Monocyte–Endothelial Adhesion in Chronic Rheumatoid Arthritis

In Situ Detection of Selectin and Integrin-dependent Interactions

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Abstract

Blood monocytes are the principal reservoir for tissue macrophages in rheumatoid synovitis. Receptor-mediated adhesive interactions between circulating cells and the synovial venules initiate recruitment. These interactions have been studied primarily in cultured endothelial cells. Thus the functional activities of specific adhesion receptors, such as the endothelial selectins and the leukocytic integrins, have not been evaluated directly in diseased tissues. We therefore examined monocyte–microvascular interactions in rheumatoid synovitis by modifying the Stamper-Woodruff frozen section binding assay initially developed to study lymphocyte homing. Specific binding of monocytes to venules lined by low or high endothelium occurred at concentrations as low as 5×10^5 cells/ml. mAbs specific for P-selectin (CD62, GMP-140/PADGEM) blocked adhesion by > 90% in all synovitis specimens examined. In contrast, P-selectin–mediated adhesion to the microvasculature was either lower or absent in frozen sections of normal foreskin and placenta. mAbs specific for E-selectin (ELAM-1) blocked 20–50% of monocyte attachment in several RA synovial specimens but had no effect in others. mAbs specific for LFA-1, Mol/Mac 1, the integrin $\beta 2$-chain, and L-selectin individually inhibited 30–40% of adhesion. An mAb specific for the integrin $\beta 1$-chain inhibited the attachment of elutriated monocytes up to 20%. We conclude that P-selectin associated with the synovial microvasculature initiates shear-resistant adhesion of monocytes in the Stamper-Woodruff assay and stabilizes bonds formed by other selectins and the integrins. Thus the frozen section binding assay permits direct evaluation of leukocyte–microvascular adhesive interactions in inflamed tissues and suggests a prominent role for P-selectin in monocyte recruitment in vivo. (*J. Clin. Invest.* 1993. 91:2609–2619.) Key words: inflammation • monocytes • vascular endothelium • cell adhesion

Introduction

Rheumatoid synovitis is characterized by marked mononuclear infiltration and microvascular proliferation. Identifiable histologic patterns include heavily infiltrated lymphocyte-rich areas, transitional zones populated by macrophages, plasma cells, and lymphocytes (1), and noninfiltrated collagenous interstitial areas containing macrophages and fibroblasts (2). A superimposed infiltrate of polymorphonuclear leukocytes, particularly in synovial fluid, occurs during acute exacerbations further compromising tissues. The relapsing acute and progressive chronic inflammation leads to development of an erosive pannus which eventually destroys the joint.

A number of observations suggest that mononuclear cells derived from circulating monocytes play a central role in joint pathology. Synovial fluid monocytes and their tissue counterparts display an activated phenotype including high density expression of MHC class II antigens (3, 4). In tissues, macrophages capable of antigen presentation are intimately associated with T cells in acellular, transitional, and immunologically active areas of the synovium (5). Monocyte products such as IL-1β, IL-6, and TNF-α contribute to a variety of the immunopathologic features including T cell activation, microvascular proliferation (6), and induction of degradative enzyme secretion by chondrocytes and synovial fibroblasts (7, 8). Finally, recent studies show that OKM-1 (CD11b)–positive mononuclear cells (i.e., monocytes and monocyte-derived macrophages) account for the bulk of IL-1β and TNF-α gene expression in RA synovium (9). Thus recruitment of circulating monocytes contributes to both the immune and inflammatory components of rheumatoid synovitis.

The entry of circulating leukocytes into inflammatory and immune reactions is commonly referred to as recruitment. The process encompasses several stages. Attachment to the lumenal surface of postcapillary venules in target organs initiates the process. The immobilized leukocytes then migrate through the endothelial layer and penetrate the basement membrane. Each stage involves adhesive interactions between the leukocyte and the vessel wall (reviewed in [10, 11]). These adhesive interactions target cells to inflamed tissues and account, in part, for the makeup of the inflammatory infiltrate. Leukocyte adhesion receptors implicated in recruitment include the $\beta 2$ integrins (leukocyte functional antigen-1 [LFA-1],[1] Mo-1/MAC-1, and

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1. *Abbreviations used in this paper:* CMF-PBS, PBS without Ca^{++} and Mg^{++}; F-H, Ficoll-Hypaque; HEV, high endothelial venules; HUVE, human umbilical vein endothelial; ICAM, intercellular adhesion molecules; LFA-1, leukocyte functional antigen-1; SV, synovial venules; S-W assay, Stamper-Woodruff frozen section binding assay; VCAM-1, vascular adhesion molecule-1; VLA-4, very late antigen-4.
2. The $\beta 2$-integrins or leucams have been assigned the cluster designation CD18 for their common beta chain. The individual alpha chains are designated CD11a (LFA-1), CD11b (Mol/Macl) and CD11c (p150,95). An alternative nomenclature specifies the individual chains-$\alpha_L/\beta 2$ (LFA-1), $\alpha_M/\beta 2$ (Mol/Macl), and $\alpha_X/\beta 2$ (p150/95).

p150,95)[2] (reviewed in [12]), the β1 integrin very late antigen-4 (VLA-4)[3] (reviewed in [13]) and L-selectin[4] (14, 15). The inducible endothelial adhesion receptors include the inter-cellular adhesion molecules (ICAMs)[5] (16), vascular adhesion molecule-1 (VCAM-1)[6] (17, 18), and the two remaining members of the selectin family, E-selectin (19) and P-selectin (20).

Monocytes express a broad spectrum of adhesion receptor and counter-receptor implicated in recruitment. L-selectin–specific mAbs block monocyte attachment to the high endothelial venules (HEV) of inflamed murine lymph nodes and inhibit their migration into peritoneal exudates (21). In addition, recent studies implicate L-selectin in monocyte attachment to cytokine-treated human umbilical vein endothelial cells (HUVE) (22). Monocytes also carry ligands for both P-selectin and E-selectin. The former mediates binding to thrombin activated platelets (23) while the latter permits attachment to E-selectin transfectants and cytokine-treated HUVE (24). These ligands have not been characterized but are presumably related to the sialylated, fucosylated binding sites identified on neutrophils and cell lines (25–31). The β1 integrin VLA-4 (CD49d) is constitutively expressed, enabling monocytes to interact with a second, cytokine-induced endothelial adhesion molecule termed VCAM-1 (24). Finally, all three members of the β2 (CD18) integrin family, LFA-1 (CD11a), Mo-1/Mac-1 (CD11b) and p150,95 (CD11c), are expressed (reviewed in [32]). Partial blockade of monocyte attachment to HUVE by mAbs specific for the common β2 (CD18) chain indicate that one or more of these receptors may participate in recruitment (24). Thus studies with cultured human endothelium and rodent models identify a broad array of monocyte adhesion receptors of potential importance in human disease. The current study uses a modified Stamper-Woodruff assay to explore adhesive interaction between mononuclear leukocytes and the microvasculature in frozen sections of rheumatoid synovitis and other tissues. We show that monocytes are the principal endothelial-binding cells in mixed mononuclear leukocyte populations, define the microvasculature supporting monocyte binding, and demonstrate that P-selectin is the principal mediator of adhesion in all tissues. E-selectin, the integrins, and L-selectin also contribute to attachment in rheumatoid synovitis.

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3. The β1-integrins or VLAs have been assigned the cluster designation CD29 for their common beta chain. The individual alpha chains are designated CD49a-f corresponding to VLAs 1–6. An alternative nomenclature specifies the individual chains for each molecule-α1–6/β1 (VLA 1–6).

4. The selectin or LEC-CAM family consists of three structurally related membrane antigens termed L-selectin, E-selectin, and P-selectin. In this nomenclature, L-selectin replaces a myriad of names, including the lymph node homing receptor, the Mel14 antigen, gp90mel14, $HEBF_{ln}$, the TQ-1 antigen, the Leu-8 antigen, the LAM-1 antigen, and the DREG antigen; E-selectin replaces endothelial leukocyte adhesion molecule-1 (ELAM-1) and P-selectin replaces granule membrane protein-140 and PADGEM.

5. The ICAM family consists of three structurally related members of the immunoglobulin supergene family, ICAM-1 (CD54), ICAM-2, and ICAM-3.

6. The VCAM family contains, at present, a single member of the immunoglobulin supergene family, VCAM-1. Several splice variants have been characterized. The terms INCAM-110 and athero-ELAM refer to the same molecule.

Methods

Chemicals. BSA (A7906), magnesium chloride (M0250), EGTA (E4378), disodium EDTA (E4931), tricine (T0377), 3-amino-9-ethylcarbazole (A5754), *N,N*-dimethyl formamide (D4254), 30% hydrogen peroxide (H1009), and Ficoll-Hypaque (Histopaque) were obtained from Sigma Chemical Co. (St. Louis, MO). Calcium chloride was purchased from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Dextrose 50% was supplied by Abbott Laboratories (North Chicago, IL). Albumin (human) 25% was obtained from the Michigan Department of Public Health (Lansing, MI). MEM and Dulbecco's PBS without Ca^{++} and Mg^{++} (CMF-PBS) were purchased from Gibco Laboratories (Grand Island, NY).

Antibodies. Dr. Rodger McEver (Oklahoma Blood Research Institute) provided Fab'2 fragments reactive with P-selectin (G1 and S12, IgG1; [33, 34]). Dr. C. Wayne Smith (Baylor University) supplied Fab'2 fragments specific for E-selectin (CL2 and CL37, IgG1; [35]). Drs. Roy Lobb and Christopher Benjamin (Biogen, Inc., Cambridge, MA) provided additional E-selectin specific Fab'2 fragments (BB11, IgG2b; [24]). Dr. Robert Todd (University of Michigan, Ann Arbor) provided mAbs specific for CD11b (44, IgG2a), CD14 (26ic, IgG2b), and HLA-DR (9-49, IgG2a) (36–38). Dr. Hergen Spits (DNAX Laboratories, Palo Alto, CA) provided anti-CD11a (L7, IgG1; [39]). Dr. Samuel Wright (Rockefeller University, New York) supplied anti-CD18 (IB4, IgG1; [40]). Dr. Peter Newman (The Blood Center of Southeastern Wisconsin, Milwakee) provided anti-CD41 (AP-3, IgG1; [41]). Dr. Carolyn Damsky (University of California, San Francisco) provided hybridoma supernatants containing rat mAbs specific for the human β1-chain (AIIB2) and α6-chain (J1B5) (42). Dr. John Harlan (University of Washington, Seattle) provided anti-VCAM-1 (4B9, IgG1; [24]). Dr. M. Jutila (Montana State, Bozeman, MT) provided the L-selectin–specific mAb DREG 200 (IgG1, [43]). Antibodies with the following specificities were purchased from commercial sources: anti-CD45R (GAP 8.3, IgG2a; American Type Culture Collection, Rockville, MD), anti-GPIb/IX (SZ1, IgG2a; AMAC, Inc., Westbrook, ME), ICAM-1 (clone 84H10, IgG1; AMAC, Inc.) and anti–von Willebrand factor (cat. no. AXL-205, rabbit affinity-purified polyclonal; Accurate Chemical and Scientific Corp., Westbury, NY). Dr. David Fox provided the mAb to von Willebrand factor (1G1G12, IgG1).

Specimens. Synovial tissues were obtained at the time of total knee or hip arthroplasty from adult patients with chronic rheumatoid arthritis. Specimens of inflamed tonsil, newborn foreskin, and placenta were obtained after routine procedures and processed in the same manner as synovium. After procurement, the specimens were promptly snap-frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use.

Fractionation of peripheral blood. Monocytes were prepared from EDTA-anticoagulated blood of healthy volunteers using either counter current centrifugal elutriation or Percoll fractionation. In brief, equal volumes of anticoagulated whole blood and 0.9% NaCl were mixed, layered on Ficoll, and centrifuged (400 *g*; 30 min; room temperature). Before elutriation, the mononuclear fraction was washed twice (200 *g*; 15 min; 5°C) in "elutriation buffer" consisting of CMF-PBS, pH 7.3, dextrose, human albumin, and EDTA (44). A counterflow centrifugal elutriation system (J2-21M centrifuge, JE-6B elutriation system and rotor; Beckman Instruments, Inc., Palo Alto, CA) was used to fractionate cells according to the following method. Mononuclear cells (1×10^8) suspended in 10 ml elutriation buffer were loaded into the 4.5-ml standard chamber at a flow rate of 4 ml/min with a rotor speed of 2,030 rpm (5°C). Cells were then eluted in 50-ml aliquots at the following flow rates: 6, 9, 9, 10, 10, 10.5, 11, 11.5, 11.5, 11.5, 17, 17 (ml/min). In general, lymphocytes eluted at flow rates < 10 ml/min while monocytes eluted at flow rates > 11 ml/min. Variations in donor cells and run temperature can dramatically alter the flow rates at which leukocytes elute; therefore, the purity of fractions was monitored by light scatter (FACScan,® Becton Dickinson, San Jose, CA) and verified by marker analysis at the end of the run. The monocyte-enriched fractions were washed and resuspended at 4°C in minimal essential medium, pH 7.3, supplemented with 1 mg/ml BSA and 40 mM tricine (MEM^+) immediately after fractionation.

Alternatively, monocytes were purified over a discontinuous gradient of Percoll after the initial Ficoll fractionation of whole blood. Briefly, Ficoll-fractionated mononuclear cells were washed in CMF-PBS and then sedimented through a preformed Percoll gradient (1,500 *g*; 30 min; 5°C) as previously described (45). The monocyte and lymphocyte fractions were collected, washed with CMF-PBS, and resuspended in MEM^+.

Both fractionation protocols yielded 80–90% pure populations of monocytes and lymphocytes as judged by surface markers and morphology. Elutriation resulted in less contamination of the monocyte surface by intact platelets and platelet fragments as previously described (46). FACS analysis showed a 10–50-fold reduction of the CD41 epitope on elutriated versus Percoll-fractionated monocytes (Fig. 1).

The density and distribution of monocyte attachment to microvessels did not differ significantly for the two preparative techniques. In addition, mAbs directed at the $\beta 2$ integrins inhibited attachment to the same degree. Inhibition studies with mAbs directed at the selectins were conducted with elutriated monocytes exclusively.

Microvascular binding assays. 12-μm frozen sections of synovial tissue were applied to three-well glass slides (Carlson Scientific Inc., Peotone, IL) precoated with 6% BSA in PBS, and air-dried for 2 h. The sections were then overlaid with cell suspensions, cooled to 4°C, and placed on a rotary agitator (Tek-Tator V; 3/8" radius of rotation, 40–60 rpm; Tek-Pro American Dade, Miami, FL) for 30 min. Optimal incubation conditions for monocytes were $1\text{–}3 \times 10^5$ cells/section and a temperature of 7–10°C. After the incubation, the cell suspensions were gently decanted and the sections were fixed in glutaraldehyde (3% in PBS; 30 min; 4°C) before staining with toluidine blue. Binding to the microvasculature was quantitated by determining the average number of cells attached to postcapillary venules per ×200 microscopic field. The mean and SEM were calculated from counts of 200–1,000 cells (40–60 fields) per section and five replicate sections per condition.

The assay buffer consisted of minimal essential medium buffered with 40 mM tricine, pH 7.3, and supplemented with 1 mg/ml BSA (MEM^+). MEM^+ was used for all washes, incubations, and dilutions in the adhesion assay. In some experiments, assay buffer was applied to the tissue sections and then decanted immediately before addition of test cells. This procedure further reduced nonspecific attachment to glass and tissues. mAbs (60 μl) specific for endothelial antigens were preincubated with the tissue sections (30 min at room temperature) before adding cell suspensions (60 μl at $2\text{–}6 \times 10^6$ cells/ml). Removal of antibody before addition of cells gave the same results. mAbs specific for leukocyte antigens were preincubated with cells ($1\text{–}3 \times 10^6$ cells/ml, 30 min; 4°C) before applying the cell suspensions to frozen sections (120 μl/section). All mAbs were used at concentrations 5–25-fold above that required to saturate binding sites.

Immunoperoxidase studies. Randomly selected synovial specimens from 20 patients, three normal foreskin specimens, and one placenta were examined. 6-μm sections were air-dried, fixed in acetone, and then sequentially incubated with normal goat serum, the unconjugated primary antibody, the biotinylated secondary, the Vectastain ABC reagent (Nector Laboratories Inc., Burlingame, CA), and a peroxidase substrate (3-amino-9-ethylcarbazole + 30% H_2O_2 in *N,N*-dimethyl formamide, acetate buffer). The incubations were conducted in a humidified chamber at room temperature. After staining, the sections were counterstained with hematoxylin, washed in tap water, and coverslipped in Aquamount (Lerner Laboratories, Pittsburgh, PA).

Confocal microscopy. 12-μm frozen sections were fixed in either paraformaldehyde (4% freshly prepared paraformaldehyde, 5 min, 4°C) or acetone (100%, 30 s, 4°C) before indirect immunofluorescence staining for P-selectin (G1 mab). Serial 0.5-μm optical sections in the fluoroscein channel were acquired and digitized using a confocal laser scanning microscope (MRC-600; Bio-Rad Laboratories, Richmond, CA) interfaced to a Nikon Diaphot at a magnification of 60 (numerical aperture = 1.4). Confocal images with a ×3 zoom factor were produced using the COMOS software package (Bio-Rad, Cambridge, MA), displayed on a Sparc 2 workstation (Sun Microsystems, San Jose, CA) and photographed off the monitor screen using a tripod-mounted 35-mm reflex camera. The fluorescence intensity distribution along the indicated axes was measured with the ISee 2-D morphometric analysis package (Inovision Inc., Research Triangle Park, NC).

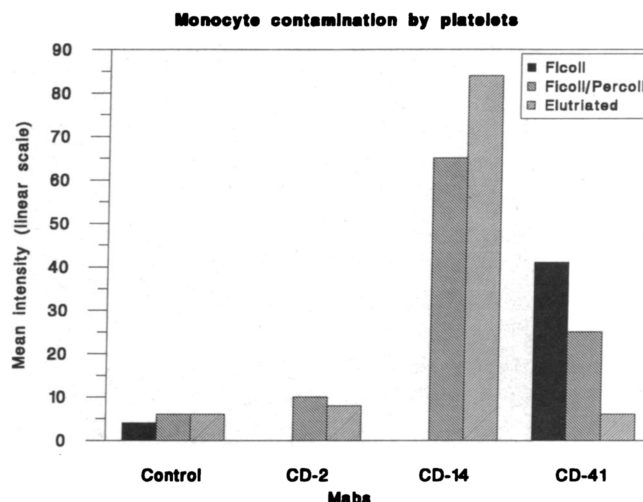


Figure 1. Expression of the platelet antigen CD41 in monocyte fractions prepared from EDTA anticoagulated peripheral blood by F/H fractionation alone ■, F/H followed by Percoll (F/H + P) ▧, and F/H followed by elutriation (F/H + E) ▨. The latter two populations consisted primarily of monocytes with strong expression of CD14 and near background levels of CD2. CD41 was associated with monocytes after F/H and F/H + P. F/H + E produced CD41-free monocytes.

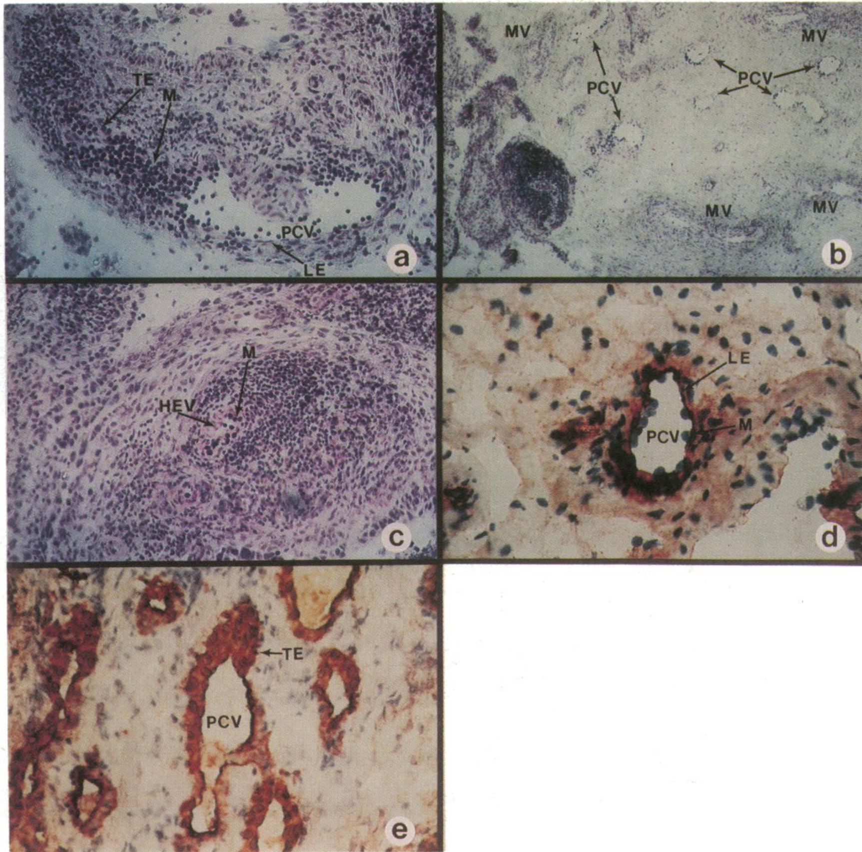
Results

Initial experiments determined optimal conditions for the in situ binding assay. Specifically, we defined conditions which maintained binding to synovial venules (SV) while minimizing attachment to stromal and synovial lining cells. Attachment to these nonendothelial tissues should not be construed as "nonspecific" and in all probability reflects receptor-mediated interactions of potential functional significance. However, our study required conditions that promoted selective interactions with the microvasculature.

Table I. Mononuclear Leukocyte Attachment to SV in Rheumatoid Arthritis

Concentration	Monocytes	Lymphocytes
	cells/hpf (sem)	
5×10^5/ml	0.91 (0.40)	ND
1×10^6/ml	1.19 (0.18)	0.08 (0.07)
5×10^6/ml	1.41 (0.80)	0.13 (0.09)
1×10^7/ml	ND	0.41 (0.14)

Air-dried frozen sections were overlaid with enriched fractions (> 90% purity) as detailed in Methods. Monocytes at 5, 10, and 50 $\times 10^5$ cells/ml were layered on sequential sections with three sections per slide and five slides in the series. Lymphocyte adhesion was measured on the next block of serial sections. Subsequent experiments followed a similar pattern with the first well of each slide containing the "control" cell population. The average number of cells bound to SV per ×200 microscopic field was calculated from counts of 200–1,000 cells/section and four to five replicate sections/condition.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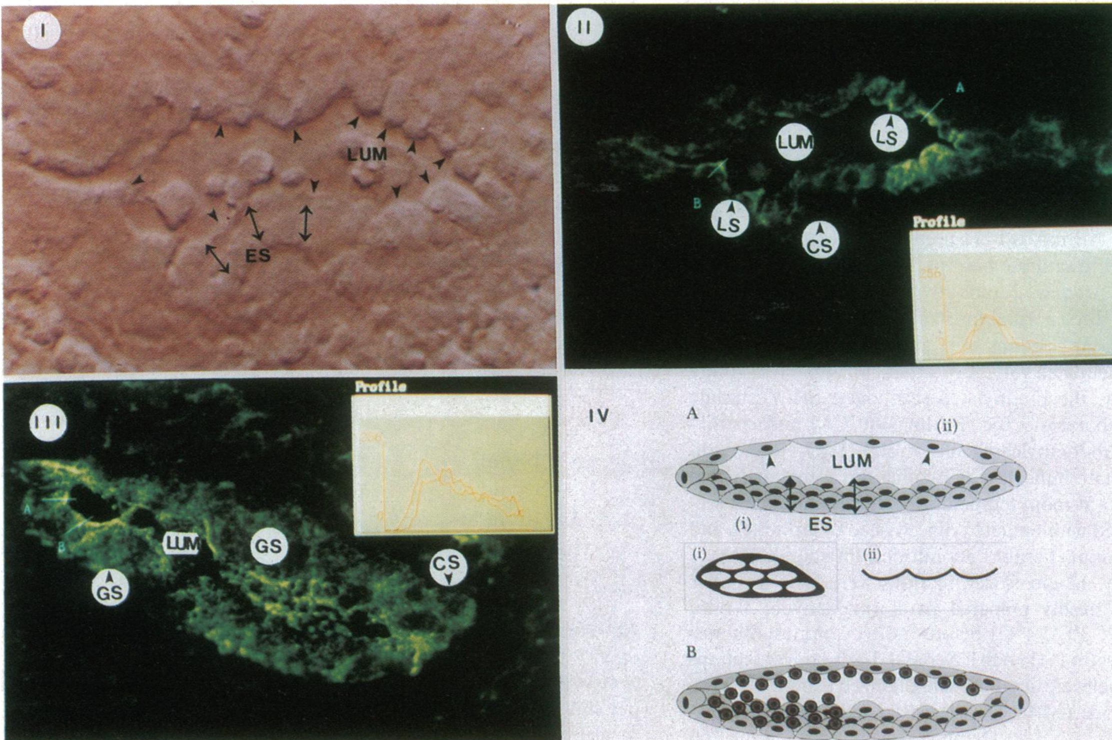
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From this standpoint, optimal adhesion was achieved using: (*a*) elutriated monocytes, (*b*) air-dried, unfixed 12–15-μm tissue sections on BSA-coated glass slides, and (*c*) 7–10°C incubations with rotary agitation. Monocytes isolated by Percoll-based techniques showed selective, high density microvascular binding. However, staining for the platelet antigen gp IIb/IIIa with mAb AP-3 (CD41) revealed substantial contamination of the monocyte surface by platelets and platelet fragments (Fig. 1). Use of centrifugal elutriation to isolate monocytes eliminated contamination facilitating interpretation of the adhesion experiments. Uncoated slides resulted in poor adherence of the sections and excessive monocyte attachment to glass. Precoating with poly-L-lysine, on the other hand, secured the sections but markedly enhanced monocyte attachment to both glass and nonvascular tissues. Incubation at 4°C reduced binding generally while room temperature markedly enhanced interactions with stromal and synovial lining cells, obscuring specific microvascular interactions.

Under optimal conditions, specific and reproducible binding of monocyte-enriched fractions to SV was evident at concentrations as low as 5×10^5 cells/ml (5×10^4 cells/section) (Table I). In contrast, the lymphocyte-enriched fraction showed significantly lower binding to SV at concentrations as high as 1×10^7 cells/ml (1×10^6 cells/section). The monocyte-enriched fraction adhered to SV lined by low endothelium in transitional (Fig. 2 *a*) and noninfiltrated interstitial areas (Fig. 2 *b*). In addition, small numbers of cells also bound to the HEV-like vessels located in nodular lymphoid aggregates (Fig. 2 *c*). Cells attached to the exposed surface of sectioned microvasculature (Fig. 2, *a–c*) as well as to the lumenal aspect of the endothelium, upon which some monocytes appeared to spread (Fig. 2 *d*). The occasional large vessels in tissue sections showed minimal binding activity (Fig. 2 *b*). In some areas the monocyte-enriched population bound to multicellular islands of homogeneous cells contiguous with the endothelium (Fig. 2 *a*). Similar structures were diffusely positive for von Willebrand factor, identifying them as sheets of contiguous endothelial cells (Fig. 2 *e*). Percoll-fractionated and elutriated monocytes showed the same distribution of attachment. Thus monocytes interact with the microvasculature in frozen sections of RA synovitis.

Immunohistochemical staining of randomly selected synovectomy specimens for endothelial adhesion molecules demonstrated the presence of ICAM-1, E-selectin, and VCAM-1 as previously reported by Koch et al. (47). ICAM-1 staining was observed on virtually all SV in 3 of 3 specimens. Staining for E-selectin with three different MAbs (BB11, CL2 and CL37) showed weak to moderate, patchy expression in 6 of 20 patients. The VCAM-1-specific epitope 4B9 was detected at low levels on SV in 8 of 18 specimens examined. In contrast, the epitope was readily detected in all specimens on synovial lining cells and mononuclear cells scattered throughout the tissues.

In contrast to E-selectin and VCAM-1, the P-selectin epitopes G1 and S12 showed uniformly strong, diffuse expression in postcapillary venules of 11 of 11 specimens (Fig. 3, *c* and *e*). A granular, cytoplasmic distribution predominated; however, linear staining along the lumenal endothelial surface was evident in some areas (*e*). P-selectin and the gp Ib/IX epitope (*f*) also stained morphologically identifiable platelets and platelet "plugs" associated with the lumenal surface. However, most P-selectin positive venules were free of the gp Ib/IX antigen (*c* versus *d*). As documented previously (48), P-selectin is a constituent of postcapillary endothelial cells in many tissues (Fig. 3, *a* and *b*). Both the dermal vascular plexus of normal human foreskin (*a*) and the microvasculature of term placenta (*b*) stained for this adhesion receptor.

Antibody blocking experiments showed > 90–95% inhibition of monocyte adhesion by Fab'2 fragments specific for a functional domain of P-selectin (mAb G1) in all synovial specimens (Fig. 4). In contrast, Fab'2 fragments directed at a nonfunctional epitope (mAb S12) had no effect. P-selectin–dependent monocyte attachment was also detected in sections of tonsillitis and normal newborn foreskin. However, the density of binding in rheumatoid synovitis and tonsillitis exceeded that in normal foreskin by 3–20 fold (Fig. 5). In addition, monocytes did not bind to the P-selectin–rich microvessels of normal term placenta. Thus P-selectin is the principal mediator of monocyte attachment in the Stamper-Woodruff assay, regardless of the tissue source. However, P-selectin dependent attachment is significantly greater in inflamed than in noninflamed tissues.

The distribution of P-selectin in the thick frozen sections used in the binding assay was mapped using confocal laser microscopy. 12-μm frozen sections of rheumatoid synovium were air-dried and fixed in either paraformaldehyde (Fig. 6, *I* and *II*) or acetone (Fig. 6 *III*) to preserve tissue structure before staining. Exposed P-selectin was stained with the G1 mAb,

Figure 2. The monocyte-SV binding assay conducted under optimal conditions as described in Methods. (*a*) Monocytes (dark blue cells above plane of section) adhered to lumenal surfaces of postcapillary venule (*PCV*) lined by low endothelium (*LE*). Sheets of monocytes (*M*) also bound to islands of cell contiguous with the endothelium. Similar islands stained for von Willebrand factor (*e*) thus consisted of either intact endothelial cells exposed by transecting the venule or tangential sections through the endothelial monolayer (*TE*). (*b*) Monocytes adhered to postcapillary venules in both cellular and acellular areas but did not adhere to muscular veins (*MV*). (*c*) Monocytes (*M*) adhered to synovial HEV-like vessels in nodular lymphocytic aggregates. (*d*) Rapid fixation in a 50:50 mixture of acetone:methanol (30 s) revealed partial spreading of monocyte membranes (*M*) on the lumenal endothelial surface (*LE*). The section was stained for vWF after conducting the binding assay to enhance visualization of the endothelium.

Figure 6. Modulation contrast and confocal images of rheumatoid synovium stained for P-selectin. 12-μm frozen sections from SYN-1 (Fig. 5) were prefixed and stained as described in Methods. (*lum*), vascular lumen (arrowheads outline the endothelial surface); (*es*), endothelial sheet; (*ls*), linear membrane staining; (*cs*), circumferential membrane staining; (*gs*), granular staining. (*I*) modulation contrast image of stained venule in (*II*). (*II*) Optical section from stained, paraformaldehyde-fixed tissue. (*III*) Optical section from stained, acetone-fixed tissue. The insets labeled "Profile" in panels II and III show fluorescent intensities along the white lines "*A*" and "*B*". In panel II, the intensity peaks over the lumenal plasma membrane and then falls to near background levels over the cell body consistent with membrane staining. In panel III, intensity peaks over the lumenal plasma membrane but remains elevated over the cell body consistent with staining of both membrane and cytoplasm. (*IV*) The circumferential, honeycomb pattern most likely occurs when the optical section transects contiguous, intact endothelial cells exposed by an oblique cut through a tortuous venule (*A, inset i*). Monocytes attached to endothelial cells exposed in this manner form sheets in the S-W assay (*B*; also see Fig. 2 *a*). In contrast, the linear staining most likely occurs when the optical section transects a single layer of endothelium (*A, inset ii*). Monocytes attached to endothelial cells in these areas line the lumenal surface in the S-W assay (*B*; also see Fig. 2, *a* and *d*).

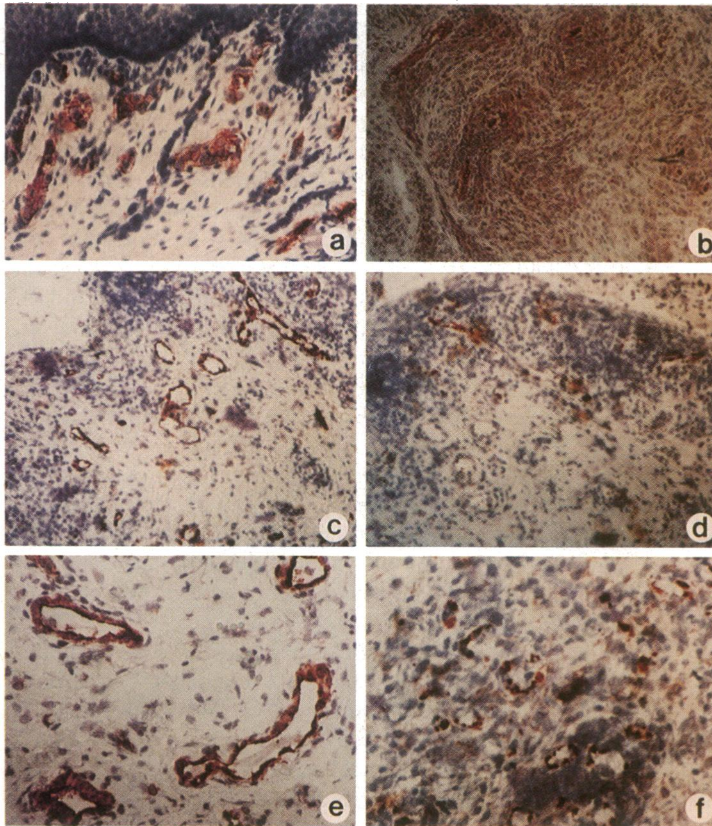

Figure 3. Immunoperoxidase stains for P-selectin and the platelet antigen gpIb/IX. (*a*) P-selectin in human newborn foreskin; (*b*) P-selectin in term placenta; (*c* and *d*) distribution of P-selectin (*c*) and gpIb/IX (*d*) in serial sections from rheumatoid synovitis; (*e* and *f*) magnified views of P-selectin (*e*) and gpIb/IX (*f*) in venules of rheumatoid synovitis.

which recognizes an epitope in the NH_2-terminal cell-binding domain of the receptor. Since paraformaldehyde does not permeabilize cell membranes, only P-selectin exposed by sectioning and available for monocyte attachment should stain. The

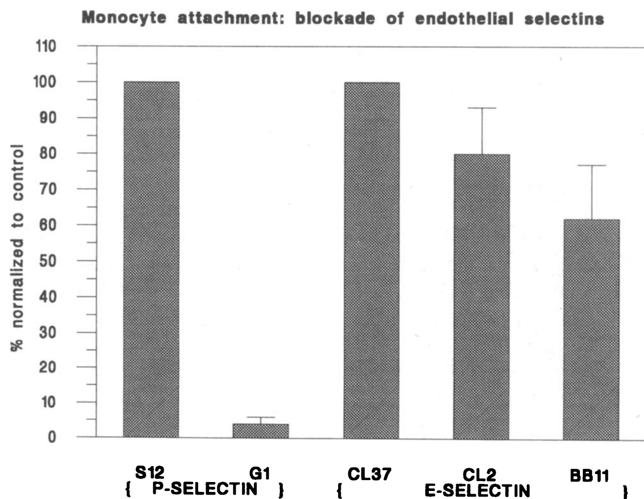

Figure 4. Selectin-dependent monocyte adhesion. Air-dried frozen sections were preincubated with mAbs (Fab'2 fragments) for 30 min at 4°C before initiation of the binding assay. The controls were as follows: nonblocking mAb S12 for G1; nonblocking mAb CL37 for CL2 and BB11. The mean and standard error of the mean were calculated from 11 independent experiments conducted on synovectomy specimens obtained from four patients. Each experiment consisted of five replicate determinations per mAb.

modulation contrast image of the stained venule (Fig. 6 *I*) indicates that the lumenal surface of the endothelium was exposed in the thick frozen sections. The confocal image (Fig. 6 *II*) shows linear staining along the lumenal endothelial surface and circumferential staining around individual endothelial cells suggesting association of P-selectin with plasma membranes. The circumferential or "honeycombed" pattern most likely results when the optical section, with an effective resolution of 0.5 μm, cuts across groups of contiguous endothelial cells exposed in the section. Both the linear and circumferential patterns are less apparent in sections permeabilized with acetone before staining (Fig. 6 *III*). In contrast, a granular pattern within endothelial cell bodies predominates consistent with staining of the cytoplasmic pool. Thus confocal fluorescence microscopy suggests that the cell-binding domain of P-selectin is associated with endothelial plasma membranes in air-dried nonpermeabilized frozen sections of rheumatoid synovium. The proposed distribution, and the resulting patterns of monocyte adhesion, are illustrated in Fig. 6 *IV*.

Fab'2 mAbs specific for functional domains of E-selectin (mAbs CL2 and BB11) partially inhibited monocyte attachment when compared with Fab'2 fragments specific for a nonfunctional epitope (CL37). In contrast to P-selectin, blockade of E-selectin inhibited from 0% to a maximum of 50% in individual experiments. Variation occurred between patients, between blocks from a single patient and, in some cases, between sections cut from a single block. This behavior is consistent with the patchy, relatively weak expression of E-selectin in our series.

The integrins and L-selectin also participated in monocyte-SV adhesion in frozen sections (Fig. 7). mAbs specific for

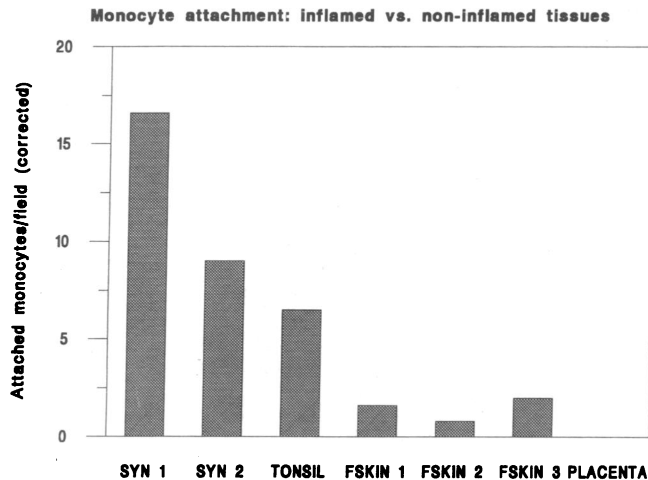

Figure 5. Monocyte-endothelial adhesion in inflamed and noninflamed tissues. *SYN 1, 2,* rheumatoid synovium; *TONSIL,* tonsillitis (acute and chronic); *FSKIN* 1-3, normal newborn foreskin; *PLACENTA,* normal term placenta. Binding was corrected for differences in the cross-sectional area (CA) of P-selectin–positive venules as follows. The CAs of P-selectin–positive venules were measured in 40–80 nonoverlapping fields (covering ~ 50% of each section) using an image analyzer (System IV; BioQuant, Inc.). A "correction factor" was determined by dividing the average CA/field by the average CA/field in SYN 1. The number of monocytes bound/high power field multiplied by this factor yielded "attached monocytes per field (corrected)." Different tissues were run in parallel with a minimum of three independent experiments, conducted in triplicate, for each. The P-selectin–specific mAb G1 inhibited attachment by > 90% in all tissues.

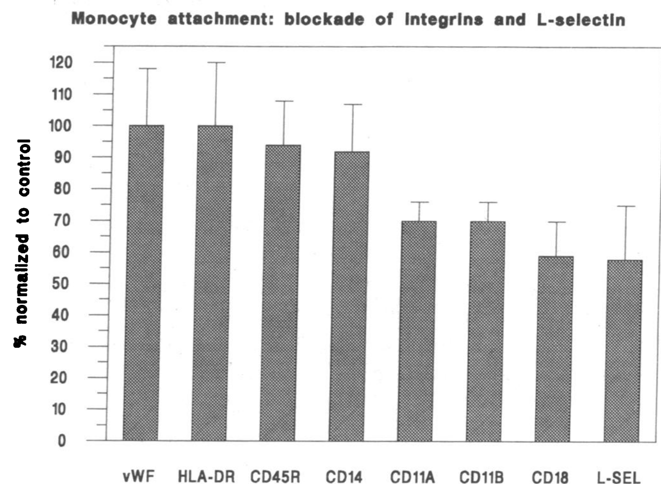

Figure 7. β2-integrin and L-selectin dependent monocyte adhesion. Percoll-fractionated or elutriated monocytes were incubated with mAbs for 30 min and then layered onto air-dried frozen sections as described. Blocking activity of integrin and L-selectin specific mAbs were compared to isotype-matched mAbs run in parallel (see Table II). The mean and SEM for inhibition by each mAb (with the exception of the L-selectin specific reagent) was calculated from four to six independent experiments on tissues from four patients. Inhibition by the L-selectin–specific mAb was determined in tissues from two patients. Each experiment consisted of five replicate determinations per mAb.

LFA-1 (CD11a) and Mo1 (CD11b) chains each inhibited attachment 30–35% while mAb to the common β2 (CD18) subunit inhibited ~ 40% relative to a class-matched control directed at von Willebrand factor. mAbs directed at CD14, CD45R, and HLA-DR, epitopes expressed at equal or greater densities than the integrins (Table II), failed to inhibit significantly. The same degree of inhibition was observed with both Percoll-fractionated and elutriated monocytes. In contrast, an mAb specific for the common β1 chain of the VLA subfamily, AIIB2 (49), inhibited the attachment of Percoll-fractionated monocytes by 30–50% while inhibiting attachment of elutriated cells by < 20% (data not shown). Thus platelet rosetting contributes to the β1-dependent monocyte adhesion observed with Percoll-fractionated cells. However, elimination of monocyte-associated platelets via elutriation does not completely eliminate β1-dependent adhesion. Finally, an mAb specific for L-selectin (DREG 200) inhibited attachment by ~ 40% in tissues from two patients with RA synovitis. We conclude that monocyte attachment in frozen sections involves P-selectin primarily. E-selectin, the β2-integrins, the β1-integrins, and L-selectin augment adhesion but appear less able to initiate monocyte attachment in the Stamper-Woodruff assay.

Discussion

This study used a modified Stamper-Woodruff frozen section binding assay (S-W assay) to investigate leukocyte-microvascular adhesion in rheumatoid synovitis and other tissues. In rheumatoid synovitis, monocyte-enriched fractions bound to venules lined by low endothelium in areas with both dense and sparse infiltrates of mononuclear cells. In addition, cells attached to the rare HEV which developed in areas of nodular lymphocytic infiltration (50). Jutila et al. also observed monocyte adhesion to HEV in murine lymph nodes following an inflammatory stimulus (21). In previous studies, mononuclear cells isolated from peripheral blood over Ficoll-Hypaque (F-H) gradients bound primarily to venules lined by "high" endothelium (51, 52). One explanation for the apparent discrepancy is

Table II. Binding of Control and Integrin-specific Monoclonal Antibodies to Monocytes

mAb	Antigen	Mean fluorescence intensity
1G1G12	vWF	74
9-49	HLA-DR	152
GAP 8.3	CD45R	194
26ic	CD14	174
L7	CD11a	151
44	CD11b	156
IB4	CD18	153

Relative binding of control and integrin-specific mAbs. Gradient-fractionated monocytes were stained using an indirect immunofluorescence technique as described in Methods. The mean fluorescence intensity is proportional to the density of mAbs at the cell surface with a doubling of signal intensity every 24 channels (logarithmic relationship). Thus CD45R, CD14, and HLA-DR bound to monocytes as well or better than mAbs with blocking activity.

that lymphocytes, the predominant population in F-H fractions, adhere preferentially to HEV-like vessels. Venules with this structure are generally associated with dense, nodular lymphocytic infiltrates in rheumatoid synovitis and other tissues, implying specialization for lymphocyte trafficking (53–55). Binding sites for monocytes, in contrast, are widely distributed in the synovial microvasculature in keeping with the broad distribution of monocyte derivatives within the synovial pannus (5, 50).

The mononuclear cells used in our study consisted of 85–90% monocytes as judged by light scatter and expression of CD14. The remaining cells are primarily lymphoid with < 2–3% granulocytes (neutrophils, eosinophils, and basophils). Thus a portion of the cells attached to the microvasculature may not be monocytes. Mononuclear cells are readily distinguished from granulocytes after staining with toluidine blue since the stain highlights nuclear configuration. Toluidine also stains basophilic leukocyte granules. Fig. 2, *a*, *c*, and *d*, shows that bound cells are primarily agranular and mononuclear. In addition, virtually all of the mononuclear cells remaining attached to the microvasculature after staining for peroxidase activity were positive consistent with monocytes (data not shown). However, the repeated washings required to detect enzyme activity resulted in loss of many bound cells thus may not accurately reflect the ratio of bound monocytes to lymphocytes in the standard assay. Therefore, the microvascular binding densities of equally enriched populations of monocytes and lymphocytes were compared in the standard S-W assay (Table I). Monocyte-enriched fractions bound to the microvasculature in the S-W assay 10–15-fold better than equally enriched lymphocyte fractions when present at the same cell concentration. Since the ratio of monocytes to lymphocytes was 5–9:1 (granulocyte-depleted, 85–90% monocytes) in the populations used in this study, we conclude that at least 90–95% of the cells attached to synovial venules are monocytes. These data also suggest that monocytes will makeup a significant percentage of the mononuclear cells attached to the microvasculature even if they comprise < 5% of the population used in the S-W assay. Therefore, F-H fractions of mononuclear cells, the previous standard for human studies (51, 52), are not optimal for assay of either monocyte or lymphocyte adhesive interactions in inflamed tissues.

Blocking experiments with monoclonals identified P-selectin as the principal mediator of monocyte-endothelial adhesion in the S-W assay. Contributions from the monocytic integrins, E-selectin, and L-selectin were also detected. As noted previously, platelet rosettes invariably form during gradient fractionation of monocytes. Such rosettes complicate interpretation of blocking experiments with mAbs to P-selectin and the β1-integrins since the adherent platelets carry these receptors (56, 57). Therefore, the current study used counter current centrifugal elutriation to isolate monocytes essentially free of contaminating platelets. These cells bound as well or better than Percoll-fractionated cells which showed 10–30-fold higher levels of platelet contamination. In addition, mAb to P-selectin blocked > 90% of monocyte attachment to SV when prebound to the tissue section and washed off prior to the start of the adhesion assay. Thus adhesion molecules on the monocytes and the microvasculature, not receptors expressed by artifactually rosetted platelets, mediated attachment in the S-W assay.

The known pool of P-selectin in Weibel-Pallade bodies raised the possibility that exposed cytoplasmic stores in tissue sections accounted for the observed adhesion (48). In addition, the tissue ischemia and trauma associated with procurement might activate thrombin or release free radicals resulting in artifactual mobilization of P-selectin to the cell surface (58, 59). We controlled for these possibilities by measuring monocyte adhesion to the P-selectin positive venules of surgically removed newborn foreskin and normal term placenta. One would expect monocyte adhesion to all P-selectin–containing venules in tissue sections if exposed cytoplasmic contents were the principal binding sites. Similarly, if ischemia or trauma before snap freezing induced adhesion to P-selectin, then one would expect high levels of P-selectin–dependent adhesion in a variety of tissues. Fig. 3, *a*, *b*, and *e*, shows strong staining for P-selectin in the microvasculature of foreskin and placenta as well as rheumatoid synovitis. Yet, monocytes did not adhere to placental venules, and P-selectin–dependent adhesion was 5–20-fold greater in chronic rheumatoid synovitis than in newborn foreskin. Monocytes also bound avidly to venules in acutely inflamed tonsil at densities three- to ninefold greater than that in foreskin. It should be noted that mild inflammation in normal foreskin is not uncommon and may account for the low levels of monocyte adhesion observed. In any event, artifactual exposure of cytoplasmic stores or mobilization of P-selectin during collection of the rheumatoid synovium is, therefore, an unlikely explanation for the high density of monocyte adhesion. We propose that the S-W assay detected P-selectin mobilized to the lumenal endothelial surface in response to inflammation.

The confocal laser microscopy studies support this hypothesis. Confocal microscopy generates high resolution optical sections permitting mapping of P-selectin expression in the thick tissue sections used in the S-W assay (60–62). The pattern of staining with the G1 mAb suggested that the cell-binding domain of P-selectin was primarily associated with endothelial plasma membranes in nonpermeabilized, thick frozen sections of RA. In contrast, the granular pool of P-selectin in the cytoplasm of endothelial cells stained most intensely after permeabilization of tissue membranes with acetone. Since the S-W assay used air-dried nonpermeabilized sections, we conclude that monocytes most likely bound to P-selectin associated with plasma membranes. The illustration in Fig. 6 *IV* indicates how this distribution could account for both the linear and sheetlike arrangement of monocytes attached to the microvasculature in the S-W assay (Fig. 2). We propose that the marked increase in P-selectin–dependent attachment observed in rheumatoid synovitis and tonsillitis reflects its participation in monocyte recruitment in vivo.

Staining of RA synovial specimens for gpIb/IX revealed platelets associated with variable numbers of venules. gpIb/IX–positive material occupied the lumens of small vessels and formed small, discrete plugs within the endothelium of larger vessels consistent with platelet containing thrombi. These findings support previous studies showing accumulation of radiolabeled platelets in active lesions of rheumatoid arthritis (63). In addition, extravascular deposits of platelets and platelet antigens have been observed in sections of rheumatoid synovium (64). Thus, activated platelets associated with the microvasculature may be a source of P-selectin in some venules. However, gpIb/IX has recently been shown to stain cytokine-activated endothelial cells in culture (65). Therefore, activated endothelial cells in tissue sections may contribute to the observed staining with gpIb/IX resulting in an overestimate of platelet deposi-

tion. Nonetheless, the fact that most P-selectin positive vessels in our sections were negative for gpIb/IX still implies that the principal source for P-selectin in rheumatoid synovitis is the endothelium.

In studies of neutrophil adhesion, Lawrence and Springer found that P-selectin initiated binding under shear-stress (66). The S-W assay also measures adhesion under shear stress. The geometry of the assay does not lend itself to precise quantitation of shear. However, Ley et al. calculated the maximal shear stress generated by rotory agitation in a similar assay system (67). These investigators calculated that a gyratory rate of 50 rpm and a 4.3-mm radius of rotation yields a shear stress of 0.44 dyn/cm^2 in media containing 1% fetal calf serum at 37°C. The current study used a gyratory rate of 50 rpm, a 7-mm radius of rotation, and media containing 1 mg/ml BSA at 7°C. Aqueous buffers are approximately twofold more viscous at 7°C than at 37°C; therefore, both the radius of rotation and the viscosity of the medium in the S-W assay exceed that in the Ley system. As a result, 50 rpm in the S-W assay should generate a maximal shear stress approaching 1 dyn/cm^2 at the interface between monocytes and the frozen section (67). Thus, the ability of P-selectin to initiate contact with leukocytes under shear stress may account for its predominance in the S-W assay.

By approximating the monocyte and endothelial cell membranes, P-selectin may foster the development of secondary attachments through receptors with shorter working distances. Thus, blockade of P-selectin may inhibit completely because it prevents the initial contact of monocyte and endothelial cell membranes in the S-W assay. In contrast, mAbs to E-selectin, the β2-integrins, and L-selectin may inhibit partially because they eliminate only one of several potential strengthening interactions. Alternatively, the partial inhibition by mAbs to L-selectin and the β2-integrins may indicate a direct interaction between P-selectin and oligosaccharide sidechains on these receptors. L-selectin on neutrophils, for example, reportedly interacts with both E- and P-selectin through sidechains carrying the sialylated Lewis^x tetrasaccharide (68). In addition, the β2 integrin family carries the structurally related Lewis^x trisaccharide (69, 70) which binds to P-selectin with low affinity (25). Whether or not the β2-integrins also express the high affinity sialylated form of Lewis^x remains to be determined; however, the sialylated and nonsialylated forms are frequently coexpressed on leukocytic glycoproteins (71–73). On the other hand, affinity chromatography and blotting with purified P-selectin suggests that its principal attachment site on neutrophils is a sialylated glycoprotein distinct from L-selectin (31). This molecule behaves like a homodimer consisting of two, 120,000-kD subunits thus differing from the heterodimeric β2-integrins. Determination of the binding sites for P-selectin on the monocyte and L-selectin on synovial endothelium is needed to clarify the interactions amongst adhesion receptors observed in the current study.

The detection of P-selectin–dependent monocyte adhesion in the S-W assay implies a previously unsuspected role for this receptor in the pathophysiology of chronic rheumatoid arthritis. However, the significance of its predominance over other adhesion receptors in the S-W assay cannot be determined for several reasons. The current series examined specimens from patients undergoing therapeutic synovectomy and joint replacement surgery. These individuals have long standing disease generally treated with multiple antiinflammatory agents. A recent immunoperoxidase study showed that antiinflammatory therapy reduced the expression of E-selectin in rheumatoid synovitis (74). Therefore, the low level of E-selectin expression and function in our group may reflect treatment history rather than a selective association of P-selectin with monocyte recruitment in RA. The expression of endothelial counter-receptors for L-selectin and the β1-integrins may be underestimated for similar reasons. Finally, the relative contribution of the β2-integrins cannot be assessed with the S-W assay since their function is temperature dependent and may require soluble mediators for optimal activity. Nonetheless, our series suggests that P-selectin is a major and, in some patients, a predominant endothelial attachment site for monocytes in chronic RA. Thus therapeutic strategies aimed at leukocyte recruitment in this disease should target P-selectin as well.

The rapid and transient expression of P-selectin on cultured HUVE after treatment with inflammatory mediators implies that this receptor mediates the initial phase of leukocyte recruitment in "acute" inflammation (34). The detection of functional P-selectin on the microvasculature in RA, on the otherhand, implies that it contributes to recruitment in "chronic" inflammatory diseases as well. How might a transiently mobilized adhesion receptor contribute to recruitment in a chronic disease? Firstly, RA is a mixture of acute and chronic inflammation in which neutrophil-rich joint effusions occur in conjunction with predominantly mononuclear infiltrates in the synovium (64). Thus the soluble mediators of acute inflammation, which induce transient P-selectin mobilization in vitro, must be generated in rheumatoid synovitis as well. Secondly, oxygen-derived free radicals induce prolonged expression of P-selectin on cultured HUVE at noncytotoxic concentrations (59). These products have been implicated in the pathophysiology of inflammatory arthritis in humans (75) and in streptococal cell-wall mediated arthritis in Lewis rats (76). In the latter, intraarticular injection of superoxide dismutase or catalase decreases phagocyte accumulation in the synovium and inhibits the development of chronic arthritis. Thus toxic metabolites generated by infiltrating phagocytes may act directly on the microvasculature to induce prolonged P-selectin expression and augment leukocyte recruitment. Finally, recent studies show that TNF-α increases the synthesis and expression of both E- and P-selectin on murine microvascular endothelial cells (77). Thus the chronic production of TNF-α in the rheumatoid pannus may lead to sustained expression of P-selectin on the synovial microvasulature.

It should be noted that all granulated leukocytes and some lymphocytes bind to P-selectin (34, 78, 79) and E-selectin (80–82) in vitro. In fact, a recent study found that lymphocytes isolated from rheumatoid synovitis show enhanced binding to soluble P-selectin (83). Thus one would expect neutrophils and some lymphocytes, as well as monocytes, to interact with the selectins expressed on the microvasculature in RA. The contributions of these adhesive interactions to recruitment in inflammatory arthritis is currently under investigation.

In summary, our study demonstrates that synovial venules in rheumatoid arthritis express functional P-selectin and E-selectin as well as counter-receptors for monocytic L-selectin, β2 and β1 integrins. P-selectin is the predominant adhesion molecule detected by the S-W assay in tissue sections. The marked increase in its activity on the microvasculature in rheumatoid synovitis suggests a role for P-selectin in the pathophysiology of this disease.

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