ROLE OF ADHESION MECHANISMS IN THE PATHOGENESIS OF CHRONIC SYNOVITIS

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SUMMARY
The hallmark of many rheumatic conditions, including rheumatoid arthritis (RA) and other seronegative inflammatory arthropathies (OIA), is a persistent inflammatory process that mainly affects synovial joints. Although the aetiology of the synovitis remains elusive, the pathogenesis is thought to be immune mediated. There are several reasons to believe that synovial T lymphocytes (S-TL) play a central role both as regulatory and effector cells in the initiation and perpetuation of the inflammatory process. In early studies, we demonstrated that the majority of S-TL are of the CD45R0 'memory' phenotype, while CD45RA 'naive' T cells are virtually absent [1,2]. Various mechanisms can be responsible for such preferential accumulation. In this dissertation, I will present a number of studies, carried out over several years, investigating the relative role of adhesion and migration in the pathogenesis of the CD45R0 accumulation in inflamed tissues. Since the first step in lymphocyte extravasation is adhesion to endothelium, the ability of purified CD45R0 or CD45RA T cells to adhere to human umbilical vein endothelial cells (HUVEC) in vitro was analysed [3]. Second, to examine the migration process itself, an in vivo model of cell migration into suction-induced skin blisters raised over delayed-type hypersensitivity reactions was developed [4]. Third, the role of tissue-specific homing mechanisms in the regulation of T-cell migration into different inflammatory sites was investigated [5]. Finally, the adhesion of T cells to extracellular matrix (ECM) components, as a mechanism for preferential cell retention in inflamed tissues, was examined [6]. These studies demonstrate that the critical mechanisms leading to CD45R0 lymphocyte accumulation in chronic inflammatory foci include (a) an increased adhesion to endothelium [3], (b) an increased migratory capacity [4] and (c) an increased adhesion to ECM components [6]. I also present evidence to suggest that besides these general mechanisms, organ-specific homing may be of relevance in determining the selective accumulation of distinct CD45R0 T cells in different inflamed tissues [5].

KEY WORDS: Rheumatoid Arthritis, Pathogenesis, T lymphocytes, Synovitis, Adhesion, Migration, CD45R0.

The understanding of the pathogenesis of rheumatoid arthritis (RA) and other chronic arthritides is likely to be advanced by studying the cellular component infiltrating the synovium, the main site of inflammation in these conditions. These inflammatory cells are thought to be responsible for the initiation and perpetuation of the pathological process. Although there is still controversy on which cell type (macrophages or T cells) is the major player, there are several reasons strongly supporting the view that T cells may be responsible for the maintenance of the immune response while macrophages may be more important for the effector mechanisms that lead to joint destruction [7-10]. The understanding of the distinctive properties of synovial T cells may be crucial since local immunoregulatory events are likely to determine the generation and resolution of inflammation.

For many years, researchers were puzzled by the apparent discrepancy between the poor suppressor function [11] of synovial T cells and the seemingly adequate numbers of suppressor (CD8+) cells. The development of monoclonal antibodies (mAb) 2H4 (CD45RA) [12], UCHL1 (CD45R0) [13] and 4B4 (CD29) [14] facilitated a more extensive correlation between phenotype and function. For example, contrary to the dogma that CD4+ lymphocytes exert a specific helper function for B-cell immunoglobulin production, peripheral blood (PB) CD4+ T cells expressing the CD45RA phenotype actually induce suppressor activity in CD8+ lymphocytes and do not provide B-cell help [12]. This cell subset also proliferates maximally to the mitogen concanavalin A (Con-A), but does not proliferate in response to antigen [12]. Vice versa, CD4+ CD45R0+ T cells provide B-cell help, do not induce suppressor function, proliferate to soluble antigens but only poorly to Con-A [13,14]. CD8+ cells can be similarly subdivided phenotypically, but the functional correlates of this are not as well defined. In the PB, approximately half of the T cells, whether CD4 or CD8, express CD45R0, while the reciprocal half express CD45RA.

Since the functions of CD45R0 T cells in the PB are remarkably similar to the functional characteristics of T lymphocytes found in the RA synovium, we postulated that synovial T cells would express this phenotype. In early studies [1], confirmed independently at the same time by Emery et al. [15], we provided the first formal evidence that RA synovial T cells overwhelmingly express the CD45R0 phenotype (Fig. 1). Furthermore, we demonstrated that this phenotypic distribution is not typical of RA, but is common to all types of synovitis [2] and, in general, represents a universal feature of inflammation (Fig. 2). These findings extend in a critical way the
understanding of the functional characteristics of synovial T cells; namely, the poor suppressor activity and the exuberant production of immunoglobulins can be explained on the basis of the phenotype of the lymphocytes infiltrating the synovium.

The most relevant question arising from this observation concerns the mechanisms by which the preferential CD45R0 accumulation occurs in inflamed tissues. There are several not mutually exclusive possibilities. One possibility is that CD45R0 T cells proliferate preferentially to local antigens. However, this mechanism seems unlikely to play a major role since only a small percentage of synovial T cells are actively proliferating [16, 17], although they are known to be partially activated [18, 19]. A second possibility that needs to be considered is the phenomenon of 'phenotype conversion' by which CD45R0-negative cells (CD45RA-positive) acquire the CD45R0 phenotype upon activation [20–22]. In other words, T lymphocytes could arrive in the synovium expressing the CD45RA phenotype but, under the influence of the inflammatory/activating stimuli present locally, would convert their phenotype to CD45R0. This mechanism is certainly possible, given the multiple factors which can activate lymphocytes in the joint. However, chronic synovial inflammation is analysed not during evolution, but at a single point in time, making it very difficult to prove the role of this mechanism in vivo. Furthermore, it remains unclear whether CD45RA and CD45R0 represent stable phenotypes with a unidirectional conversion from CD45RA to CD45R0 upon maturation [21, 22], or whether phenotype reversibility from CD45R0 to CD45RA can take place.
A third possibility may relate to a decreased apoptotic cell death in the synovium. Programmed cell death, or apoptosis, is an important biological phenomenon which controls, among other functions, uncontrolled cell proliferation. The mechanisms controlling apoptosis are complex and not yet completely clarified, but they are thought to be the result of a balancing act of death (APO-1/Fas) and survival (Bcl-2) signals [25–27]. A detailed discussion of this phenomenon is not directly relevant to the work presented here, but the hypothesis that a dysregulation of Fas/Bcl-2 may be responsible for a prolonged survival of CD45R0 in the synovium is certainly important to consider [28, 29].

The studies presented in this dissertation were designed to investigate the three other mechanisms which, we hypothesized, were most likely to play a critical role. First, since, in order to leave the circulation, leucocytes must adhere to and cross the endothelial vascular lining, the preferential tissue accumulation of CD45R0 T cells may relate to their increased capacity to adhere to and migrate through endothelium. Second, since leucocyte migration to different tissues is not a random process, but a phenomenon involving, among other factors, organ-specific migration, the entry to the synovium of distinct lymphocyte subsets may be regulated by specific homing mechanisms. Third, since the cellular make-up of infiltrating the synovial membrane (SM) results from the balance between cells entering and exiting the SM, the selective adhesion to extracellular matrix (ECM) may contribute to the preferential retention of CD45R0 T cells in the synovium. The relative role of each of these mechanisms will be considered in turn.

ROLE OF SELECTIVE ADHESION TO AND MIGRATION THROUGH THE ENDOTHELIAL IN THE ACCUMULATION OF CD45R0 T CELLS IN INFLAMED TISSUES

Lymphocyte migration is a complex phenomenon regulated by a cascade of molecular events that take place in an ordered series of steps involving close interactions between adhesion receptors expressed by migrating lymphocytes and endothelial cell (EC) counter-receptors. A consensus model of leucocyte migration in four sequential steps is now generally accepted [30–33]. In the first step (tethering/rolling), some of the flowing leucocytes come into brief contact with the vessel wall, slow their movement, and roll on the endothelium. This step is transient, activation independent and mediated by constitutively expressed selectin molecules and their cognate oligosaccharide ligands [34–36]. In the second step (triggering/activation), rolling leucocytes can sample the local endothelial microenvironment for the presence of inflammatory mediators (e.g. chemokines) which, in turn, deliver activating signals to the leucocytes by binding to a specific G-protein-linked receptor [37, 38]. The third step (strong adhesion) is primarily mediated by activated (step two) β1 (VLA4) and β2 (LFA1 and Mac-1) integrins [39, 40] which bind to their counter-receptors belonging to the immunoglobulin superfamily, VCAM-1 and ICAMs. During this phase, cells change shape, acquiring a flattened morphology, and within minutes they actively extravasate (fourth step, trans-endothelial migration) into the tissues [34]. Furthermore, it is also known that, in the lymphoid tissue, lymphocyte migration takes place at specialized post-capillary vascular sites, called 'high endothelial venules' (HEV) because of their particular cuboidal morphology [41, 42]. In non-lymphoid tissues, cell migration also takes place in post-capillary venules where blood flows at lower speed but, in contrast to lymphoid HEV, these vessels are lined by flat endothelial cells. However, HEV-like vessels have also been described in non-lymphoid tissues affected by chronic inflammation, such as the rheumatoid synovium [43–45]. Here, HEV are localized in areas where the infiltrating lymphocytes are organized in lymphoid follicle-like structures, as compared to the areas where the lymphocytic infiltrate is diffuse and where the endothelium remains flat [45, 46]. This observation suggests that HEV formation can be induced outside the lymphoid tissues in response to local factors (e.g. inflammatory cytokines) and this is the site of preferential lymphocyte traffic.

Since the first moment in lymphocyte migration into inflammatory lesions is represented by adhesion of circulating cells to endothelium, the question of whether the prevalence of CD45R0 + T cells might be due to differential adhesiveness to EC was examined first. Because of the difficulties in growing synovial microvascular endothelium (MVE) in vitro, we elected to use human umbilical vein EC (HUVEC) in the classic lymphocyte–HUVEC binding assay. Using this system we demonstrated, both by a sequential panning technique (Fig. 3) and, directly, using CD45R0 and CD45RA sorted cells (Table I), that CD45R0 bind 2- and 4-fold better to HUVEC compared to CD45RA lymphocytes. Furthermore, it was also demonstrated that although CD45R0 T cells express higher levels of the leucocyte-associated molecule-1 (LFA1, CD11a/CD18) on their cell surface (data not shown), the addition of LFA1 mAb to the adhesion assay (Fig. 4) inhibited binding by 50%, but did not alter the difference between CD45R0 and CD45RA. This latter experiment suggested that LFA1-independent mechanisms, such as the subsequently discovered VLA4 pathway (see later), were also involved in the preferential CD45R0 endothelial adhesion.

The question of most relevance, of course, is whether the 4-fold difference in the ability of CD45R0 + compared to CD45RA + T cells to adhere to EC under static (selectin-independent) conditions in vitro accounts for the vast preponderance of CD45R0 + cells within the inflammatory lesions in vivo. Whether or not a circulating lymphocyte adheres to endothelium and migrates into the perivascular space is likely to depend on a number of factors, including lymphocyte adhesiveness to EC, alteration in EC adhesiveness in response to cytokines [47–49], chemotactic factors [50], vascular permeability [51] and local blood flow [52]. In
The percentage of CD45R0-positive (solid bars) and CD45RA-positive (open bars) lymphocytes in the original peripheral blood T-cell population (i) compared with the T-cell fractions adherent (ii) and non-adherent (iii) to an unstimulated endothelial cell monolayer (UEC). T cells which did not adhere UEC were allowed to adhere to an IL-1-stimulated endothelial cell monolayer (SEC). The T-cell fraction adherent to SEC is shown in (iv), while the non-adherent fraction is shown in (v). Results are expressed as the mean percentage of T cells ± S.E.M. from four experiments. It can be seen that the proportion of CD45R0-positive T cells is higher by 30–40% in the adherent fractions compared to CD45RA T cells. Vice versa, in the non-adherent fractions there is a prevalence of CD45RA T cells. (Reproduced with permission from [3].)

the rheumatoid synovium, because of the local physical conditions and the large amounts of inflammatory mediators present, flow rates are very slow and synovial blood vessels exhibit an increased permeability. This, together with the induced high expression of endothelial ligands [53], would facilitate lymphocyte adhesion to EC in an integrin-dependent fashion similar to the static conditions in vitro. Furthermore, it has been shown that the β1 integrin (VLA4), highly expressed by synovial T cells (see later), can mediate attachment and rolling independently from selectins [54]. Within this complex framework, therefore, it is reasonable to suggest that the 4-fold increased adhesion capacity to endothelium of CD45R0 lymphocytes could result in their preponderance in chronically inflamed tissues.

However, these studies, by their intrinsic nature, did not demonstrate that CD45R0 lymphocytes could actually preferentially migrate into inflamed tissues in vivo. To investigate this directly, we developed an experimental model of lymphocyte migration into epidermal suction blisters raised over purified protein derivative (PPD)-induced delayed-type hypersensitivity (DTH) skin lesions in normal volunteers. The acantholytic skin blisters generated in this way (Fig. 5A and B) fill up with fluid and cells which can be sampled at different time points for phenotypic/functional studies. Using this system, it was shown that the vast majority of blister lymphocytes expressed the CD45R0

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>EC</th>
<th>UF*</th>
<th>CD45RA negative (x)</th>
<th>CD45R0 negative (y)</th>
<th>Binding ratio†</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Unstimulated</td>
<td>15.1 ± 1.1</td>
<td>23.3 ± 2.0</td>
<td>6.2 ± 0.5</td>
<td>3.8</td>
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<tr>
<td>2</td>
<td>IL-1 stimulated</td>
<td>30.9 ± 2.0</td>
<td>52.6 ± 2.7</td>
<td>21.6 ± 1.3</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>Unstimulated</td>
<td>9.7 ± 1.9</td>
<td>15.0 ± 1.7</td>
<td>4.2 ± 0.9</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>IL-1 stimulated</td>
<td>18.6 ± 1.6</td>
<td>41.7 ± 1.9</td>
<td>18.9 ± 2.5</td>
<td>2.2</td>
</tr>
<tr>
<td>5</td>
<td>Unstimulated</td>
<td>17.3 ± 1.6</td>
<td>28.1 ± 2.8</td>
<td>7.4 ± 0.4</td>
<td>3.8</td>
</tr>
<tr>
<td>6</td>
<td>IL-1 stimulated</td>
<td>33.4 ± 1.7</td>
<td>45.5 ± 2.1</td>
<td>25.0 ± 1.8</td>
<td>1.8</td>
</tr>
</tbody>
</table>

*Unfractionated.
†Binding ratio = % (x) cells bound
†Results expressed as mean ± 1 S.E.M.

It can be seen that, in the adhesion assay to unstimulated HUVEC, CD45R0 (CD45RA negative = x) bind 3- to 4-fold better than CD45RA (CD45R0 negative = y). (Reproduced with permission from [3].)
phenotype as early as 24 h after the blisters were raised, reaching the peak between 48 and 72 h (Fig. 6A). In addition, the level of expression of the CD45R0 molecule on the surface of blister lymphocytes, compared with circulating PB CD45R0+ T cells, was found to be elevated and this increased further as the inflammation progressed (Fig. 6B). In contrast, the mean intensity of fluorescence of the CD45RA molecules remained static (Fig. 6B). The significance of this is still uncertain.

These experiments not only provide evidence that CD45R0+ T cells preferentially migrate into inflammatory lesions, but also allow some conclusions to be drawn on the pathogenetic mechanisms potentially responsible for their accumulation. Similar to other sites of inflammation, the mechanisms leading to CD45R0+ lymphocyte accumulation in experimental skin blisters could include lymphocyte proliferation and phenotype conversion. As mentioned earlier, CD45R0+ T cells are known to respond preferentially to recall antigens such as PPD [13, 14], and to convert from CD45RA+ cells upon activation [20, 22]. However, it seems unlikely that either of these two mechanisms could account for the overwhelming predominance of CD45R0+ T cells in the blisters since this subset starts to prevail as early as 24 h. Such a time scale, according to experiments in vitro, is too short to permit either antigen-induced proliferation [55] or acquisition of the CD45R0 marker (peak between 5 and 7 days) [21, 23]. Hence, the predominance of CD45R0 T cells in evolving inflammatory lesions, such as blisters overlaying DTH skin reactions, is almost certainly related to their enhanced migration capacity.

In the same study, it was also demonstrated that CD4-positive cells predominate over CD8-negative cells in the blisters compared to the PB (Fig. 7), although the difference was far less significant than between CD45R0- and CD45RA-positive T cells. This is in agreement with immunohistological data which show a modest prevalence of CD4 vs CD8 lymphocytes at inflammatory sites [56]. The analysis of blister T cells from a naturally occurring immune-mediated blistering disease, bullous pemphigoid (Table II), revealed very similar results to those of the model blisters and was also comparable to the data on T cells from chronic inflammatory lesions, confirming the reproducibility of the observation.

In summary, the increased ability of CD45R0 lymphocytes to adhere to and migrate through endothelium, by favouring a preferential extravasation of this subset into inflamed tissues, is one of the most important pathophysiological mechanisms responsible for their preponderance at sites of inflammation. However, the jury is still out as to whether, within the CD45R0 population, distinct sub-subsets can selectively home to different inflamed organs. The evidence for this will be discussed in the next section.

ROLE OF SPECIFIC HOMING MECHANISMS IN THE ACCUMULATION OF DISTINCT CD45R0 T CELLS IN DIFFERENT INFLAMED TISSUES

As mentioned, lymphocytes leave the circulation and enter various tissues by adhering to and migrating through post-capillary vessels with the characteristic HEV-like morphology. However, lymphocyte migration into various tissues is not a random process and
lymphocytes homing into lymphoid organs are known to be different from those migrating into non-lymphoid tissues. There is general agreement that, under resting conditions, CD45RA lymphocytes migrate mainly to lymphoid organs, while CD45R0 lymphocytes migrate primarily to peripheral tissues [57, 58]. Lymphocyte entry into different organs is thought to be regulated by specific receptor/counter-receptor pairs of adhesion.

![Figure 5](https://academic.oup.com/rheumatology/article-abstract/35/12/1198/1782372)
molecules expressed by migrating lymphocytes and by the MVE of various tissues [59]. The lymphocyte-associated molecules, determining the selective interaction with the MVE of a given organ, are termed ‘homing receptors' (HR), while the cognate MVE ligands are defined as ‘vascular addressins' (VA) [60, 61]. Well-characterized examples, within lymphoid organs, of HR/VA interactions are represented by L-selectin/GlyCAMs [62], which mediate migration to peripheral lymph nodes (PLN), and by α4β7/MadCAM1 [63], which mediate migration to mucosal-associated lymphoid tissue (MALT). As far as peripheral tissues are concerned, further specialization is indicated by early in vivo animal studies which demonstrated that immunoblasts from the gut and skin, as well as PLN, preferentially home to the same type of tissue from which they were isolated [41, 64, 65]. In humans, such

direct proof is still lacking, but indirect evidence of the existence of a lymphocyte-endothelial recognition system specific for different organs was provided by Jalkanen et al. [66]. Furthermore, the recent report that 62% of synovial lymphocytes express the α4β7 HR [67], also expressed by gut lymphocytes [68], has revived the hypothesis that there might be preferential trafficking between these two organs, and that this may be an important step in the pathogenesis of inflammatory arthropathies.

The development of mAb HECA-452, which identifies a cell surface antigen (Ag) expressed by the majority of skin-infiltrating lymphocytes of several inflammatory dermatoses, but not by lymphocytes present in other inflamed non-cutaneous tissues [69], has prompted us to investigate its distribution in psoriatic arthritis (PSA). In this disease, inflammation affects two different organs within the same individual. It therefore offers a unique opportunity to address the question of the relative contribution that tissue-specific homing and inflammatory-related mechanisms play in controlling lymphocyte entry into skin and joints.

<table>
<thead>
<tr>
<th>Marker</th>
<th>% of CD3+ T cells bearing marker</th>
<th>mif of marker</th>
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<tr>
<td>CD4</td>
<td>92.8</td>
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</tr>
<tr>
<td>CD8</td>
<td>4.8</td>
<td>144.4</td>
</tr>
<tr>
<td>CD45R0</td>
<td>99.0</td>
<td>214.6</td>
</tr>
<tr>
<td>CD45RA</td>
<td>4.3</td>
<td>93.6</td>
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</table>

mif, mean intensity of fluorescence.

It can be seen that, similarly to experimental blisters and to chronically inflamed tissues, the majority of T cells express the CD45R0 marker (99.0%) at high level (mif = 214.6). (Reproduced with permission from [4].)
HECA-452 mAb recognizes several heavily glycosylated molecular species, including sialyl Lewis\(^x\) and other closely related carbohydrate moieties \cite{69,70}. The HECA-452 Ag (comprehensively defined as 'cutaneous lymphocyte Ag' or CLA) strongly binds to the endothelial ligand E-selectin \cite{71,72} and, on this basis, it was proposed that CLA represents the homing receptor and E-selectin the vascular addressin specific for the skin. In the original study of Picker \textit{et al.} \cite{69}, although psoriatic skin was analysed among different inflammatory dermatoses, the SM studied were from patients with RA who had no skin inflammation. That study, therefore, could draw no conclusions on disease specificity, nor could it exclude the possibility that skin lymphocytes could migrate to other inflamed organs within the same individual. This is particularly important in PSA where, if the same inflammatory/immune process were responsible for the disease in skin and joints, one would expect to find the same type of immune cells at the two sites, as indeed is the case for cells of the CD45R0\(^+\) phenotype. Furthermore, the low number of CLA\(^+\) T cells in the SM could be due to the failure of these cells to enter the joint or to their inability to persist in the synovial tissue with consequent accumulation in the synovial fluid (SF), as happens for polymorphonuclear cells.

Using standard immunoperoxidase staining on sequential sections of skin and paired SM of patients with PSA (Fig. 8), we demonstrated in 16 samples (10 skin and six SM) that the percentage of CLA\(^+\) lymphocytes in the skin (57.4 ± 13.0) was significantly higher \((P < 0.0002)\) compared to the SM (4.8 ± 2.4) (Table III). In order to examine disease specificity, we repeated the same type of analysis in skin (DTH reaction sites) and paired SM of patients with RA (Fig. 9). Similar to the findings in PSA, the majority of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Skin (%)</th>
<th>Synovial membrane (%)</th>
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<tbody>
<tr>
<td>1</td>
<td>52.5</td>
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<td>35.0</td>
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<tr>
<td>3</td>
<td>32.7</td>
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<td>5</td>
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\textit{Mean ± S.D.} 57.4 ± 13.0 4.8 ± 2.4

Skin and SM histological samples were analysed using a standard immunoperoxidase technique. The results are expressed as the percentage of double-positive CD3\(^+\)/HECA-452\(^+\) out of the total CD3\(^+\) cells. The difference between skin and synovial membrane was highly significant \((P < 0.0002)\). ND, not determined. (Reproduced with permission from [5].)
CD3+ cells in the RA DTH skin reactions were CLA+, whilst only a very few CD3+ cells in the SM were CLA+ (Table IV). These results strongly suggest that the preferential accumulation of CLA+ lymphocytes in the skin is not a disease-specific phenomenon, but is due to the general homing properties of this subset.

To investigate the role of E-selectin as a specific VA for CLA+ lymphocytes, using the same technique, we analysed its expression in skin and SM of both PSA and RA patients (Fig. 10). We found that in the majority of cases there was a similar E-selectin expression in the skin and in the SM (Table V), with no direct correlation between disease activity and E-selectin expression in skin and synovium of either PSA or RA patients. These results imply that the lack of CLA+ T cells in the SM is not due to the lack of the putative CLA ligand E-selectin, since its expression is approximately to the same degree in dermal and synovial microvascular endothelium. Furthermore, these data would suggest that E-selectin is not a specific skin VA.

The analysis of the proportion of CLA+ cells in the SF (Table VI), which was shown to be similarly low to the PB (Table VI), suggests that the small number of CLA+ cells found in PSA and RA SM (Tables III and IV) is most likely to be due to the failure of these cells to enter the synovium, related to the lack of the appropriate homing specificity, rather than to the alternative possibility, namely, the inability of CLA+ cells to persist in the SM and accumulate in the SF.

### Table IV

<table>
<thead>
<tr>
<th>Patient</th>
<th>Skin (%)</th>
<th>Synovial membrane (%)</th>
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<td>3</td>
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<tr>
<td>10</td>
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Mean ± s.d. 48.8 ± 3.7 4.2 ± 2.2

Skin and SM histological samples were analysed using a standard immunoperoxidase technique. The results are expressed as the percentage of double-positive CD3+/HECA-452+ out of the total CD3+ cells. Patients 4 and 5 were PPD non-responders, hence they had a poor DTH reaction. Since only very few T cells were found in the skin, the results for these two patients are given as absolute numbers rather than percentages.

†Total CD3+ cells counted: 6; CD3+/HECA-452+: 4.

The difference between skin and synovial membrane was highly significant (P < 0.007). ND, not determined. (Reproduced with permission from [5].)
Finally, to address the question of whether the increased prevalence of CLA+ T lymphocytes in the skin is due to the acquisition of the CLA antigen following cell differentiation in the tissue or to preferential migration, we turn again to our model of lymphocyte migration into epidermal suction-induced skin blisters. Skin blister (SB) lymphocytes were double labelled with CD3 and CLA (HECA-452) mAbs and analysed by FACS (Fig. 11). For comparison, paired PB and SF (available only in three patients) lymphocytes were studied. The results (Table VII) show a remarkable similarity between the proportion of CLA+ T lymphocytes migrating into SB and the proportion found in the underlying DTH lesions and in the PSA skin. These findings corroborate the immunohistological data. More importantly, these results suggest that the principal mechanism for the accumulation of CLA+ cells in the skin relates to preferential migration rather than local differentiation. The time (48 h) at which SB lymphocytes were analysed is too short to allow the de novo expression of CLA. Although the expression of the CLA Ag can be induced

![Image](https://academic.oup.com/rheumatology/article-abstract/35/12/1198/1782372/121968/1782372)
after mitogen stimulation in the presence of transforming growth factor β (TGFβ) in vitro, this takes ~6 days [73]. Since TGFβ is produced in the skin in large amounts, it has been suggested that this factor may be responsible for the high proportion of these cells in this compartment. However, this is unlikely since TGFβ is also produced abundantly in the SM and yet there is no induction of CLA expression in synovial T cells. Nonetheless, the possibility that CLA expression in vivo may be regulated with a different time scale from in vitro, and that unknown local differentiating factors may be important for the induction of the high proportion of CLA+ cells in the skin, cannot be ruled out.

Although the studies presented earlier showed a predominance of CD45R0 memory T cells in several sites of inflammation, including skin of various inflammatory dermatoses [74] and in the synovium of several chronic arthritides [2], the results of this latter study, on the basis of the expression of the CLA phenotype, clearly imply that the infiltrating lymphocytes in the two compartments are different. This study, therefore, supports the concept that lymphocyte homing to chronic skin and synovial inflammatory sites is regulated by organ-specific as well as general mechanisms of migration related to inflammation.

It is, however, important to say that whereas there is little doubt of the critical importance of HR/VA adhesive interactions, it has become clear that the molecular control of lymphocyte homing is considerably more complex than implied by the model of 'single HR/VA pair, single tissue specificity' [75]. Some HR, for example, facilitate lymphocyte adhesion to more than one tissue and some of the proposed VA molecules also have a widespread distribution in different organs [75]. This has prompted the proposal of the more sophisticated 'area code model' [32]. According to this model, similarly to the telephone exchange, multiple digits would be required for access into different 'districts'. Whether such a model applies to the real in vivo situation, or whether lymphocytes expressing a combination of adhesion molecules 'probabilistically' have an increased chance of extravasating into a given tissue, is not known. However, it is entirely plausible to hypothesize that, since immune cells have the ability to develop memory for an extraordinary number of cognate antigens, they might also have the capacity to recognize those microenviron-

The distribution of CLA+ T cells in epidermal skin blisters (SB), peripheral blood (PB) and synovial fluid (SF) of patients with rheumatoid arthritis (RA) is shown in Table VII.

<table>
<thead>
<tr>
<th>Patient</th>
<th>SB%</th>
<th>PB%</th>
<th>SF%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46.1</td>
<td>4.9</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>40.8</td>
<td>8.8</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>68.6</td>
<td>1.1</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>46.3</td>
<td>9.1</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>41.6</td>
<td>12.3</td>
<td>ND</td>
</tr>
<tr>
<td>Mean ± s.d.</td>
<td>48.6 ± 11.4</td>
<td>7.2 ± 4.3</td>
<td>3.6 ± 0.7</td>
</tr>
</tbody>
</table>

Samples were examined by double immunofluorescence and FACS analysis. The results are expressed as the percentage of double-positive CD3+/HECA-452+ out of the total CD3+ population. The difference between skin blisters, peripheral blood and synovial fluid was highly significant, P < 0.004 and P < 0.001, respectively. ND, not determined.

It should be noted that the proportion of CLA+ cells in the SB (48.6 ± 11.4) is remarkably similar to the proportion found in the underlying RA DTH lesions (48.8 ± 3.7) reported in Table IV and in psoriatic plaques (57.4 ± 13.0) reported in Table III. (Reproduced with permission from [5]).
ments where they are more likely to meet the same antigen again. Better understanding of the mechanisms regulating lymphocyte entry to the synovium may have important implications for the development of specific anti-adhesion therapies.

ROLE OF PREFERENTIAL ADHESION TO EXTRACELLULAR MATRIX IN THE ACCUMULATION OF CD45R0 T CELLS IN INFLAMED TISSUES

The RA synovial membrane, like other chronic inflammatory lesions, shows a characteristic monocellular cell (MNC) infiltrate constituted by macrophages, B and predominantly CD45R0+ T lymphocytes, but very few polymorphonuclear cells. This is in contrast to SF, where neutrophils are very abundant. This naturally occurring pathophysiological experiment illustrates the relevance of cell adhesion to ECM in determining the nature of the cells present at sites of chronic inflammation. In fact, while neutrophils lack the specific molecules to adhere to the ECM, MNC have the capacity of adhering to ECM constituents such as collagen, proteoglycans and fibronectin (FN). FN plays an essential role in MNC-ECM interaction, being a multifunctional protein capable of homotypic and heterotypic adhesion with, among other molecules, collagen, heparin and proteoglycans [76]. Since FN also possesses binding sites for cell surface receptors, it can function as an anchor molecule between MNC and ECM components [76].

FN, in its soluble or plasma form, is a dimer with a mol. wt of 550 kDa [76, 77] whose two chains are linked by two disulphide bonds near the COOH-terminus. Tissue FN differs slightly from this structure due to alternative splicing of its precursor mRNA [78] and polymerization into fibrils. Despite these structural differences, FNs from different sources are remarkably similar in their functional properties [76].

T cells interact with FN mainly via two members of the very late activation antigen (VLA) family of proteins, VLA4 and VLA5, which belong to the β1-integrin superfamily [79–82]. VLA molecules comprise six heterodimeric glycoproteins each with a different α chain (α1–α6), linked to a common β1 chain. VLA4 and VLA5 recognize and bind to different parts of the FN molecule. VLA4 binds to the third connecting segment (IIICS) region of FN [79, 80], which includes at least two cell-binding sequences, CS1 and CS5 [83, 84]. VLA5 recognizes a key short peptide sequence, RGDS, within the central cell-binding domain of FN [85].

For all these reasons, binding to FN was the method chosen to test the hypothesis that an increased adhesion to ECM may contribute to the accumulation of CD45R0 T cells in chronically inflamed tissues. The classical in vitro lymphocyte–FN binding assay, where FN is bound to a plastic surface, was used. This system was validated using purified T cells stimulated with the mitogen Con-A. It proved to be accurate, reproducible and specific since T-cell/FN interactions were inhibited both by mAbs to FN and by the FN peptide RGDS (Fig. 12).

Using this assay, it was shown that SF T cells (mostly CD45R0+) from patients with RA and other arthritides bind better to FN than paired PB T cells in vitro (Fig. 13). In addition, such increased adhesion was accompanied by upregulation, measured by fluorescence analysis, of the cell surface expression of VLAα4 and VLAβ1, but not VLAα5 on SF T cells (Fig. 14). In trying to dissect the relative contribution of these VLA molecules, FN adhesion experiments were repeated with and without the addition of mAb against VLAα4, VLAα5 and VLAβ1 (Fig. 15). It can be seen that mAb against all three molecules, α4, α5 and β1, were able to inhibit SF T-cell binding to FN in vitro, but to a different degree, α5 being the most inhibitory. In addition, although both VLA4 and VLA5 share a common β1 chain, the inhibitory effect of our anti-VLAβ1 was never greater than that produced by anti-VLAα5 alone, supporting the idea that the main contribution to this binding came from VLA5 and that VLA4 interactions may occur only in the presence of VLA5/FN binding. This is in agreement with previous observations [81, 86].

Interestingly, as mentioned, despite the profound inhibitory effect of the anti-VLAα5 mAb, there was no quantitative change in the number of VLA5 molecules

Fig. 12.—Concanavalin A-activated T-cell binding to fibronectin in the presence of (a) various dilutions of the mouse monoclonal antifibronectin antibody HFN 7.1 (●), a goat polyclonal antibody against fibronectin (●) and an irrelevant antibody directed against HTLV1 protein coat (▲); (b) various concentrations of the tetrapeptide RGDS (●), the reverse sequence SDGR (●) and the irrelevant peptide leucine enkephalin (▲). Results are expressed as a percentage of cells binding ±1 s.D. (Reproduced with permission from [6].)
on the surface of SF T cells with respect to PB T lymphocytes; in contrast, the levels of VLAα4 and VLAβ1 were increased (Fig. 14). This might have important pathophysiological consequences in the generation of the synovial infiltrate since VLAα4 has been shown to mediate at least three different T-cell activities. Besides its role in lymphocyte binding to FN, VLAα4 is also important in homotypic aggregation [87] and in adhesion to EC through binding to VCAM1 [88] and the CS-1 peptide of FN, which was recently found to be present and functional on the luminal surface of synovial EC [89, 90]. The increased expression of VLAα4 on SF T cells might, therefore, be important in T-cell migration into the synovium, the formation of the perivascular aggregates and, albeit to a smaller degree than VLAα5, to the retention of T cells in the inflamed joint.

Another issue which was addressed in this study was whether the increased ability of SF T cells to bind to FN was related to their state of activation and/or to their preponderant CD45R0 phenotype. Using the same lymphocyte–FN binding assay, it was found that resting T cells, whether CD45R0 or CD45RA, bind very poorly to FN (data not shown). This is in contrast with the described adhesion of resting lymphocytes to HUVEC, which was found to be higher. Whether this is due to a true low adhesion affinity to FN, in resting conditions, or whether the disparity is related to technical differences between the assays, remains to be determined. Since the binding of resting CD45R0 and CD45RA lymphocytes to FN was at the detection limit of the assay, in order to enhance the sensitivity, 0.5 mM Mn^{2+} was added to the assay medium [91]. Divalent cations, such as Mn^{2+}, are known to activate surface integrins and therefore increase binding avidity [91, 92]. In this way, it was possible to observe that CD45R0 T cells bind better to FN than CD45RA T cells (Experiment A: 4.7% vs 3.2%; Experiment B: 16.2% vs 9.4%). However, whether these results are influenced by a differential integrin activation between the two subsets cannot be excluded. Nonetheless, the most important entity that these experiments demonstrated was that, even in the presence of Mn^{2+}, binding of resting CD45R0 T cells to FN was still not as high as that observed for SF T cells (Fig. 13).

Since SF T cells have been shown to be activated by several different criteria [93] and the experiments described above indicated that activated T cells bind better to FN than resting cells, the interactions of SF T cells with FN were compared with that of Con-A-activated T cells. As in SF T cells, the expression of VLAα4 and VLAβ1 but not VLAα5 molecules was upregulated on Con-A-activated T cells (Fig. 16a). It would appear, therefore, that VLA5 is already maximally expressed even in resting conditions and that the increased FN binding by activated cells dependent on this molecule is mediated via qualitative rather than quantitative changes. Similar findings were shown by Shimizu et al. who also noted a rapid increase in CD4 T-cell binding to FN after activation with 12-O-tetradecanoylphorbol-13-acetate (TPA) not associated with quantitative changes in surface expression of VLA molecules [94]. Ligand-binding avidity has also been shown to be rapidly modulated
for other integrins including LFA1 [95] and VLA6 [94]. The mechanisms by which this occurs are not completely clear, but surface redistribution leading to integrin clustering and/or conformational changes in the tertiary molecular structure are thought to be the most important [39, 40, 95, 96]. The rapid ability to modulate the adhesion–de-adhesion integrin status is a very efficient mechanism for T cells to respond swiftly to microenvironmental signals coming from other cells and/or from the ECM, and to prevent random adhesion in the blood stream.

In these experiments (Fig. 16b), it was also demonstrated that the pattern of inhibition of Con-A-activated T cell binding to FN by anti-VLA mAbs was very similar to that observed for SF T cells (Fig. 15). Again, all three mAbs inhibited binding to FN, but the most inhibitory one was anti-VLA5. Thus, mitogen activation of PB T cells led to the development of characteristics similar to that of SF T cells, including binding capabilities, expression and use of VLA molecules. This suggests that the increased binding of SF T cells to FN compared to PB T cells is more related to their state of activation than to their phenotype. However, it must be remembered that SF T cells are both activated and of the CD45R0 phenotype; therefore, both elements may contribute to their interaction with ECM in vivo. In actual fact, the largely proinflammatory nature of CD45R0 T cells may be responsible, together with both Ag-dependent and independent stimuli, for the activation status of these cells within the synovium. In line with this is the fact that CD45RO T cells express high levels of LFA1 and VLA integrins [94], which make them more susceptible to activation.

There is increasing evidence that lymphocyte–ECM interactions are important not only in cell retention within the tissues, but also in cell activation. FN has been shown to synergize with T-cell receptor (TCR) ligation in stimulating the proliferation of CD4 T cells [97]. Activation signals are mediated by FN interactions with VLA molecules since this synergism can almost be completely abolished by anti-VLA5 and, to a lesser extent, anti-VLA4 mAb [86, 98]. One of the intracellular changes in CD4 cells associated with the VLA5 and VLA4 interaction with FN is the activation of transcription factors (AP-1 and NFAT-1) which in turn induce interleukin 2 gene expression and subsequent cell proliferation [99].

In conclusion, the adhesion to ECM components certainly plays an important role in the retention of CD45RO T cells within the synovium. Furthermore, the abundance of FN in the synovium and other

Fig. 15.—Peripheral blood (a) and synovial fluid (b) T-cell binding to fibronectin in the presence of mAbs to VLAa4, VLAa5 and VLAβ1. Results are expressed as the normalized mean of five experiments ± 1 S.E. None of the inhibition values for peripheral blood are significantly different from that of the irrelevant monoclonal antibody. For the synovial fluids, the values are $P = 0.002, 0.02$ and 0.005 for anti-VLAβ1, VLAa4 and VLAa5, respectively. Statistical comparisons were made by Student’s $t$-test. (Reproduced with permission from [6].)

Fig. 16.—(a) Kinetics of expression of VLAa4, VLAa5 and VLAβ1 molecules on concanavalin A-activated T cells. Results are expressed as the percentage of cells expressing the VLAa5 marker, and bright for VLAa4 and VLAβ1. (b) Concanavalin A-activated T-cell binding to fibronectin in the presence of mAbs to VLAa4, VLAa5 and VLAβ1. Results are expressed as the normalized mean of six experiments ± 1 S.E. (Reproduced with permission from [6].)
inflammatory foci may perpetuate the immune/inflammatory response not only by favouring T-cell retention, but also by a direct effect on T-cell activation [97, 99].

SUMMARY AND CONCLUSIONS

In summary, the studies presented here have demonstrated that of the various potential pathogenetic mechanisms which could lead to CD45R0 accumulation at different inflammatory sites, preferential adhesion to and migration through endothelium is certainly one of the most important. Furthermore, evidence was presented to support the concept that along with migration mechanisms regulated by inflammatory events, organ-selective mechanisms contribute to control access of distinct CD45R0 lymphocytes into different tissues. Finally, data were presented which demonstrate that adhesion to ECM components also plays an important role in the preferential retention within the tissues of CD45R0 T cells. I believe that these studies have contributed to highlighting the crucial role played by adhesion mechanisms in the pathogenesis of chronic synovitis and this may have major therapeutic implications.

The general realization of the importance of adhesion mechanisms has, in fact, prompted several groups to develop 'anti-adhesion' therapies in arthritis as well as other inflammatory conditions and transplantation. For example, the therapeutic potential of mAb against adhesion molecules has been amply demonstrated in a variety of animal models, including allograft rejection, cardiac reperfusion injury and experimental autoimmune encephalomyelitis [100–102]. In addition to the use of mAb, several other ways of modulating cell adhesion are currently being investigated. Of great interest is the use of synthetic oligosaccharides to block the terminal sugars on selectin molecules [103, 104], and the use of soluble and recombinant ICAM-1 constructs [105].

Going back specifically to arthritis, in animal models, blocking either the LFA1 or the VLA4 pathways has been shown to inhibit disease [106, 107]. As a result of these studies, a trial using anti-ICAM-1 mAb has been conducted in RA [108]. The patients showed a transient improvement which correlated with the development of a peripheral blood lymphocytosis, suggesting that inhibition of lymphocyte migration into inflammatory sites was occurring. However, the therapeutic effect could also have occurred by inhibiting other adhesion-dependent immune functions in which ICAM-1 plays a major role, such as T-cell activation. Further understanding of organ-specific recognition systems regulating lymphocyte extravasation into different tissues may provide a more selective therapeutic target. It may also allow greater insight into the complex events which control organ-specific lymphocyte recirculation.

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