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Vascular cell adhesion molecule 1 and α4 and β1 integrins in lymphocyte aggregates in Sjögren's syndrome and rheumatoid arthritis

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Abstract

***Objectives*—Interactions between vascular cell adhesion molecule 1 (VCAM-1) and its ligand, the α4/β1 integrin, have been shown to be important in a number of cellular events in vitro. To assess the importance of such interactions in the development of lymphocytic infiltration in diseased tissue the distribution of the two ligands has been studied immunohistochemically.**

***Methods*—Cryostat sections of labial tissue from patients with Sjögren's syndrome, normal labial tissues, rheumatoid synovia, and normal tonsils were stained using antibodies to VCAM-1, α4 and β1 integrin chains, and markers for T cells, B cells, macrophages, and follicular dendritic reticulum cells (FDRCs), visualised using alkaline phosphatase and fast red.**

***Results*—Staining patterns for VCAM-1 and integrin chains in lymphocyte aggregates in synovial and labial tissues were similar. VCAM-1 staining was found on both vascular and ramifying dendritic cells at the centre of large T cell aggregates and in all aggregates where there was a central clustering of B cells. VCAM-1 colocalised with, but also extended beyond, staining for the FDRC marker R4/23. Staining for the α4 and β1 integrin chains was more widespread than staining for VCAM-1, with no significant increase in staining at sites of maximum VCAM-1 staining. In tonsils VCAM-1 and R4/23 codistributed in germinal centres, but staining for the α4 and β1 integrin chains was chiefly seen in T lymphocyte areas.**

***Conclusions*—VCAM-1 may be more important in determining the distribution of B than T lymphocytes in lymphocytic infiltration of non-lymphoid tissue. Unlike the follicles of lymphoid tissue, ectopic follicle-like structures in non-lymphoid tissues may form by immigration of B cells via VCAM-1+ vessels at the centre of T cell aggregates.**

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Lymphocytes pass in small numbers through normal non-lymphoid tissues and in chronic inflammation may accumulate in large numbers.[1] In certain situations, including the salivary glands of patients with Sjögren's syndrome[2] and in the synovium of those with rheumatoid arthritis,[3] infiltrating lymphocytes form aggregates which take on a number of the structural features of the follicles seen in lymph nodes, tonsils, or the spleen. Small aggregates contain mostly T cells, but with increasing size B cells appear at the aggregate centre, and in the largest aggregates in synovium true germinal centre formation is seen.

The subpopulation of lymphocytes infiltrating these tissues has been studied in detail. Most of the CD3+ (T cell) population in labial salivary gland tissue from patients with Sjögren's syndrome is CD4+/CD8– and the ratio of 'primed' (CD45RA–/CD45RO+) to 'naive' (CD45RA+/CD45RO–) cells is higher in focal than diffuse areas of infiltrate.[4 5] Most of the T cells carry the αβ T cell receptor.[6] This contrasts with findings in the peripheral blood of patients with Sjögren's syndrome in which the γδ receptor predominates.[7 8] In rheumatoid synovium most of the T cells are also CD4+/CD8– and primed cells predominate over naive cells.[9]

There is evidence of oligoclonality among B cells in labial tissues in Sjögren's syndrome on the basis of κ and λ chain expression.[10] The B cells in rheumatoid synovium appear to be polyclonal and have a wide range of antibody affinities including rheumatoid factor activity.[11 12]

Despite this knowledge, the mechanisms responsible for the accumulation of lymphocytes in such lesions are still unclear. Recent interest has focused on the possible role of the vascular cell adhesion molecule 1 (VCAM-1) and its ligand, the α4/β1 integrin heterodimer (VLA-4, CD29/49d),[13] in rheumatoid arthritis.[14 15] VCAM-1 was first described as being present on activated endothelium.[16] It is now known to be present on dendritic cells[17] and fibroblast-like synoviocytes.[15] It has been implicated in T cell emigration.[18] The adherence of lymphocytes to high endothelial venules in cryostat sections of rheumatoid synovia is dependent on an interaction between the β1 integrin chain CD49d and VCAM-1.[14] VCAM-1 is also implicated in CD3 dependent T cell proliferation, in conjunction with the α4/β1 integrin,[19] and in B cell adherence to dendritic cells (shown in cryostat sections of lymphoid tissue).[20] In normal lymphoid tissue, however, germinal centre B cells are negative or only weakly positive for both chains (α and β) of the integrin ligand.[21] Thus the significance of VCAM-1 expression cannot

entirely be explained on the basis of an interaction with α4/β1 integrin in vivo.

This study aimed to investigate the distribution of VCAM-1 and the α4 and β1 integrin chains on cells within lymphocyte aggregates in non-lymphoid tissues to assess the likely significance of an interaction between these molecules in the development of lymphocyte aggregates and to compare findings with those in follicles in lymphoid tissue.

Materials and methods

TISSUES

Synovial tissue was obtained at arthroplasty from the hip, knee, or wrist of five patients with rheumatoid arthritis (definite or classical by the American Rheumatism Association criteria[22]). Labial biopsy samples were obtained for diagnostic purposes. Of these, six were from patients with Sjögren's syndrome and showed characteristic histological features. Six were from patients with unrelated symptoms and were judged to be histologically normal. Of the patients with Sjögren's syndrome two had no associated rheumatic disease (primary cases) and four had associated rheumatoid arthritis (secondary cases). Patients with rheumatoid arthritis all had a disease duration of more than five years. They had received various combinations of non-steroidal anti-inflammatory drugs and disease modifying drugs, but not systemic corticosteroids. Two samples of tonsil were obtained from a routine tonsillectomy. Where necessary, the tissues were trimmed of fat and fibrous tissue, snap frozen immediately after the operation, and stored at −70°C until required for sectioning. Serial crysostat sections (5 μm thick) were cut on a Bright's cryostat, taken up on to slides, air dried, and stored individually wrapped in aluminium foil in a hermetically sealed box at −70°C until required for immunochemistry.

PRIMARY ANTIBODIES

All monoclonal antibodies used were of the IgG class, with the exception of R4/23, which is IgM. 1·4C3 recognises VCAM-1,[23] EBM11 recognises CD68 on macrophages,[24] UCHT1 recognises CD3 on T lymphocytes,[25] 4KB128 recognises CD22 on B lymphocytes,[26] R4/23 recognises a non-CD designated epitope on follicular dendritic reticulum cells (FDRCs),[27] HP2/1 recognises CD49, the α4 integrin chain,[28] and B-D15 recognises CD29, the β1 integrin chain.[29]

IMMUNOHISTOCHEMISTRY

Sections were removed from storage at −70°C and allowed to reach ambient temperature before being unwrapped. Samples were fixed in cold acetone for ten minutes before incubation with primary monoclonal antibodies at optimum dilution for one hour at room temperature, followed by washing in buffer. All dilutions and incubations were performed in freshly prepared TRIS buffered saline, pH 7·6. For IgG monoclonal antibodies rabbit antimouse immunoglobulins (Dako, High Wycombe, Buckinghamshire, United Kingdom) in 20% normal human AB serum were then applied for 30 minutes, followed by alkaline phosphatase-antialkaline phosphatase complexes (Dako), also in 20% normal human serum, for 30 minutes. For R4/23, biotinylated goat antimouse IgM (Sera Lab) was applied, followed by streptavidin alkaline phosphatase (Dako), both in 20% normal human serum for 30 minutes. Alkaline phosphatase conjugates were developed using naphthol-AS phosphate/fast red to yield a red product. Sections were lightly counterstained in Harris's haematoxylin, washed, and mounted in Apathy's medium.

The lymphocyte infiltration seen in labial tissue in Sjögren's syndrome occurs in the context of little or no other evidence of inflammation. In rheumatoid synovium chronic inflammatory changes such as macrophage infiltration and fibrin deposition are prominent. To gauge the importance of associated chronic inflammation to the pattern of VCAM-1 and integrin chain expression the relative numbers of macrophages (CD68+) and T cells (CD3+) were counted within 10 fields with a ×20 objective for each tissue.

Results

The table gives an overview of the patterns of staining. The normal labial tissues showed small numbers of T cells and macrophages. Staining for VCAM-1 and the integrin chains was minimal. There was no staining with R4/23.

Summary of immunohistochemical findings

Tissue/area	*Staining for adhesion molecules*		
	VCAM-1	*α4 integrin*	*β1 integrin*
Normal labial tissue			
All sites	Minimal	Minimal	Minimal
Sjögren's labial tissue			
Small T cell aggregates	Central on small blood vessels and a few nearby cells	Throughout	Weakly throughout
Larger aggregates	Mesh in central B cell area extending beyond R4/23	Throughout	Weakly throughout
Tissue stroma	A few scattered cells	Most mononuclear cells	Weakly throughout
Rheumatoid synovium			
Small T cell aggregates	Central on small blood vessels and a few nearby cells	Weakly throughout	Throughout
Larger aggregates	Mesh in central B cell area extending beyond R4/23	Weakly throughout	Throughout
Aggregates with germinal centres	Mesh in central B cell area codistributing with R4/23	Weakly throughout	Throughout
Tissue stroma	A few scattered cells	Most mononuclear cells	Throughout
Synovial lining	Prominent	Minimal	Prominent
Tonsils			
T cell areas	Scattered cells, venules	Most cells	Most cells
Follicle centre	Dense mesh throughout	Minimal	Minimal

Most of the infiltrating cells in rheumatoid synovium were macrophages, despite the presence of well developed lymphocytic aggregates. In the labial tissue from patients with Sjögren's syndrome T cells predominated (figure A, B), though the aggregates tended to be smaller than in the rheumatoid tissue. Mean counts of CD68+ and CD3+ cells in 10 fields at ×20 objective magnification were: normal labial tissue CD68+ 48, CD3+ 26·5; Sjögren's syndrome labial tissue CD68+ 108, CD3+ 153; and rheumatoid synovium CD68+ 558, CD3+ 156. B Lymphocytes made up less than 10% of infiltrating mononuclear cells in all instances, being present chiefly at the centres of larger lymphocyte aggregates (figure C).

The amount of staining for VCAM-1, the α4 and β1 integrins, CD22, and R4/23 reflected the numbers of infiltrating cells present. Thus in lesional tissue from patients with either Sjögren's syndrome or rheumatoid arthritis, where there was a paucity of T cells and macrophages, little staining was seen. Where large lymphocyte aggregates were present in either type of tissue, however, VCAM-1 appeared at the centre of the aggregate in an area occupied by B cells and R4/23 positive FDRCs. In smaller aggregates VCAM-1 staining was often limited to one or more central blood vessels and a few nearby cells (figure D), but in larger aggregates VCAM-1 formed a network throughout the whole central B cell containing area (figure E). R4/23 staining for FDRCs was sparse in the Sjögren's syndrome labial tissues, with the exception of the tissue with the largest T cell infiltrate. In Sjögren's syndrome labial tissues and rheumatoid synovial tissues VCAM-1 staining colocalised with, but extended beyond, R4/23 staining in many smaller aggregates. Where fully formed germinal centres were present in rheumatoid arthritic tissue, however, the two markers showed a complete and precise codistribution.

VCAM-1 expression in tonsils was seen most strongly within follicle centres and co-distributed with R4/23, forming a continuous reticulum in the B cell rich area. Individual VCAM-1 positive cells were also present in the outer T cell zone of follicles and VCAM-1 was present on small venules.

Although the distribution of VCAM-1 closely paralleled that of CD22 and R4/23, there was no relation between the distributions of VCAM-1 and either α4 or β1 integrin chains. In the Sjögren's syndrome labial tissue there was expression of α4 within the central areas of lymphocyte aggregates, but extending beyond the VCAM-1 staining and at a similar level to that on macrophages (figure F) and T cells scattered within the tissue. Expression of the β1 integrin was ubiquitous on infiltrating cells but weak, being barely detectable in four of the tissues.

In rheumatoid synovium little α4 integrin was present diffusely in the lymphocyte aggregates, but the intensity of staining was weaker than in Sjögren's syndrome labial tissue, though the pattern was similar, with no preferential expression in areas containing VCAM-1 expressing cells. The β1 chain was, on the other hand, expressed more strongly, particularly on endothelial cells, diffusely in lymphoid aggregates, and within the synovial intima.

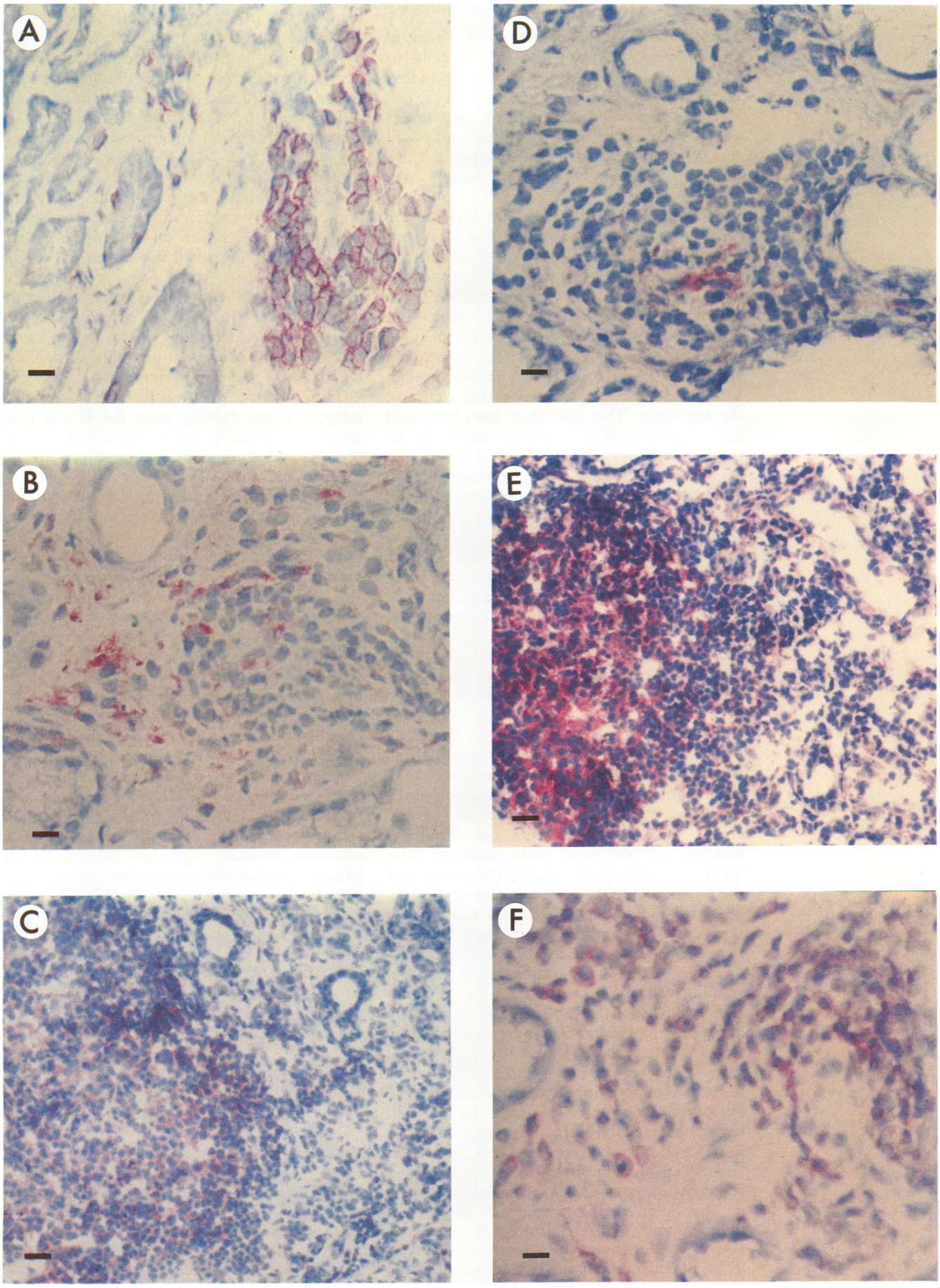
In tonsils α4 and β1 integrin staining was absent from, or only weakly present in, the central VCAM-1 rich areas of follicles.

Discussion

The role of integrins and their ligands in human inflammatory and autoimmune processes remains uncertain. Initially, VCAM-1 was chiefly implicated in the adherence of leucocytes to endothelium, but some studies suggest other possible roles, including the promotion of the proliferation and maturation of lymphocytes.[13–21] Direct evidence for the functional potential of adhesion molecules tends to come from in vitro studies, however. Immunohistochemical analysis plays an important complementary part in assessing the relevance of putative functional interactions to the whole tissue.

Dense lymphocyte aggregates can develop outside lymphoid tissues as part of a chronic inflammatory response, which also includes events such as vascular proliferation, fibrin deposition, and macrophage and polymorph infiltration. This is perhaps best seen in synovial tissue in rheumatoid arthritis. Similar lymphocyte aggregates also occur in the salivary glands of patients with Sjögren's syndrome in the absence of significant inflammation. In this situation there may be a specific disorder of lymphocyte traffic, possibly related to a low grade lymphoproliferative process.[10]

The findings of this study indicate that large lymphocyte aggregates developing in non-lymphoid tissues, in either of the above contexts, mimic the development of lymphoid follicles in terms of the central accumulation of B cells and the presence of associated VCAM-1 and R4/23 positive cells. These latter cells are probably FDRCs, which, unlike other 'dendritic cells', are macrophage marker and class II negative.[30] The implication is that FDRCs can be differentiated from local perivascular fibroblastic cells in non-lymphoid tissues subject to chronic T cell infiltration. VCAM-1 expression within central areas of lymphocyte aggregates often extended beyond that of R4/23 and was often present where R4/23 was absent. This suggests that VCAM-1 expression appears on FRDCs or other supporting cells at an earlier stage of differentiation than R4/23, which is in keeping with ontological studies[31] which suggest R4/23 is a 'late' FDRC marker. (Other markers of FDRCs which might clarify this are, unfortunately, unhelpful in a histological study such as this because they are also present on the adjacent B cell.) Although the factors responsible for T cell infiltration in chronic synovitis and in the labial tissue of patients with Sjögren's syndrome are thought to be different, it appears that the cellular patterns within lymphocyte aggregates follow the same rules.



Sections of labial gland from patients with Sjögren's syndrome stained using an indirect alkaline phosphatase-antialkaline phosphatase technique, developed with fast red, and counterstained with haematoxylin. (A) Staining for CD3 showing that most lymphocytes in aggregates found between salivary acina are T cells. Bar = 20 μm. (B) Staining for CD68 showing macrophages scattered within the tissue stroma. Bar = 20 μm. (C) Staining for CD22 showing an area of positive cells in the centre of a lymphocyte aggregate. Bar = 40 μm. (D) A small T cell cluster showing staining for VCAM-1 on a few central cells, probably including a small vessel. Bar = 20 μm. (E) Extensive VCAM-1 staining at the centre of a large lymphocyte aggregate. Bar = 40 μm. (F) Staining for α4 integrin chain showing positive cells diffusely within a lymphocyte aggregate, and also scattered among the tissue stroma. Bar = 20 μm.

Despite the demonstration that VCAM-1 binding to the T cell surface of α4/β1 integrin can increase the adhesion of T cells to endothelial cells in vitro, it seems unlikely that VCAM-1 expression is important in T cell infiltration in the tissues studied. In samples where lymphocyte infiltrates were composed almost exclusively of T rather than B cells, VCAM-1 expression on the vessels was minimal.

In contrast, B cells were not seen at the centre of aggregates unless VCAM-1 positive cells were also present. There is evidence that in lymphoid tissues B cells migrate to the centre of follicles from the surrounding tissue through the outer T cell rich zone.[32] In inflamed tissues, however, it seems possible that B cells appearing at the centre of aggregates have entered the tissue through VCAM-1 expressing venules at the centre of the aggregate. This difference from lymphoid tissue would not, perhaps, be surprising. In lymph nodes lymphocyte entry is thought to occur chiefly through the specialised high endothelial venules of the paracortical T cell areas. High endothelial venules are not present constitutively in synovium, but appear in rheumatoid arthritis vessels with similar properties, though only in areas rich in T cells.[33] It seems reasonable to suggest that once T cells have accumulated in sufficient numbers to form substantial aggregates they induce VCAM-1 expression on cells in and around the walls of venules lying within the aggregates, and that the influx of B cells is then facilitated at this site.

The most unexpected finding of the study was the lack of correlation between the distribution of VCAM-1 expression and that of the integrin chains. This could suggest that the degree of expression of α4 and β1 integrin chains on lymphocytes does not determine whether or not these cells associate with VCAM-1 positive cells, raising the question of the function of VCAM-1 expression at this site. The explanation may be complex and the lack of apparent codistribution of such adhesion ligands should perhaps not be too surprising.

The intensity of immunohistochemical staining will not necessarily reflect the functional activity of the molecule identified. The apparent staining intensity may be affected by technical and geometrical factors such as cell size. Integrins may be present as non-functional newly synthesised protein within the cytoplasm, and once expressed on the cell surface their avidity may be modulated by a number of other molecules. α4 and β1 integrin chains can be combined with other (β and α respectively) partners. β1 can combine with α1–3, α5–8, and αV to form heterodimers that do not bind VCAM-1, and will do so on activated T cells and monocyte/macrophages. This provides a simple explanation for the ubiquitous presence of β1 carrying cells in the tissues examined.

α4 can pair with β7, but this also has an affinity for VCAM-1, and it is the failure of codistribution of α4 with VCAM-1 which is the most puzzling. One possibility is that T lymphocytes use the α4/β1 heterodimer as an assistance to cell migration. The α4/β1 combination also binds fibronectin and it may be that the T cell responds to the binding of α4/β1 integrin to a ligand by increased movement rather than by continued attachment. In contrast, when α4/β1 integrin on B cells comes into contact with VCAM-1, the B cell may respond by a reduction or end of movement and, if concomitant antigen specific signals are appropriate, by proliferation. There is also an outside possibility that an alternative ligand for VCAM-1, not involving either α4 or β1 integrin chains, could be preferentially expressed on B lymphocytes. Either way it would appear that B cells allow VCAM-1 positive cells to envelope them, giving close correspondence between the extent of staining for VCAM-1 and the B cell marker.

The absence of prominent α4 and β1 integrin chain staining from the B cell rich areas in the tissues studied may not mean that the B cells did not carry larger amounts of the integrin on arrival at the site. α4 and β1 integrin chains have been found to be absent or only weakly expressed on the B cells in lymphoid tissue of normal human tonsils, Peyer's patches, and peripheral lymph nodes,[21] but circulating B cells and B cells activated in vitro express more of α4 and β1. The fact that staining for the integrin chains was present in any amount in the B cell rich areas of diseased tissue (in contrast with a virtual absence in B cell rich areas of tonsils) suggests that in diseased tissue a higher proportion of B cells may have recently immigrated from the circulation or be undergoing activation.

VCAM-1 is expressed strongly on fibroblastic synoviocytes, where it may interact with integrin on the surface of the closely associated macrophages. The function of such an interaction is unknown. Thus it appears that a molecule initially implicated specifically in leucocyte/endothelial interactions has a number of roles in diseased non-lymphoid tissues which remain to be elucidated.

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