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THE VLA-4/VCAM-1 PATHWAY IS INVOLVED IN LYMPHOCYTE ADHESION TO ENDOTHELIUM IN RHEUMATOID SYNOVIUM[1]

ANNA C. H. M. VAN DINTHER-JANSSEN,[2]* EVELIENE HORST,[3]* GERRIT KOOPMAN,[3]* WALTER NEWMANN,† RIK J. SCHEPER,* CHRIS J. L. M. MEIJER,* AND STEVEN T. PALS[3]*

*From the *Department of Pathology, Free University Hospital, Amsterdam, The Netherlands; and †Otsuka America Pharmaceutical, Inc., Research Laboratories (Maryland), Rockville, MD*

Lymphocyte migration to inflammatory sites is an essential factor in the pathogenesis of chronic inflammation. An ensemble of adhesion receptors mediating lymphocyte-endothelial cell recognition and binding are thought to play a crucial role in this process. In the present study, we have explored the molecular basis of lymphocyte adhesion to endothelium in the synovial membrane of patients with rheumatoid arthritis. We established that the very late antigen-4 [VLA-4 (CD49d)] and the vascular cell adhesion molecule-1 (VCAM-1) are important mediators of binding to synovial endothelium of resting and, to a greater extent, of activated T lymphocytes, whereas the leukocyte-function associated antigen-1 [LFA-1 (CD11a/18)]/intercellular adhesion molecule-1 [ICAM-1 (CD54)] pathway is less important in this interaction. In contrast to its prominent role in lymphocyte interaction with endothelium in rheumatoid synovium, the VLA-4/VCAM-1 pathway does not significantly contribute to lymphocyte adhesion to peripheral lymph node high endothelial venule. Thus, the VLA-4/VCAM-1 pathway may be of primary importance in mediating lymphocyte adhesion to inflamed endothelium and in lymphocyte homing to rheumatoid synovium.

Lymphocytes play a central role as regulatory and effector cells in inflammation but are also essential mediators of tissue damage in, for example, RA[4] and other autoimmune diseases. Elucidation of the mechanisms that control lymphocyte trafficking to sites of immunologic assault might provide new tools to control such damage. The exit of circulating lymphocytes from the blood into the lymphoid tissues is regulated by interactions with specialized venules lined by HEV through adhesion molecules, termed "homing" receptors (1–5). Distinct sets of homing receptors/ligands are thought to mediate lymphocyte migration to various anatomical sites (2–5). HEV are constitutively present in lymph nodes and mucosa associated lymphoid tissues. In addition, vessels with phenotypic and functional characteristics of HEV can also develop at sites of chronic inflammation, including rheumatoid synovium (2, 5–9).

It is not at present known which molecules mediate the lymphocyte-endothelial cell recognition and binding in rheumatoid synovium. However, two different molecular pathways are known that affect lymphocyte adhesion to in vitro cultured human endothelial cells. The leukocyte integrin LFA-1 (CD11a/18) is responsible for most of the basal binding of lymphocytes to unstimulated endothelium via its ligands ICAM-1 (CD54) and/or ICAM-2 (10, 11). Although ICAM-1 is strongly induced on cytokine-stimulated endothelium, evidence obtained both in vivo and in vitro indicates that one or more other adhesion molecules are required for lymphocyte recruitment. Leucocyte adhesion deficiency patients, who lack LFA-1 and hence the known ligand of ICAM-1/2, demonstrate essentially normal migration of lymphocytes in vivo (12). In vitro, binding of T lymphocytes to cytokine-stimulated endothelium can be inhibited only partially by anti-LFA-1 mAb, indicating that at least one more inducible molecule mediates binding (10, 11). One such molecule, VCAM-1, has recently been identified (13–18); it is induced strongly on cytokine stimulated in vitro cultured endothelium and like the ICAM is a member of the Ig superfamily (13). It binds lymphocytes and monocytes via the β1-integrin VLA-4 (14, 15). In the present study we have explored the molecular basis of lymphocyte homing to rheumatoid synovium by analysis of adhesion receptor expression on HEV-like vessels in the synovium of RA patients and by studying lymphocyte-endothelial cell binding.

MATERIALS AND METHODS

Mab. The mAb used were CLB-CD45 (IgG1), specific for CD45 (19); NKI-P1 (IgG1), specific for anti-CD44 (20); Dreg-56 (IgG1), specific for LECAM-1 (LAM-1) (21); HP 2/1 (IgG1) and HP1/3 (IgG3), specific for the α-chain of VLA-4 (CD49d) (22); 2G7 (IgG1) and 4B9 (IgG1), specific for VCAM-1 (15, 16); and CLB-LFA-1/2 (IgG1), specific for the α subunit of LFA-1 (CD11a) (19).

In vitro HEV-binding assay. The in vitro binding of human lymphocytes to HEV was measured by means of the frozen section assay (23). Briefly, 1×10^6 lymphocytes were incubated with mild rotation for 30 min at 4°C on freshly cut frozen sections of PLN or synovial membranes of RA patients. After incubation, the sections

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[1] This work was supported by Grant 88/CR/032/89 from the Dutch Rheumatism Foundation.
[2] Address correspondence and reprint requests to A. C. H. M. van Dinther-Janssen, Department of Pathology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
[3] Current address: Department of Pathology, University of Amsterdam, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.
[4] Abbreviations used in this paper: RA, rheumatoid arthritis; HEV, high endothelial venule; VLA-4, very late antigen-4; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function associated antigen-1; LECAM-1, lectine adhesion molecule-1; PLN, peripheral lymph node; SM, synovial membrane; PE, peritoneal exudate; CAPD, continuous ambulant peritoneal dialysis; PB-T, peripheral blood T lymphocytes; SM-T, synovial membrane T lymphocytes; PE-T, peritoneal T lymphocytes.

were fixed in cold PBS containing 2% glutaraldehyde and briefly stained with 1% toluidine blue in aquadest. HEV adherent cells were quantified per standard unit of HEV length by means of a graphic tablet fitted to a microcomputer (MOP-Videoplan, Kontron, Eching, FRG) as described previously (24). Per tissue section, 20 to 50 vessel cross-sections were examined. mAb concentrations used were based on titration studies to obtain optimal inhibitory effects. Tissues used

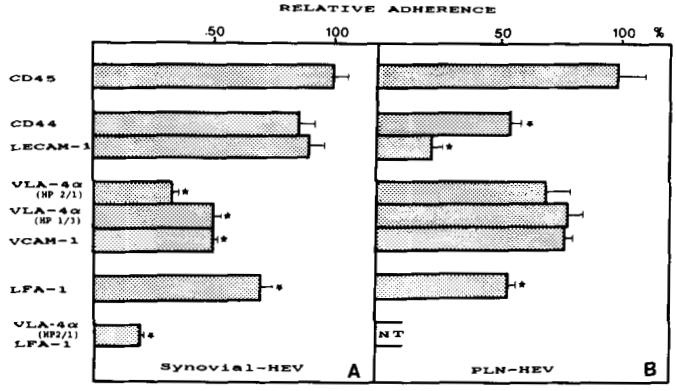

Figure 1. Inhibition of adhesion of normal human PB-T to synovial (*A*) and PLN HEV (*B*) by mAb against various adhesion molecules. Adherence is expressed as percentage of the control (no mAb added). Each value represents the mean ± SE of at least three experiments. The anti-VCAM-1 mAb 2G7 and 4B9 gave identical results. Absolute binding of PB-T: SM HEV, 5.75 ± 0.52/mm HEV; PLN HEV, 18.59 ± 2.94/mm HEV. *, significantly different from control by two-tailed Student's *t* test.

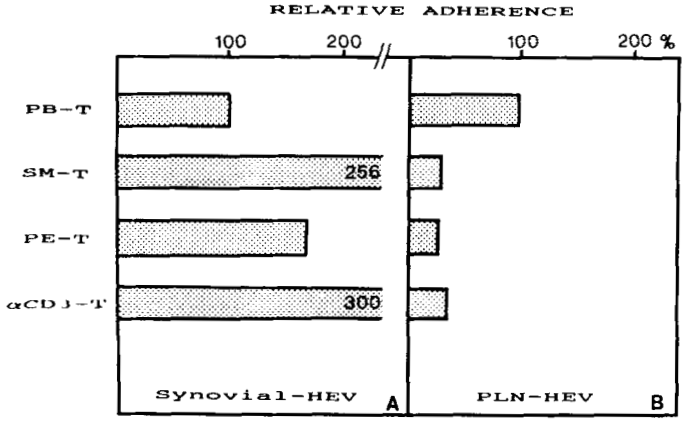

Figure 2. Binding of activated T lymphocytes to synovial (*A*), and PLN HEV (*B*). PB-T, normal (nonactivated) peripheral blood T cells; SM-T, T cells from rheumatoid synovium; PE-T, peritoneal T cells; anti-CD3-T, anti-CD3 stimulated T cell. Adherence is expressed as percentage of the binding of PB-T. Each value represents the mean ± SE of at least three experiments. Absolute binding of different T cell populations to synovial and PLN HEV is shown in Table I.

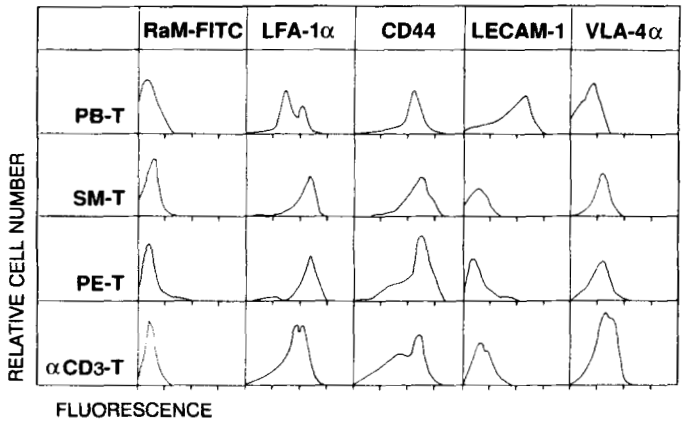

Figure 3. Expression of adhesion receptors on resting and activated T lymphocytes from various sources.

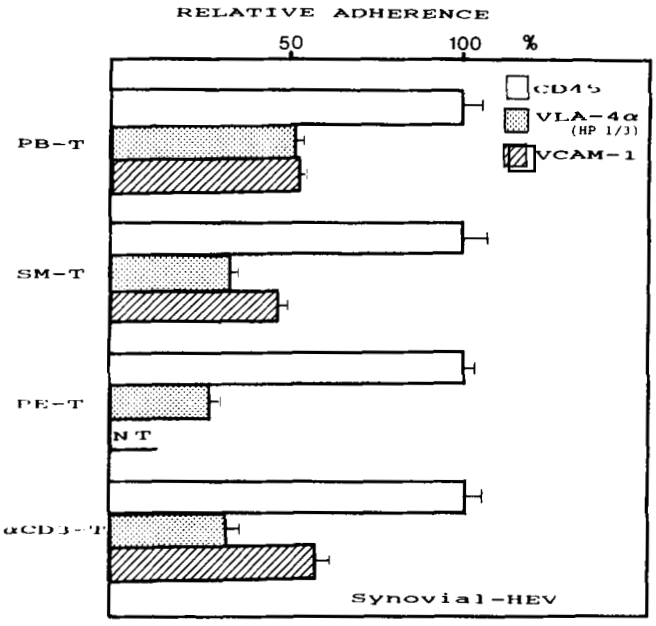

Figure 4. Inhibition of adhesion of activated T lymphocytes from various sources by mAb against VLA-4α and VCAM-1. Adherence is expressed as a percentage of the control (no mAb added). Each value represents the mean ± SE of at least three experiments.

TABLE I

Binding of T cells to synovial and PLN HEV

Cell Type	SM HEV	PLN HEV
PB-T	5.75 ± 0.52[a]	18.59 ± 2.94
SM-T	15.28 ± 0.91	5.08 ± 1.06
PE-T	9.66 ± 0.62	4.82 ± 0.79
α-CD3-T	15.39 ± 3.50	6.54 ± 0.98

[a] Results are expressed as the number of T cells/mm HEV length. Each value represents the mean ± SE of at least three experiments.

were synovial membranes from joints of patients with classic or definite RA according to the criteria of the American Rheumatism Association and were obtained at reconstructive joint surgery. Non-rheumatoid synovial membranes were obtained from amputated limbs of patients with vascular insufficiency. In addition, normal or reactive PLN were also used.

Isolation of lymphocytes. PB-T (n = 10) were isolated by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes were removed by adherence on plastic petri dishes (Falcon Plastics, Oxnard, CA) for 1 h at 37°C and the nonadherent cells were depleted of B cells by a panning technique (24). PB-T contained >95% $CD2^+$ cells and <5% surface Ig^+ cells as determined by immunofluorescence. SM-T (n = 10) were recovered by enzymatic digestion of synovial tissue fragments with medium (0.5 g tissue/ml) containing DNAase (1 mg/ml), collagenase (2 mg/ml), and hyaluronidase (2 mg/ml) (Boehringer, Mannheim, FRG) for 1 h at 37°C. The supernatant was collected and fresh enzymes were added to the tissue for a second incubation. The supernatants were pooled and T cells were isolated as described above. Cells isolated from synovial membranes contained >80% $CD2^+$ cells that were >90% $CD4^+$, <5% surface Ig^+ cells, and <15% synoviocytes and monocytes. Although the latter cells also bound to HEV, they could readily be discriminated from lymphocytes on morphologic grounds. In control experiments it was established that enzymatic treatment of PB-T did not effect expression of the adhesion molecules studied or T-cell HEV interaction. PE-T (n = 3) were isolated from the peritoneal exudate of CAPD patients as described above. T cells isolated from PE contained >90% $CD2^+$ cells and <5% surface Ig^+ cells. PB-T from normal donors were activated in vitro by culturing with anti-CD3 mAb OKT3 (0.01%, Ortho Diagnostic Systems Inc, Raritan, NJ) and rIL-2 (α-CD3-T, n = 4).

FACS analysis. Cell surface expression of adhesion molecules was analyzed on a FACS-Star flow cytometer (Becton-Dickinson, Mountain View, CA) by indirect immunofluorescence.

Immunohistochemistry. Immunoperoxidase staining was performed on 4-μm acetone-fixed cryostat sections of synovium by an indirect immunoperoxidase procedure as described previously (20). The anti-VCAM-1 antibodies used were 2G7 (16) and 4B9 (17), and

gave identical results. The second step antibody was horseradish peroxidase-conjugated goat anti-mouse Ig (DAKO, Glostrup, Denmark).

RESULTS AND DISCUSSION

Synovectomy specimens from patients with definite or classical RA showing marked lymphocytic infiltration were selected for study. They contained a spectrum of vessel types; in addition to venules with a flat endothelial lining, venules lined by endothelial cells morphologically resembling HEV in lymph nodes were present. Many of these activated vessels stained with mAb against VCAM-1 (data not shown). The intensity of VCAM-1 expression was variable, ranging from weak to moderate, but was highly inflammation-specific, as the Ag was not detectable on vessels in normal synovium. Also, VCAM-1 was only sporadically detectable on HEV of PLN.

In agreement with previous reports (5, 8), we observed that normal PB-T, when overlaid on frozen sections of RA synovium, bind preferentially to HEV-like synovial vessels. Binding to vessels in control synovia was not observed. To explore the molecular basis of this adhesion, we examined a panel of mAb for inhibition of lymphocyte binding to these synovial vessels. For comparison, and to expose the specificity of the inhibition, parallel experiments measuring inhibition of lymphocyte interaction with PLN HEV were performed. Adhesion of unstimulated PB-T to synovial vessels was strongly inhibited by antibodies against VLA-4α (mAb HP2/1) and VCAM-1 (% inhibition, 67 ± 3 and 48 ± 4, respectively, $p < 0.001$) and to a lesser extent by antibodies against LFA-1α (% inhibition, 27 ± 6, $p = 0.05$) (Fig. 1*A*). Inhibition by the anti-VLA-4α mAb HP2/1 (67 ± 3%) was significantly ($p < 0.05$) stronger than by the anti-VLA-4α mAb HP1/3 (48 ± 3%), inhibition by the latter mAb being comparable with that by anti-VCAM-1 (Fig. 1*A*). This difference between the two VLA-4 mAb might be explained by the fact that the epitope recognized by HP1/3 is uniquely involved in VCAM-1 binding, whereas the HP2/1 epitope of VLA-4 is involved in binding to both VCAM-1 and the CS1 domain of fibronectin (14).

Indeed, it has recently been shown by Ager and coworkers (25) that synthetic CS1 peptides can partially inhibit lymphocyte binding to rat HEV. In addition, CS1 peptides also partially block lymphocyte adhesion to rheumatoid synovium (A. C. H. M. van Dinther-Janssen, S. T. Pals, R. J. Scheper, and C. J. L. M. Meijer, manuscript in preparation). Antibodies to the other adhesion receptors and cell surface antigens examined, i.e. the lymphocyte homing receptor CD44 (20), the peripheral lymph node homing receptor LECAM-1 (21), and the common leukocyte Ag CD45 (19), had no significant effect on lymphocyte binding to synovial HEV (Fig. 1*A*). These findings are in marked contrast to lymphocyte binding to PLN HEV that is largely dependent on LECAM-1 (% inhibition, 77 ± 6, $p < 0.001$), although LFA-1 and CD44 (Fig. 1*B*) are involved as well (% inhibition, 45 ± 5 and 42 ± 6, respectively, $p < 0.05$), as was described before by us and others (5, 20–22, 24). mAb against VLA-4α and VCAM-1 slightly but not significantly inhibited lymphocyte binding to PLN HEV, suggesting that this pathway is, if at all, of minor importance in this interaction. T lymphocyte binding to non rheumatoid synovial vessels was not observed.

Our data indicate that the VLA-4/VCAM-1 pathway is important in the binding of normal (nonactivated) PB-T to synovial endothelium, while binding to PLN-HEV is mediated primarily through LECAM-1. In patients with active RA, however, activated T lymphocytes are found in the peripheral blood and inflamed joints (26–29). These cells, which are believed to play a pivotal role in the disease process, conceivably possess homing characteristics that differ importantly from those of nonactivated PB-T. In support of this notion, activated or previously activated (memory) T cells have been shown to have an increased ability to adhere to activated cultured endothelium, and comprise the great majority of T cells recirculating through extra-lymphoid inflammatory sites (29–31). Compared to virgin T cells they express increased levels of adhesion receptors like CD44, LFA-1, and VLA-4 (32) but, on the other hand, expression of the PLN homing receptor (LECAM-1) is often decreased (3, 21, 33).

Hence, to create a situation more reminiscent of RA in vivo, we subsequently studied the adhesion to synovial and PLN-HEV of T lymphocytes that had been isolated from the synovia of patients with active RA. In the same experiments, normal (nonactivated) PB-T, and activated T cells from two different sources other than synovium, i.e., PB-T stimulated in vitro with anti-CD3 mAb and IL-2, and PE-T from patients undergoing CAPD, were also studied. In comparison with normal PB-T, RA SM-T bound much more efficiently to synovial HEV (Fig. 2*A*, relative adherence 256%). Similarly, PE-T and anti-CD3 stimulated T cells showed high relative binding to synovial vessels (Fig. 2*A*, relative adherence 170 and 300%, respectively). In sharp contrast, all three of these activated cell populations showed poor binding to PLN HEV, the relative adherence compared to PB-T being 20 to 30% (Fig. 2*B*). These results indicate that after activation, either in vivo or in vitro, T lymphocytes undergo changes in adhesiveness that facilitate their binding to venules in inflamed RA synovium but greatly reduce their adhesiveness for PLN HEV.

To clarify the molecular basis of this altered adhesiveness, we studied the expression of cell surface adhesion molecules by the activated T cells using FACS analyses, and determined the role of these molecules in synovial-HEV binding. Compared to resting PB-T, synovial, peritoneal and anti-CD3-stimulated T cells showed a two- to three-fold increase in CD44 and LFA-1 expression. Moreover, a 5- to 10-fold increase in VLA-4α expression was found (Fig. 3); the latter molecule largely mediated the binding of the activated cells to synovial endothelium (Fig. 4). These results are consistent with the observations of Shimizu et al. (34) that within the CD4 population only activated memory cells bind to VCAM-1 transfected L cells. By contrast, expression of the PLN homing receptor LECAM-1 on the activated T cells was almost completely down-regulated (Fig. 3), a finding which readily explains their poor binding to PLN HEV (Fig. 2*B*).

In conclusion, the data suggests that the VLA-4/VCAM-1 pathway functions as an inflammation-specific homing receptor/ligand system mediating preferential recruitment of activated lymphocytes to inflammatory sites. This finding may have clinical relevance since it is conceivable that blocking of this pathway, e.g., by mAb, might selectively interfere with lymphocyte trafficking to

inflamed tissues in vivo and hence with a pivotal step in the pathogenesis of chronic inflammatory the diseases.

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