IMMUNOHISTOLOGIC ANALYSIS OF THE DISTRIBUTION OF CELL ADHESION MOLECULES WITHIN THE INFLAMMATORY SYNOVIAL MICROENVIRONMENT

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Antigen-independent binding of T lymphocytes to a variety of cell types has been shown to be mediated by receptor–ligand pairs of adhesion molecules. In forms of inflammatory synovitis (including rheumatoid arthritis), T cells home to synovium, become activated, and participate in the generation of chronic synovitis. Using indirect immunofluorescence assays on synovial frozen tissue sections and on synovial fibroblast cell lines, we studied the distribution of cell adhesion molecules on components of the synovial microenvironment in inflammatory synovitis. We reasoned that analysis of the cell types within synovium that express adhesion molecules might provide clues to lymphocyte–stromal interactions that occur in inflammatory synovitis. We found that antibodies against the lymphocyte function–associated antigen 3 (LFA-3) molecule and the intercellular adhesion molecule 1 (ICAM-1) both reacted with macrophage-like type A synovial cells and synovial fibroblasts, as well as with tissue macrophages and vessel endothelium. Using flow cytometry, we found that anti–LFA-3 and anti–ICAM-1 (but not antibodies against their ligands CD2 and LFA-1) reacted with synovial fibroblast cells cultured in vitro. Thus, these data demonstrate that the ligands for lymphocyte LFA-1 molecules (ICAM-1) and for T cell CD2 molecules (LFA-3) are widely distributed among cell types of the synovial microenvironment and provide numerous cell types with which lymphocytes can interact via these 2 adhesion pathways during the course of inflammatory synovitis.

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by inflammation of synovium and by a myriad of extraarticular manifestations (reviewed in ref. 1). The mediators of joint inflammation have been shown to be both cellular and soluble (1–3). The interaction of lymphoid cells with cells in the synovial microenvironment has been suggested to be important in the pathogenesis of RA (4). Recently, cell adhesion molecules that mediate interactions between lymphocytes and various accessory or target cells have been described (reviewed in ref. 5). Importantly, many of these interactions mediated by adhesion molecules and their ligands may have functional consequences for T cells as well as for the cells to which they bind (6,7).

The intercellular adhesion molecule 1 (ICAM-1) has recently been shown to be a ligand for the lym-

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Supported by NIH grants CA-28936, AR-34808, and AI-23308.

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Submitted for publication February 17, 1988; accepted in revised form August 23, 1988.

phocyte function–associated antigen 1 (LFA-1) molecule (8,9). The binding of T lymphocytes to the endothelium of vessels has been suggested to occur via ICAM-1 on endothelial cells (6) and via LFA-1 on T cells (6,10). Moreover, the LFA-3 molecule has recently been shown to be an endogenous ligand for the CD2 (E-rosette receptor, T11, LFA-2) molecule (11–15) and to mediate antigen-independent T cell activation (16,17). In studies of T cell–microenvironment interactions, it has been shown that developing thymocytes bind to thymic epithelial (TE) cells via CD2 molecules on T cells and LFA-3 on TE cells (7,16,17). Whereas binding of TE cells to immature thymocytes in vitro activates them to proliferation and clonal expansion (18), binding of thymocytes to TE cells via TE LFA-3 molecules results in TE cell production of interleukin-1 (IL-1) (19) and the induction of TE expression of major histocompatibility complex (MHC) class II antigens (20). Thus, the concept has arisen that T cells can modulate the expression of MHC class II molecules and cytokine production by stromal cells of the thymic microenvironment (7).

In the present study, to identify cell types that might use these cell adhesion molecules in synovial microenvironment–T cell interactions, we used monoclonal antibodies (MAb) and indirect immunofluorescence assays to determine the expression of adhesion molecules by nonlymphoid cells (synovial cells, macrophages, endothelial cells) in the inflammatory synovial microenvironment. We found that anti–LFA-3 and anti–ICAM-1 antibodies bound to synovial fibroblasts, synovial type A macrophage-like cells, tissue macrophages, and synovial vessel endothelial cells.

PATIENTS AND METHODS

Patients. All patients were seen at Duke University Medical Center from December 1986 to March 1987. Synovial tissue was obtained at the time of reconstructive joint surgery from 5 patients with classic RA according to the criteria of the American Rheumatism Association (21), 3 patients with osteoarthritis (OA), and 1 patient with systemic lupus erythematosus (SLE). Clinical characteristics of these patients are summarized in Table 1.

Synovial cells grown in culture. Synovial fibroblast cultures were initiated by an explant technique (22) and propagated in enriched culture medium (23,24) containing a 3:1 (volume/volume) mixture of Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) and Ham's F-12 nutrient mixture (Gibco) with the following supplements at the final concentrations indicated: 5% fetal calf serum (Gibco), 0.4 μg/ml hydrocortisone (Calbiochem-Behring, La Jolla, CA), 20 ng/ml epidermal growth factor (Collaborative Research, Waltham, MA), $10^{-10}M$ cholera enterotoxin (Schwarz-Mann, Orangeburg, NJ), 5 μg/ml insulin (Sigma, St. Louis, MO), $1.8 \times 10^{-4}M$ adenine (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Gibco). Primary cultures were subcultured into 75-cm^2 tissue culture flasks.

Histopathologic techniques. Portions of each synovial specimen were formalin-fixed, paraffin-embedded, and stained for light microscopy using hematoxylin and eosin. Additional portions of each specimen were snap-frozen in ethanol–dry ice slurry and then embedded in OCT compound (Miles Scientific, Naperville, IL). Sections (4 μm) were cut at −20°C, fixed in cold acetone for 5 minutes, and stored at −110°C. In some experiments, frozen sections

Table 1. Clinical characteristics of patients studied*

Patient no.	Age/sex	Diagnosis (disease duration, yrs.)	Source of synovial specimen	Arthritis medications (dosage)
7	62/F	RA (8)	Hand	ASA (3.5 gm/day); AUR (6 mg/day); Pred (5 mg/day); Napr (375 mg/day)
16	61/F	RA (6)	Foot	Pirox (20 mg/day); MTX (7.5 mg/week)
18	41/F	RA (16)	Hand	SSZ (1 gm/day); Napr (500 mg/day); ASA (3.25 gm/day)
22	63/F	RA (10)	Knee	Pirox (20 mg/day); Pred (2 mg/day); MTX (5 mg/week)
38	69/F	RA (16)	Knee	ASA (2.5 gm/day); Pirox (20 mg/day)
11	58/F	OA (10)	Knee	None
13	67/F	OA (11)	Knee	Indomethacin (150 mg/day)
19	72/F	OA (13)	Knee	None
12	55/F	SLE (9)	Hand	Pred (7 mg/day); Pirox (20 mg/day); HCQ (200 mg/day)

* RA = rheumatoid arthritis; ASA = acetylsalicylic acid; AUR = auranofin; Pred = prednisone, Napr = naproxen; Pirox = piroxicam; MTX = methotrexate; SSZ = sulfasalazine; OA = osteoarthritis; SLE = systemic lupus erythematosus; HCQ = hydroxychloroquine sulfate.

were incubated with MAbs without prior acetone fixation. Cultured synovial cells were removed from flasks using 0.2% trypsin in phosphate buffered saline containing 0.02% EDTA, and 2×10^4 cells were applied to gelatin-coated slides using a cytocentrifuge. Slides were fixed for 5 minutes in cold acetone and stored at −110°C. Indirect immunofluorescence was performed as described previously (25).

Monoclonal antibodies. The following monoclonal reagents were used (Table 2): Leu-M3 (anti-macrophage lineage) (26), T4/19THY5D7 (anti-CD4) (27), 3A1 (anti-CD7) (28), B1 (anti–B cell) (29), L243 (anti–MHC class II) (30), 3F10 (anti–MHC class I) (31), TS2/9 (anti–LFA-3) (32), OKT11 (anti-CD2) (33), RR1/1 (anti–ICAM-1) (34), TS1/22 (anti–LFA-1) (32), and M38 (binds to C-terminal region of type I procollagen) (35). In each experiment, the negative control used was the IgG1 paraprotein secreted by the myeloma line P3X63Ag8 (36).

Flow cytometry. Flow cytometric analysis was performed on synovial cell suspensions using an Ortho 50H flow cytometer in indirect immunofluorescence assays, as described previously (37).

Criteria for distinguishing components of the synovial microenvironment. MAbs (in indirect immunofluorescence assays) and hematoxylin and eosin–stained frozen tissue sections were used to identify structures and cell types within each synovial specimen. Four major cell types were studied in detail: macrophage-like (type A) synovial cells, synovial fibroblasts, tissue macrophages, and endothelial cells. Type A synovial lining cells express MHC class II and macrophage surface antigens (38), are phagocytic, and are derived from bone marrow stem cells (39). Fibroblast-like type B synovial lining cells do not express macrophage antigens; they do secrete collagen, and are derived from mesenchymal cells (38,40,41). Cells with these characteristics predominate in our in vitro synovial cell cultures (38,42).

Table 2. Monoclonal reagents used to identify structures and cell types within specimens*

Monoclonal antibody	Specificity	Ref.
Leu-M3	CD14, monocyte/macrophage lineage	26
T4/19THY5D7	CD4, T inducer cells, macrophages	27
3A1	CD7, pan-T cell marker	28
B1	CD20, mature B cell marker	29
L243	Nonpolymorphic determinant on human class II MHC antigens	30
3F10	Nonpolymorphic determinant on class I MHC	31
TS2/9	LFA-3	32
OKT11	CD2, T cell marker	33
RR1/1	ICAM-1	34
TS1/22	LFA-1	32
M38	Type I procollagen	35
Leu-4	CD3, T cell marker	47

* In each experiment, the negative control used was IgG1 paraprotein secreted by the myeloma line P3X63Ag8 (36). MHC = major histocompatibility complex; LFA = lymphocyte function–associated antigen; ICAM-1 = intercellular adhesion molecule 1.

It is not possible, however, to determine whether the fibroblast-like cells that grow out in culture of synovial tissue explants originate in the intima or in the sublining tissue. Therefore, these cells obtained from culture will simply be called synovial fibroblasts. Using MAbs (i.e., Leu-M3 for macrophages, OKT11 for T cells), side-by-side comparisons were performed on sequential tissue sections of synovium, to show macrophage and lymphocyte reactivity with antibodies against adhesion molecules.

RESULTS

Cell types in inflammatory synovium. Using T cell, B cell, monocyte, MHC class I and class II, and type I procollagen MAbs, we defined the location of cell types in synovial tissue sections. Identification of each cell type was made by its location within synovium by light microscopy and by the surface antigen expression determined by indirect immunofluorescence on tissue sections (Figure 1). As shown, the subintimal layer was densely infiltrated by mononuclear cells, often in nodular aggregates (Figure 1A–C). Perivascular accumulations of lymphoid cells were common (Figure 1C). The macrophage-like type A synovial cells were readily identified by their reactivity with anti-macrophage antibody Leu-M3 (Figure 1D) and were found to make up the intimal layer of synovium. A subset of synovial fibroblasts was identified in synovial tissue sections by reactivity with anti-procollagen antibody M38. These fibroblasts were generally located in the subintimal layer of inflammatory synovium (Figure 1F). As reported previously (38,43), we found that many components of inflammatory synovium express HLA–DR antigens, as indicated by reactivity with L243 antibody. Figure 1E shows HLA–DR+ macrophage-like type A synovial cells and synovial fibroblasts, as well as DR+ endothelial cells and tissue macrophages. Leu-M3+ cells below the type A synovial cell layer within synovial tissues were classified as tissue macrophages (Figure 1D).

Immunohistologic localization of adhesion molecules in synovium. Synovial tissue sections were characterized using MAbs specific for ligands involved in interactions between lymphocytes and nonlymphoid cells. Anti–LFA-3 antibody reacted with type A macrophage-like synovial cells, with synovial fibroblasts, with a subset of endothelial cells, and with tissue monocyte/macrophages, as well as with infiltrating

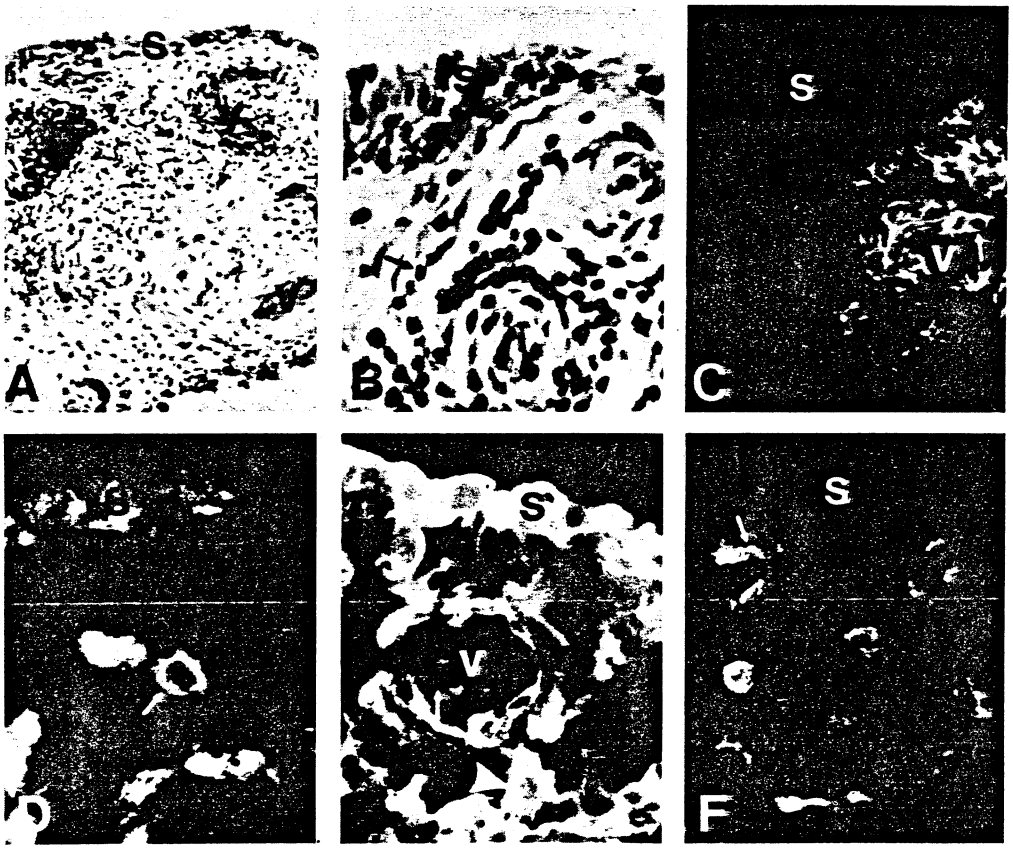

Figure 1. Identification of cell types within the synovial microenvironment. Frozen sections of synovial tissue obtained from patients with inflammatory synovitis were stained with hematoxylin and eosin (**A** and **B**) or with monoclonal antibodies (**C–F**) and viewed under light microscopy (**A** and **B**) or fluorescence microscopy (**C–F**). **A,** Low power view of synovium in inflammatory synovitis, showing lymphoid nodule (**L**). **S** = synovial lining; **V** = vessel. **B,** Higher magnification view of section shown in **A**. **Arrow** indicates a representative lymphocyte. **C,** Reactivity of inflammatory synovium with the anti-CD3 antibody Leu-4. Leu-4+ T cells surround a vessel. **D,** Tissue macrophage reactivity with antibody Leu-M3 (**arrow**). Synovial lining type A cells also reacted with antibody Leu-M3. **E,** Reactivity of inflamed synovium with L243 antibody, against HLA–DR markers. **Arrow** indicates L243+ endothelial cell. **Arrowhead** points to synovial macrophage. **F,** Reactivity of M38 antibody against type I procollagen with collagen-producing synovial fibroblasts (**arrow**). (Original magnification × 100 in **A**, × 400 in **B–F**.)

Table 3. Adhesion molecule expression in the inflammatory synovial microenvironment*

Cell type	Reactivity (no. reactive/no. studied)			
	LFA-3	CD2	ICAM-1	LFA-1
Type A†	+ (8/9)	− (0/9)	+ (9/9)	+ (2/7)
Synovial fibroblasts	+ (6/7)	− (0/9)	+ (9/9)	− (0/3)
Endothelial cells	+ (6/9)	− (0/9)	+ (8/9)	− (0/8)
Macrophages	+ (8/8)	− (0/9)	+ (8/8)	+ (6/8)
Lymphocytes	+ (7/9)	+ (9/9)	+ (9/9)	+ (9/9)

* LFA = lymphocyte function–associated antigen molecule; CD2 = E-rosette receptor (T11, LFA-2) molecule; ICAM-1 = intercellular adhesion molecule 1.
† Macrophage-like synovial lining cells.

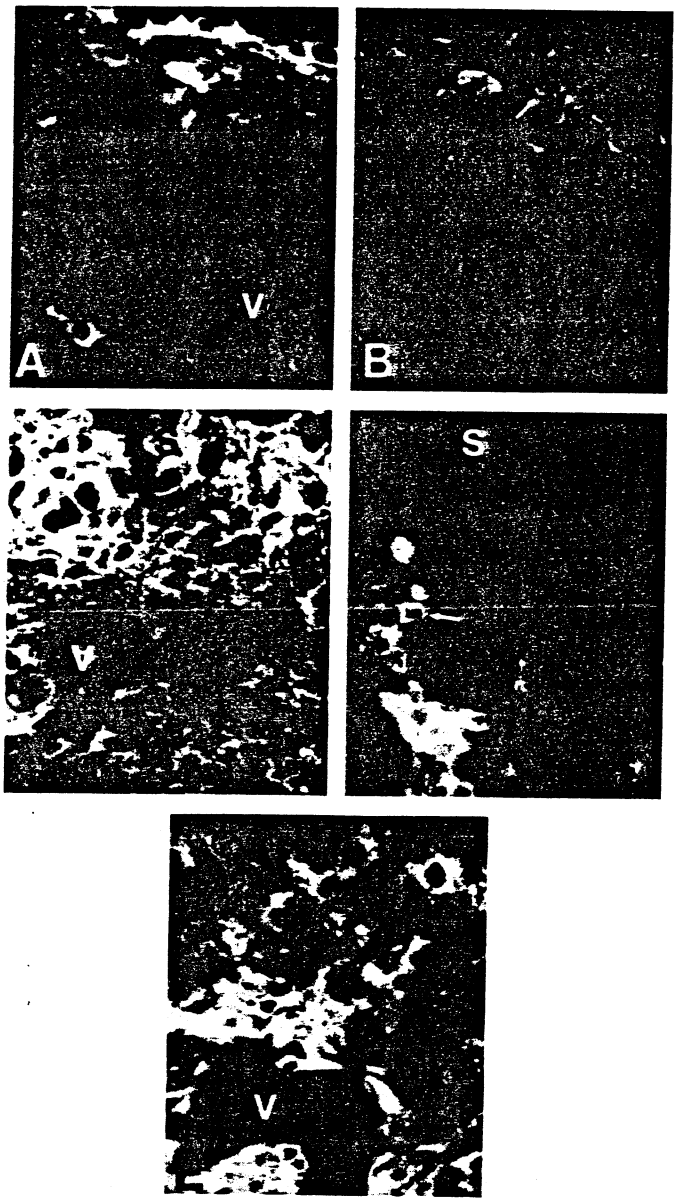

Figure 2. Immunohistologic identification of reactivity of antibody against adhesion molecules within inflammatory synovium. Frozen sections of synovial tissue obtained from patients with inflammatory synovitis were reacted with the indicated antibodies and viewed under indirect immunofluorescence. **A,** Reactivity with TS2/9 (anti–lymphocyte function–associated 3 [LFA-3]) antibody. Note the positive reaction with synovial (S) lining cells and with vessel (V) endothelium. **B,** Reactivity with OKT11 (anti-CD2) antibody. **Arrow** indicates CD2+ cell. Synovial lining was nonreactive with this antibody (not shown). **C,** Reactivity with RR1/1 antibody against intercellular adhesion molecule 1 (ICAM-1). Synovial lining, vessel endothelium, and infiltrating mononuclear cells all reacted with anti–ICAM-1. **D,** Reactivity of inflammatory synovium with antibody TS1/22 (anti–LFA-1). Synovial lining cells were weakly reactive. **Arrow** indicates LFA-1+ mononuclear cell. **E,** Reactivity of anti–LFA-3 antibody, showing synovial tissue in an area of intense inflammation. Note the strong reactivity with synovial lining, vessels, and mononuclear infiltrate. (Original magnification × 400.)

lymphocytes (Table 3 and Figure 2). Not all vessels were positive in each specimen, and not all specimens had vessels reactive with anti–LFA-3 antibody in the area sampled. In areas of intense inflammation, however, virtually all vessels were reactive with anti–LFA-3 antibody. CD2, a ligand for LFA-3, was found to be present only on T lymphocytes within the synovium (Table 3 and Figure 2B).

RR1/1 antibody against the ICAM-1 molecule was reactive with macrophage-like type A synovial cells and with synovial fibroblasts, as well as with macrophages, endothelial cells, and synovial lymphocytes (Table 3 and Figure 2). Reactivity of anti–ICAM-1 was particularly intense with vascular endothelium and with the type A synovial lining cells in areas with inflammatory infiltrates (Figure 2). Anti–LFA-1 reacted with type A synovial cells from 2 of 7 patients, and with synovial macrophages and lymphocytes, but not with endothelial or fibroblast-like synovial cells (Table 3 and Figure 2). LFA-1+ mononuclear cells were frequently seen in and around vessels, with LFA-1+ cells juxtaposed to ICAM-1+ endothelial cells (Figure 3).

A comparison of expression of adhesion and functional molecules on synovial tissue derived from patients with RA and those with other forms of synovitis (OA, SLE) showed no major qualitative differences in expression of the markers studied. Tissue from RA patients and from OA patients showed evidence of inflammation and/or its sequelae, often with large infiltrates of mononuclear cells or extensive fibrosis (44).

Next, to confirm the reactivity of these reagents with synovial fibroblasts, cytocentrifuge preparations or cell suspensions of synovial fibroblast cells grown in vitro were assayed for reactivity with the panel of antibodies (Table 4). In indirect immunofluorescence assays of cytocentrifuge preparations, cultured synovial fibroblasts were nonreactive with antibodies against CD2 and LFA-1, but reacted with anti–LFA-3 and anti–ICAM-1 antibodies. Flow cytometric analysis of viable synovial fibroblasts in suspension confirmed this reactivity with anti–ICAM-1 and anti–LFA-3 MAbs (Figure 4).

DISCUSSION

In this report, we have shown that the adhesion molecules ICAM-1 and LFA-3 (the ligands for lymphocyte LFA-1 and CD2 molecules, respectively) are present on macrophage-like synovial cells, synovial

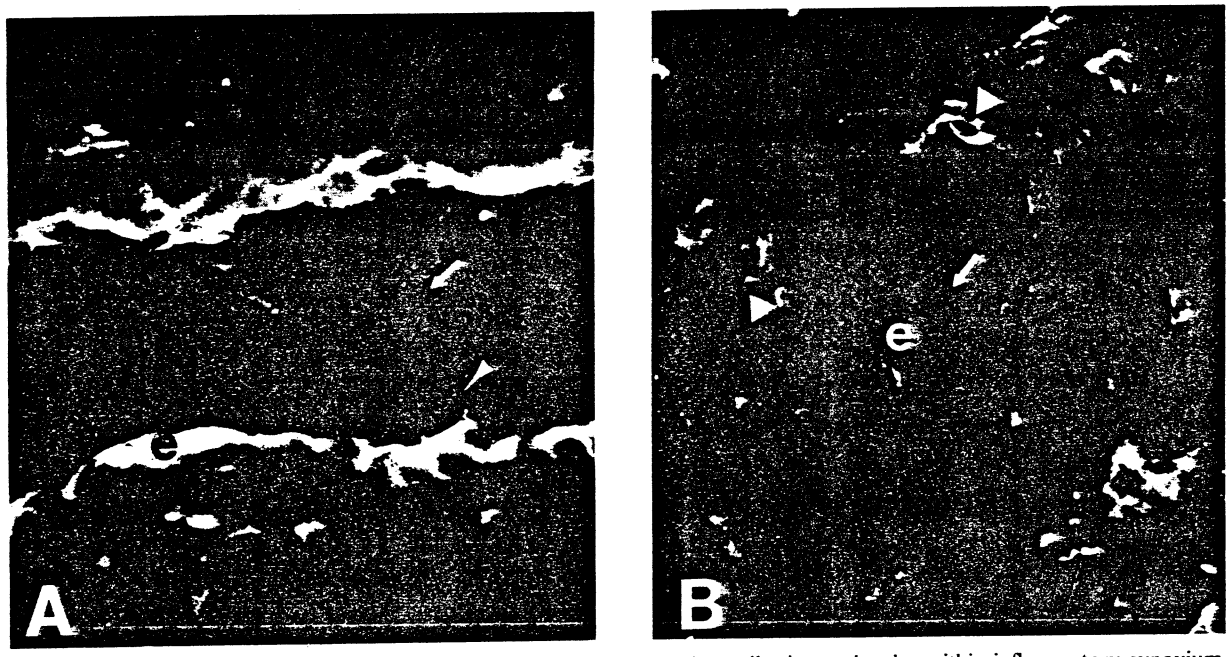

Figure 3. Immunohistologic identification of reactivity of antibody against adhesion molecules within inflammatory synovium. Frozen sections of synovial tissue obtained from patients with inflammatory synovitis were reacted with the indicated antibodies and viewed under indirect immunofluorescence. **A,** Reactivity with RR1/1 (anti–ICAM-1) antibody. Note the positive reaction with vessel endothelium (**e**) and with mononuclear cells inside the vessel (**arrow**). **Arrowhead** shows an adherent mononuclear cell. **B,** Reactivity with TS1/22 (anti–LFA-1) antibody. Vessel endothelium was nonreactive; however, mononuclear cells surrounding (**arrowheads**) and inside the vessel did react with anti–LFA-1 antibody. **Arrow** indicates an LFA-1+ cell adherent to vessel endothelium. See Figure 2 for definitions. (Original magnification × 400.)

fibroblasts, macrophages, and endothelial cells in the inflammatory synovial microenvironment.

ICAM-1 is a cell surface glycoprotein, originally described by Rothlein and coworkers (34), which has been shown to be involved in adhesion of lymphocyte blasts to human dermal fibroblasts. Using MAb RR1/1, ICAM-1 was shown to be expressed on vascular endothelial cells, thymic and skin epithelial cells, fibroblasts, and on hematopoietic cells such as tissue macrophages, mitogen-activated T lymphocytes, and dendritic cells from germinal centers in lymphoid tissue, as well as being present in low

Table 4. Reactivity of monoclonal antibodies with cultured synovial cells*

Antibody specificity	% positive	
	Mean ± SEM	Range
LFA-3	38.5 ± 4.6	28–49
CD2	0.2 ± 0.2	0–1
ICAM-1	33.9 ± 7.6	6–58
LFA-1	0.3 ± 0.2	0–1
CD3	0	0
Type I procollagen	65.0 ± 17.6	30–84
MHC class II	53.2 ± 16.8	22–67

* Reactivity with antibodies was determined by indirect immunofluorescence on acetone-fixed cytocentrifuge preparations, as described in Patients and Methods. MHC = major histocompatibility complex; see Table 3 for other definitions.

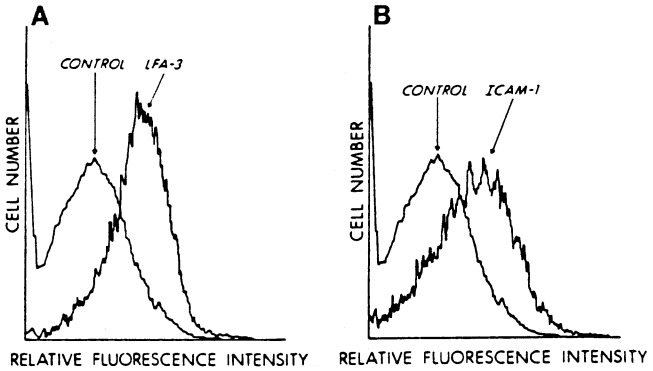

Figure 4. Cytofluorographic analysis of cultured synovial fibroblasts with **A,** anti–lymphocyte function–associated antigen 3 (LFA-3) and **B,** intercellular adhesion molecule 1 (ICAM-1) antibodies. The mean ± SEM percent positivity in cultured synovial cells was 39 ± 5% for LFA-3 and 34 ± 8% for ICAM-1. Control curves using P3X63/Ag8 ascites (1:1,000 dilution) are indicated.

amounts on peripheral blood lymphocytes (45). The expression of ICAM-1 on fibroblasts was shown to be induced by γ-interferon (γ-IFN) and IL-1 (45). Recently, ICAM-1 expression on keratinocytes, fibroblasts, and endothelial cells has been shown to be synergistically induced by γ-IFN and tumor necrosis factor (TNF) (9,45,46).

In a separate series of experiments, we have recently shown that ICAM-1 expression on synovial fibroblasts is indeed induced by both γ-IFN and TNF (Hale LP, Haynes BF, Singer KH: unpublished observations). ICAM-1 staining on vascular endothelial cells has been previously found to be more intense in T cell areas of lymph nodes and tonsils showing reactive hyperplasia (45).

Results presented here indicate that ICAM-1 is expressed on all major components of the synovial microenvironment in RA and other inflammatory synovium. The strong expression of this antigen on the intimal synovial lining (type A cells) and on the vascular endothelium within areas of active inflammation is consistent with its induction by inflammatory mediators released at these sites. Cultured synovial cells expressed variable amounts of ICAM-1, as indicated by the range of ICAM-1+ cells demonstrated on indirect immunofluorescence (Table 4). The presence of ICAM-1 on inflammatory synovial tissue in situ lends further support to the hypothesis that the LFA-1–ICAM-1 adhesion pathway could be involved in the migration of lymphocytes to synovium and could participate in the maintenance of T and B cell responses in synovium (6).

The LFA-3 molecule has been implicated in T lymphocyte adhesion to a variety of cells. In the thymus, lymphocyte adhesion to TE cells occurs via CD2–LFA-3 interaction, with resulting functional consequences for both lymphocytes and TE cells (11,16,19,20). The demonstration of LFA-3 expression on synovial cells, macrophages, and endothelial cells suggests that a number of cell types may participate in the activation of T cells within synovium. The significance of the expression of LFA-3 by synovial endothelial cells in a subset of patients with both RA and OA is not clear, but could relate to T lymphocyte diapedesis and activation of T cells within vessel walls in inflamed tissue.

The presence of LFA-3 on synovial cell types suggests that synovial cell–T cell interactions can occur. Recently, we tested this hypothesis, using LFA-3+ synovial fibroblasts, and showed that thymocytes indeed bind to LFA-3+ synovial cells (42). Moreover, thymocyte binding to synovial fibroblasts is inhibited by anti-CD2 and anti–LFA-3 antibodies (42).

Taken together, these data suggest that numerous cell types in synovium express ligands for lymphocyte LFA-1 and CD2 molecules. The distribution of these cellular adhesion molecules suggests that lymphocyte interactions with numerous cells types in synovium could occur and could participate in the maintenance of chronic synovial inflammation.

ACKNOWLEDGMENT

The authors thank Kim R. McClammy for expert secretarial assistance.

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