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Research Article

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Functional Activation of Lymphocyte CD44 in Peripheral Blood is a Marker of Autoimmune Disease Activity

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Abstract

Interactions between complementary receptors on leukocytes and endothelial cells play a central role in regulating extravasation from the blood and thereby affect both normal and pathologic inflammatory responses. CD44 on lymphocytes that has been "activated" to bind its principal ligand hyaluronate (HA) on endothelium can mediate the primary adhesion (rolling) of lymphocytes to vascular endothelial cells under conditions of physiologic shear stress, and this interaction is used for activated T cell extravasation into an inflamed site in vivo in mice (DeGrendele, H.C., P. Estess, L.J. Picker, and M.H. Siegelman. 1996. *J. Exp. Med.* 183:1119–1130. DeGrendele, H.D., P. Estess, and M.H. Siegelman. 1997. *Science.* 278:672–675. DeGrendele, H.C., P. Estess, and M.H. Siegelman. 1997. *J. Immunol.* 159: 2549–2553). Here, we have investigated the role of lymphocyte-borne–activated CD44 in the human and show that CD44-dependent primary adhesion is induced in human peripheral blood T cells through T cell receptor triggering. In addition, lymphocytes capable of CD44/HA-dependent rolling interactions can be found resident within inflamed tonsils. In analysis of peripheral bloods of patients from a pediatric rheumatology clinic, examining systemic lupus erythematosus, and a group of chronic arthropathies, expression of CD44-dependent primary adhesion strongly correlates with concurrent symptomatic disease, with 85% of samples from clinically active patients showing elevated levels of rolling activity (compared with only 4% of inactive patients). These rolling interactions are predominantly mediated by T cells. The results suggest that circulating T lymphocytes bearing activated CD44 are elevated under conditions of chronic inflammation and that these may represent a pathogenically important subpopulation of activated circulating cells that may provide a reliable marker for autoimmune or chronic inflammatory disease activity. (*J. Clin. Invest*. 1998. 102:1173–1182.) Key words: adhesion • inflammation • hyaluronate • SLE • arthritis

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Introduction

Autoimmune diseases result from the failure of regulatory mechanisms that maintain self-tolerance. Although the etiologic agents that initiate autoimmunity are generally not defined, the result at an established chronically inflamed site is persistent infiltration of mononuclear effector cells, among which T cells frequently play a prominent role. While the pathogenic role of the T cell has not been precisely defined, it has long been considered that most forms of autoimmune disease have dysregulation of T cell function as an initiating mechanism and/or in maintenance of the autoimmune state. Activated T cells serve as the trigger for a cascade of events that lead to amplification of the inflammatory process with the eventual resultant tissue damage at target sites. It is clear that T cells can also play an overt role in the inflammatory process subsequent to the inciting events. For example, in rheumatoid arthritis the synovium is infiltrated with mononuclear cells which are predominantly T cells, and these may be largely responsible for the sequelae, which result in the characteristic features of rheumatoid arthritis (1). However, whether for initiation or maintenance of the autoimmune state, it is likely that T cells indeed gain access to such sites in autoimmune diseases. Thus, direct homing of effector T cells to target sites may represent an important control point of immune regulation.

That memory/effector and lymphoblast populations engage in discrete tissue-specific homing pathways has long been appreciated (2, 3). Recirculation of these subsets tends to primarily involve extralymphoid (tertiary) tissues, i.e., those inflammatory sites at which final effector cells exert their function. Examples of preferred homing of memory/effector subsets have been described for inflamed skin, intestinal lamina propria, and inflamed synovium. A relationship of activated or memory/effector lymphocyte populations to increased expression of the proteoglycan link protein family member CD44 has been described, and the importance of such CD44^{hi}-expressing cells in the effector arm of the immune response in vivo has been reported (4). Moreover, increased CD44 expression has been shown on the inflammatory infiltrate in human and mouse arthritis (5, 6), and anti-CD44 antibodies have been shown to inhibit contact hypersensitivity (7) and collagen-induced murine arthritis (6, 8). The mechanism by which CD44 exerts its influence, however, has not been clarified.

We have recently described a novel interaction between lymphocytes and endothelial cells that initiates lymphocyte contact and primary adhesion, or rolling, on endothelial cells under conditions of physiologic laminar flow. This is mediated by CD44 on lymphocytes "activated" to bind its primary ligand, the glycosaminoglycan hyaluronan (9). Direct signaling via the T cell receptor results in the activated form of CD44, which enables primary adhesion (10), and in vivo superantigen stimulation leads to peripheralization and accumulation of antigen-specific T cell subsets at the inflamed site of challenge in a CD44-dependent fashion (11). These observations in mice

have led us to propose that the activated form of CD44 is induced on lymphocytes as a result of antigen stimulation within secondary lymphoid sites and that this is followed by release of activated cells from secondary lymphoid sites into the peripheral circulation. Activated CD44 then functions to initiate lymphocyte extravasation at sites of antigen localization or inflammation, where HA can be upregulated on the endothelium of appropriate vascular beds (12).

An essential element of our model is the demonstration that cells in the peripheral circulation exhibit CD44-dependent rolling under appropriate circumstances. The evidence presented here establishes that this activity is indeed present in humans, has similar characteristics under shear stress to those described for selectins and for CD44 in the mouse, and is inducible through T cell activation. The occurrence of cells engaging in CD44-dependent primary adhesion in peripheral blood strikingly correlates with exacerbations of autoimmune disease in SLE and arthritis. The cumulative data is consistent with the view that activated CD44 selectively participates in the well-described enhanced homing of activated lymphocytes to sites of inflammation, that this may be an indicator of existing autoimmune activity, and that manifestations of increased CD44/HA interactions in autoimmune disease may have significant monitoring and therapeutic implications.

Methods

Reagents and antibodies. The following reagents were used in in vitro analyses: PMA, rooster comb hyaluronic acid (HA),[1] and bovine trachea chondroitin sulfate A (CSA; Sigma Chemical Co., St. Louis, MO); Ionomycin (Calbiochem, La Jolla, CA); hamster anti–human CD3, mouse anti–human CD28, and control hamster anti-TNP (PharMingen, San Diego, CA); tetanus toxoid (Connaught Laboratories, Swiftwater, PA). 515, an HA-blocking mouse anti–human CD44 monoclonal antibody, was kindly provided by G. Kansas (Northwestern Medical School) (13, 14), and Hermes-3, a non-HA–blocking anti-CD44 antibody, was provided by Dr. L. Picker (UTSWMCD) (15). Fluorescein-conjugated HA (Fl-HA) was prepared using fluoresceinamine as described (16).

The following reagents were used for FACS analysis: IM7-PE anti-CD44 (PharMingen); anti–CD69-PE, anti–HLA-DR-biotin, anti–CD3-biotin, anti–CD3-FITC, anti-CD25-PE (Becton Dickinson, San Jose, CA); Streptavidin-R670 (GIBCO BRL, Grand Island, NY).

Additional reagents used for blood and tonsil cell depletion or fractionation were: anti–CD69-FITC, anti–CD19-FITC, anti–CD33-FITC (Becton Dickinson); anti-FITC–conjugated magnetic particles (Miltenyi Biotec, Sunnyvale, CA); anti-CD2, anti-CD19, and Streptavidin-conjugated magnetic beads (Dynal, Lake Success, NY); anti–FITC-biotin (Sigma Chemical Company); T cell enrichment columns (R & D Systems, Minneapolis, MN).

Fluorescence-activated cell sorter analysis. Cells were analyzed by two-color immunofluorescence for expression of activated CD44 using Fl-HA followed by the anti-CD44 mAb IM7-PE. For blocking studies, saturating amounts of unlabeled mAb 515, which does not cross-block the staining of IM7, were preincubated with cells before addition of Fl-HA. Other reagent combinations were used similarly with addition of fluorochrome-labeled Streptavidin as indicated. Data was collected on a FACScan™ analytical instrument and analyzed using Lysis II™ or CELLQuest™ software (Becton Dickinson).

Adhesion assay under flow conditions. Physiological flow conditions were produced using a parallel plate flow chamber coated with 500 μl of 2.5 mg/ml soluble rooster comb HA, as described (9). All experiments were carried out at 3×10^6 cells/ml and a wall shear stress of 2.0 dynes/cm^2, unless otherwise noted. For blocking studies, 515 anti-CD44, Hermes anti-CD44, isotype matched control antibody (anti-TNP), soluble HA, or soluble CSA were added at saturating concentrations to the cell suspension before flow. Interaction of lymphoid cells with the HA substrate after equilibration of flow was monitored with an inverted phase contrast microscope connected to a video camera and recorder, changing the field of view every minute. Rolling cells were scored visually, and data is reported as the average number of interacting cells/mm^2/min, based on an actual field of view of 0.6×0.8 mm and an actual viewing time of at least 5 min. For patient peripheral blood samples, results are reported as the cumulative number of interacting cells over a 4-min period. This was accomplished by systematically scanning the entire surface of the chamber (0.9 cm^2), beginning from the outlet side of the chamber and progressing toward the inlet side; since cells are moving toward the outlet side, they are not reencountered during the observation period (11). Following enrichment or depletion, samples were readjusted to 3×10^6 cells/ml before assaying under flow.

Collection and processing of human peripheral blood and tonsils. Peripheral blood used for in vitro studies was obtained from healthy donors using heparinized syringes (120 mls/donor), and PBMCs were purified by Ficoll-Hypaque gradient centrifugation. For enrichment before in vitro activation, T cells were isolated using T cell purification columns according to manufacturer's instructions (R & D Systems).

Pediatric tonsils were obtained fresh from Surgical Pathology, Children's Medical Center, UTSWMCD, processed into single-cell suspensions, depleted of red blood cells with NH_4Cl lysis buffer, and used for FACS® or in rolling analysis. Separation of tonsil cells into subpopulations was accomplished via T cell purification columns to enrich for T cells. Anti–CD19-FITC was used for B cells or anti–CD69-FITC for activated cells, followed by enrichment of the antibody binding populations by using anti-FITC–conjugated magnetic particles and MiniMACS separation columns (Miltenyi Biotec) according to manufacturer's instructions. Control peripheral lymph nodes (cervical, periaortic, and inguinal) were obtained from Forensic Pathology, UTSWMCD, and were harvested at autopsy from individuals 20–30 yr of age with no known underlying diseases, deceased from traumatic events.

Patient PBMC were purified by Vacutainer™ CPT cell separation tubes (Becton Dickinson) according to manufacturer's instructions. Fractionation of blood to examine the rolling populations in active patients was accomplished using anti-CD2–conjugated magnetic beads to deplete T cells, or anti-CD19–conjugated magnetic beads to deplete B cells. Monocyte depletion was performed by incubating the cells with anti–CD33-FITC, followed by anti–FITC-biotin and Streptavidin conjugated magnetic beads. Control blood samples were obtained as random consecutive samples drawn simultaneously with blood donations at Carter BloodCare (Dallas, TX). Normal blood donors ranged in age from 18–59 yr old.

In vitro activation of cells. PMA/Ionomycin stimulation of normal peripheral blood T cells was accomplished by incubating cells with 1 ng/ml PMA plus 500 ng/ml Ionomycin for 18 h at 37°C. For anti-T cell receptor stimulation, wells of 96-well polystyrene tissue culture plates were coated with 5 μg/ml anti-CD3 plus 1 μg/ml anti-CD28. 0.2 ml of 2×10^6/ml peripheral blood T cells were incubated in complete RPMI1640/5% FCS at 37°C with immobilized antibody and collected after 48 h for analysis. Following collections, cells were washed, and an aliquot was stained with Fl-HA plus anti–CD44-PE; the remainder was analyzed for rolling on HA-coated plates. For an-

1. *Abbreviations used in this paper:* CBC, complete blood cell count; CSA, chondroitin sulfate A; EC, endothelial cell; ESR, erythrocyte sedimentation rate; Fl-HA, fluorescein conjugated hyaluronate; HA, hyaluronate acid; NSAID, nonsteroidal anti-inflammatory agent; SLEDAI, SLE Disease Activity Index; UA, urine analysis; WSS, wall shear stress.

tigen-specific secondary activation, 2×10^6 PBMC were placed in culture with 0.5 Lf/ml tetanus toxoid. Control cells were incubated in parallel without antigen. After 4 d, harvested cells were analyzed by FACS and for rolling on HA-coated plates.

Patients. The patient sample population consisted of 78 outpatients (134 samples) from pediatric rheumatology clinic (see Table I) visiting between March 1996 and February 1998. SLE and arthritis patients were enrolled, and samples were collected randomly. There were 26 SLE patients, all of whom had an established diagnosis of SLE, as defined by the 1982 revised criteria established by the American College of Rheumatology for the classification of SLE (17). Of 52 arthritis patients, 17 were polyarticular, 21 pauci-articular, 6 systemic, 3 reactive, 2 ankylosing spondylitis, and 3 psoriatic arthritis. All arthritis patients met the American College of Rheumatology criteria for the diagnosis of JRA (18) or the European Spondyloarthropathy Study Group criteria for juvenile SpA (19). Patients were classified according to their age at disease onset, presence or absence of systemic features, and number of joints involved in the first 6 mo of disease (20). The mean age at the start of the study was 11.5 years. Diagnosis was made at a mean of 3.5 yr before the onset of the study. Blood samples (3–10 cc) were collected directly into acid citrate dextrose tubes. All adhesion and flow cytometry analyses were performed without knowledge of the patient's clinical status. Studies were approved by and all samples were collected in accordance with the guidelines of the Institutional Review Boards at UTSWMCD and Texas Scottish Rite Hospital for Children.

Clinical evaluation and disease activity. In patients with juvenile chronic arthritis, the duration of morning stiffness and the patient/parent and physician global scores of disease activity were recorded at each clinic visit. Rheumatoid factor level and antinuclear antibodies were obtained at baseline and repeated in patients with unconventional clinical courses. Laboratory data, including complete blood cell count (CBC), erythrocyte sedimentation rate (ESR), and urine analysis (UA) were performed during each visit. The degree of functional ability, number of joints with active arthritis, number of joints with decreased range of motion, and ESR were scored by the physician to yield a global assessment of disease (21). In this study, a patient was considered active if at least one joint was swollen and/or there was limitation to motion with pain or tenderness on movement. Patients with systemic arthritis additionally were considered active in the absence of joint involvement if they exhibited systemic symptoms such as rash and/or fever or laboratory findings of elevated ESR or white blood cell count. Treatment modalities included daily nonsteroidal anti-inflammatory agents (NSAIDs), and/or second line drugs Methotrexate (0.3–1 mg/Kg q week), Hydroxychloroquine (3–7 mg/Kg q day), and low dose Prednisone ($<$ 1 mg/Kg q day).

For SLE, patients were evaluated during each clinic visit for the presence of signs and symptoms of SLE, reviewing multiple organ systems and laboratory tests including CBC, ESR, UA, electrolytes, creatinine, BUN, CH50, C3, C4, and anti-dsDNA. Positive clinical and laboratory findings were recorded in keeping with the criteria of the SLE Disease Activity Index (SLEDAI) (22), which includes both clinical and laboratory features of SLE and is weighted to account for "severity". Because of the high frequency of nephritis and its chronic persistence in pediatric SLE relative to adults, resulting in a general constitutive high score in UA in most of these patients, a SLEDAI score of $>$ 10 was considered to represent "active" disease, and $\leq$ 10 was considered inactive. A flare was defined as an increase of at least three points in the SLEDAI scoring system from the previous visit or from a visit within the last 3 mo (23). SLE patients were treated with variable doses of oral Prednisone (0.5–2 mg/Kg q day) and Hydroxychloroquine (3–7 mg/Kg q day). Disease exacerbations with major organ involvement were treated with intravenous Methylprednisolone (30 mg/Kg for 3 consecutive days), and/or intravenous Cyclophosphamide (0.5–1 g/m^2 q 1–3 mo). Most SLE patients received oral antihypertensive medications (calcium blockers and/or angiotensin converting enzyme inhibitors) during the period of study.

Results

Polyclonal and antigen-specific stimulation induces activated CD44 and CD44-mediated primary adhesion in normal human peripheral blood T cells. Purified peripheral blood T cells isolated from normal human donors were analyzed by FACS for expression of HA-binding CD44 before and after stimulation with PMA/Ionomycin. After 18 h of stimulation, there was a significant (30%) population of CD44$^+$/HA-binding cells not seen in the untreated population (Fig. 1 *A*). The Fl-HA binding was shown to be CD44- and HA-dependent by blocking with the HA-blocking anti-CD44 mAb 515. To more directly assess stimulation via the TCR, T cells were treated with immobilized anti-CD3/anti-CD28. Again, a prominent population of CD44$^+$/Fl-HA–binding cells comprising 9% of total cells was induced, and Fl-HA binding was CD44 dependent as shown by blocking with anti-CD44 (Fig. 1 *B*). Analysis of the blast population based on forward and side scatter characteristics showed an enriched subpopulation (22%) of blast cells expressing the HA-binding form of CD44, further consistent with this phenotype being associated with the activated cells. We also examined the response of PBMC from individuals previously immunized to tetanus toxoid. After 4 d in culture with antigen, overall CD44 expression had increased and a distinct population of lymphoblasts bearing activated CD44 was identified, with $\sim$ 4% of the cells binding HA in a CD44-dependent fashion (data not shown). Virtually all of the Fl-HA–binding cells were CD3$^+$ T cells as well as positive for the early activation marker CD69 (Fig. 1 *C*).

Activated cells were further analyzed in a laminar flow parallel plate assay at physiologic wall shear stress to assess CD44-mediated primary adhesion. Each form of stimulation was associated with rolling interactions on HA substrate. The rolling interaction of PMA/Ionomycin stimulated cells was shown to be CD44 dependent by inhibition with HA-blocking anti-CD44 mAb 515 and with soluble HA but not by the non–HA-blocking anti-CD44 mAb Hermes-3, isotype control antibody or the control glycan chondroitin sulfate (Fig. 1 *D*). Rolling of anti-CD3/CD28 and tetanus toxoid stimulated cells was likewise inhibited by 515 anti-CD44 but not by control antibody. The interactions with HA substrate occurred over a range of wall shear stresses from 1.0 to 2.5 dynes/cm^2 (Fig. 1 *E*), similar to those observed for the selectin family of adhesion molecules interacting with their endothelial carbohydrate ligands (24–26), as well as for CD44/HA interactions in the mouse (9). This analysis suggests that the CD44-mediated rolling has the potential to operate, as do selectins, at postcapillary venular wall shear stresses (1–4 dyn/cm^2). It should also be noted that even under lower shear stress, the CD44/HA interaction did not convert to firm adhesion. The results indicate that this activation phenotype and potential for CD44 mediated primary adhesion under physiologic flow extends to humans.

Lymphocytes from inflamed tonsils show CD44-mediated primary adhesion. One prediction of our model is that in secondary lymphoid sites, ongoing antigen presentation and lymphocyte stimulation will promote activation of CD44. We initially examined freshly isolated cells from inflamed pediatric tonsils, which have previously been reported to be capable of rolling on HA substrate (27). In each of the inflamed tonsil samples analyzed, CD44-dependent rolling activity was observed, while no such activity was seen in noninflamed normal peripheral lymph node samples (Fig. 2). Rolling was inhibited

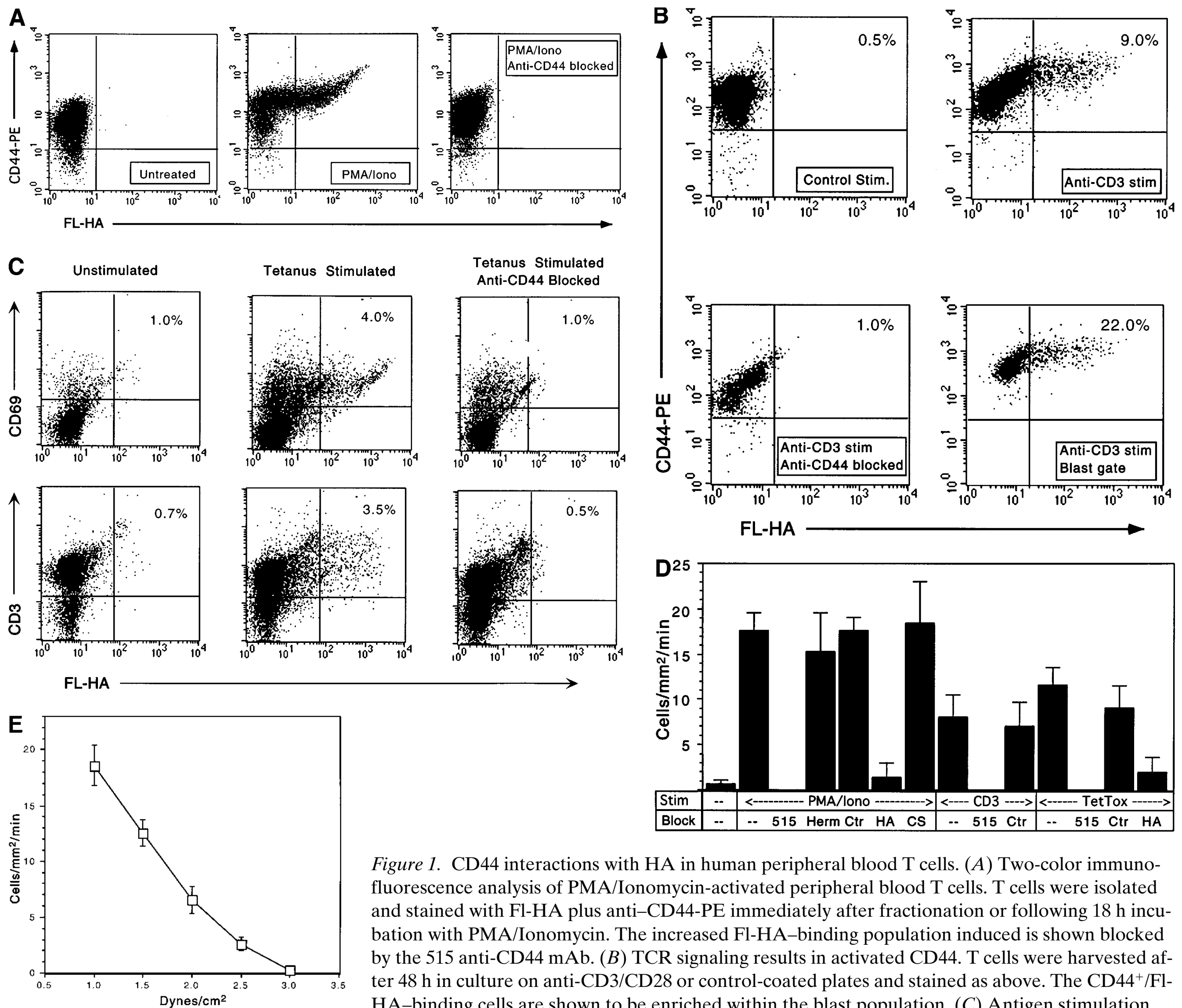

Figure 1. CD44 interactions with HA in human peripheral blood T cells. (*A*) Two-color immunofluorescence analysis of PMA/Ionomycin-activated peripheral blood T cells. T cells were isolated and stained with Fl-HA plus anti–CD44-PE immediately after fractionation or following 18 h incubation with PMA/Ionomycin. The increased Fl-HA–binding population induced is shown blocked by the 515 anti-CD44 mAb. (*B*) TCR signaling results in activated CD44. T cells were harvested after 48 h in culture on anti-CD3/CD28 or control-coated plates and stained as above. The $CD44^+$/Fl-HA–binding cells are shown to be enriched within the blast population. (*C*) Antigen stimulation also results in activation of CD44. PBMC were cultured with or without tetanus toxoid for 4 d and stained with Fl-HA, anti–CD69-PE, and anti–CD3-biotin/SA-R670 for FACS analysis. (*D*) Rolling of activated lymphocytes on HA-coated plates under conditions of shear stress. Cells were assayed at 2.0 dynes/cm^2 on HA-coated plates after in vitro activation, as indicated. HA blocking anti-CD44 antibody (515), control non-HA–blocking anti-CD44 antibody (Hermes-3), isotype control antibody (anti-TNP) (all 20 μg/ml), soluble HA (50 μg/ml), or soluble CSA (50 μg/ml) were included, as indicated. (*E*) Rolling interaction of anti-CD3/CD28–activated T cells with HA at varying wall shear stress (WSS). Activated cells were applied to feed solution already equilibrated under flow at an initial WSS of 4.0 dynes/cm^2 and perfused over HA-coated plates in the parallel plate flow assay. The flow rate of the feed solution was incrementally decreased to effect altered WSS, as previously described (9, 25). The number of cells/mm^2/min rolling across the monolayer was determined for each WSS after equilibration.

by the 515 anti-CD44 mAb but not by control antibody. Tonsil preparations were further fractionated by T cell purification columns or magnetic particle separation into T and B cell populations and assayed for rolling. The bulk of the rolling activity resided predominantly in the T cell fraction (Fig. 2), even though such tonsil preparations were 60–70% B cells. However, some activity was seen in the B cell fraction as well. This is consistent with previous reports indicating some B cell lines can engage in CD44-mediated rolling and HA binding (9, 28). Furthermore, enriching for those cells bearing the early cell activation marker CD69 (29) indicated that the rolling activity, as with HA binding in tetanus toxoid–stimulated T cells (Fig. 1 *C*), was predominantly a property associated with $CD69^+$ cells (Fig. 2). Thus, cells with the capacity to engage in CD44-dependent primary adhesion occur in situ under conditions of inflammation in the human, and in the tonsil, this phenotype is expressed primarily by T cells.

CD44-mediated primary adhesion in a population with autoimmune disease. An important aspect of our model is that cells with the capacity to undergo CD44-mediated adhesion are released into the peripheral blood to then localize within target sites of inflammation. We thus sought to examine whether such cells could be found in the peripheral circulation in the context of chronic inflammatory disease. Peripheral blood from a cohort of 78 patients diagnosed with either SLE or arthritis attending pediatric rheumatology clinic was exam-

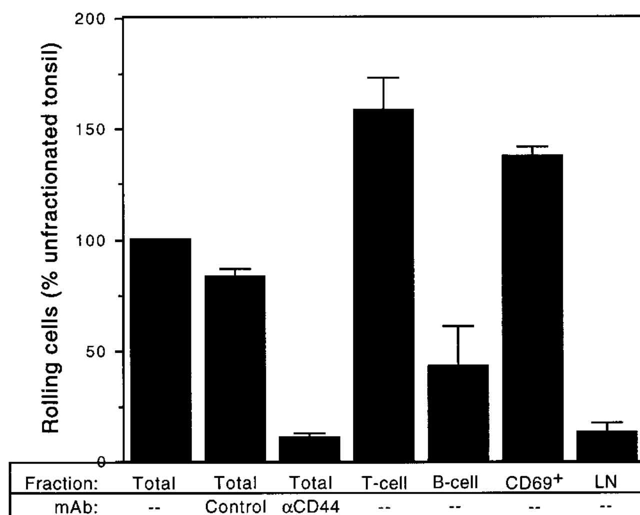

Figure 2. CD44-dependent rolling activity in inflamed human tonsils. Freshly isolated cells from human tonsils (*Total*) or control lymph nodes (*LN*) were applied directly to HA-coated plates in the parallel plate flow assay with or without antibody, as indicated. Tonsil cells were also assayed after fractionation into T cell, B cell, and $CD69^+$ populations, as shown. Rolling results are shown as the mean percent of unfractionated tonsil ($n = 3$; 100% = 20, 10, and 8 cells/mm^2/min). Control normal lymph node results are also shown as percent of unfractionated tonsils ($n = 5$; lymph node values ranged from 0–2 cells/mm^2/min).

ined for CD44-mediated rolling activity by laminar flow analysis. A total of 134 samples were tested; 66 samples were from arthritis and 68 from SLE patients (Table I; see Methods). Normal blood bank donors were used as a control population. All but one of these 43 control samples showed rolling activity of 0–1 (Fig. 3 *B*), and we thus consider this to represent background levels of rolling in this assay. As shown in Fig. 3 *A*, < 5% of the samples with 0–1 rolling cells had active disease, while 82% of samples with 2–5 rolling cells were from patients with active disease; among samples with ≥ 6 rolling cells, 100% of patients had active disease. The majority of samples, 95 (71%) showed background levels of rolling of 0–1. Of these, only 6 (6.3%) derived from patients categorized as clinically active. In contrast, of the 39 samples that had two or more rolling cells, 35 (89%) were from patients with active disease. The amount of rolling in samples from patients with active disease was highly significantly different from that in patients with inactive disease ($P < 0.0001$, Mann-Whitney U test), as well as from the normal population. The presence or absence of rolling did not significantly correlate with measures of circulating levels of leukocytes or lymphocytes (Table II), indicating that rolling did not simply reflect absolute levels of these subsets. Indeed, there appeared a trend toward lower lymphocyte and T cell counts among rolling samples, perhaps owing to more aggressive immunosuppressive therapy in these patients. Thus, rolling activity within peripheral blood has a striking correlation with active disease.

Table II. Peripheral Blood White Blood Cell Values for Pediatric Rheumatology Patients

	WBC*	% Neuts	% Lys	Abs Lys*	% Monos	% $CD3^+$
Roll < 2	7.4±3.6	58.8±14.0	30.9±11.7	2.2±1.2	7.2±2.8	61.5±16.2
Roll ≥ 2	6.7±2.2	65.7±18.8	24.7±13.5	1.6±0.9	5.9±2.5	56.2±21.4
P	0.051	0.010	0.002	< 0.001	0.003	0.312

*Cells × 10^3/μl; WBC, white blood cells.

Grouping of samples from SLE and arthritis patients by disease activity is shown in Fig. 3 *B*. A total of 41 samples were from clinically active patients, 16 from arthritis and 25 from SLE patients. 12 of the active arthritis (75%) and 23 of the active SLE samples (92%) showed rolling activity of ≥ 2, while only 1 of the inactive arthritis (2%) and 3 of the inactive SLE samples (6.9%) showed rolling ≥ 2. The differences between active and inactive samples were significantly different for both disease groups (Fig. 3 *B*; $P \leq 0.0001$ for arthritis and SLE groups, Mann-Whitney U test). While the sensitivity for the arthritis group appears somewhat less than that for SLE, it is of note that of the four arthritis samples from patients categorized as active, which rolled at background levels (0–1), three were described as "improving" or "resolving" from a recent prior active state, although some joint swelling was still present. Thus, while this assay may not be sufficiently sensitive to detect activity in some patients, an alternative explanation is

Table I. Characteristics of Pediatric Rheumatology Patients

Disease	Patients			Age range (yr)	Duration (yr)	Samples			Rx*
	Total	M	F			Total	Act	Inact	
Arthritis (52)									
Ankylosing spondylitis	2	2	0	16–18	5–8	3	2	1	Mtx, NSAID, Pred
Pauciarticular arthritis	21	4	17	1.5–17	2 mo–7	24	2	22	NSAID
Polyarticular arthritis	17	6	11	3–17	0.5–10	22	10	12	Mtx, NSAID, Pred
Psoriatic arthritis	3	1	2	12–17	2–5	4	0	4	Mtx, NSAID
Reactive arthritis	3	1	2	6–15	1–9	4	0	4	NSAID
Systemic arthritis	6	2	4	5–10	1.5–3.5	9	2	7	Mtx, NSAID, Pred
SLE (26)	26	5	21	6–18	0.5–8	68	25	43	Cy, Mtx, Pred
Total	78	20	58	1.5–18	2 mo–10	134	41	93	

*Cy, cyclophosphamide; Mtx, methotrexate; NSAID, nonsteroidal anti-inflammatory drug; Pred, prednisone.

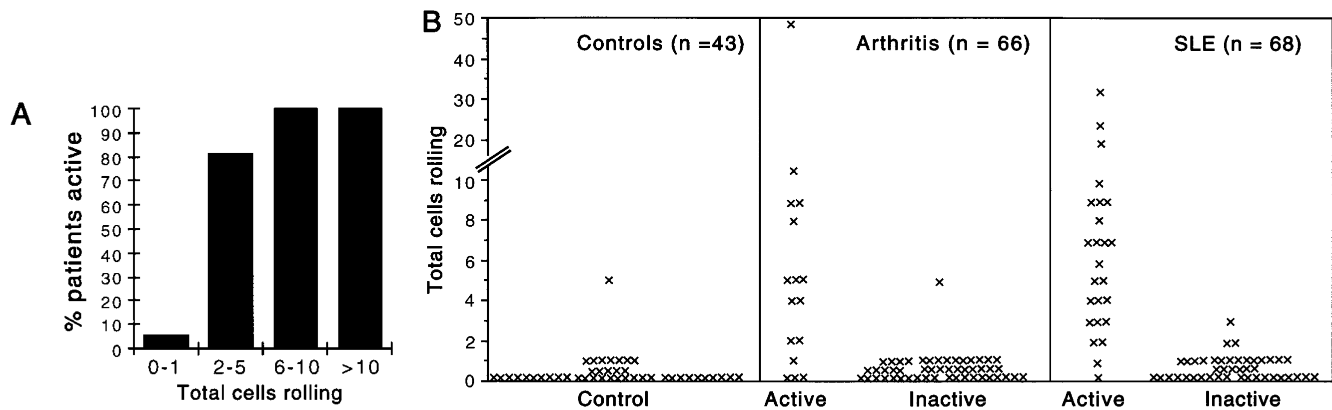

Figure 3. Peripheral blood rolling values in normal individuals and patients with autoimmune disease. PBMC were rolled directly in the parallel plate flow assay, as described. Patients were categorized as active or inactive at the time of clinic visit and sample collection. (*A*) Samples are grouped according to cumulative number of rolling cells per 4 min, and the fraction of patients with active disease in each group was calculated. (*B*) Scatter plots of cumulative rolling numbers from normal and patient bloods. The increased rolling in active patients maintained for both arthritis and SLE groups.

that the resolution phase of exacerbations is due to the characteristic persistent and prolonged inflammatory state within the synovial tissues. This state may no longer be reliant on the recruitment of cells from the periphery.

T cells are the predominant population mediating primary adhesion on HA in active SLE patients. Our previous studies demonstrated that T cells use a CD44-dependent mechanism to access an inflammatory site in a mouse model (11). However, the T cell superantigen used in this system favored the stimulation of a particular and large T cell subset. Since any of the mononuclear constituents in patient peripheral blood could be responsible for the CD44-mediated rolling, we examined which cells mediate this adhesion under autoimmune conditions. The very low frequency of the HA-binding population even in active patients precluded direct measurement of the relevant HA-binding population by flow cytometry. Therefore, sufficient blood was taken from a small number of patients with active disease for depletion of subsets followed by examination of adhesion under flow. PBMC were depleted of T cells, B cells, or monocytes using appropriate antibodies and magnetic bead separation. The results of four representative patient samples with active disease, three SLE and one systemic arthritis, are shown in Fig. 4. Two examples of T and B cell depletion and two of T and monocyte depletion are given. Depletion of T cells routinely removed 80–100% of rolling activity, while depletion of monocytes or B cells resulted in a slight decrement (Fig. 4), or occasionally enriched for rolling activity (data not shown). Thus, while a role for monocytes or B cells cannot be excluded, T cells appear to be the primary mediators of the rolling activity in all patients thus far examined.

General T cell activation markers do not bear a clear relationship to clinical disease activity or rolling. We have shown that activation of CD44 to bind HA is an early marker of T cell activation initiated by TCR signaling (Fig. 1) (10) and is coordinately regulated with other activation markers such as CD69. Furthermore, previous studies have reported a relationship between activation/memory markers and rheumatologic autoimmune diseases (30–33). It was therefore of interest to examine the relationship of other T cell activation markers with both disease and rolling activity in this study set. A total of 112 peripheral blood samples were stained with anti-CD3 and the activation markers anti-CD25, anti-CD69, or anti–HLA-DR. 26 normal control blood donors, 49 arthritis, and 37 SLE were stained. As shown in Fig. 5, the percentage of T cells bearing these activation markers reaches statistical significance between normals and patients for CD69 ($P = 0.015$) and CD25 ($P = 0.01$) but not HLA-DR ($P = 0.149$). However, there were no significant differences between active vs. inactive or rolling vs. nonrolling populations ($P > 0.6$ for pairwise comparisons of all markers). These results suggest that the relatively rare circulating cells bearing activated CD44 under conditions of disease exacerbation are unlikely to merely reflect elevated levels of larger activated subsets.

Rolling activity in individual patients followed longitudinally parallels clinical activity. While the correlation of rolling activity with clinically active disease was clear, it was of further

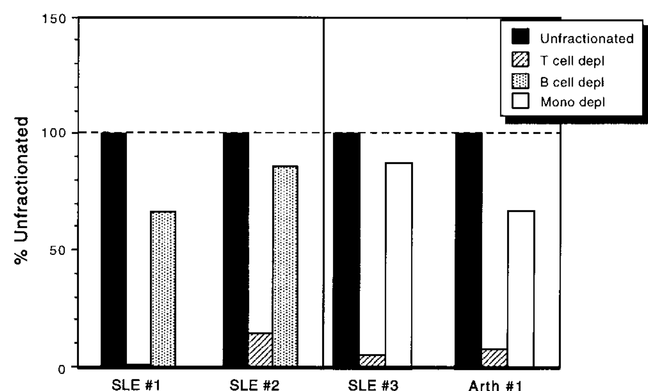

Figure 4. T cell dependence of rolling activity in PBMC from representative patients with SLE or arthritis. Cells were applied to HA-coated plates in the parallel plate flow assay without treatment (*solid bars*), or after antibody-conjugated magnetic bead depletion of T cells (*hatched bars*), B cells (*stippled bars*), or monocytes (*open bars*), as shown. Samples were indexed to the unfractionated value (100%) for each patient, and results are shown as the mean percentage of each unfractionated sample.

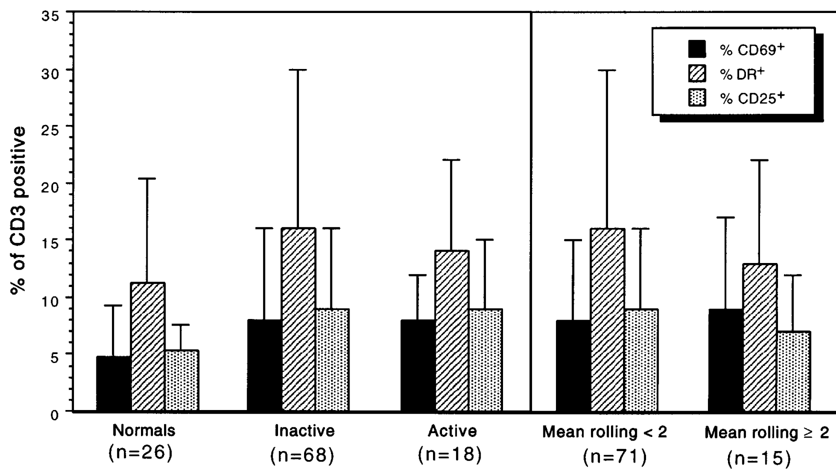

Figure 5. Peripheral T cell analysis in normal ($n = 26$) and patient ($n = 86$) samples. PBMC were stained with anti-CD3 plus either anti-CD69, anti-HLA-DR, or anti-CD25 and analyzed by two-color immunofluorescence. Results are shown as the percentage of $CD3^+$ cells staining with the indicated activation marker. No significant differences between samples from active vs. inactive patients or rolling vs. nonrolling samples were noted for any of the markers tested.

interest to examine the pattern of activity over time in individual patients. To follow the initial patient set longitudinally, samples were obtained at multiple visits over the course of the study. Individual patients were followed in clinic for up to 2 yr. Data was obtained for three or more clinic visits on 8 patients and from two clinic visits on an additional 22 patients. Three representative examples of rolling activity over time are shown along with the patient's clinical activity status and lymphocyte count at the time of each visit in Fig. 6. With each patient, there was a clear correlation between the presence of CD44-dependent primary adhesion and clinical activity. Conversely, no rolling above background was seen in blood from these patients on visits when patients' disease was under control. Patient A illustrates a patient 1 yr after initial presentation, diagnosed with SLE and membranous nephritis. The patient was in flare initially, had not responded to cytoxan, and was treated more aggressively, and this was followed by sustained remission. Patient B presented August 1997 with active SLE involving joints, central nervous system (CNS), and kidneys, responded initially to steroid pulses, was inactive by October 1997, then subsequently had a flare in February 1998. Patient C illustrates a more short-term analysis of rolling. After a long period of quiescence, this patient had a lupus flare and was hospitalized to rule out infection 9 January 1998 (Fig. 6 *C, solid horizontal bar*). The patient was given a steroid pulse that appeared to result in a rapid but short-lived drop in rolling activity. Within 1 mo of discharge, however, her rolling numbers had again risen, and she continued to be clinically active.

Discussion

The specificity and regulation of leukocyte extravasation results in large part from various types of adhesion receptors acting in sequential fashion, allowing directed leukocyte exit from the blood into diverse tissues within the organism (34, 35). The initial interaction (primary adhesion) of leukocytes with vascular endothelium generally has been attributed to the engagement of members of the selectin family with their carbohydrate ligands. Secondary (firm) adhesion is due to heterodimeric integrins interacting with their endothelial cell ligands, members of the immunoglobulin gene superfamily (36, 37). While this model was initially proposed based on observations of neutrophils, lymphocyte extravasation also has been shown to use selectin and integrin interactions (34, 36, 38). However, the compartmentalization and function of the immune system has resulted in the development of additional specialized trafficking and recirculation patterns for lymphocytes beyond that of other hematopoietic cells. For instance, $\alpha4\beta1$ and $\alpha4\beta7$ integrins on lymphocytes can mediate primary as well as secondary interactions (39), and primary adhesion of human T cells has been shown to use as yet uncharacterized molecules (40).

We have previously demonstrated that activated CD44 interacting with its major ligand HA is another participant in the extravasation of T cells. CD44 constitutes a widely distributed set of cell surface glycoproteins on hematolymphoid as well as various other cells, with the described roles for this molecule ranging from extracellular matrix binding and lymphocyte homing and activation to lymphopoiesis and metastasis (4). The prediction from our model that cells bearing "activated" CD44 would be present in peripheral blood under inflammatory conditions led us to examine a relevant patient population. These patients with rheumatologic diseases represented a spectrum of disease types and durations, clinical states, and therapeutic modalities. 29% of PBMC samples from this group of patients exhibited rolling activity, while only 2.3% of a normal control population did so. Moreover, rolling was striking among clinically active (85.4%) but not inactive (4.3%) patients, and this correlation was highly significant in both arthritis and SLE groups.

In both rheumatoid and juvenile arthritis, T cells exert a central influence in the maintenance and perpetuation of the highly complex and multicomponent inflammatory process within the inflamed synovial tissue, where there appears to be a clear enrichment of activated and memory T cells (41–44). Such cells presumably derive either from trafficking from the peripheral circulation and/or directly from proliferation within the synovial tissue (31, 45). The evidence presented here shows a close association between a very small subpopulation of activated T cells bearing activated CD44 in peripheral blood and active autoimmune disease. Thus, our observations favor the former interpretation that a subset of previously activated T cells enter the inflamed synovium. A further implication is that this may form an important element not only of the initial autoimmune response but also of subsequent inflammatory

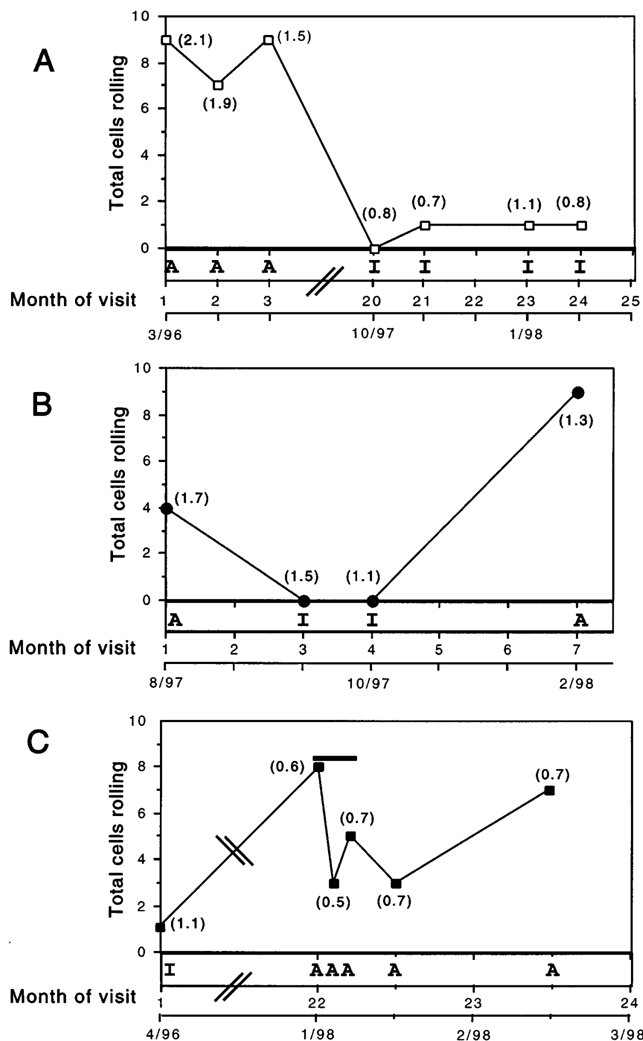

Figure 6. CD44-dependent rolling and disease activity in individual patients over time. Individual patients were followed in clinic for up to 2 yr. Clinical activity was assessed separately from rolling activity; results from three SLE patients from whom at least four separate samples were collected are shown. A, active disease; I, inactive disease. Also shown in parentheses are the absolute lymphocyte counts obtained at each clinic visit (cells $\times$ $10^3/\mu l$). The horizontal bar in (*C*) indicates a hospitalization during a flare. Therapies administered were: (*A*) Cytoxan pulses were initiated March 1996 and continued throughout the remainder of the visits; patient was also on daily oral Prednisone throughout; (*B*) weekly Solumedrol pulses at August 1997 and monthly Solumedrol pulses from the second throughout the remainder of the visits; (*C*) Cytoxan pulses and daily oral Prednisone in April 1996 and throughout, except patient had missed 2 mo of Cytoxan pulses before January 1998 visit. Upon hospital admission (*1/98*), patient received a Solumedrol pulse and a second pulse 1 mo later (*2/98*). Mean SLEDAI score at visits in which patients were active (*A*) = 25.4, inactive (*I*) = 6.5.

episodes. Thus, this activated subpopulation may have a significant influence on the evolution of an autoimmune exacerbation.

The correlation of rolling with exacerbations of SLE was also striking. 38% of 68 samples from 26 patients rolled, and 88% of these were from patients active at the time of visit. Patients with SLE have been reported to have increased numbers of circulating activated T cells (30, 33, 46), and clonal expansions of T cells found in blood have been identified in pleural and pericardial fluids of patients with active lupus serositis (47). Although many of the symptoms and much of the tissue damage in SLE is thought to be secondary to autoantibody production and immune complex deposition, production of anti-dsDNA antibody is a T-dependent process (48–53), and in the NZB/NZW murine model, anti-T cell mAb therapy has been reported to reverse disease (54). In addition, aberrant activation and signaling of T cells in SLE has been widely described (55), and blocking of T cell activation also prevents autoantibody production and disease (56, 57). Thus, flares of lupus may be coordinately regulated with and causally related to the peripheralization and entry into target sites of such activated T cells for either direct T cell mediated damage and/or T cell cytokine production and help to B cells.

Alterations in circulating T cell subsets have been described in a variety of autoimmune diseases, and in many instances, this has been associated with relative increases in activated T cells (30–33, 58–63). Elevated expression of HLA-DR has been found in PBL from rheumatoid and juvenile arthritis patients as compared to normal populations (31, 33, 64), and increased CD25 and HLA-DR has been reported in PBL from SLE patients (30, 46). Our data generally agree with the findings of slightly elevated levels of activated T cells compared with a normal population (Fig. 5). However, although T cell activation induces the HA binding form of CD44 on a subset of activated cells, the common T cell activation markers used here did not distinguish between active and inactive patients. Because the presence of the CD44-dependent rolling population we have described was detectable by laminar flow analysis but not by multiparameter flow cytometry, these cells clearly reflect a very small subpopulation within the subset of activated T cells. This is consistent with our observations in the mouse, where superantigen stimulation resulted in activation of CD44 on only 8% of the appropriate Vβ8-expressing T cells (11). The means of maintenance and regulation of this small population found in patients is not clear, but since the frequency of these cells is probably on the order of $<$ 1:1,000 (the limits of flow cytometry), a low frequency generally associated with antigen-specific stimulation, it is possible that these cells represent recent (auto)antigen-stimulated cells. Further characterization of this subset will require isolation and detailed examination.

While we have proposed that the presence of cells bearing activated CD44 is related to extravasation into inflamed sites during autoimmune exacerbations, it should be considered that subsidiary events unrelated to lymphocyte trafficking could result in the activation and/or mobilization of such cells; i.e., this could be a secondary rather than a primary event during exacerbations of such diseases. Since a general inflammatory state would be in effect in such patients, it is possible that such cells in the periphery could somehow derive from already inflamed or other sites. We should note that while rolling activity was generally associated with laboratory correlates of active disease such as elevated ESR, four samples from patients with clinically active disease demonstrated rolling in the absence of an elevated ESR. Cells did not roll in 27 samples from patients judged to be clinically inactive but who had an elevated ESR, indicating some independence from these acute phase reactants. Thus, while the mechanism for the association

remains to be fully clarified, rolling activity appears to be a highly correlated marker for clinically active autoimmune disease.

A number of adhesion receptor pairs have been implicated in the recruitment of T cells and other inflammatory cells into inflamed sites, and as a result, potential therapies based on these mechanisms are being explored. For example, the interacting ligands CD18 and ICAM-1 have both been shown to be important in the onset and perpetuation of chronic arthritis in animal models (65, 66). A clinical trial targeting leukocyte trafficking into the synovium in patients with severe rheumatoid arthritis has been performed using an mAb to endothelially expressed ICAM-1 (67). The mechanism for firm adhesion following contact with the vessel wall and subsequent primary adhesion mediated by CD44 has not been determined but may also be regulated by integrin molecules and their endothelial ligands. Thus, a more selective therapy targeting the CD44/HA interaction of circulating activated T cells could represent a viable approach for intervention.

In summary, we have described a rolling interaction of human lymphoid cells that has as its basis the interaction of activated CD44 with its principal ligand, the glycosaminoglycan hyaluronan. The activation of CD44, which enables rolling subsequent to T cell stimulation, the evidence that cells with this interactive potential are found in the peripheral circulation during the course of chronic inflammatory diseases, and the correlation of their appearance with disease exacerbations all strongly support the notion that CD44 selectively participates in the enhanced homing of activated lymphocytes to target sites of autoimmunity. Further studies to examine the generality of this principle will be needed to determine whether similar cell populations exist in other immunologically mediated diseases, whether this measure serves as a prognostic indicator, and whether strategies targeting this interaction may be of therapeutic value.

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