draining intestinal and prescapular lymph nodes of the fetal sheep are almost all thymus-derived cells, but B cells could always be detected. Furthermore, it was shown that these cells are long-lived (25). In contrast to observations made in adult animals (39,40), it was not possible to demonstrate non-random recirculation of fetal intestinal or prescapular lymph cells through the gut of the fetus (22,24) until after birth (25). This line of investigation was later extended by a series of experiments which yielded rather startling results. Kimpton and Cahill (69) transfused maternal or fetal intestinal and prescapular lymph cells into fetal sheep and measured the localization of these cells in a variety of tissues, including the small intestine. The proportions of transfused cells which localized in the fetal gut were similar to values reported earlier in adult sheep (40), when adult lymphocytes were used. When fetal lymph cells were used, preferential localization of intestinal lymph cells in the small intestine could be demonstrated. The effects could not be explained by the presence of lymphoblasts in the adult population, however. This observation

suggested that the mechanism allowing for the selective migration of 'gut-seeking' lymphocytes into the intestine was present in the fetus, but that fetal intestinal lymph did not contain a population of lymphocytes which was capable of migrating preferentially through the small gut. This idea proved to be correct, since intestinal lymph cells obtained from a 10 week old lamb recirculated preferentially into intestinal lymph when transfused into a fetus. Although these experiments involved allogeneic pairings, an important consideration in view of the observation that allogeneic lymphocytes recirculate very poorly in adults of any species (70-77), the experimental observations strongly support the idea that the mechanism allowing the selective migration of small lymphocytes through the gut-associated lymphoid tissues develops before birth. Since fetal intestinal lymph does not contain a population of lymphocytes which recirculates preferentially through these tissues, it is reasonable to conclude that tissue-specific endothelial specialization is the 'mechanism' which is present before birth. That this mechanism should develop in the absence of extrinsic antigen or maternal immunoglobulin further underscores the complexity of the biology of lymphocyte recirculation and raises important questions about the interrelationship of lymphocyte recirculation and the development of immune competence.

LYMPHOCYTE SUBSET-SPECIFIC PATTERNS OF LYMPHOCYTE MIGRATION

In contrast to the extensive literature on tissue-specific lymphocyte and lymphoblast migration, very little is known about the migratory properties of distinct sIg^-(T) and sIg^+(B) lymphocyte populations. Studies performed in rodents in the late 1960's and early 1970's demonstrated that the ability to recirculate from blood to lymph is a property of mature thymus-derived (T, sIg^-) cells (78,79) and bone-marrow-derived (B, sIg^+) cells (80,81) and indicated, in an indirect manner, that presumed B cells recirculate more slowly than T cells (8,80-82). A study in thymectomized fetal lambs also supported, in a similar way, the idea that B cells recirculate more sluggishly than T cells (20). Before proceeding further, it should be made perfectly clear that the B cells of the sheep appear to be produced in the Peyer's patches of the ileum and jejunum (83-85) by a process that is not influenced by the presence of extrinsic antigen (86,87). When Kuttner and Woodruff (88) examined the binding of T and B lymphocytes to the HEV of rat lymph nodes *in vitro*, no differences in the affinities of the two cell types for HEV could be detected. However, in studies involving the use of murine lymphoid tissues, it was possible to demonstrate tissue-specific differences in the binding of T and B lymphocytes *in vitro*: T lymphocytes were found to bind preferentially to the HEV of peripheral lymph nodes, whereas B lymphocytes were observed to display a preferential affinity for the HEV of Peyer's patches (89). Qualitatively similar observations were made in *in vivo* experiments: transfused T lymphocytes distributed preferentially to peripheral lymph nodes, whereas transfused B lymphocytes localized preferentially in Peyer's patches (89). The correlation between the *in vitro* and *in vivo* data corroborated the data obtained in a previous study of tissue-specific lymphocyte migration (49 – described above) and lent further support to the premise that the migratory properties of lymphocyte populations could be inferred from examinations of their affinities for the HEV of lymphoid tissues *in vitro*. However, it needs to be remembered that the cells of interest were subjected to considerable manipulation, *in vitro*, prior to the evaluation of their migratory or HEV-binding properties. It is possible that this handling of the cells may have influenced these functional activities.

The kinetics and tissue-specificity of the recirculation of T and B lymphocytes in the rat were recently examined by Pabst and his

colleagues (90, 91). B lymphocytes appeared to recirculate more slowly into thoracic duct lymph than T cells and demonstrated a preference for migration through Peyer's patches. The kinetics of the recirculation of lymph-borne T and B lymphocytes were recently examined in sheep under conditions which were considered to be physiological (92). It was not possible to demonstrate differences in the tempo of recirculation of these subsets in this species. However, the recovery of B cells was lower when the transfused lymphocytes were obtained from lymph draining mesenteric lymph nodes and other gut-associated lymphoid tissues than when they were obtained from lymph draining subcutaneous lymph nodes. It is conceivable that the apparent discrepancy between the rat and sheep data might be attributed to species-specific factors, since both groups used lymph-borne cells. It is also possible that technical considerations might be implicated, since cells in lymph draining single lymph nodes were used in the sheep experiments, whereas thoracic duct lymph cells, which are a mixture of cells borne in lymph from mesenteric, peripheral, and other lymph nodes, were employed in the rat studies.

Over a decade ago, it became clear that the T lymphocytes could be classified into subsets on the basis of their functional properties. T lymphocytes which provided 'help' in humoral immune responses and participated in delayed-type hypersensitivity reactions were designated as T 'helper' (T_h) cells. T cells which displayed cytotoxic activity, suppressed humoral immune response, and participated in allograft rejection were designated as T 'cytotoxic/suppressor' (T_s) cells. It was later found that these subsets could be distinguished on the basis of the expression of one or the other, but not both, of two cell-surface antigens defined by polyclonal antisera. T_h cells in the mouse expressed the Lyt-1 molecule but not the Lyt-2 antigen. T_s cells expressed Lyt-2 but not Lyt-1. The emergence of hybridoma technology extended this line of investigation in a very significant manner and led to the adoption of a new system of nomenclature for molecules expressed by lymphoid cells. These molecules are now referred to as "CD" antigens (CD=cluster designation). Monoclonal antibodies specific for human (93), rat (94), and murine (95) $CD4^+$ and $CD8^+$ T lymphocytes were described in the early part of this decade. Lyt-1 is the murine analogue of human CD4, while Lyt-2 is the analogue of CD8. Reagents specific for $CD4^+$ and $CD8^+$ T cells in pigs (96), sheep (97-99), and cattle (100,101) have also been described. Note that, whereas it used to be thought that all mature T cells express either the CD4 antigen or the CD8 antigen, but not both, it has recently become clear that mature T cells which express neither antigen ($CD4^-$ $CD8^-$T cells) can be found in extrathymic tissues, i.e., in the periphery, in several species (98,102-104). It is particularly noteworthy that, in the sheep system, these cells have been identified by monoclonal antibodies (mAbs) (98,102).

Monoclonal reagents specific for distinct T lymphocyte subsets have been used in only a few studies of lymphocyte migration; therefore, the migratory properties of $CD4^+$ and $CD8^+$ T lymphocyte subsets are less well established than those of bulk populations of T lymphocytes. Nevertheless, it is apparent that the migratory properties of T lymphocyte subsets defined by mAbs are non-random. In a study in mice, it was found that $CD4^+$ T lymphocytes localized in Peyer's patches more efficiently than did $CD8^+$ lymphocytes. However, these $CD4^+$ and $CD8^+$ T cells localized with comparable efficiencies in peripheral lymph nodes (105). These results were corroborated by data obtained from studies of the binding of these lymphocyte subsets to the HEV of murine lymphoid tissues *in vitro* (105). An *in vitro* study involving the use of human tissues yielded different results. $CD4^+$ T cells were found to bind to the HEV of Peyer's patches less efficiently, but to the HEV of peripheral lymph nodes more efficiently, than $CD8^+$ T cells (106). Although species differences might again be implicated, it is also possible

that technical considerations explained the discrepancies. For example, the cells used by Kraal and his colleagues (105) were obtained mainly from lymph nodes and Peyer's patches; the cells used in the human study were prepared from peripheral blood and spleen (106). It is also worth noting that, as in previous studies of tissue-specific lymphocyte migration in mice (49, 89), only localization, not *recirculation*, was measured. Pabst and his colleagues in Hannover described the kinetics of the recirculation of $CD4^+$ and $CD8^+$ T lymphocytes into thoracic duct lymph in the rat (90,91). Small, $CD4^+$ T cells recirculated into thoracic duct lymph at a faster rate than small, $CD8^+$ T cells in the first 12 hours following transfusion. After this time, however, there was no difference in the overall recovery of these subsets in the lymph compartment examined. $CD4^+$ and $CD8^+$ T lymphocyte subset localization differed in a number of organs in the first 3-6 hours after transfusion, but these differences were neither consistent nor tissue-specific and, by 24 hours, they were not detectable.

The results of several recent studies in sheep convincingly demonstrate that the migratory properties of small, $CD4^+$ and $CD8^+$ T lymphocytes are non-random. First, steady state studies have shown that the distribution of these subsets between the blood, the efferent and afferent lymph of the popliteal lymph node, and the lymph node, itself, is non-random (61). Specifically, the relative proportion of small, $CD4^+$ T cells to small $CD8^+$ T cells, that is, the CD4/CD8 ratio, is higher in the lymph node and in efferent and afferent lymph than in blood. This phenomenon is not peculiar to the popliteal lymph compartments, since a study of the distribution of $CD4^+$ and $CD8^+$ T cells between the blood and the efferent and afferent lymph of prescapular lymph nodes yields quantitatively similar data (62). Additional data lends considerable support to the hypothesis that lymphocyte subset-specific lymphocyte-endothelial cell recognition mechanisms play a role in directing the recirculation of lymphocytes from blood to lymph. A comparison of the delivery of lymphocytes to prescapular lymph nodes via the blood (107) and the output of lymphocytes in afferent and efferent lymph has led to the conclusion that $CD4^+$ T lymphocytes are extracted from the blood by specialized vascular endothelium with greater efficiency than $CD8^+$ T cells (62). This hypothesis has been tested by directly monitoring the recirculation of fluorochrome-labeled, small $CD4^+$ and $CD8^+$ lymphocytes on a single transit from blood to lymph (108). In 3 experiments employing efferent prescapular lymph cells, 3 experiments employing efferent intestinal lymph cells, and 2 experiments employing afferent intestinal lymph cells, the mean CD4/CD8 ratio of the recirculated, fluorochrome-labeled population was higher than the CD4/CD8 ratio of the transfused starting population. Furthermore, in 6 experiments in which lymph compartments draining at least two different tissues were examined simultaneously, the recirculation of lymphocyte subpopulations according to tissue specificity (e.g., intestinal vs. subcutaneous) occurred independently of their reassortment according to lymphocyte subset specificity (109). These experimental findings indicate that tissue-specific and lymphocyte subset-specific lymphocyte-endothelial cell recognition mechanisms independently direct the recirculation of lymphocytes from blood to lymph. In addition, more recent data indicate that the differences in the relative extraction efficiencies of small, $CD4^+$ and $CD8^+$ cells are significantly altered in conditions of chronic inflammation (110).

LYMPHOCYTE TRAFFIC ENDOTHELIUM

By the mid 1960's, it had been established that lymphocytes migrate out of the blood by traversing specialized postcapillary venules (7,111). The original view that lymphocytes migrate through the specialized endothelial cells which line these vessels (111) did not sit well with many in the field. Eventually, Schoefl (112) reexamined the phenomenon in a meticulous

electron microscopic study of sections of rodent Peyer's patches and concluded that most, if not all, small lymphocytes leave the bloodstream by passing between adjacent endothelial cells. This finding was confirmed and extended by the elegant studies of Anderson and Anderson (113,114). Although there are still those who believe that lymphocytes may migrate directly *through* an individual endothelial cell, it is generally accepted that the physiological extravasation of lymphocytes within lymphoid and non-lymphoid tissues is an *inter-endothelial cell* event.

The postcapillary venules of rodent lymph nodes are lined by cuboidal, or 'high' endothelial cells; hence, the term 'HEV'. When lymphocyte migration was discovered to occur across these vessels (7), it was naturally assumed that this morphological peculiarity (7,111,115), was key to the large-scale emigration of lymphocytes (116-120). Yet, HEV are exceptionally rare in the lymph nodes of the athymic, nude rat, through which there is an extensive recirculation of lymphocytes from blood to lymph (121,122). Moreover, HEV are absent from mice raised under germ-free conditions, although their presence can be induced by immune interferon (123). Furthermore, although HEV are found in bovine lymph nodes (124), they are not normally present in any lymphoid tissue of the adult sheep (125). Curiously, blood vessels lined by cuboidal endothelial cells can be detected early in the development of fetal ovine lymphoid tissues (125), but they disappear prior to the establishment of lymphocyte recirculation. Finally, in experiments in which the supply of afferent lymph to subcutaneous lymph nodes in the rat was surgically interrupted, HEV appeared morphologically normal at a time when lymphocyte trafficking through the lymph node was markedly depressed (126,127); however, these HEV eventually flattened considerably (125-128). Although it is now very difficult to hold the position that the morphology of the endothelial cells which line the postcapillary venules of lymph nodes is intimately related in a mechanistic way with physiological lymphocyte recirculation (1,129), it is quite possible that this unique morphology may have an important, alternative physiological function, such as providing a mechanism for minimizing the leakage of plasma which occurs as lymphocytes extravasate between the endothelial cells in these tissues (17,18). To this date, the cause and function of the apparent intense metabolic activity of these specialized cells, as revealed by electron microscopic and histochemical studies (113,130-133), have not been elucidated.

Recently, Ager, (134,135) and Ise and colleagues (136) report the successful isolation, culture and characterization of high endothelial cells (HEC) from rat lymph nodes. These cells bind lymphocytes as efficiently as HEV in frozen sections and up to 50 times more efficiently than large vessel endothelial cells, suggesting that rat lymph node HEC constitutively express lymphocyte-binding molecules *in vitro*. Abernethy and Hay (137) recently report the isolation and culture of endothelial cells derived from the postcapillary venules of sheep mesenteric lymph nodes and Peyer's patches. *See Fig. 2.* Surprisingly, sheep lymph node microvascular endothelial cells and large vessel endothelial cells display similar lymphocyte-binding properties *in vitro*. Further studies involving cultured lymphoid tissue endothelial cells may yield useful insights into the adhesive interactions which mediate the migration of lymphocytes out of the bloodstream.

CELL-SURFACE MOLECULES INVOLVED IN LYMPHOCYTE MIGRATION

Studies on the Role of Cell-Surface Carbohydrates

The idea that the attachment of lymphocytes to specialized vascular endothelial cells is mediated by specific cell-surface structures was proposed in the 1960's. Since, by then, it was well known that cell adhesion could be mediated by the interaction of sugars and lectin-like

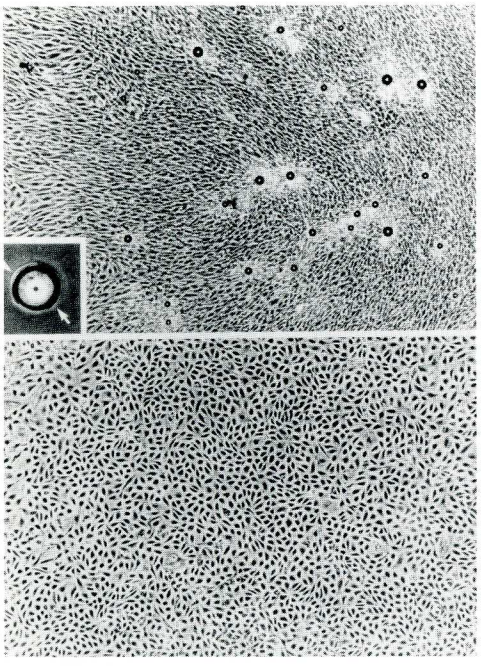

Fig. 2. Appearance of endothelial cells derived from the post capillary venule of sheep mesenteric lymph nodes, in culture. Upper panel: confluent primary culture showing elongated cells and many firmly anchored beads 3 weeks post-isolation, x26. Inset: single microcarrier bead with attached endothelial cells (arrows) 3 hours after perfusion and plating, x130. Lower panel: confluent subculture, fifth passage, showing cobblestone-like' morphology, x26. (Ref. 137, reprinted by permission of Oxford University Press).

molecules, a number of groups investigated the possibility that similar mechanisms were operative in lymphocyte-endothelial cell interactions *in vivo*. Essentially, three types of experiments were performed. In one, lymphocytes were exposed to one or the other of several glycosidases or sulphated polysaccharides, infused into the blood of syngeneic recipients, and their localization in lymphoid and non-lymphoid tissues examined (138-141). The second approach involved treating the recipient animal with various sulphated polysaccharides prior to transfusion of the lymphocytes (141). In these ways, it was possible to demonstrate altered patterns of lymphocyte migration. However, the interpretation that cell-surface sugars are involved in lymphocyte recirculation has always been subject to the criticism that the observed reduced localization of transfused lymphocytes in a particular lymphoid tissue may have resulted from an increased sequestration by non-lymphoid tissues, such as the liver and lung. This issue was addressed in the third experimental approach, which was engineered by Sedgley and Ford, who combined *in vivo* transfusion experiments with studies involving the perfusion of chains of mesenteric lymph nodes with lymphocytes *in vitro* (142). When lymphocytes were pretreated with trypsin, they failed to migrate into isolated, perfused mesenteric lymph nodes. Lymphocytes treated with neuraminidase migrated into the isolated lymph nodes in slightly increased numbers. Based on these observations and on data obtained from *in vivo* experiments, Ford and his colleagues concluded that the effect of neuraminidase was to increase the localization of transfused cells in the liver; this occurrence led to a decreased localization in lymphoid tissues (143). Using somewhat refined techniques, the original experiments of the 1970's were recently repeated (144,145) and qualitatively similar results were obtained. The results of a recent physiological study in sheep (146) strongly suggest that much of the effect of exogenous sulphated polysaccharides on the recirculation of lymphocytes is neither direct nor specific, but secondary to an increased secretion of corticosteroid hormones. Nevertheless, there is convincing evidence, obtained from recent studies of the binding of lymphocytes to endothelial cells in frozen sections of lymphoid tissue *in vitro*, that saccharide moieties, particularly phosphomannosyl molecules, may indeed be involved in lymphocyte-endothelial cell recognition (147-152). Additional studies, *in vivo* and *in vitro*, implicate a role for sialic acid in this process (153,154).

The first description of an endothelial cell molecule which might be important in lymphocyte recirculation arose out of

studies performed in rats by Andrews and Ford and their colleagues, who investigated the hypothesis that sulphated, cell-surface proteoglycans are important in the process of lymphocyte migration. It was found that the HEC of rat popliteal lymph nodes selectively incorporated subcutaneously injected ^{35}S-sulphate (155). The finding that the radiolabel was incorporated into a glycolipid, as opposed to a glycoprotein, was somewhat unexpected (155,156). Since the radiolabeled species could be found only in HEC, it was concluded that the sulphated molecule might have an important role in the process of lymphocyte recirculation. However, the incorporation of radioactive sulphate was found to be markedly reduced in the nude rat, despite approximately normal levels of lymphocyte recirculation. On the other hand, this molecule has been quite useful for identifying HEC grown *in vitro* (134-136).

An In Vitro *Model of Lymphocyte-Endothelial Cell Recognition: The Woodruff Assay*

A major development in lymphocyte migration research in the late 1970's was the description of an *in vitro* model, alluded to earlier, for studying the interaction between recirculating lymphocytes and specialized vascular endothelium (51,52). It was found that when rodent lymphocytes were layered onto 8 µm-thick sections of frozen lymph nodes, the lymphocytes obtained derived from thoracic duct lymph, lymph nodes, and spleen, but not cells from thymus or bone marrow. Since earlier studies, described above, had shown that thymocytes and bone marrow cells recirculate very poorly, it was felt that the *in vitro* assay provided meaningful, i.e., physiologically relevant, results. However, lymphocytes did not adhere well at temperatures above 7°C and, essentially, not at all at physiological temperatures. Because of this discrepancy, many researchers remained skeptical of its usefulness in providing insights into *in vivo* migration phenomena. In any event, the *in vitro* system offered a new and simple method for studying the interaction between lymphocytes and HEV and Woodruff and her colleagues were quick to capitalize on their novel development by showing that certain enzymes and drugs could modulate this interaction (158).

Lymphocyte "Homing Receptors"

In 1980, Woodruff's group described the isolation of a factor from thoracic duct lymph of the rat, which inhibited the binding of lymphocytes to HEV *in vitro* (159,160). A polyclonal antisera raised against this molecule stained thoracic duct lymph cells, but not thymocytes, and neutralized the inhibitory effects of the inhibitory factor. These data suggested that the molecule was "derived from shed TDL (thoracic duct lymphocyte) surface components that play a role in adherence to HEV of lymph nodes" and that it "binds to endothelial cell structures identical or closely related to those involved in lymphocyte attachment" (159). Later, it was found that transfusion of anti-inhibitor antibody into rats blocked lymphocyte migration into peripheral and mesenteric lymph nodes by 70% and 50% of control values, respectively, but had no effect on lymphocyte localization in Peyer's patches (161). This observation led to the conclusion that different HEV adherence molecules mediate lymphocyte entry into different types of lymphoid tissues. The inhibitory factor was eventually isolated from lysates of thoracic duct lymph T and B cells and termed "high endothelial binding factor" (HEBF). Shortly thereafter, another inhibitory factor was isolated from thoracic duct lymph. Unlike the first molecule, this factor modulated interactions between lymphocytes and the HEV of Peyer's patches, but not peripheral lymph nodes (163). This molecule was designated as $HEBF_{PP}$ (PP=Peyer's patches) and the original molecule was renamed $HEBF_{LN}$ (LN=lymph node). Later reports described mAbs raised against these molecules and their effects on the interactions of lymphocytes with the HEV of lymph nodes and Peyer's patches (164,165). It is important to

note that $HEBF_{LN}$ and $HEBF_{PP}$ are expressed on only 50-60% and 55-65% of rat thoracic duct lymph cells, respectively (dual expression apparently was not studied).

Weissman and his colleagues at Stanford were among those investigators who believed that physiologically relevant data could be garnered from studies employing the Woodruff assay. By modifying the assay for studies involving murine tissues (50), it was possible to study tissue-specific and lymphocyte subset-specific lymphocyte-endothelial cell recognition phenomena (49,89). In 1983, Gallatin, Weissman, and Butcher (166) described the production of mAb, MEL-14, which selectively blocked the interaction of lymphocytes with the HEV of peripheral lymph nodes, but not other tissues, *in vitro* and *in vivo*. It is interesting to note that the antibody was raised against a cloned murine B cell lymphoma line (38C-13), but did not inhibit lymphocyte interactions with the HEV of Peyer's patches, even though B cells were previously found to migrate preferentially through these structures. The finding that the antigen recognized by MEL-14 is heavily ubiquitinated (167,168), which is a trait common to many cell-surface proteins on diverse cell types, initially caused some confusion. However, the recent discovery of structural relationships between lymphocyte "homing receptors" and receptors for extracellular matrix molecules, described below, has shed new light on this finding and there is little doubt that MEL-14 is a physiologically relevant molecule in lymphocyte recirculation. Indeed, the recent discovery of a lectin domain in the primary structure of this molecule (169,170) explains several previous observations on the effects of carbohydrates on lymphocyte-HEV interactions mediated by MEL-14 (150).

The MEL-14 mAb has been widely used to study tissue-specific and species-specific lymphocyte-endothelial cell interactions. The antibody selectively blocks the interaction of human lymphocytes with lymph node HEV, but not with mucosal or synovial HEV (171,172). Also, there appears to be a significant degree of evolutionary conservation in MEL-14 mediated interactions with HEV (173). Recently, it was found that the selective recognition of mucosal lymphoid tissue high endothelium by murine gut intraepithelial lymphocytes is not mediated by the MEL-14 molecule (174). Finally, the expression of different forms of the MEL-14 antigen on other leukocytes, including neutrophils, monocytes, and eosinophils, as well as the inhibition of neutrophil extravasation at acute dermal inflammatory sites by MEL-14 mAb (175), suggests that this molecule is involved in directing the migration of a diverse array of blood-borne cells.

Several years after the discovery of MEL-14, Butcher and his colleagues described a mAb specific for a lymphocyte cell-surface glycoprotein (Hermes-1) presumed to be involved in endothelial cell recognition and lymphocyte homing in man (176) and, more recently, in the macaque (177). Although the Hermes-1 epitope is not involved in endothelial cell recognition, another mAb raised against the Hermes-1 antigen and the determinant recognized by the second antibody (Hermes-3) does selectively block the binding of lymphocytes to mucosal HEV (172,178). The Hermes-1 glycoprotein has a molecular weight (85-95 kilodaltons) similar to the MEL-14 antigen (80-92 K) and MEL-14 antibody cross-reacts with Hermes-1, findings which suggest a close evolutionary relationship between these two antigens. However, recent nucleic acid sequencing data has conclusively established that the MEL-14 and Hermes-1 molecules are completely unrelated (169,179-181). In fact, it has recently been found that mAbs against the CD44 and Pgp-1 antigens in man recognize the Hermes glycoprotein (182) and it now appears that the Hermes antigen is a member of a family of cartilage link proteins (179-181). On the other hand, the primary structure of MEL-14 encodes a lectin domain, an epidermal growth factor-like domain, and an extracellular domain, precisely duplicated tandem repeat homologous to that seen in complement regulatory proteins (170).

Holzmann, McIntyre and Weissman (183) have reported the identification of murine Peyer's patch-specific lymphocyte "homing receptors" (LPAM-1 and LPAM-2). One of these heterodimers (LPAM-2) appears to be the murine analogue of VLA-4 (179), which is a member of the integrin superfamily of adhesion molecules (185-190). LPAM-1, which shares the same subunit as LPAM-2, was initially thought to be the analogue of VLA-4 (183), but more recent data indicate that it is distinct from other integrin molecules (184).

Finally, a putative lymphocyte "homing receptor" (CT4) has been identified in the guinea pig (191). This molecule (molecular weight: 32-36 K) appears to be completely unrelated to any of the other "homing receptors" which have been described to date. Whether or not this molecule is structurally related to the subunits of integrin- or immunoglobulin-like molecules is not known.

Vascular Endothelial "Addressins"

Monoclonal antibodies which recognize antigens on endothelial cells involved in lymphocyte recirculation have been described only in the last two years (192-196). MECA-325 is an antigen expressed only on HEV in the mouse (192). The antigen is not normally expressed on endothelial cells lining blood or lymphatic vessels in any non-lymphoid organ examined so far. However, the antigen is expressed by HEV induced in dermal granulomas about one week after initiation of such lesions. MECA-325 appears *not* to be directly involved in lymphocyte-endothelial cell recognition, since the mAb specific for this antigen does not inhibit the binding of lymphocytes to MECA-325$^+$ HEV (192).

The murine endothelial cell molecules MECA-367 and MECA-79 appear to be crucially involved in tissue-specific lymphocyte-endothelial cell recognition *in vitro* and *in vivo*. As a result, they have been termed vascular "addressins" by Butcher and his colleagues. MECA-367 is an antigen expressed on HEV in mucosal lymphoid tissues, such as Peyer's patches and mesenteric lymph nodes, but not in non-mucosal lymphoid tissues, such as peripheral lymph nodes (194). Three lines of evidence indicate that MECA-367 is involved in tissue-specific lymphocyte-endothelial cell recognition *in vivo*. First, mAb to MECA-367 almost completely inhibits the binding of lymphocytes to Peyer's patches, but has no effect on the binding of lymphocytes to peripheral lymph nodes. Second, transfused MECA-367 mAb completely inhibits lymphocyte localization in Peyer's patches, but has no effect on localization of lymphocytes in peripheral lymph nodes. Third, lymphocytes bind to supported phospholipid planar membranes containing MECA-367, but not to membranes containing other, unrelated molecules, such as glycophorin (195), and this binding is selectively blocked by anti-MECA-367 mAb. MECA-79 appears to be the peripheral lymph node analogue of MECA-367. Monoclonal antibody to MECA-79 defines an antigen expressed at high levels on the HEV of peripheral lymph nodes and almost completely blocks recognition events involving lymphocytes and peripheral lymph node, but not Peyer's patch, HEV *in vitro* and *in vivo* (197). Because the mAb specific for MECA-79 is of the IgM isotype and has a low affinity for the MECA-79 antigen, it has not been possible to immunoprecipitate the antigen and insert it into planar membranes. Nevertheless, the evidence obtained thus far convincingly suggests that this molecule is involved in tissue-specific lymphocyte recirculation *in vivo*.

The identification of murine vascular "addressins" has been particularly useful in explaining previously reported observations on tissue-specific lymphocyte-endothelial cell recognition in this species (49). Cells obtained from either Peyer's patches or peripheral lymph nodes interact with HEV in the other tissue very poorly, if at all. However, cells from both of these tissues interact with the HEV of mesenteric lymph nodes to an intermediate extent. It is now

known that subsets, or portions, of HEV in mesenteric lymph nodes stain exclusively with either MECA-79 mAb or MECA-367 mAb, but that most high endothelial cells in mesenteric lymph nodes express *both* antigens (197). One would expect, then, both Peyer's patch-specific and peripheral lymph node-specific lymphocytes to migrate through the mesenteric lymph nodes. This is precisely what is observed: both mAbs only partially (50%) inhibit the interactions of lymphocytes with the HEV of mesenteric lymph nodes *in vitro* and *in vivo*. The immunological significance of the dual expression of the vascular "addressins" in mesenteric lymph nodes is not known, but it has been proposed that the non-uniform expression of MECA-367 on the HEV of mesenteric lymph nodes represents clonal or microregional regulation of the antigen, possibly by mucosa-derived factors arriving in the lymph nodes via afferent intestinal lymph (194).

Accessory Cell Adhesion Molecules

There is convincing experimental evidence that additional cell adhesion molecules are involved in the specific lymphocyte-endothelial cell interactions which mediate lymphocyte trafficking. One of these accessory molecules is lymphocyte function-associated antigen-1 (LFA-1). Originally described in the early part of this decade as an effector molecule required for the adhesion of cytotoxic T cell clones to their targets (198,199), this heterodimer shares the same b subunit (95 K), later designated as CD18, as the complement receptor C3bi (Mac-1) and p150,95 (200) and is now known to be a member of the integrin superfamily of adhesion molecules (185). Significantly, the latter glycoprotein heterodimer is absolutely required for neutrophil emigration *in vivo* (201-205) and is involved in endothelial cell binding of monocytes (205), basophils (206), and eosinophils (207,208) *in vitro*. The binding of lymphocytes to cultured human umbilical vein endothelial cells (HUVEC) is inhibited by mAb to LFA-1 by 60-75% (209,210). Since lymphocyte extravasation occurs exclusively across the endothelium of postcapillary venules, the physiological relevance of this finding is questionable. However, it has recently been shown that a mAb specific for LFA-1 partially inhibits the interactions of lymphocytes with HEV in both mice (211) and humans (212). Unlike the patterns of inhibition observed with lymphocyte "homing receptors", such as MEL-14 and Hermes-3, the LFA-1-mediated lymphocyte-endothelial cell interaction is not tissue-specific.

It is now known that there are least two ligands for LFA-1, neither of which is related to any of the lymphocyte "homing receptors" described to date. One of these is the ubiquitous and heavily glycosylated, intercellular adhesion molecule-1 (ICAM-1, CD54). ICAM-1 was identified (213) in 1986 as a molecule induced on HUVEC stimulated with interleukin 1 (IL-1) or gamma interferon (IFNγ), and recognized as a ligand for LFA-1 shortly thereafter (214-216). A particularly surprising development (214,217,218), but one which certainly excited students of neuroimmunology, was the discovery that ICAM-1 is homologous to neural cell adhesion molecule (NCAM), which has recently been shown to be a member of the Ig supergene family of adhesion molecules (219). The expression of ICAM-1 *in vivo* raises questions about the physiological relevance of this molecule in lymphocyte recirculation. Most notably, under normal conditions, ICAM-1 is expressed only at very low levels on endothelial cells lining the postcapillary venules of lymph nodes (213). However, the endothelial cells of blood vessels in T cell areas of lymph nodes and tonsils showing reactive hyperplasia express relatively high levels of ICAM-1. It is conceivable, therefore, that this molecule and, by association, LFA-1, might be of greater significance in lymphocyte-endothelial cell interactions during pathological conditions. A second ligand for LFA-1 in lymphocyte-HUVEC interactions, ICAM-2, has recently been identified through a beautiful series of

experiments (220). Sequencing and molecular weight data indicate that this molecule is not a member of the Ig superfamily.

Potentially one of the most relevant molecules to lymphocyte migration is vascular cellular adhesion molecule 1 or VCAM 1. This substance may turn out to be more than an accessory molecule. It is inducible on endothelial cells and it binds to VLA 4 on the surface of lymphocytes and monocytes (221). It has also been cloned from human and animal sources (222) and the effects of blocking this molecule on the migration of cells *in vivo* is being attempted in several systems.

Trying to achieve a unifying nomenclature Hynes and Butcher recently proposed the following selection naming scheme. The term for the peripheral lymph node homing receptor (formerly MEL 14), recently designated LECAM-1, becomes the L-selection (for leukocyte selection), GMP140 the P- (for platelet) selection, and ELAM-1 the E- (for endothelial) selection.

THE ROLE OF INFLAMMATORY CYTOKINES IN LYMPHOCYTE MIGRATION

Inflammatory cytokines comprise another group of molecules which may be involved in the regulation of lymphocyte migration. Of the many soluble mediators which have been described thus far, tumor necrosis factor alpha (TNFα), interferon alpha and gamma (IFNα and IFNγ) appear to be the most likely candidates with respect to modulation of lymphocyte traffic *in vivo* . While there is one report which describes altered lymphocyte migration following treatment of lymphocytes with cytokines (223), most of the data on this subject has come from experiments in which lymphocyte migration has been measured following pretreatment of the recipient with one or the other of these molecules. Following intravenous infusion, IFNα causes a generalized peripheral lymphadenopathy in mice (224). In studies performed in rats, radiolabeled, peritoneal exudate lymphocytes migrate, in a dose-dependent manner, into skin sites containing IFNα, IFNγ, and TNFα (225-227). Of these molecules, only recombinant bovine IFNγ and recombinant human or bovine TNFα recruit lymphocytes into the skin of sheep (228). *See Fig. 3.* While TNFα is much more potent than IFNγ in this respect, human TNFα is at least as effective as bovine TNFα. It is not yet known whether sheep TNFα will be more potent. Sheep TNFα has been cloned by Young et al (229) from our laboratory but sufficient material is not yet available to test *in vivo*. Intravenously infused or intradermally injected IFNα inhibits the migration of lymphocytes out of, but not into, lymph nodes (230, 231), thereby corroborating the findings of Gresser et al (224) in mice. How these cytokines exert their effects has not been conclusively established; however, it is widely suspected that they induce or up-regulate the expression of lymphocyte-

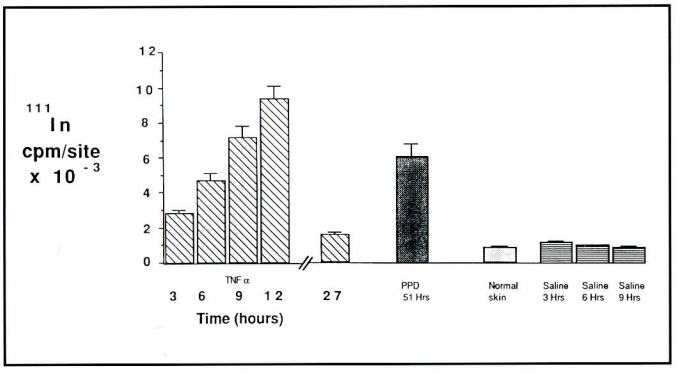

Fig. 3. The localization of 111-indium labeled lymphocytes in skin test sites of sheep. The maximal entry into TNFα sites is near 12 hours. The positive control (PPD) is a tuberculin reaction near the time of maximal lymphocyte entry produced following BCG priming. (Ref. 230, reprinted by permission of Elsevier Science Publishers).

binding, endothelial cell-surface molecules. Much of the evidence in support of this view has come from studies on the binding of lymphocytes to cytokine-treated HUVEC (232,233) and dermal (234) or cerebral (235) microvascular endothelial cells *in vitro*. There is evidence that the increased binding of lymphocytes to cytokine-treated, cultured endothelial cells is mediated by the interaction of LFA-1 with endothelial cell-surface ICAM-1 (209,210,213,215). Two lines of evidence initially raised serious doubts about the physiological relevance of the results of these *in vitro* studies to mechanisms involved in lymphocyte migration *in vivo*. First, in both normal and pathological conditions, lymphocyte extravasation occurs across specialized, postcapillary venule endothelium, not across large vessels or arterial microvessels (16,27,111,114). Second, lymphocyte traffic through the skin is an order of magnitude, and through the brain several orders of magnitude, less than that through lymphoid tissues (14,16). However, two other lines of evidence indicate that studies of the binding of lymphocytes to 'irrelevant' endothelial cells may provide useful insights into *in vivo* phenomena. First, such studies have led to the identification of an inducible endothelial cell molecule (ELAM-1, endothelial-leukocyte adhesion molecule-1) involved in neutrophil emigration *in vivo* (236-242). Second, the endothelia of lymphoid tissues showing reactive hyperplasia express high levels of ICAM-1 (213). It is therefore reasonable to suggest that cytokines exert their effect on lymphocyte migration *in vivo* by inducing or up-regulating the expression of ICAM-1 and related molecules on the surface of endothelial cells involved in lymphocyte extravasation.

Memory Cells

From cell transfer experiments it has been evident that specific immunological memory in a variety of systems is a property of recirculating lymphocytes. Memory cells are present at higher frequencies compared with their naive precursors. Two models of memory have been described. The first states that memory is due to the long-lived nature of such lymphocytes. The second states that persistent antigens continually stimulate the proliferation of memory cells. Memory cells proliferate *in vitro* in response to recall antigens, they proliferate in the presence of monoclonal antibodies directed against CD3 and CD2, and although a complete distinction from naive cells is not seen, memory cells appear to show differences in cytokine production compared to naive cells. For example, memory cells appear to synthesize considerably more IL-4 and gamma interferon and less IL-2 than naive cells (243).

In a variety of species including man, rat, mouse, and sheep, naive and memory T cells can be distinguished on the basis of 2 different isoforms of the leukocyte common antigen (CD45). One population of T cells express the RA isoform, and such cells exhibit low levels of molecules known to elicit cell-cell adhesion. These are the naive cells. The other cells which express the RO isoform of CD45 also express increased levels of adhesion molecules including, CD2, LFA-1, LFA-3, CD44, ICAM-1, and the very late antigens VLA-4,5 and 6 (243,244).

By taking advantage of these identifying surface markers, Mackay has been able to compare the migratory characteristics of these two populations in sheep (245). These data show that T cells in peripheral afferent lymph draining tissues such as skin are entirely of the memory phenotype but that the T cells in efferent lymph are overwhelmingly of the naive phenotype. Most of these naive cells migrate from the blood via the postcapillary venules within the lymph node. He suggests that it is significant that memory cells predominate at places like epithelial surfaces. There is even the suggestion that the gut/non-gut pattern of selective migration is due to memory and naive T cells respectfully. As previously discussed, this selective pathway does not develop until after birth, and it is probably antigen driven.

Memory is a fundamental aspect of the

immune response and the migratory properties of memory cells is clearly interesting and important. However, recent data from Cahill et al (246) demonstrate that there is a non-random distribution of T cells in the sheep fetus which cannot easily be explained by a memory/naive scheme. They found a very high concentration of CD8 cells circulating through the ileal lymph node and the ileum in the fetus in a situation where they are clearly not memory cells. They argue therefore that one cannot explain T cell migratory behavior solely in terms of antigen recognition and immune effector functions as we presently think of them. Another very interesting subset of T cells are those bearing the gd T cell receptor phenotype. In adult animals they are thought to be involved in a surveillance system for infected or transformed cells at epithelial surfaces (247). During ontogeny, these cells form very close associations with Hassall's corpuscles in the fetal thymus. The ratio of gd to ab T cells in the blood of the fetus increases 4-5 fold in the month before birth and doubles in the first month of postnatal life at which time 70% of the T cells in the blood express the gd receptor (248).

The design of new experiments to better understand the complexities of lymphocyte migration depends upon the application of new technologies. Over the years our laboratory and many others have used radioactive labels such as 111-indium or 51-chromium to follow lymphocytes *in vivo*. More recently, direct, vital labeling with fluorescent dyes has made possible experiments which permit the simultaneous use with monoclonal antibodies directed against lymphocyte subsets to define subset migratory patterns. These labels, for the most part, bind to proteins in and on cells. Fluorescence intensity is lost as proteins turn over in cells. This means that such cells can be followed *in vivo* for about one week before the intensity is too low. This of course refers to situations where optimal labeling conditions are used. It is well known that overlabeled cells are incapable of migrating from blood to lymph and it is therefore an important aspect of these types of experiment that recovery data are presented.

A newly developed and marketed family of dyes (PKH dyes) which intercolate in the plasma membrane (Zynaxis, Cell Science) permit experimental protocols which have previously not been possible. We have shown that lymphocytes can be followed *in vivo* for several weeks and probably for 3 months using these PKH dyes (249). *See Fig. 4.* We have not found that such labeled cells behave differently than unlabeled cells. It is now possible to measure the circulating half life of various subsets of lymphocytes and to begin to study the effects of a variety of cytokines on the migratory behavior of the individual subsets.

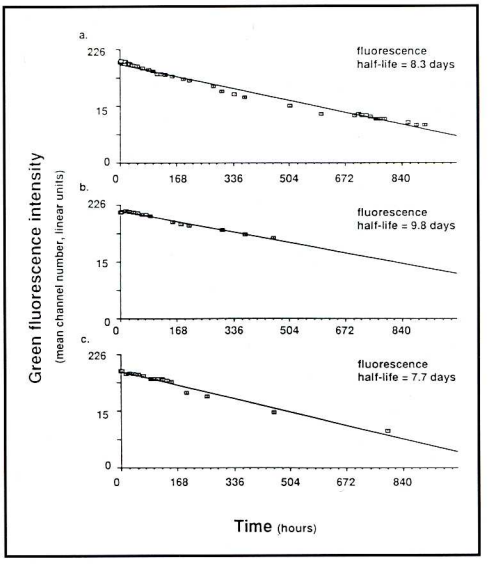

Fig. 4. Mean fluorescence intensity of recirculating lymphocytes labeled in 10μM PKH-2. Lymphocytes collected by chronic lymphatic drain were fixed in paraformaldehyde and analyzed by flow cytometry. Results from three experiments in two separate sheep are shown; (a) sheep T04, first injection of labeled lymphocytes; (b) sheep T04-labeled lymphocytes injected 26 days after the first injection; (c) sheep T05. The half-life of fluorescence intensity is remarkably consistent between experiments, with a mean of 8.6 days. (Ref. 249, reprinted by permission of Academic Press).

CONCLUSIONS

The lymphocyte "homing receptors" and vascular endothelial "addressins" which have been described only in the last few years provide a molecular basis for some of the patterns of lymphocyte trafficking which have been observed over the years. However, the recirculation of lymphocytes from blood, through the tissues, and into lymph is a physiological process which involves more than the specific recognition of a vascular endothelial "addressin" by the "homing receptor" of a blood-borne lymphocyte. This recognition event, which is dynamic in its own right (250), is only the first in a sequence of events which must occur prior to the subsequent export of the cell into lymph. Once firmly attached to the endothelial cell surface, the lymphocyte must then migrate between adjacent endothelial cells and penetrate the underlying basement membrane to gain access to the parenchyma of the tissue. The stimuli and mechanisms involved in this directed migration are largely unknown. In the case of lymphoid tissues, such as lymph nodes, Peyer's patches, and the spleen, distinct T and B cell subsets appear to segregate into specific compartments. How and why this differential compartmentalization occurs is a complete mystery. Furthermore, the interactions which most assuredly occur between an extravasated lymphocyte and the various stromal cells or extracellular matrix components of the tissue will no doubt determine, perhaps to a large extent, how long the cell remains within the substance of the tissue prior into its exit in lymph. When one considers how antigen sequestered in lymph nodes alters patterns of lymphocyte migration (251,252), the situation becomes even more complicated. If the migration of many different lymphocyte populations is mediated by the interaction of cellular adhesion molecules on the surface of both the lymphocytes and the endothelium then there must be many more molecules or combinations of molecules than are presently apparent. There will always be a need for more hypothesis testing and the design and execution of well controlled experiments *in vivo* as well as a continuation of a reductionist approach identifying more molecules and lymphocyte subset differences. There is very little basic information on the migration of lymphocytes in humans and, with the development of new methodologies for tracking cells, this should be a fruitful area for the future.

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