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Recirculation of Lymphocytes: Its Role in Implementing Immune Responses in the Skin

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Summary

A number of recent investigations have suggested that the subset of lymphocytes which migrate into non-lymphoid tissue to appear in peripheral lymph may be partly different from the major recirculating pool migrating through lymphoid tissues. We report results on the migration from the blood of thoracic duct lymphocytes labelled with ^{51}Cr and three subsets-accredited recirculators, activated lymphocytes and „long-lived" lymphocytes. The localization of these populations was studied in normal skin, in a contact sensitivity lesion and in a site of non-immune inflammation. All four populations localized in the contact sensitivity lesion in increased numbers compared to normal skin but „long-lived" lymphocytes appeared to discriminate between cell-mediated immunity and non-immune inflammation; activated lymphocytes migrated most efficiently into the non-immune inflammatory site (Table 4).

Introduction

Lymphocyte recirculation refers to the process by which lymphocytes leave the blood by crossing vascular endothelium and later return to the blood by a different route. The operational term „localization" simply refers to the presence of cells at a particular time and place. Higher localization at a particular site means that more cells were found there without prejudice to the processes involved which may be increased influx, decreased efflux or the net effect of changes in both influx and efflux.

The major sites of lymphocyte recirculation are the spleen, the bone marrow, lymph-nodes and gut-associated lymphoid tissue (Fig. 1). This symposium is principally concerned with lymphocyte migration through nonlymphoid tissue as an essential process in immunity especially in cell-mediated immune responses. The implementation of such responses, for example in the skin, is thought to depend on the migration of sensitized T lymphocytes into regions of antigen deposition. If the T lymphocytes leaving the blood for all sites are considered probably only about 10% of them migrate into non-lymphoid tissue. Since the spleen and lymph-nodes account for about 1% body weight the migration of lymphocytes into these organs per unit weight is greater than into non-lymphoid tissue by a factor of 1000 or more (1).

There are also consistent differences between different non-lymphoid tissues in the flux of lymphocytes leaving blood-vessels. The greatest traffic is in the liver, small-intestine and ovary; the least detectable flux is in the brain (2). In general lymphocytes return from the interstitium of tissues via the afferent lymphatics to lymph-nodes and subsequently via efferent lymphatics to the blood. The average time taken for lymphocytes to traverse a lymph-node from afferent to efferent lymphatic is substantially shorter than the average sojurn in lymph-nodes of the majority of recirculating cells which leave the blood by crossing high-endothelial venules (HEV) in the paracortex (3). This may possibly be because the afferent to efferent route is completely within lymphatic spaces whereas the HEV to efferent lymphatic route involves negotiating a labyrinth of sessile cells and reticulin.

The question of whether the minority traffic through non-lymphoid tissue is a completely representative sample of the whole recirculating lymphocyte pool is of crucial importance but the data on this point are limited. It is easiest to obtain lymphocytes in large numbers from efferent lymph but of course these cells had previously arrived at the node by two different routes, afferent lymphatic and HEV (4), and their properties may therefore be the resultant of contributions from two unequal populations. However there is a body of circums-

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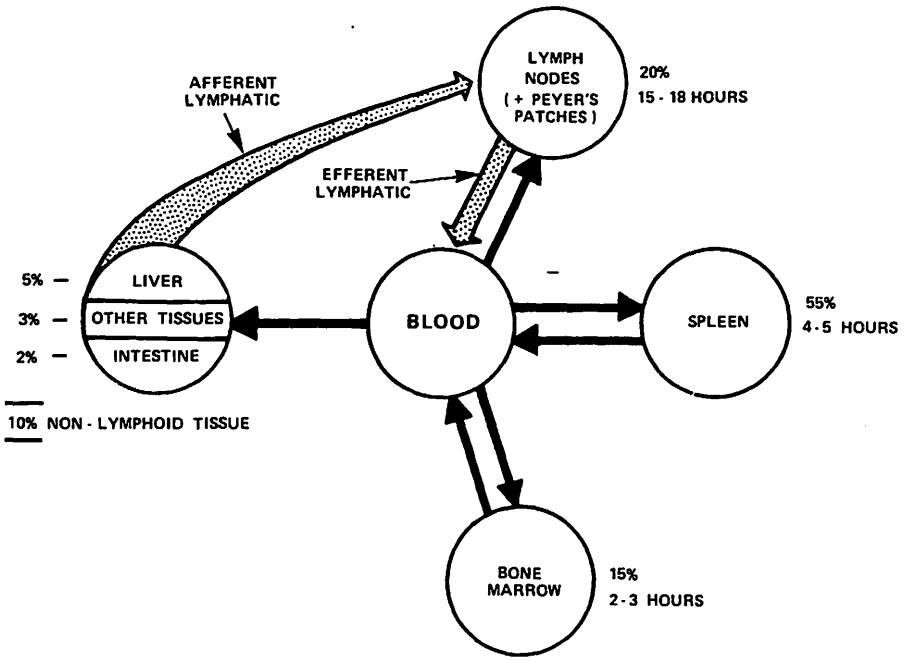

Fig. 1 Simplified diagram of the recirculating lymphocyte pool with five main compartments. The values refer to recirculating T lymphocytes in an adult rat. The upper figure is the approximate % of T cells which leave the blood to enter that compartment. The lower figure is the approximate modal transit time.

tantial evidence that distinct subsets of lymphoid recirculators and non-lymphoid recirculators do exist probably with some overlap. First whereas B lymphocytes migrate well into the lymph-nodes and spleen leaving the blood by the same route as do T lymphocytes (5, 6) they migrate poorly into non-lymphoid tissue since a much smaller proportion of Ig + ve small lymphocytes is found when the cells in afferent lymph of sheep (7) and of man (8) are compared to efferent lymph. Second the Ig -ve cells of afferent lymph in sheep display much reduced immune reactions to histocompatibility antigens compared to the Ig -ve cells in efferent lymph (7). Third there is a greatly increased proportion of T cells bearing Ia antigens in human afferent lymph compared to T cells in the blood (9). Finally the discriminatory migration of Ig -ve lymphocytes in the sheep can best be explained by subsets of T cells which migrate preferentially across either HEVs into lymph-nodes or into the gut (and perhaps other non-lymphoid tissues) and so reach lymph-nodes via afferent lymphatics (10).

Most quantitative studies of the traffic of lymphocytes in non-lymphoid tissues have been performed by cannulation of peripheral lymphatics as pioneered in the sheep by *Morris* and his colleagues (11) or alternatively in smaller animals by transferring radioactively labelled lymphocytes by i.v. injection followed by excision of certain non-lymphoid tissues for radioactive counting and autoradiography at different time intervals (e.g. 12). A recent methodological study (2) suggested that in this context ^{51}Cr sodium chromate was the radioactive label with fewest disadvantages for lymphocyte migration studies. The need for extensive vascular perfusion of the whole carcase in order to minimise radioactivity that is no longer associated with the injected cells and also to remove intravascular lymphocytes was stressed. The results of applying this technique to lymphocyte traffic into normal and inflamed skin of the rat are reported here.

Materials and Methods

Animals Adult rats of either of the inbred strains AS or (DA x PVG/c)F_1 were used as donor and

recipients of lymphocytes. Cells were transferred between syngeneic rats.

Isotopes Sodium chromate (^{51}Cr) (CJS.IP), 5-(^{125}I) iodo-2-desoxyuridine (IM.352) and (6-^{3}H)-thymidine (TRA.61) were obtained from the Radiochemical Centre, Amersham, U.K.

Thoracic duct cannulation was performed and lymphocytes were collected as described by Ford (13).

Whole body perfusion Immediately after death all recipients were perfused via an aortic cannula with 250 ml PBS as previously described (2).

Cell preparation and labelling Thoracic duct lymphocytes (TDL) were collected on ice for periods of up to 16 hours. After centrifugation these were resuspended at 3 x 10^7/ml in PBS, layered on to a 5:2 mixture of 14% Ficoll (Pharmacia) and Hypaque (45% Na diatrozoate – Winthrop) and centrifuged at 1200 g for 20 min at 15 °C (14). The TDL in the interface were then free of contaminating erythrocytes. They were washed in PBS and resuspended for labelling at 10^8/ml in RPMI 1640 + 10% FCS (Gibco-Biocult). The TDL were incubated for 1 h at 37 °C in a gently shaking waterbath in the presence of 10 μCi/ml of ^{51}Cr *or* 1 μCi/ml ^{125}IUdR. The cells were then washed x 3 in PBS before being layered onto 5 ml of 50% FCS/PBS and centrifuged for a final time at 350 g for 10 mins before resuspension in 2.0 ml for i.v. injection.

Accredited recirculating lymphocytes were obtained by i.v. injection of ^{51}Cr labelled TDL into a syngeneic rat with a pre-existing thoracic duct fistula. The TDL collected from this intermediate rat over the next 2–16 hours were used. They included approximately 10% of the injected radioactivity.

Long-lived recirculating lymphocytes were labelled in vivo by administration of ^{3}H-thymidine (1 μCi/g body wt/day i.p.) daily for three weeks to prospeotive donors (15). These were cannulated 3 weeks after the last injection of isotope. TDL were centrifuged once before injection.

Contact sensitisation and challenge

Table 1 lists the agents, dilutions and diluents used.

Tab. 1

Agent	Sensitizing Dose	Challenge Dose
1, Chloro 2, 4. Dinitrobenzene[a] (DNCB)	5%[c]	0.5%[c]
Picryl Chloride[a] (TNCB)	7%[c]	1.0%[c]
4 Ethoxymethylene-2-Phenyl[a] Oxazolone (OXAZ)	5%[c]	0.5%[c]
Croton Oil[b]	10%[d]	10%[d]

a) B.D.H. Chemicals Ltd., Poole, Dorset.
b) Sigma Chemical Co., P.O. Box 14508, St. Louis, U.S.A.
c) dissolved in absolute ethyl alcohol.
d) dissolved in Di-n-butylphthalate – a).

Rats were sensitized by application of 0.1 ml of one of the agents to the clipped abdominal skin. Secondary lesions were elicited by application of 0.1 ml of the appropriately diluted agent to a circular area (Ca. 1.5 cm diam) of clipped skin on the back twelve days later. At this time similar areas were painted with challenge doses of the other agents. Thus recipients were challenged with the agent to which they had been sensitized, two other sensitizing agents and croton oil which is an irritant but shows no evidence of increased response on secondary challenge. Challenge was normally 24 hrs before injection of labelled cells. DNCB was usually used as the specific sensitizing agent although all three agents induced an increased localization of lymphocytes in skin sites of secondary challenge.

The specific contact sensitivity lesion was an area of erythema with little induration. Histologically a mononuclear cell infiltrate of moderate intensity was seen throughout the dermis with occasional superficial foci of polymorphonuclear leucocytes. Lymphocytes were present but did not predominate. The maximum intensity of the inflammation was seen 24–48 hrs after challenge and the lesion

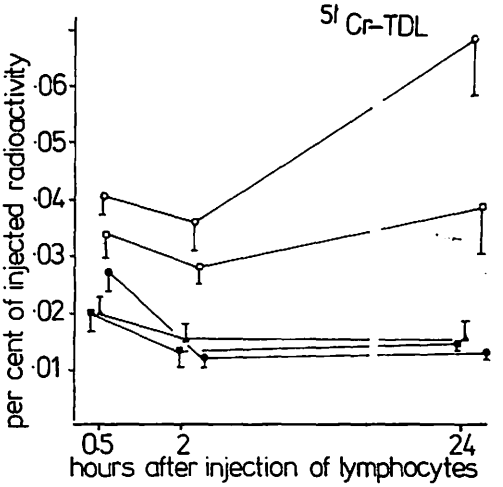

Fig. 2 Radioactivity measures in skin at different intervals after the injection of labelled lymphocytes. Mean ± S.E.M. ●——● – normal skin. ○—○ – site of challenge with DNCB (secondary application). □—□ – site of croton oil application. ▲—▲ – site of primary application of OXAZ. ■—■ – site of primary application of TNCB. 2a – TDL labelled with ^{51}Cr sodium chromate.

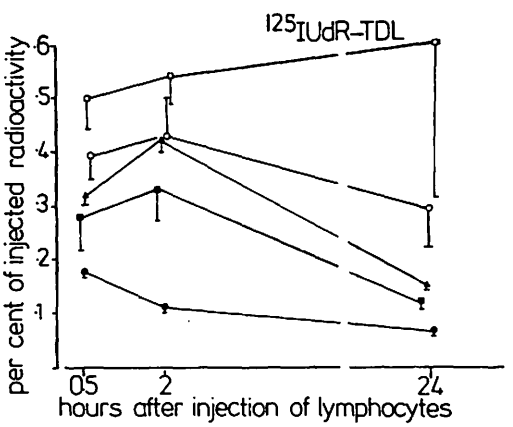

Fig. 2b TDL labelled with ^{125}IUdR.

remained roughly the same for a period of 48 hrs.

Skin painting with croton oil produced erythema and oedema. Secondary application of this agent produced a lesion of similar intensity as primary application with no increased localization of labelled cells.

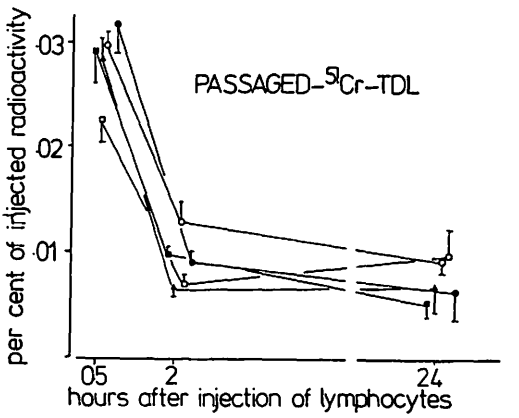

Fig. 2c TDL labelled with ^{51}Cr-sodium chromate before passage from blood to lymph in an intermediate rat.

Primary application of challenge doses of DNCB, TNCB or OXAZ produced no response detectible by inspection.

Tissue sampling and measurement of radioactivity

After whole body perfusion tissues were sampled by careful dissection, weighed and assayed for total gamma radioactivity in an LKB Wallac Ultragamma dual channel deep well -scintillation spectrometer which allowed simultaneous counting of ^{125}I and ^{51}Cr.

Heparinized blood samples of 4.0 ml were taken under ether anaesthesia just before death; 2.0 ml was diluted and centrifuged over *Ficoll/Hypaque* for the leucocyte layer; the other 2.0 ml was centrifuged for plasma. The draining perfusate was also assayed for radioactivity in both cells and fluid. Liquid scintillation counting of tissues for ^{3}H activity was performed as previously described (13).

Results

The localization of labelled lymphocytes in normal skin and areas which had been painted with various inflammatory agents (Table 1) is represented in Fig. 2. Three populations of TDL were studied – a) TDL labelled with ^{51}Cr which is not known to discriminate between different cell-types except in so far as large cells are labelled more intensely than

small cells, b) ^{125}IUdR which labels only activated cells in S-phase (a small minority of TDL), and c) ^{51}Cr labelled TDL which had recirculated from blood to lymph in an intermediate recipient. This manouevre removed not only most of the activated cells but also those small lymphocytes which did not recirculated by this route during the collection period. Recipients were killed at 0.5 hours after injection – when the tissue distribution largely reflected the initial distribution of cells from the blood; 2 hours – when the spleen and bonemarrow were near their maximum uptake; and at 24 hours – when the injected cells were approaching equilibrium with the recirculating lymphocyte pool.

Considering first the whole population labelled with ^{51}Cr the highest values for skin localization were found at 0.5 h with a definite fall at 2 h followed by little change at 24 h. This pattern of maximum activity early after injection was found in most non-lymphoid tissues (2). It suggested that many of the lymphocytes which had entered the tissue at the time of the high concentration in the blood had already left it a few hours later. The is concordant with the rapid blood → tissue → afferent lymph transit time recorded in the sheep (16). The sites of application of contact sensitizers in non-sensitized recipients gave almost the same values as normal skin. Croton oil-induced inflammation produced higher values with a similar temporal pattern, whereas the DNCB challenge site produced the highest values at all time intervals. In this case there was a smaller relative fall between 0.5 h and 2 h and the maximum value was reached at 24 hours. The progressive increase in the augmented localization due to challenge suggested that as well as the influx from the blood being increased migratory lymphocytes were retained in the lesions for longer than in normal skin.The increased localization of ^{51}Cr labelled lymphocytes in sites of contact sensitivity has been found in previous investigations (17, 18).

In other experiments the localization of ^{51}Cr labelled TDL was measured at 48 and 72 hours as well as at 24 hours after injection. The values in normal skin and the specific recall DNCB lesions remained almost the same over that period with approximately 10 times higher values in the lesions. This is attributable to equilibration with the recirculating pool having been almost reached by 24 hours. Labelled cells presumably continued to migrate through the DNCB lesions increased numbers over 72 hours. The localization in the croton oil lesion had progressively decreased by 48 h and 72 h no doubt because the inflammation was subsiding. (Detailed results not given).

Since activated or large lymphocytes incorporate somewhat more ^{51}Cr than do small lymphocytes (19, 20) all of the difference between the inflamed sites might be attributable to this minority. After i.v. injection of ^{125}IUdR lymphocytes the proportion localizing in normal skin was about ten times greater than with ^{51}Cr labelled TDL; this localization fell progressively at 2h and 24 h. Croton oil induced the highest values for the localization of ^{125}IUdR lymphocytes and the contact sensitizing agents produced intermediate values. The agent to which the recipients had been sensitized produced greater localization than the other agents at 0.5 h and 24 h but the difference was not as marked as with ^{51}Cr TDL.

In an experiment on passaged ^{51}Cr-labelled TDL the localization was maximal at 0.5 h and there was a much greater decline by 2 h than was the case with whole TDL (Fig. 2). There were only small differences in the values found at different sites although at 2 h and at 24 h the localization in the site of DNCB challenge was 40–50% greater than in normal skin or sites painted with the other sensitizing agents. However croton oil induced an equal small increase in localization at 24 hours.

The data presented so far do not fit with the assumption that with respect to their migratory properties ^{51}Cr-TDL are equivalent to the sum of the ^{125}IUdR subset plus the passaged ^{51}Cr TDL. For example there is higher localization of whole TDL in the site of DNCB challenge than in the croton oil lesion but this was not found with either subset. A further experiment of similar design (but limiting observation of localization to 24 hours after injection) was performed by transferring TDL from a donor which had been labelled in vivo

Tab. 2 Localization of ^{3}H-labelled Lymphocytes in Skin Lesions*

Expt No.	% of injected activity /gm normal skin	Factor of increase over normal skin			
		TNCB	OXAZ	DNCB[1]	CROTON
B. 1 A	.041	0.8	1.0	9.2	1.0
B	.031		0.8	9.4	1.4

*Donor of TDL labelled by daily injection of ^{3}H-thymidine with a 3 week "cool off" period before cannulation. AO recipients were challenged 12 days after sensitization with DNCB and localization was measured 24 hours after challenge and labelled cell transfer.
[1] Second application of DNCB.

Tab. 3 Localization of S-Phase Cells compared to all Thoracic duct Lymphocytes*

$$\text{Ratio of } \frac{\text{(mean \% of injected } ^{125}\text{I activity/gm)}}{\text{(mean \% of injected } ^{51}\text{Cr activity/gm)}}$$

Tissue	Interval after Labelled cell transfer		
	0.5 HR	2 HR	24 HR
Skin normal	6.5	9.1	5.3
DNCB[1]	9.4	11.8	4.3
Croton	14.5	19.3	16.4
TNCB	14.1	24.8	8.0
OXAZ	16.1	27.7	9.2

*TDL labelled in vitro with ^{125}IUdR and ^{51}Cr-sodium chromate respectively.
[1] Second application of DNCB.

with ^{3}H-thymidine by a regime intended to confine the label to long-lived lymphocytes (15). In this case the selectivity of the excessive localization in a site of DNCB challenge was the most impressive of all the populations studied being 9 times higher than in the other sites including the croton oil lesion. Previous experience with this labelling regime has shown that about 20% of small lymphocytes are heavily labelled on autoradiographs while no large lymphocytes are perceptibly labelled.

One important technical reservation should be noted with respect to this last result. A contribution by the radioactivity found in the sites of DNCB challenge by reutilization of ^{3}H-thymidine from the minority of lymphocytes which may have died after transfer was not excluded. Proliferating mononuclear cells in the challenge site may have taken up some label in this way but it seems most unlikely that this could explain the 9 fold difference because the i.v. injection of ^{3}H-thymidine into 6 sensitized rats challenged either 24 or 48 hours previously with DNCB produced an increase in incorporation into the challenge sites compared to normal skin by a factor of only 1.38 ± 0.28 (Marilyn E. Smith and W.L. Ford, unpublished).

Discussion

This preliminary study of certain lymphocyte populations migrating into normal skin, a cell-mediated immune lesion (contact sensitivity to DNCB) and an acute inflammatory lesion (croton oil) revealed striking and unexpected differences between the localization of these lymphocyte subsets. Previous work has indicated that large cells recently activated by antigen localize more effectively in inflammatory lesions than do small lymphocytes (21, 22). This observation has been confirmed here in that as a fraction of the injected dose 4–12 times as much ^{125}IUdR as ^{51}Cr was found in the DNCB recall lesions, the exact value depending on the time of examination (Table 3). Since ^{125}IUdR is associated exclusively with the minority of proliferating TDL and ^{51}Cr is associated with both the non-proliferating majority of small lymphocytes and the proliferating minority the relative efficiency of large cell localization must be substantially greater than the ratios suggest.

However the superiority of activated lymphocyte localization was not confined to cell-mediated lesions; it was almost the same with respect to normal skin (Table 3). This is similar to the observation of *Braendstrup* and *Werdelin* (personal communication) that a higher proportion of activated lymphocytes compared

to small lymphocytes localize in the peritoneal cavity of rats whether the peritoneum is inflammed or not. In fact the superiority of activated lymphocytes is significantly greater in the case of croton oil induced inflammation which apparently has no immune component. The same enhanced superiority is seen in the sites of primary application of TNCB and oxazolone probably because they induced a mild non-immune inflammatory response not obvious to inspection (Table 3).

Despite the fact that more ^{125}IUdR labelled cells localized in the croton oil lesion than in the site of contact sensitivity there was a substantially higher localization of whole ^{51}Cr-TDL in the latter. The excess of selectively localizing cells in the whole population was pressumably attributable to small lymphocytes, i.e. non ^{125}IUdR incorporating, but what was their nature? When B and T lymphocytes were first distinguished the notion became popular that thoracic duct small lymphocytes were all both long-lived and recirculating (23). The present results on long-lived TDL (defined by a ^{3}H-thymidine labelling regime) and recirculating TDL (defined by blood to lymph passage in intermediate recipients) emphasizes that these populations are not identical. Recirculating small lymphocytes localized poorly in the skin in all the situations tested with only a slight excess in the inflammatory lesions possibly because of "contamination" with the small proportion of large lymphocytes which make the passage from blood to lymph (24).

By contrast long-lived lymphocytes discriminated between the contact sensitivity and croton oil lesions with remarkable accuracy. Taken at face value these results signify that the increased localization of lymphocytes in cell-mediated immune lesions compared to other inflammatory sites is at least partly attributable to a long-lived small lymphocytes in the thoracic duct population which does not recirculate from blood to lymph through lymph-nodes within a 16 hour period. It must be admitted that for practical reasons this conclusion is based on limited data since to obtain sufficient labelled lymphocytes for skin localization studies either by in vivo labelling of the donor or passage through an intermediate recipient is exceptionally laborious.

One difficulty with this unorthodox conclusion is the lack of corroborative evidence for such a strange population. However a completely different approach has recently incriminated a population of lymphocytes with remarkably similar properties as being responsible for the accelerated second-set rejection of heart allografts in rats (25, 26, 27). In the case of heart allografts across an MHC barrier irradiation of the recipient before grafting allows very long graft survival and lymphoid populations from immunized donors are many times more potent in restoring rejection times to a minimum value than are non-immune lymphocytes. By exploiting this system as an assay for memory T cells Roser and his colleagues found that these cells were present in the thoracic duct lymph and in the spleen of immunized rats but were completely lost on passage from blood to lymph through either irradiated or normal intermediates (27). Moreover the functional activity of the population was not diminished by treatment of the donors with vinblastine for 48 h to destroy the dividing cells (26). The memory cells were therefore long-lived, although by a different operational test from that described here. While it should be remembered that *Roser* was using a functional test on an immune population and we have measured selective localization of a non-immune population there is a remarkable correspondence of the properties of what appears to be the key cells in each system.

Gentle treatment of lymphocytes in vitro with trypsin is known to impair their capacity to localize in lymph-nodes without affecting the splenic localization suggesting that different mechanisms of selective localization are responsible (28, 29). The effect of brief exposure of lymphocytes to trypsin on their capacity to migrate into normal skin; croton oil lesions, cell-mediated immune (CMI) lesions and other non-lymphoid tissues was studied as part of the same project as included the present experiments (30). The enzyme treatment prevented the localization of lymphocytes in two forms of CMI – DNCB sensitivity and a Fruend's adjuvant granuloma – but did not impair the localization of lymphocytes in croton oil lesions, normal skin or in other non-lymphoid tissues. This suggested

Tab. 4 Summary of studies of lymphocyte migration into skin*

Skin Site	^{51}Cr-TDL (Whole population)	^{125}IUdR-TDL (Activated-cells)	Passaged ^{51}Cr-TDL (Accredited recirculators)	^{3}H-Thymidine TDL ("long lived")	Trypsinization of TDL
Normal skin	+	++	+	+	No effect
Croton oil inflamed	++	++++	++	+	No effect
DNCB contact Sensitivity	+++	+++	++	+++	Inhibition of excess

*The number of +s allow vertical or horizontal comparisons of relative localization. They indicate higher or lower localization but are no more quantitative than this.

that different mechanisms are involved for migration into the spleen and non-lymphoid tissues (trypsin resistant) and for migration into lymph nodes and sites of CMI (trypsin sensitive). We favoured the notion that trypsinization discriminates between different mechanisms of selective migration which may be used by the same cell. While this is almost certainly true for the differential effect on the migration into the spleen and lymph-nodes (because the same population of lymphocytes is believed to recirculate indiscriminately through the spleen and through lymph-nodes (31)) in the case of normal skin and CMI lesions the possibility remains open that the subset localizing preferentially in CMI lesions is impaired by trypsinization while a different subset localizing in normal skin is unaffected.

In conclusion these studies of cell localization in normal and inflammed skin have revealed several differences between cell-mediated immunity and non-immune inflammation (Table 4). No doubt this and similar studies are no more than a beginning to the mass of painstaking work required to unravel the complexities of lymphocytes subsets which a) migrate to the lymph-nodes and spleen b) migrate physiologically into non-lymphoid tissue c) migrate into sites of non-immune inflammation and d) migrate preferentially into CMI lesions. Possibly selective lymphocyte migration will be used as a tool to clarify the nature of certain pathological processes,e.g. are the lesions of adjuvant arthritis examples of CMI or not? Of course it must be kept in mind that the lymphocytes are often in a small minority in such lesions and their role in promoting the migration and accumulation of monocytes, granulocytes and fibroblasts is another question. Future work will be concerned not only with the life history of lymphocyte subsets as defined by their migratory properties but also with their role in modifying locally the function of vascular endothelium because it is in the interaction of endothelium and lymphocyte subsets that the mechanism of selective migration from the blood must be sought (32).

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