CD44 and Hyaluronan-dependent Rolling Interactions of Lymphocytes on Tonsillar Stroma

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Abstract. Little is known about how lymphocytes migrate within secondary lymphoid organs. Stromal cells and their associated reticular fibers form a network of fibers that radiate from high endothelial venules to all areas of the lymph node and may provide a scaffold for lymphocyte migration. We studied interactions of lymphocytes with cultured human tonsillar stromal cells and their extracellular matrix using shear stress to distinguish transient interactions from firm adhesion. Tonsillar lymphocytes and SKW3 T lymphoma cells tethered and rolled on monolayers of cultured tonsillar stromal cells and their matrix. A significant proportion of these rolling interactions were independent of divalent cations and were mediated by CD44 binding to hyaluronan, as shown by inhibition with mAb to CD44, soluble hyaluronan, hyaluronidase treatment of the substrate, and O-glycoprotease treatment of the rolling cells. O-glycoprotease treatment of the substrate also blocked binding completely to stromal matrix and partially to stromal monolayers. SKW3 cells tethered and rolled on plastic-immobilized hyaluronan, confirming the specificity of this interaction. By contrast, monolayers of resting or stimulated human umbilical vein endothelial cells failed to support CD44- and hyaluronan-dependent rolling. SKW3 cells added under flow conditions to frozen sections of human tonsil bound and rolled along reticular fibers in the presence of EDTA. Rolling was blocked by either CD44 mAb or hyaluronan. We propose that lymphocytes migrating through secondary lymphoid organs may use CD44 to bind to hyaluronan immobilized on stromal cells and reticular fibers.
Materials and Methods

Culture of Stromal Cells from Human Tonsil

Tonsils obtained from pediatric patients undergoing tonsillectomy (Massachusetts General Hospital, Boston, MA) were processed as described (26), except that the cells obtained from the second collagenase treatment were cultured without passage through a steel screen. Briefly, tonsils were minced on a steel mesh screen (200 mesh = 74 μm, Type 316; Tylinter, Mentor, OH) and washed with L15 medium (Sigma Chemical, St. Louis, MO) to remove released lymphocytes. Material retained on the screen was treated with 0.5% collagenase (collagenase L 187 U/mg; Worthington Biochemical Corp., Freehold, NJ) and 1 U/ml DNAse (Promega, Madison, WI) for 30 min at 37°C. Material was then minced again and treated with 0.5% collagenase and DNAse for 60 min at 37°C. The cell suspension was washed three times in RPMI 1640 medium (JRH Biosciences, Lenexa, KS), 10% FBS (JRH Biosciences), and 5 × 10^5 cells were seeded in 10 ml of medium per 150-mm Falcon tissue-culture dishes (Becton Dickinson Labware, Lincoln Park, NJ). Nonadherent cells were removed on days 2 and 4 by rinsing with fresh RPMI 1640, 10% FBS. Dendritic cells, macrophages, endothelial cells, and adherent lymphocytes, identified by morphology and expression of specific surface markers, were present in newly established primary cultures at day 2 but were not present after 5 d. Cells were routinely subcultured up to three times at confluence by removal with trypsin/EDTA and 1:10 dilution into fresh medium. Cells were grown to confluence in six-well plates for flow studies. Stromal cells in early passages grew quickly and formed confluent monolayers, while cells passed four times or more failed to form monolayers and instead grew in clumps. For matrix experiments, confluent monolayers were removed with 5 mM EDTA in HBSS at 37°C for 10 min, and wells were rinsed with HBSS.

Isolation of Lymphocytes from Human Tonsil

Lymphocytes were released from tonsil by mincing the tissue on a steel mesh screen and rinsing with L15 medium. Cells that passed through the screen were washed three times with RPMI 1640 and 10% FBS, once with HBSS and 5 mM EDTA, and resuspended in HBSS, 1 mM MgCl₂, and 2 mM CaCl₂ for flow experiments. Separation of tonsillar lymphocytes into B and T cell populations by immunomagnetic-negative selection was done as previously described (58) with the substitution of the mAb CD3 (Coulter Corp., Hialeah, FL) for the CD20 mAb in purification of B cells.

Antibodies

2F3 mAb (53) was a kind gift of R. Kannagi (Aichi Cancer Center, Nagoya, Japan); MECA-79 mAb (71) was a kind gift of E. Butcher (Stanford University School of Medicine, Stanford, CA); and PUJ4 (α4 integrin mAb) and PUJ5 (α5 integrin mAb) ascites were both kindly given by M. Hemler (Dana Farber Cancer Institute, Boston, MA). Mab 13 (β1 integrin mAb) was kindly provided by K. Yamada (NIH, Bethesda, MD), and GoH3 (α6 integrin mAb) was graciously donated by A. Sonnenberg (University of Amsterdam, The Netherlands). CBRP150/2E1 (65), recognizing P150,95, was used as a control mAb in flow antibody-blocking assays. M138 mAb was produced by immunizing mice with human tonsillar stromal preparations and screening the resulting mAb by immunohistochemistry on sections of human tonsil. The protocol for fusion and hybridoma maintenance has been described in detail (64). M138 recognizes type IV collagen, as evidenced by its immunohistochemical reactivity pattern and recognition of immobilized type IV collagen in dot blots (data not shown). All other mAbs were obtained from the Fifth International Workshop on Human White Cell Differentiation Antigens. mAbs to integrin subunits from the Fifth Leukocyte Workshop were IB3.1 (α1), G9 (α2), T145 (α3). mAb were used for flow cytometry at 5 μg/ml or at 1:100 dilution for workshop mAb. For flow assay mAb-blocking studies, input cells were incubated 40 min at 4°C with a 1:20 dilution of mAb from the Fifth International Workshop on Leukocyte Differentiation Antigens, a 1:1000 dilution of PUJ4 or PUJ5 ascites, 25 μg/ml of Mab 13, 50 μg/ml of CBRP150/2E1, or neat supernatant of GoH3.

Flow Experiments

Cell tethering and rolling was measured in a parallel-plate flow chamber that was placed into one well of a six-well plate (35-mm wells) such that cell monolayers or matrix formed the floor of the chamber (35, 42). All experiments were in HBSS with or without divalent cations as described. Hyaluronic acid (1 mg/ml or 0.5 mg/ml, 25 μl vol; Sigma Chemical Co.) was adsorbed on 100 × 15-mm plastic petri dishes (Nunc Inc., Naperville, IL) overnight at 4°C and blocked with 2% human serum albumin (Calbiochem-Novabiochem Corp., San Diego, CA) for 3 h at 37°C. Video microscopy, analysis of videotape, measurements of cell accumulation in shear flow, and measurement of detachment of cells and rolling velocities were as described (42). Unless otherwise stated, rolling experiments used the following shear stresses: cells were accumulated at 2.7, 5.3, 8.0, and 11.1 dynes/cm² for 40 s each, followed by rinsing with cell-free medium at 1.6, 2.1, 2.7, 3.7, 4.8, 5.8, 7.9, 10.1, 12.2, 17.5, 22.8, and 28.1 dynes/cm² for 10 s each. Adherent cells were counted after the .80 dynes/cm² accumulation step. For a cell to be counted as rolling, it had to roll a distance of at least five times the cell diameter. This was determined by marking each cell's location at the time of initial attachment, placing a second mark at the site of detachment or at the final cell position at the end of the 28.1 dynes/cm² step, and then measuring the distance traveled.

Flow Cytometry

Flow cytometry was done as previously described (17).

Enzyme Treatments

Cells (2 × 10⁶ cells in 200 μl) or substrates (in six-well plates overlaid with 500 μl of medium) were treated with 0.1 U/ml of neuraminidase (from Vibrio cholerae; Sigma Chemical Co.), 60 μg/ml (0.3 U/ml) O-glycosidease (Accurate Chemical and Scientific Corp., Westbury, NY), 0.5 U/ml of
Epithelial, and Dermal Fibroblast Assays

Human Umbilical Vein Endothelial Cell (HUVEC), before flow experiments and were present throughout the experiments. HUVEC were grown to confluence on six-well plates and stimulated with 200 U/ml from human umbilical cord (Sigma Chemical Co.) at 200 μg/ml, or 5 mM Chondroitin sulfate B from bovine mucosa (Sigma Chemical Co.), chon-
exo-13-galactosidase (grade VIII from Sigma Chemical Co.) diluted in RPMI 1640 and 0.1% BSA for 40 min at 37°C.

Soluble Inhibitors

Chondroitin sulfate B from bovine mucosa (Sigma Chemical Co.), chon-
droitin sulfate C from shark cartilage (Sigma Chemical Co.) or hyaluronan from human umbilical cord (Sigma Chemical Co.) at 200 μg/ml, or 5 mM EDTA were added to the cell suspension and the flow chamber for 10 min before flow experiments and were present throughout the experiments.

Human Umbilical Vein Endothelial Cell (HUVEC), Epithelial, and Dermal Fibroblast Assays

HUVEC were isolated and cultured as previously described (25, 33). Cells were grown to confluence on six-well plates and stimulated with 200 U/ml tumor necrosis factor α (TNF-α) (Genzyme, Boston, MA) for 24 h. Epider-
cell line A-431 (CRL 1555) and dermal fibroblasts (CCD-32Sk) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in six-well plates in RPMI 1640 and 10% FBS.

Rolling on Tonsil Sections

Freshly cut 5-μm sections of human tonsil were placed on 70 × 50-mm glass slides (Corning Glass Works, Corning, NY) precoated with 0.1 mg/ml poly-l-lysine (Sigma Chemical Co.), allowed to dry 1-3 h at room temper-

ture, and then stored at 4°C for up to 2 h until used. The flow chamber was attached directly to the glass slides. A serial section was stained with collagen type IV mAb M138. Video images were captured using NIH Image software version 1.59 written by Wayne Rasband, and unbound cells were darkened by comparing two consecutive frames and using the mini-
imum value for each pixel.

Results

Tonsillar Lymphocytes Roll on Monolayers of Cultured Tonsil Stromal Cells

Cultured tonsillar stromal cells of passage number 1-3 were grown to confluence on six-well plates and used as a rolling substrate in flow studies. Freshly isolated tonsill lymphocytes introduced under flow adhered to the mono-

layer at a wall shear stress of 0.8 dynes/cm², began to roll at a wall shear stress of 1.06 dynes/cm², and continued to roll as shear was increased. The ability of cells to form rolling adhesions was only partially inhibited by EDTA (Fig. 1). In the continued presence of EDTA, stromal cells began detaching, and tonsill lymphocytes were seen to roll on the underlying extracellular matrix. Stromal ECM, produced by removing confluent stromal cells with EDTA, was therefore used as an additional rolling substrate in subsequent experiments (see below). Separation of tonsillar lymphocytes into T and B cell populations by negative selection demonstrated that both T and B cells bound to stromal monolayers under flow (data not shown). There was significant variability between tonsill lymphocyte preparations in the number of lymphocytes adhering per field and in the amount of inhibition by EDTA, perhaps due to variation between the freshness of tissue and the degree of activation of lymphocytes in different tonsillar specimens. Lymphocytes harvested from tonsil contain activated cells that express the activation antigens CD26, CD30, CD39, CD69, CD70, and CD71 (8, 9, 20, 59, 60, 68, 69). Because T lymphoblastoid cell lines are useful models for activated lymphocytes, cell lines were also evaluated for their ability to form rolling attachments to stromal cell monolayers (Fig. 1). Jurkat cells and HL-60 cells failed to bind, despite the presence by flow cytometry of L-selectin on Jurkat cells and the ability of HL-60 cells to bind to immobilized L-selectin (data not shown). SKW3 cells, in contrast, bound to the monolayer at 0.8 dynes/cm² and rolled as shear was increased. EDTA inhibited SKW3 cell binding by 32% (Fig. 1). When SKW3 cells were pretreated with 0.1% azide for 10 min, all cells rolled in a sustained manner. Without azide treatment, most cells rolled only a short distance before firmly attaching. Azide treatment was included in subsequent experiments to facilitate examination of the rolling component of cell adhesion.

Tonsillar Stromal Cells Lack Known Selectins and Selectin Ligands

Cultured tonsillar stromal cells were examined by flow cy-
tometry for their expression of surface molecules (Table I). Stromal cells expressed no E-, L- or P-selectin, nor did they express carbohydrate antigens associated with selectin ligands such as sialyl Leα, sialyl Leβ, CLA, and PNA. The FH6 mAb did stain these cells, suggesting that stromal cells may bear a difucosylated form of sialyl Leα. However, stromal cells detached from their substrate by EDTA failed to form rolling adhesions on E-selectin and L-selectin substrates (data not shown), suggesting that this structure is not a functional selectin ligand. Stromal cells expressed high levels of Thy-1 and CD44, as well as moderate to low levels of VLA-4, Limaxflavus agglutinin (LFA)-3, VCAM-1, intracellular adhesion molecule (ICAM)-1, and CD21. Stromal cultures were negative for B cell, T cell, and mono-
cyte/macrophage markers showing lack of contamination with these cell types.

Enzymatic Sensitivity of Rolling Interactions

To study the nature of the rolling interactions, SKW3 and stromal cell monolayers and matrix were treated with a panel of glycosidases and proteases before their use in flow...
by >99% to matrix and by 53% to stromal monolayers. These results suggest that there are two classes of structures with which hyaluronan is associated on stromal cells, one resistant to O-glycoprotease and one whose susceptibility to this enzyme indicates that it is a glycoprotein containing O-linked sugar moieties. Stromal cell matrix contains only the latter class of structures.

Treatment of SKW3 cells with O-glycoprotease inhibited the interaction with stromal cells by 98% and extracellular matrix by 96%, while the other enzymes had little effect. This suggests that the receptor on SKW3 cells is a glycoprotein that bears O-linked carbohydrates.

Rolling Is Blocked by Anti-CD44 mAb and Soluble Hyaluronan

CD44 binds hyaluronan (4, 15, 44, 49, 51) and is susceptible to cleavage by O-glycoprotease (72). To determine if CD44 mediates rolling on stromal cells, anti-CD44 mAbs from the Fifth International Leukocyte Workshop were tested for their ability to block cell attachment and rolling (Fig. 3). Studies were done in the presence of EDTA. BRIC235, 3F12, and A3D8 mAbs inhibited attachment to stromal cell monolayers by 52, 38, and 53%, respectively. A combination of all three mAbs reduced binding by 1% of control levels. On stromal cell matrix, BRIC235 reduced binding by 99%, while 3F12 and A3D8 inhibited binding by 61 and 94%, respectively. These results suggest SKW3 cells use CD44 to roll on stromal cells and matrix.

To clarify the identity of the hyaluronidase-sensitive ligand on stromal cells, soluble chondroitin sulfates B and C and hyaluronan were tested for their ability to block rolling (Fig. 3). Binding to stromal monolayers was unaffected by chondroitin sulfates B and C; binding to matrix was not reduced by chondroitin sulfates B and reduced by only 24% by chondroitin sulfate C. Soluble hyaluronan reduced binding under flow to stromal monolayers and stromal matrix by 96 and 94%, respectively. These results confirm that the divalent calcium-independent rolling interactions are mediated by CD44 on the rolling cells and hyaluronan on the substrate.

Of the three leukocyte cell lines used in this study, imm-

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### Table I: Characterization of Cultured Tonsillar Stromal Cells by Flow Cytometry

| Antigen       | CD assignment | mAb        | Mean fluorescence |
|---------------|---------------|------------|-------------------|
| LFA-1         | CD11a         | TS1/22     | 2.4               |
| Mac-1         | CD11b         | CBRM1/29.1 | -0.8              |
| p150,95       | CD11c         | CBRp150/2E1| -0.4              |
| ICAM-1        | CD54          | CBR-IC1/11.2| 20.0              |
| ICAM-2        | CD102         | CBR-IC2/2  | 1.1               |
| ICAM-3        | CD30          | CBR-IC3/1  | -1.2              |
| VCAM-1        | CD106         | E1/6       | 31.0              |
| VLA-4         | CD49d         | 6G1        | 110.0             |
| LFA-3         | CD38          | TS2/9      | 31.0              |
| PECAM-1       | CD31          | SG 134     | 0.4               |
| Thy-1         | CDw90         | SE10       | 540.0             |
| CD44          | CD44          | Hermes-3   | 920.0             |
| CR1           | CD15          | 13D3       | 2.0               |
| CR2           | CD21          | HB5        | 60.0              |
| CD2           | CD2           | TS2/18     | 1.0               |
| CD45          | CD45          | S62.1OD3   | 9.0               |
| HLA Class II  |              |            |                   |
| E-selectin    | CD62E         | H18/7      | -1.2              |
| L-selectin    | CD62L         | LAM1-3     | -0.8              |
| P-selectin    | CD62P         | SZ-51      | -1.4              |
| CD34          | CD34          | QBEnd-10   | -0.8              |
| Sialyl LeX    | CD15s         | CSLEX-1    | 0.0               |
| Sialyl LeX    | CD15s         | 2F3        | 0.6               |
| Di-fucosyl SLeX| CD15         | FH6        | 120.0             |
| Sialyl LeX    | CD15          | CSLEX-1    | 1.0               |
| LeX           | CD15          | PM-3E      | -2.3              |
| PNAd          | CD15          | MEMA-79    | 2.5               |
| CLA           |               | HEECA-452  | 0.0               |

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### Figure 2. Inhibition of accumulation of rolling SKW3 cells by enzyme pretreatment. (a) Stromal cell monolayers. Control binding of untreated cells was 86 cells per ×100 field. (b) Stromal cell matrix. Control binding of untreated cells was 197 cells per ×100 field. The substrate or 0.1% azide-treated SKW3 cells were treated as indicated with control medium (Control), neuraminidase (Neur.), neuraminidase and exo-β-galactosidase (Neur+B-gal), O-glycoprotease (OG), or hyaluronidase (Hydase). Cells were accumulated at 0.27, 0.53, 0.80, and 1.1 dynes/cm² for 40 s each, followed by rinsing with cell-free medium at 1.6, 2.1, 2.7, 3.7, 4.8, 5.8, 7.9, 10.1, 12.2, 17.5, 22.8, and 28.1 dynes/cm² for 10 s each. Adherent cells were counted after the 0.8 dynes/cm² accumulation step; all adherent cells rolled at higher shear stresses. The mean values of two identical experiments are shown with the ranges indicated by brackets.
munofluorescence flow cytometry revealed that SKW3 T lymphoblastoid and HL-60 myelomonocytic cells expressed high levels of CD44, while Jurkat T lymphoblastoid cells did not express CD44 (data not shown). HL-60 cells did not bind to stromal cells or matrix even though they express CD44; similar results have been observed with many cells including murine splenic lymphocytes and bone marrow myeloid cells that express high levels of CD44 but do not bind purified hyaluronan (44). Human peripheral blood lymphocytes also failed to bind stromal cells under flow although they express CD44 (data not shown), in keeping with the finding that resting human peripheral blood lymphocytes do not bind hyaluronan (23, 46). Human T cells can be induced to bind hyaluronan by stimulation with anti-CD3 for 24 h (23), and murine B cells can be induced to bind hyaluronan by culture in IL-5 or by a chronic graft-vs-host disease in vivo (31, 51, 52). The ability of stimulated human B cells to bind hyaluronan has not been tested directly, but mitogen stimulation of human B cells induces expression of CD44 V6 isoforms that are associated in T cells with increased hyaluronan binding (24, 39). We therefore postulate that the activated cells present in tonsillar lymphocyte preparations possess CD44 which is capable of binding hyaluronan and it is this difference that accounts for the observation that tonsillar lymphocytes adhere under flow, while peripheral blood lymphocytes do not.

**Tonsillar Lymphocytes Also Use CD44 to Form Rolling Adhesions on Stromal Matrix**

We tested whether CD44--hyaluronan interactions also mediate the rolling of tonsillar lymphocytes on stromal matrix (Fig. 4). Treatment of lymphocytes with O-glycosidase reduced adhesion to stromal matrix by 90%. Soluble hyaluronan and the CD44 mAb BRIC235 completely blocked binding to substrate in the presence of EDTA. Hyaluronidase treatment of the substrate likewise eliminated binding in the presence of EDTA. Unlike SKW3 cells, lymphocytes adhered to and rolled on the hyaluronidase-treated substrate in the presence of divalent cations. Thus, additional, cation-dependent mechanisms may exist by which lymphocytes interact with these substrates; these mechanisms are unlikely to involve selectins for the reasons cited above. Taken together, these studies show that tonsillar lymphocytes also use CD44 and hyaluronan to roll on stromal matrix, but additional cation-dependent mechanisms may also play a role.

**Rolling on Plastic-immobilized Hyaluronan via CD44**

Hyaluronan was immobilized on plastic and used as a substrate in flow assays to test whether it can support rolling. SKW3 cells bound to immobilized hyaluronan under flow conditions (Fig. 5 a). Ability of cells to tether decreased...
with increasing shear. CD44 mAb blocked tethering and rolling by 95%, and soluble hyaluronan blocked it by 80%. Tethering and rolling were blocked by anti-CD44 mAb BRIC235 and soluble hyaluronan (Fig. 5 b), confirming the specificity of these interactions. Rolling interactions formed by SKW3 to immobilized hyaluronan were surprisingly shear resistant (Fig. 5 c). A minority of cells flattened out yet kept rolling; these cells had the highest shear resistance. Detachment proceeded in a roughly linear manner, but complete detachment was not seen until shear stress reached an impressive 273 dynes/cm². SKW3 cells rolling on hyaluronan did not arrest and form firm attachments like cells rolling on stromal matrix or stromal monolayers; azide treatment of the cells was thus unnecessary in these experiments and was not done unless noted.

The rolling velocities of SKW3 cells on stromal monolayers, matrix, and purified hyaluronan were on the order of 1 μm/s (Fig. 6). In the case of stromal matrix, mean rolling velocity decreased between 7 and 17 dynes/cm² shear stress, as the majority of cells sped up and detached, leaving a particularly adherent population behind that continued to roll at slow velocities.

Blocking mAb Recognize Hyaluronan-binding Region of CD44

To define the region of CD44 responsible for mediating rolling, a panel of mAbs from the Fifth International Leukocyte Workshop was tested for their ability to block rolling on plastic-immobilized hyaluronan in the presence of EDTA (Table II). mAbs to CD44 have been clustered to three distinct epitope groups by competitive inhibition (18, 19).

Figure 5. Accumulation of rolling SKW3 cells on purified hyaluronan. (a) SKW3 cell tethering to plastic-immobilized hyaluronan at various shear stresses. Cells were accumulated for 30 s. (b) Inhibition of rolling on hyaluronan plated at 1.0 mg/ml by soluble inhibitors chondroitin sulfate B (CS B), chondroitin sulfate C (CS C), hyaluronan (HA), and CD44 mAb BRIC235. (c) Detachment of cells rolling on hyaluronan plated at 1.0 mg/ml under increasing shear stress. Cells were initially accumulated at .53 dynes/cm² shear stress for 30 s. Unless noted, cells were not treated with azide; all adherent cells rolled as shear stress was increased. One of two similar experiments are shown in a and c, while mean values of two experiments are shown in b with the ranges indicated by brackets.

Figure 6. Rolling velocities of SKW3 cells on different substrates. (a) Stromal cell monolayers. (b) Stromal cell matrix. (c) Hyaluronan adsorbed to plastic at 1.0 mg/ml. SKW3 cells used in monolayer and matrix experiments were treated with azide to prevent firm adhesion through CD44-independent mechanisms. Cells were initially accumulated at 0.8 dynes/cm² for monolayer and matrix experiments and at 0.5 dynes/cm² for hyaluronan experiments, and subsequently subjected to increasing wall shear stresses at 10 s per interval. Values were obtained from averaging the rolling velocities of all cells on the field; the SEM is indicated by brackets.
Table II. Inhibition of Accumulation of Rolling SKW3 Cells on Plastic-immobilized Hyaluronan by CD44 mAb*

| mAb     | Control accumulation | CD44 epitope |
|---------|----------------------|--------------|
| BRIC235 | 0                    | 2a           |
| CD44-6B6| 41                   | 2            |
| BU75    | 45                   | 2a           |
| CD44-9F5| 70                   | 2            |
| 50B4    | 77                   | 2a           |
| 3F12    | 98                   | 2a           |
| HP2/9   | 100                  | 2a           |
| Hermes-3| 110                  | 3            |
| 156-3C11| 113                  | 3            |
| KZ1     | 114                  | 3            |
| 212.3   | 114                  | 1            |
| 4C3     | 116                  | 1            |
| BRIC222 | 137                  | 1            |
| A3D8    | 154                  | 1            |
| F10-44-2| 330                  | 1            |

* Experiment was as described in Fig. 5 legend.
1 Epitope groups defined by references 18 and 62.
2 The mAb shows characteristics that overlap more than one epitope group.

62). Some members of epitope group 1 inhibit T cell rosetting (3, 30, 61), while some mAbs of epitope group 3 block lymphocyte binding to high endothelial venules (34, 54). Epitope 2 contains mAbs that interfere with hyaluronan binding (5); mAb that inhibit hyaluronan binding in solution were assigned to epitope group 2a, which is a subset of epitope group 2, although this subset partially overlaps other groups. Five out of seven mAbs in epitope group 2 and 2a inhibited rolling on purified hyaluronan, while mAbs in group 3 had little effect, and three out of five mAbs in group 1 increased binding. One mAb, F10-44-2, greatly increased binding; this mAb also increased binding of CD44-transfected, activated Jurkat cells to soluble hyaluronan (47). It was surprising that the mAb A3D8, which maps to epitope group 1, inhibited attachment to stromal cell monolayers and matrix but not to purified hyaluronan (Fig. 3 and Table II). There may be a difference in the way purified hyaluronan bound to plastic and hyaluronan bound to proteoglycans in stromal matrix are presented to CD44, such that an antibody that binds outside of the hyaluronan-binding region may have an effect on the binding of cells to one substrate but not to the other.

**Dermal Fibroblasts But Not A-431 Cells or HUVEC Support Hyaluronan-dependent Rolling of SKW3 Cells**

To determine if other cell types produce hyaluronan-like ligands that are capable of supporting rolling, we expanded our flow chamber studies to include dermal fibroblasts, the epithelial cell line A-431, and HUVEC.

SKW3 cells formed hyaluronan-inhibitable rolling interactions with monolayers of dermal fibroblasts and their matrix (Fig. 7 a). However, binding to dermal fibroblasts was twofold lower than binding to stromal cell monolayers. A-431 monolayers supported SKW3 cell attachment at a level fourfold lower than stromal cell monolayers (Fig. 7 a). These interactions were not inhibited by EDTA, chondroitin sulfate B, or hyaluronan, and thus seem unlikely to involve CD44. SKW3 cells did not bind to A-431 matrix.

HUVEC monolayers were tested either under resting conditions or after stimulation with TNF-α for 24 h. While we did observe rolling of SKW3 cells on TNF-α-stimulated HUVEC monolayers at a level twofold higher than on stromal cell monolayers, these interactions were completely blocked by EDTA and unaffected by soluble hyaluronan (Fig. 7 b). Furthermore, no interactions of SKW3 cells were seen with the matrix of either stimulated or unstimulated HUVEC, nor with unstimulated monolayers.

**Divalent Cation-sensitive Aspects of SKW3 Cell Interactions with Stromal Cells**

Although the CD44–hyaluronan ligand pair are clearly capable of mediating cell rolling, the number of rolling adhesions formed by SKW3 and tonsillar lymphocytes decreased in the presence of EDTA. When SKW3 cells bound to stromal matrix in the presence of divalent cations at 0.3–0.5 dynes/cm², rolling was not evident. When shear stress was increased to 0.8 dynes/cm², ~15% of adherent cells began to roll. When shear stress reached 1.1 dynes/cm², the majority of cells rolled (Fig. 8). In the absence of divalent cations, fewer cells adhered to the substrate at 0.3–0.8 dynes/cm², and those that did adhere began rolling immediately. When EGTA and Mg²⁺ were used in the assay instead of EDTA, cells that adhered also began rolling immediately, suggesting that the initial firm adhesion seen in the presence of divalent cations was Ca²⁺ dependent. To examine these effects, we conducted antibody-blocking studies in the presence of divalent cations.

BRIC23 5 CD44 mAb inhibited binding of SKW3 cells to stromal matrix by 95% (Fig. 9). However, in the absence of CD44 mAb, mAb to the β₁ integrin subunit inhibited binding of SKW3 cells to stromal matrix by 50%; EDTA inhibited binding by a similar amount. mAb to the β₁ integrin subunit, like EDTA, also abolished stationary, nonrolling adhesion of cells to stromal cell matrix under low shear (Fig. 8). β₁ blockade has an effect equivalent to EDTA in blocking binding to matrix (Figs. 8 and 9), suggesting that β₁ integrins mediate the majority of cation-dependent interactions with this substrate. Comparison of blocking mAb to integrin α subunits showed inhibition of adhesion to stromal matrix only by mAb to the α6 subunit (Fig. 9 b). The α6β₁ integrin did not appear to play a role in initial cell tethering to substrates because binding under flow was negligible after hyaluronidase treatment of both stromal monolayers and matrix (Fig. 2), and, as mentioned above, mAb to CD44 almost completely blocked binding. However, the α6β₁ integrin clearly participated in strengthening adhesion to the substrate once contact was established. mAb to the β₁ integrin subunit had less effect on binding to stromal cell monolayers, inhibiting by only 25%. In addition, 20% of tethering and rolling of SKW3 cells on stromal cell monolayers was not blocked by a combination of mAb to CD44 and the β₁ integrin subunit, suggesting that other divalent, cation-dependent adhesion interactions contributed to binding to monolayers.

**Tonsillar Lymphocytes and Lymphoblastoid Cells Roll via CD44 on Frozen Sections of Human Tonsil**

To study the interactions of tonsillar lymphocytes and SKW3 cells with tonsillar stromal cells and their matrix products in situ in the tonsil, freshly cut frozen sections of
Figure 7. Formation of rolling interactions of SKW3 cells with monolayers and matrix of various cell types. (a) Tonsillar stromal cells, dermal fibroblasts, and A-431 cells. (b) HUVEC with and without stimulation with 200 U/ml TNF-α for 24 h. Interactions of azide-treated SKW3 cells with monolayers or the ECM after monolayer removal were inhibited with chondroitin sulfate B (CS B), chondroitin sulfate C (CS C), and hyaluronan (HA). Adherent cells were counted after the 0.8 dynes/cm² accumulation step; all adherent cells rolled as shear was increased. Results shown are from one of two similar experiments.

Human tonsil were used as the substrate for rolling experiments. The locations of T cell and B cell areas, HEV, and reticular fibers in the T cell areas could be identified by light microscopy and were verified by fluorescent staining of serial sections. In the presence of divalent cations, tonsillar lymphocytes and SKW3 cells bound under flow to both HEV, as evidenced by ringlike accumulation of cells in the T cell areas, and also to other structures in the T cell areas and near crypts. Under increasing shear, cells bound to putative HEV detached, while those bound to structures in the T cell areas were able to roll a considerable distance along the section. When EDTA was included in the experiments, binding to HEV-like structures was greatly attenuated, while binding to other structures in the T cell areas and near crypts was only partially inhibited. Tonsill lymphocytes and SKW3 cells behaved similarly, but SKW3 cells were more easily visually discriminated from the underlying frozen section because of their larger size. In regions of the tonsil where reticular fibers were visually discernible, cells were observed to roll along single fibers oriented parallel to the direction of flow. When a fiber diverged from the direction of flow, cells were seen to pause at that location, and then either detach under increasing shear or transfer onto a neighboring fiber (Fig. 10 a). Inclusion of EDTA in the assay decreased the number of SKW3 cells stably rolling across the section from 234 to 220 cells and eliminated the transient adhesions of cells seen in the presence of divalent cations (Fig. 10 b). How-

Figure 8. Binding of SKW3 cells to stromal cell matrix. Adhesion of azide-treated SKW3 cells to matrix was done in the presence of 2 mM Ca²⁺ and 1 mM Mg²⁺ and no inhibitors (a) or the β1 integrin subunit mAb Mab 13 (b) or 10 mM EDTA (c). A cell was designated as “stationary” if its rolling velocity was <0.25 μm/s. Results are from one of three similar experiments.
Discussion

Our studies demonstrate that stromal cells cultured from human tonsil produce hyaluronan-containing ligands that support CD44-dependent lymphocyte tethering and rolling along the fibers in the presence of EDTA to six and five cells, respectively (Fig. 10, c and d). A serial section stained with collagen type IV mAb confirmed that the field contained an extensive network of reticular fibers (Fig. 10, e and f). Tonsillar lymphocytes attached and rolled along reticular fibers at lower levels than SKW3 cells. In one representative experiment, 46 tonsillar lymphocytes bound per ×100 field in the presence of divalent cations; 21 cells bound in the presence of EDTA, and no cells bound when BRIC235 or hyaluronan were combined with EDTA (data not shown). These experiments illustrate that lymphocytes are able to bind and roll along structures actually present in the human tonsil, using the CD44-hyaluronan interaction examined in this study.

![Figure 9. Inhibition of accumulation of SKW3 cells with mAb and EDTA. (a) Stromal cell monolayers and matrix. Control binding of untreated cells was 155 cells per ×100 field to stromal monolayers and 157 cells per ×100 field to stromal matrix. (b) Stromal cell matrix. Control binding of untreated cells was 224 cells per ×100 field. Binding of azide-treated SKW3 cells was inhibited by 5 mM EDTA, CD44 mAb BRIC235, 3F12, and A3D8 (CD44 mAb mix), by BRIC235 mAb alone, by β1 integrin mAb (β1 mAb); and by mAb to the integrin α1β3 (α1 mAb). Gi9 (α2 mAb), J143 (α3 mAb), PUJ4 (α4 mAb), PUJ5 (α5 mAb) and GoH3 (α6 mAb). Adherent cells were counted after the 0.8 dynes/cm² accumulation step; all adherent cells rolled as shear was increased. The mean values of two identical experiments are shown. Ranges are indicated by brackets.](image-url)

Ever, treatment of SKW3 cells with mAb BRIC235 or hyaluronan reduced tethering and rolling along the fibers in the presence of EDTA to six and five cells, respectively (Fig. 10, c and d). A serial section stained with collagen type IV mAb confirmed that the field contained an extensive network of reticular fibers (Fig. 10, e and f). Tonsillar lymphocytes attached and rolled along reticular fibers at lower levels than SKW3 cells. In one representative experiment, 46 tonsillar lymphocytes bound per ×100 field in the presence of divalent cations; 21 cells bound in the presence of EDTA, and no cells bound when BRIC235 or hyaluronan were combined with EDTA (data not shown). These experiments illustrate that lymphocytes are able to bind and roll along structures actually present in the human tonsil, using the CD44-hyaluronan interaction examined in this study.

Our studies demonstrate that stromal cells cultured from human tonsil produce hyaluronan-containing ligands that support CD44-dependent lymphocyte tethering and rolling. The ligand for CD44 was identified as hyaluronan by its sensitivity to hyaluronidase, by inhibition with soluble hyaluronan, and by reconstitution of CD44-dependent binding and rolling on substrates bearing immobilized hyaluronan. CD44 has been previously shown to bind hyaluronan in static assays (4, 15, 44, 49, 51). Hyaluronan is produced by fibroblasts and other cells as a soluble molecule on the inner surface of the plasma membrane and is translocated into the pericellular space during synthesis (57). It can remain bound to cells directly, presumably through continued attachment to the synthase (56) or indirectly, via hyaluronan-binding proteins (37). Our studies show that hyaluronan-like ligands produced by stromal cells were present both on the cell surface and incorporated into the ECM. We found two separate classes of association of CD44 ligands with the surface of stromal cells. One class was susceptible to cleavage by O-glycosidase, suggesting the structure that associated hyaluronan with the cell surface contained O-linked glycans. One such molecule is CD44 itself, which is highly expressed by stromal cells. Hyaluronan produced by the stromal cells may be immobilized and presented on the cell surface by stromal cell CD44. The O-glycosidase-insensitive ligand activity on stromal cells may represent either hyaluronan directly associated with the stromal cell membrane or bound to another uncharacterized structure on the cell surface. O-glycosidase treatment of stromal cell ECM almost completely removed ligand activity. The structure with which hyaluronan associates in the stromal cell ECM is unlikely to be CD44 because fluorescent staining of the ECM revealed CD44 was present only in membrane fragments that remained after cell removal. This CD44 was not distributed in a continuous manner and seemed unlikely to support the sustained rolling that was observed (data not shown). Several ECM components are known to bind hyaluronan, including the cartilage proteins aggrecan (50) and link protein (27) and the fibroblast protein versican (43). Fluorescent staining of the stromal cell ECM demonstrated a laminar deposition of versican, and this protein is also a component of the reticular fibers of the lymph node and tonsil (data not shown). Versican contains putative attachment sites for both N- and O-linked carbohydrates (78), but the susceptibility of this protein to O-glycosidase is not known. Our findings suggest that the proteins responsible for assembly of hyaluronan in the matrix of stromal cells possess both the hyaluronan-binding properties of proteins like versican and O-glycosidase-sensitive, mucin-like proteins.

Although blocking of CD44-hyaluronan interactions by either CD44 mAb or hyaluronidase treatment almost completely inhibited tethering to stromal substrates under shear stress, the α6β1 integrin was seen to participate in adhesion by stabilizing the binding of adherent cells to the substrate, mAb to the α6 and β1 integrins subunits decreased cell attachment to stromal cell monolayers and matrix and caused adherent cells to begin rolling at lower shear stresses. CD44-hyaluronan interactions appeared to mediate initial cell tethering under shear stress, while α6β1 increased the avidity of binding, thereby decreasing the rolling velocity of cells and enhancing cell binding by...
Figure 10. Video images of SKW3 cells rolling on frozen sections of human tonsil. (a) Cell adhesion and rolling in the presence of divalent cations. (b) Adhesion and rolling in presence of EDTA. (c) Inhibition of adhesion with CD44 mAb BRIC235 and EDTA. (d) Inhibition of adhesion with soluble hyaluronan and EDTA. Counts of adherent cells were made from the videotape with the full field of view; only a portion of the field is shown. SKW3 cells were not treated with azide. (e) Serial tonsil section stained with collagen type IV mAb M138. The photo represents only part of the field shown in a–d; the region shown is outlined in f. (f) The same field as shown in a–d averaged over 50 frames in 5 s so that images of rolling cells are lost. The area shown in e is outlined.
enabling cells that would otherwise have tethered only transiently to remain adherent. The complementary relationship of these two adhesion systems is similar to that proposed for the selectins, which mediate binding under shear stress, and integrins such as LFA-1 and Mac-1, which mediate firm adhesion once contact between cells and rolling substrates has been established (42). However, we suppressed development of a true firm adhesion in our experiments by treating cells with azide. This allowed for weaker integrin-dependent adhesion that did not prevent cells from rolling when the wall shear stress was increased above 1 dyne/cm².

Dermal fibroblast monolayers and ECM but not HUVEC endothelial or A431 epithelial monolayers or their secreted ECM supported hyaluronan-dependent lymphocyte rolling. SKW3 cells bound under flow and rolled on monolayers and matrix of dermal fibroblasts at lower levels than that seen with cultured tonsillar stromal cells. Stromal cells of the secondary lymphoid organs may be specialized to produce high levels of hyaluronan, a characteristic in keeping with the production of reticular fibers. The findings with HUVEC are surprising given that CD44-hyaluronan interactions mediate binding to cultured endothelium in static assays in both the human and mouse (4, 45, 66). Additional studies are needed to determine if hyaluronan produced by cultured endothelium is able to bind CD44 in static assays but not under conditions of flow.

The first step in lymphocyte recirculation involves the entry of cells into the secondary lymphoid organs by transmigration through HEV. CD44 has been implicated in lymphocyte extravasation by studies in which binding of lymphocytes to HEV of human frozen sections was inhibited by the CD44 mAb Hermes-3 and by polyclonal antisera raised against CD44 (34). However, Hermes-3 did not block hyaluronan-dependent adhesion of a CD44-transfected B cell line to cultured rat endothelium (66), and this mAb recognizes a portion of the CD44 molecule that is not required for hyaluronan binding (28, 32, 55). These results suggest that the observed binding to human HEV may not be hyaluronan dependent. In the mouse, lymphocyte binding to HEV was insensitive to hyaluronidase treatment and mAb known to block CD44 binding to hyaluronan (15). Adhesion to mouse HEV was found to be mediated by L-selectin binding to carbohydrates other than hyaluronan (45). Moreover, human lymph nodes treated with CD44 chimera gave weak definition of HEV but intense reactivity with connective fibers organizing the parenchyma (4), indicating that hyaluronan is expressed more highly in reticular fibers than in HEV. The low expression of hyaluronan on lymph node HEV, the lack of clear mAb blocking data, and the findings of this study that HUVEC monolayers do not support CD44- and hyaluronan-dependent rolling raise questions as to whether CD44 interactions with hyaluronan play a role in lymphocyte extravasation.

After entry into the secondary lymphoid organs, lymphocytes migrate to specific T and B cell areas to contact distinct classes of antigen-presenting cells. To examine the possible contributions of CD44-hyaluronan interactions to migration within secondary lymphoid organs, we developed an assay in which cells bind under conditions of flow to frozen sections of human tonsil. This assay resembles the Stamper-Woodruff assay (67) in that it uses frozen sections but has the additional advantage of isolating interactions that are capable of mediating tethering and rolling under flow. We observed divalent cation-independent adhesion and rolling along reticular fibers in the T cell areas of the tonsil section that were blocked by hyaluronan or CD44 mAb. Because reticular fibers are always closely associated with stromal cells (14), it was impossible to tell if cells rolled along the stromal cells themselves or the reticular fibers. However, our results with cultured cells show that cells roll on both stromal cells and their ECM products via CD44, so both structures probably present hyaluronan to rolling cells. These findings lend credence to the hypothesis that reticular fibers act as a scaffold for the migration of lymphocytes throughout secondary lymphoid organs (14, 38).

We speculate that the observed transient interactions of lymphocyte CD44 with the hyaluronan of secondary lymphoid organs may support the migration of lymphocytes as they transit through these sites in search of specific antigen. Production of hyaluronan is known to be increased at sites of cell migration (41), and this carbohydrate surrounds migrating and proliferating cells in areas of tissue remodeling, regeneration, and healing (73). It has been suggested that hyaluronan aids cell migration by creating hydrated pathways that assist cell penetration of tissues (73) and also by directly stimulating cell locomotion (74). Moreover, the dynamic nature of the interactions we observe between lymphocytes and hyaluronan are intriguing in light of current models of cell migration. A crucial aspect of cell migration is the dynamic reversibility of bonds formed between a migrating cell and the substrate (22). The fact that lymphocytes bind to hyaluronan under flow and then roll under moderate shear stresses suggests that this ligand pair can rapidly establish bonds that are strong enough to maintain cellular contact with the substratum but not strong enough to induce permanent cell arrest. Studies of migration of smooth muscle cells on the ECM ligands fibronectin and type IV collagen found that cell migration speed was fastest at intermediate attachment strengths (19). Empirical and theoretical findings suggest that migration speed is biphasic with respect to attachment strength: low attachment strengths do not allow the motile force within the cell to be transmitted to the substratum, moderate attachment strengths produce fastest migration, and high attachment strengths result in cell arrest and spreading (19, 40). Based on these findings, it is possible that the transient rolling interactions we have observed between lymphocytes and hyaluronan may reflect an ability of this ligand pair to support cell migration.

Although we do not propose that the interaction between CD44 and hyaluronan supports rolling in vivo, its ability to do so in vitro may be instructive nonetheless. The mechanical stability of a bond, i.e., a small rather than large effect of hydrodynamic drag force on the off-rate of a bond, as well as fast on- and off-rates, have been shown to be important for selectin-mediated rolling (1). These characteristics may apply to the CD44-hyaluronan bond as well. Mechanical strength is thought to be an important characteristic of the ECM, and it may be that, given sufficiently high on- and off-rates, other ECM receptor-ligand...
systems may also exhibit adhesion and rolling in shear flow. For example, the interaction of the ECM protein laminin with the integrin α6β4 also supports rolling (75). Adhesion in shear flow may therefore prove to be an interesting assay in which to examine the mechanical characteristics of ECM-receptor bonds.

In conclusion, we demonstrate that lymphocytes form CD44- and hyaluronan-dependent rolling interactions with cultured tonsillar stromal cells, stromal cell ECM, purified hyaluronan, and reticular fibers in the T-cell areas of lymph nodes: the reticular network. This work was supported by National Institutes of Health grant CA31708.

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