

# Lymphocyte CD44 Binds the COOH-terminal Heparin-binding Domain of Fibronectin

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**Abstract.** The lymphocyte-high endothelial venule (HEV) cell interaction is an essential element of the immune system, as it controls lymphocyte recirculation between blood and lymphoid organs in the body. This interaction involves an 85–95-kD class of lymphocyte surface glycoprotein(s), CD44. A subset of lymphocyte CD44 molecules is modified by covalent linkage to chondroitin sulfate (Jalkanen, S., M. Jalkanen, R. Bargatze, M. Tammi, and E. C. Butcher. 1988. *J. Immunol.* 141:1615–1623). In this work, we show that removal of chondroitin sulfate by chondroitinase treatment of lymphocytes or incubation of HEV with chondroitin sulfate does not significantly inhibit lymphocyte binding to HEV, suggesting that chondroitin sulfate is not involved in endothelial cell recognition of lymphocytes. Affinity-purified CD44 antigen was, on the other hand, observed to bind native Type I collagen fibrils, laminin, and fibronectin, but not gelatin. Binding to fibronectin was studied more closely, and it was found to be mediated through the chondroitin sulfate-containing form of the molecule. The binding site on fibronectin

was the COOH-terminal heparin binding domain, because (a) the COOH-terminal heparin-binding fragment of fibronectin-bound isolated CD44 antigen; (b) chondroitin sulfate inhibited this binding; and (c) finally, the ectodomain of another cell surface proteoglycan, syndecan, which is known to bind the COOH-terminal heparin binding domain of fibronectin (Saunders, S., and M. Bernfield. 1988. *J. Cell Biol.* 106: 423–430), inhibited binding of CD44 both to intact fibronectin and to its heparin binding domain. Moreover, inhibition studies showed that binding of a lymphoblastoid cell line, KCA, to heparin binding peptides from COOH-terminal heparin binding fragment of fibronectin was mediated via CD44. These findings suggest that recirculating lymphocytes use the CD44 class of molecules not only for binding to HEV at the site of lymphocyte entry to lymphoid organs as reported earlier but also within the lymphatic tissue where CD44, especially the subset modified by chondroitin sulfate, is used for interaction with extracellular matrix molecules such as fibronectin.

**H**ERMES-defined human lymphocyte surface molecules were originally designated as lymphocyte homing receptors because of their involvement in lymphocyte binding to organ-specific endothelial cell determinants in lymphoid organs (Jalkanen et al., 1986; Jalkanen, S. et al., 1987). Recently, the antigen recognized by the Hermes series of mAbs has been shown to be identical to, or include, the CD44 antigen (Picker et al., 1989a; Goldstein et al., 1989; Stamenkovic et al., 1989). Expression of CD44 or antigenically related proteins is not restricted to lymphocytes (Jalkanen et al., 1986; Picker et al., 1989a,b). Other leukocytes as well as some nonhematopoietic cell types express CD44 molecules of various sizes (Picker et al., 1989b; Pals et al., 1989). CD44 molecules on different cell types seem to have different functions. For example, CD44 antigen (90 kD) from lymphoma cells is able to bind to purified mucosal endothelial cell ligand, addressin, whereas CD44 antigen (150 kD) from epithelial cells lacks such binding capability (Berg et al., 1989; Picker et al., 1989b). CD44 has also been described to be involved in T cell activation (Shimizu et al.,

1989; Huet et al., 1989), lateral movement of cells (Jacobson et al., 1984), and erythrocyte rosetting (Hale et al., 1989). Furthermore, Carter and Wayner (1988) have shown that fibroblast CD44 (extracellular matrix receptor III) binds to collagen and fibronectin, and colocalizes with vimentin. In another study (Kalomiris and Bourguignon, 1988), binding of CD44 to a cytoskeletal protein ankyrin has been demonstrated. These findings suggest that CD44 may link adhesive interactions at cell surface with the cytoskeleton. Recently, CD44 has been shown to function also as a hyaluronate receptor (Aruffo et al., 1990; Miyake et al., 1990). These examples clearly demonstrate the multifunctional characteristics of CD44.

Structurally CD44 molecules belong to the family of proteoglycans, since in addition to O-linked and N-linked carbohydrates, a subset of lymphocyte CD44 molecules contains chondroitin sulfate side chains yielding a higher molecular mass form of 180–200 kD (Jalkanen et al., 1988). Moreover, CD44 is expressed on epithelial cells with heparan sulfate side chains (Brown et al., 1991). Recent sequence data in-

dicates that CD44 has significant homology to functional domains in the cartilage link protein and the cartilage proteoglycan monomer; and despite striking biochemical similarities, it is unrelated to mouse peripheral lymph node homing receptor (MEL-14) (Goldstein et al., 1989; Stamenkovic et al., 1989; Siegelman et al., 1989; Lasky et al., 1989). Furthermore, new variants of CD44 mainly expressed on cells of epithelial origin have been sequenced (Brown et al., 1991; Günthert et al., 1991; Stamenkovic et al., 1991), but the functional roles of the variant are still unclear. Interestingly, one variant has been connected to metastatic behavior of rat carcinoma cells (Günthert et al., 1991). Thus, CD44 represents a polymorphic family of cell adhesion molecules.

Since cell surface proteoglycans are important in diverse cell-cell and cell-matrix adhesive interactions (Ruoslahti, 1988, 1989), this work was designed to study the role of chondroitin sulfate on lymphocyte CD44 antigen. Our results indicate that chondroitin sulfate is not needed for lymphocyte-high endothelial venule (HEV) interaction. However, the ability of the CD44 antigen to bind extracellular matrix molecules, especially fibronectin, via chondroitin sulfate chains suggests that the high molecular weight form of CD44 could, indeed, be involved in lymphocyte-matrix interaction subsequent to lymphocyte extravasation into tissues. By this mechanism CD44 might participate at the tissue level in lymphocyte migration, differentiation and maturation.

## Materials and Methods

### Cells

Ficoll- (Ficoll-Paque, Pharmacia Fine Chemicals, Piscataway, NJ) purified human peripheral blood lymphocytes (PBLs) from normal healthy adults were used in the HEV assays. The PBL from leukopheresis samples of patients suffering from rheumatoid arthritis were used for CD44 antigen isolation. This source was selected because it allowed relatively large quantities of CD44 to be isolated. Composition of 90-kD and 180-kD forms of CD44 on PBL of these patients did not differ from that seen on PBL of healthy individuals when tested using immunoblotting. A lymphoblastoid B cell line, KCA, that was used in binding assays to fibronectin and fibronectin peptides was a generous gift from Dr. E. Engleman (Stanford University, Stanford, CA).

### Antibodies

Production and specificity of polyclonal anti-CD44 and mAbs, Hermes-1 and Hermes-3, against distinct epitopes of CD44 have been described earlier (Jalkanen et al., 1986; Jalkanen, S., et al., 1987). Hermes-1 inhibits hyaluronate binding to CD44, whereas Hermes-3 is able to block lymphocyte binding to mucosal HEV (Culty et al., 1990; Jalkanen, S., et al., 1987). For inhibition studies Hermes-1 and Hermes-3 mAbs were used as serum-free culture supernatants and polyclonal mouse anti-CD44 (that inhibits lymphocyte binding to peripheral lymph node, mucosal and synovial HEV; Jalkanen, S., et al., 1987) at 1:10 dilution. A polyclonal rabbit antibody against the common human  $\beta$  ( $\beta$ 1) chain of VLA-integrins was a generous gift from Dr. J. Heino (Turku University, Turku, Finland) and was used at 1:50 dilution. mAb against the alpha chain of VLA-4 (HP2/1) was a generous gift from Dr. F. Sanchez-Madrid (University of Madrid, Madrid, Spain), and mAb against the alpha chain of VLA-5 was from Immunotech, Marseille, France. mAb 3E1 against the COOH-terminal heparin-binding domain of fibronectin was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Specificity of this antibody has been reported by Pierschbacher et al. (1981). Anti-CD3 from American Type Culture Collection (Rockville, MD) and 281.2 against syndecan (Jalkanen et al., 1985) were used as class-

matched negative control antibodies, and 281.2 was also used for syndecan purification. Normal mouse serum and normal rabbit serum were used as controls for polyclonal antisera. The second stage reagent for ELISA was alkaline phosphatase-conjugated goat anti-mouse IgG and IgM (Tago Inc., Burlingame, CA), for immunoblotting peroxidase-conjugated rabbit anti-mouse Ig (Dako Corp., Santa Barbara, CA) and for immunofluorescence FITC-conjugated sheep anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) and FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.).

### Immunofluorescence

Single color indirect immunofluorescence was carried out as described (Jalkanen et al., 1986) using saturating levels of the first-stage antibodies followed by the appropriate second-stage reagent, either FITC-conjugated sheep anti-mouse IgG (Sigma Chemical Co.) or FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co.). Samples were analyzed using a cytometer (FACScan, Becton-Dickinson Immunocytometry Sys., Mountain View, CA). Routinely, data from 10,000 cells were collected.

### Chondroitinase Treatment

For chondroitinase treatment human PBL ( $1.8 \times 10^7$ ) and KCA ( $4.5 \times 10^6$ ; KCA cells are approximately four times the size of PBL cells) were incubated for 2 h at 37°C with 0.25 U of chondroitinase ABC (Miles-Yeda, Elkhart, IN) in 1 ml RPMI 1640 medium containing 1% BSA and 10 mM Hepes. Control lymphocytes were maintained in the same conditions without chondroitinase. This treatment was sufficient to remove all the high molecular weight form of the CD44 antigen, as determined by SDS-PAGE of immunisolated CD44 from sulfate-labeled lymphocytes (not shown).

### In Vitro Frozen Section Assay

An in vitro frozen section assay was performed as described earlier (Stamper and Woodruff, 1976; Jalkanen and Butcher, 1985). Briefly, lymphocytes in RPMI 1640 containing 5% FCS and 10 mM Hepes, pH 7.3, were incubated on fresh gut frozen sections of human and mouse peripheral lymph nodes, human appendix, mouse Peyer's patches, and human inflamed synovium for 30 min at 7°C. Similar results were obtained both on mouse and human HEV. The preservation of tissue specificity in xenogeneic lymphocyte-HEV interactions has been previously described (Wu et al., 1988). After incubation, bound lymphocytes were fixed in cold PBS containing 1% glutaraldehyde, and cell binding was quantitated microscopically as described earlier (Jalkanen and Butcher, 1985). Approximately 130 HEV per sample were counted. In other sets of experiments, either 100  $\mu$ l (100  $\mu$ g/ml) chondroitin sulfate (Sigma Chemical Co.) or heparan sulfate (Sigma Chemical Co.) or PBS (control) was incubated on frozen sections for 30 min at 7°C before the assay. Thereafter, untreated PBL were added and the binding assay was performed in a standard way. Results are presented as relative adherence ratios, the calculated number of sample cells bound to HEV per control cell binding under identical conditions. Standard errors were determined by the delta method (Rao, 1965).

### Purification of CD44 and Syndecan Ectodomain

Lymphocytes were lysed in lysis buffer (1% NP-40, 0.15 M NaCl, 0.01 M Tris, pH 7.0, 1.5 mM MgCl<sub>2</sub>, and 1 mM PMSF). The clarified lysate was applied first to a Sepharose CL-4B (Pharmacia Fine Chemicals) column (30-ml column volume) and then sequentially to two CnBr-activated Sepharose-4B (Pharmacia Fine Chemicals) columns, derivatized respectively with normal rat serum and with Hermes-1 mAb (5 mg/ml, 3 ml column volume). The column was washed extensively with the lysis buffer. Thereafter, the material bound to the Hermes-1 column was eluted with 50 mM triethanolamine and lyophilized. Fractionation of different forms of the CD44 antigen was performed using the Elfe fractionation system (Genofit; Grand Lancy, Switzerland). Purity of isolated CD44 was analyzed by protein staining after SDS-PAGE and also by immunoblotting. Small amounts of Hermes-1 antibody eluted off the column with CD44, and was the contaminating protein in some preparations. If the purity was not satisfactory (other visible protein bands), the affinity binding with the subsequent steps was repeated. After gel fractionation (Elfe, Genofit) no proteins other than CD44 were observed.

The ectodomain of the cell surface proteoglycan (syndecan; Saunders et al., 1989) was prepared from conditioned culture medium of NMuMG mouse mammary epithelial cells by DEAE chromatography, CsCl<sub>2</sub> density centrifugation, and 281.2 mAb affinity chromatography, as has been described elsewhere (Jalkanen et al., 1985; Jalkanen, M., et al., 1987).

1. *Abbreviations used in this paper:* HEV, high endothelial venule; PBL, peripheral blood lymphocytes.

## Enzymatic Digestion of Fibronectin

Fibronectin (1 mg/ml) in 2.5 mM CaCl<sub>2</sub>, 0.5 mM EDTA, 50 mM NaCl, and 25 mM Tris/HCl (pH 7.2) was digested for 4 h at 22°C with 5 µg/ml of thermolysin (Protease X; Sigma Chemical Co.).

## Synthetic Peptides

Two peptides (YEKPGSPPREVVPRPRPGV and KNNQKSEPLIGRKKKT; residues 1,906-1,924 and 1,946-1,960, respectively) known to promote heparin binding to COOH-terminal heparin-binding domain of fibronectin (McCarthy et al., 1988) were synthesized using an automated peptide synthesizer (model 431A; Applied Biosystems Inc., Foster City, CA). In addition, negative control peptides, CSI (DELPQLVTLPHPNLHGPEILDVPST), from the COOH-terminal region of fibronectin known to contain the binding site for VLA-4 but not to promote the heparin binding and RGDS were likewise synthesized. Purification of peptides was carried out with a preparative HPLC (Applied Biosystems Inc.) using a reversed phase column, and their purity was confirmed by an analytical HPLC (Applied Biosystems Inc.).

## Binding of Purified CD44 to Matrix Molecules

Binding of the purified CD44 antigen to fibronectin, laminin, collagen type I fibrils, and gelatin was tested using an ELISA method. Polystyrene plates (M129B; Dynatech Laboratories, Inc., Alexandria, VA) were coated overnight at 37°C with the indicated amount of gelatin (Merck & Co. Inc., Rahway, NJ), of human fibronectin (Calbiochem-Behring Corp., San Diego, CA), of COOH-terminal heparin-binding fragment of fibronectin (Calbiochem-Behring Corp.), of laminin (Sigma Chemical Co.), of collagen type I fibrils (purified using a previously described method; Chandrasenan et al., 1976), or fibronectin peptides. Thereafter, the wells were washed free of unbound material and saturated with 1% gelatin for 2 h. Approximately 5 ng (in PBS) of purified CD44 antigen was applied to the wells. After overnight incubation unbound material was washed away with PBS containing 0.5% Tween-20. Bound material was detected using Hermes-3 mAb as a first stage antibody, and alkaline phosphatase conjugated goat anti-mouse IgG and IgM (Tago Inc.) was used at the second stage. mAb against CD3, CRL8001 (American Type Culture Collection), served as a class matched negative control antibody. When the inhibition assays were performed, heparin (Sigma Chemical Co.), heparan sulfate (Sigma Chemical Co.), chondroitin sulfate (Sigma Chemical Co.), synthetic RGDS peptide (negative control), isolated ectodomain of syndecan, mannan (negative control; Sigma Chemical Co.), or soluble fibronectin (Calbiochem-Behring Corp.) was added after nonspecific binding sites had been blocked with gelatin. The wells were then incubated for 1 h at 37°C before the purified CD44 antigen was applied. The experiments were performed using triplicate wells.

Lymphocyte CD44 antigen binding to intact fibronectin and fibronectin fragments was measured also using immunoblotting method. Intact fibronectin, COOH-terminal heparin-binding domain of fibronectin (Calbiochem-Behring Corp.), and thermolysin digested fibronectin fragments (30 µg) were loaded, after reduction with 2% mercaptoethanol, onto a gradient (5-17.5%) SDS-PAGE. Phosphorylase b, 94 kD; BSA, 67 kD; ovalbumin, 43 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 20.1 kD; lactalbumin, 14.4 kD served as molecular mass standards and as negative control proteins for the binding experiments. Proteins in the gel were transferred onto a nitrocellulose paper, and nonspecific binding sites on the paper were blocked with PBS containing 0.1% Tween-20. The nitrocellulose strips were then incubated overnight with affinity-purified CD44 antigen in PBS. The bound antigen was detected with Hermes-3 mAb using peroxidase-conjugated rabbit anti-mouse Ig (Dako Corp.) at the second stage, with 4-chloro-1-naphthol (Sigma Chemical Co.) as the substrate. Monoclonal anti-CD3 antibody served as a negative control antibody. Fragments of COOH-terminal heparin-binding domain of fibronectin were detected using a mAb, 3E1 (Boehringer Mannheim Biochemicals), that recognizes this domain of fibronectin.

## Binding of Cells to Fibronectin and Fibronectin Peptides

Glass slides with tissue culture chambers (Lab-Tek Div., Miles Laboratories Inc., Naperville, IL) were coated with fibronectin and fibronectin peptides (10 µg/ml) overnight at 37°C and then blocked with 1 mg/ml heat-denatured BSA in PBS for 1 h at 37°C. KCA cells (150,000/well in HB101 serum-free medium; Du Pont, Hana Biologics, Inc., Alameda, CA) were added and allowed to adhere for 2 h at 37°C. Inhibition assays were performed in the

presence of chondroitin sulfate, heparin, and affinity-purified CD44. When the inhibitory capacity of different antibodies (Hermes-1, Hermes-3, polyclonal anti-CD44, and anti-β1 and their negative controls) was tested, KCA cells were first treated with the antibodies for 30 min and then washed once before the assay, or alternatively, the assays were performed in the presence of the antibodies. After incubation, unattached cells were removed by flicking. The tops of the wells were removed and the slides were washed by dipping them in PBS. Bound cells were fixed with 1% formaldehyde in PBS. Thereafter, the cells were stained using the Diff-Quick stains (comparable to May-Grünwald-Giemsa; Merz+Dade AG, Duding, Switzerland) and mounted in DePex (BDH Chemicals Ltd., Poole, England). The number of bound cells were analyzed by counting at least 20 fields in each case using an ocular grid (at a magnification of 250). Each experiment was performed using duplicate wells.

## Results

### Chondroitin Sulfate Moieties Are Not Involved in Lymphocyte-HEV Interaction

Possible involvement of chondroitin sulfate in lymphocyte-HEV interaction was investigated using an in vitro frozen section assay (Stamper and Woodruff, 1976; Jalkanen and Butcher, 1985). First, lymphocytes were treated with chondroitinase to cleave chondroitin sulfate side chains from the CD44 molecule. The conditions of this enzymatic treatment had been tested and previously determined to be sufficient to cleave chondroitin sulfate side chains of the CD44 antigen from intact lymphocytes. Such treatment had no effect on lymphocyte binding to peripheral lymph node, mucosal, or synovial HEV (Fig. 1). To confirm this finding, frozen sections were treated with chondroitin sulfate and heparan sulfate before the assay. The potential inhibitors were also present in the incubation medium during the assay. These conditions resulted in decreased binding, but the decrease did not achieve statistical significance (Fig. 1). In contrast, a polyclonal anti-Hermes antibody (anti-gp90; Jalkanen, S. et al., 1987) inhibits nearly 90% of lymphocyte binding to HEV (data not shown). Based on these results,

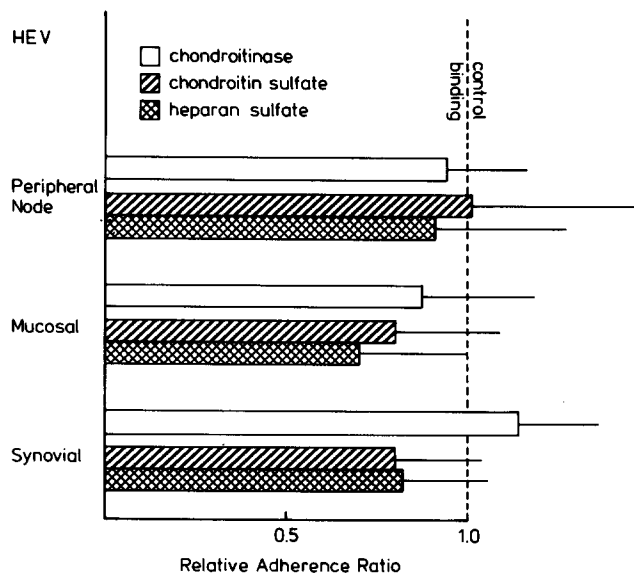
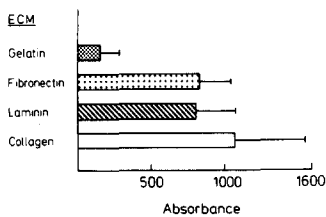


Figure 1. Chondroitin sulfate side chains play no significant role in lymphocyte-HEV interaction. In vitro frozen section binding assays were done four times on peripheral lymph nodes and mucosa-associated lymphatic tissues, and twice on inflamed (rheumatoid) synovial tissue. Data from those experiments are pooled.



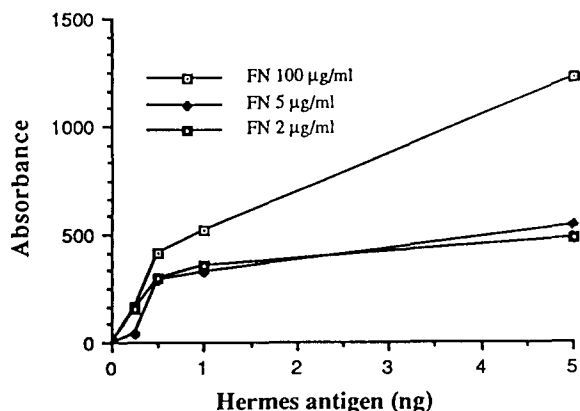
**Figure 2.** Binding of purified lymphocyte CD44 antigen to extracellular matrix molecules. Binding of purified CD44 antigen to fibronectin, laminin, collagen type I fibrils, and gelatin (100  $\mu\text{g}/\text{ml}$ ) is presented as net absorbance values with their standard deviations (net absorbance values = absorbance values with Hermes-3 - absorbance values with anti-CD3). Data from five experiments are pooled.

chondroitin sulfate seemingly plays no significant role in lymphocyte binding to their HEV ligands.

### Capacity of Lymphocyte CD44 Antigen to Bind Extracellular Matrix Molecules

An ELISA was established to test the capacity of lymphocyte CD44 antigen to bind surfaces coated with matrix molecules. In this assay, microtiter wells were first coated with fibronectin, laminin, collagen Type I fibrils, or gelatin. Then the purified CD44 was added and the immobilized CD44 was detected with primary and secondary antibodies. In this assay, purified CD44 antigen bound to fibronectin, laminin, and collagen type I, but not to denatured collagen (gelatin) (Fig. 2). Next, CD44 binding to fibronectin was studied more closely. When 2  $\mu\text{g}/\text{ml}$  of fibronectin was used for coating, the saturation level was achieved with  $\sim 1$  ng of CD44 antigen per well. When, however, 100  $\mu\text{g}/\text{ml}$  of fibronectin was used, saturation was not observed, even though the amount of CD44 antigen applied was increased to 5 ng/well. The titration curves of CD44 antigen binding to fibronectin in ELISA are shown in Fig. 3. These saturation curves clearly indicate the concentration dependency of this assay, both for the added CD44 antigen and for the binding sites on fibronectin-coated wells.

Binding of CD44 antigen to fibronectin was confirmed by immunoblotting: CD44 antigen bound to fibronectin transferred from the SDS-PAGE to nitrocellulose, whereas no binding of CD44 was observed to any of the control proteins (Fig. 4 A). Binding sites on fibronectin were studied after



**Figure 3.** Binding of purified lymphocyte CD44 antigen to fibronectin. Fibronectin (Fn) at the indicated amount was used as a ligand for coating. Binding was measured using an ELISA assay, as described in Materials and Methods. Absorbance values are presented as net absorbance values, as in Fig. 2.

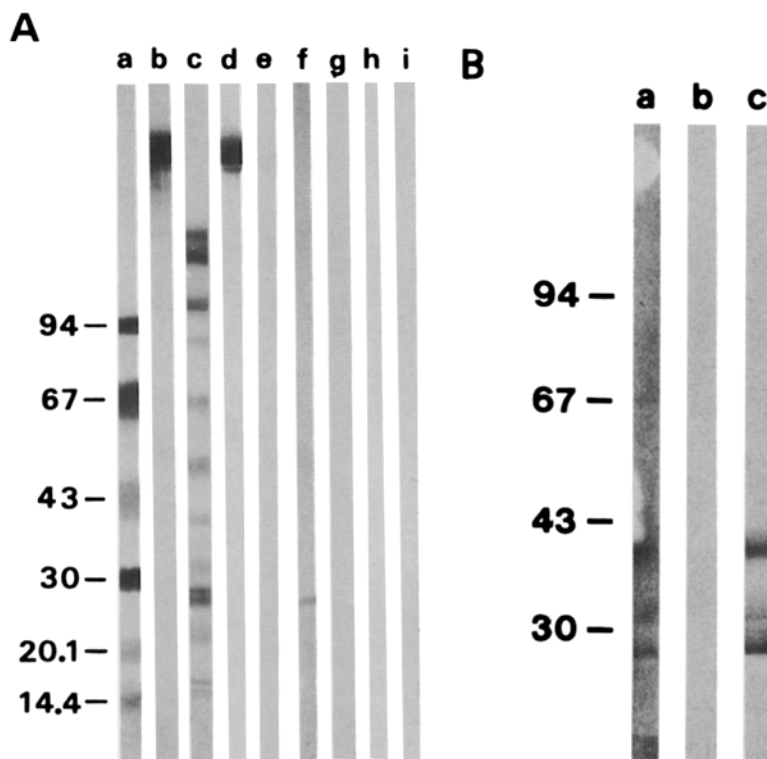
thermolysin digestion that generates well-defined fragments from intact fibronectin molecule (Sekiguchi and Hakomori, 1983; Zardi et al., 1985). Purified CD44 antigen reproducibly bound to a 29-kD fragment that is described to be one of the heparin-binding domains of fibronectin (Fig. 4 A, lane f). Very weak binding was sometimes observed to other fragments, perhaps representing partial digestion products containing still the heparin-binding domain. Since both  $\text{NH}_2$ -terminal and  $\text{COOH}$ -terminal heparin-binding fragments after thermolysin digestion are at the same molecular mass range (28–29 kD), we tested the binding to commercially available  $\text{COOH}$ -terminal heparin-binding domain of fibronectin (Chymotrypsin digested). The CD44 antigen bound to the fragments of  $\text{COOH}$ -terminal heparin-binding domain as shown in Fig. 4 B. The origin of the fragments was confirmed by using 3E1 antibody that recognizes an epitope which is located at the  $\text{COOH}$ -terminal heparin-binding site of fibronectin (Fig. 4 B). The original size of chymotrypsin digested  $\text{COOH}$ -terminal heparin-binding domain is 40 kD. The smaller fragments seen in Fig. 4 B are most likely degradation products of this larger fragment. This opinion is supported by the result that the 3E1 antibody recognized the smaller fragments, too.

McCarthy et al. (1988) have reported two peptides with heparin-binding activities from the  $\text{COOH}$ -terminal heparin-binding domain of fibronectin. Binding to the  $\text{COOH}$ -terminal heparin-binding domain of fibronectin was further confirmed by the experiments in which these heparin-binding peptides were used for coating (100  $\mu\text{g}/\text{ml}$ ). CSI peptide served as a negative control peptide. As reported below, the absorbance values given represent net absorbances: they were derived by subtracting those absorbance values obtained from the CD44 binding to the CSI control peptide from those of CD44 binding to YEKPGSPPREVVPRPRPGV or to KNNQKSEPLIGRKKKT. Mean absorbance values with their standard errors of two experiments are given. CD44 showed weak but clearly over the background binding (net absorbance  $175 \pm 64$ ) to YEKPGSPPREVVPRPRPGV. Binding of CD44 was significant to KNNQKSEPLIGRKKKT (net absorbance  $440 \pm 148$ ;  $P < 0.01$ ). The CD44 binding to KNNQKSEPLIGRKKKT represented 65.5% of the binding to intact fibronectin. The sum of these absorbance values was close to the value obtained from the binding to intact fibronectin in these particular experiments (91.5%), indicating that CD44 can bind to more than one polypeptide region.

### Chondroitin Sulfate Containing Form of the CD44 Molecule Is Responsible for Binding to Fibronectin

When the CD44 antigen was fractionated into its 90- and 180–200-kD forms (Fig. 5), only the form containing chondroitin sulfate was capable of binding to fibronectin (Fig. 5). The ELISA absorbance values are presented from an experiment in which one-sixth of the material shown in both lanes was applied to each microwell. Neither aggregation nor oversaturation accounted for the lack of binding of the material in lane 1, since, in fact, decreasing the amount of the material applied did not increase the binding (data not shown).

Involvement of chondroitin sulfate moieties was further tested in inhibition studies. In the ELISA system, heparin, heparan sulfate, and chondroitin sulfate inhibited binding of CD44 antigen to fibronectin in a dose-dependent manner. Maximum inhibition of 53% was observed at the level of 50



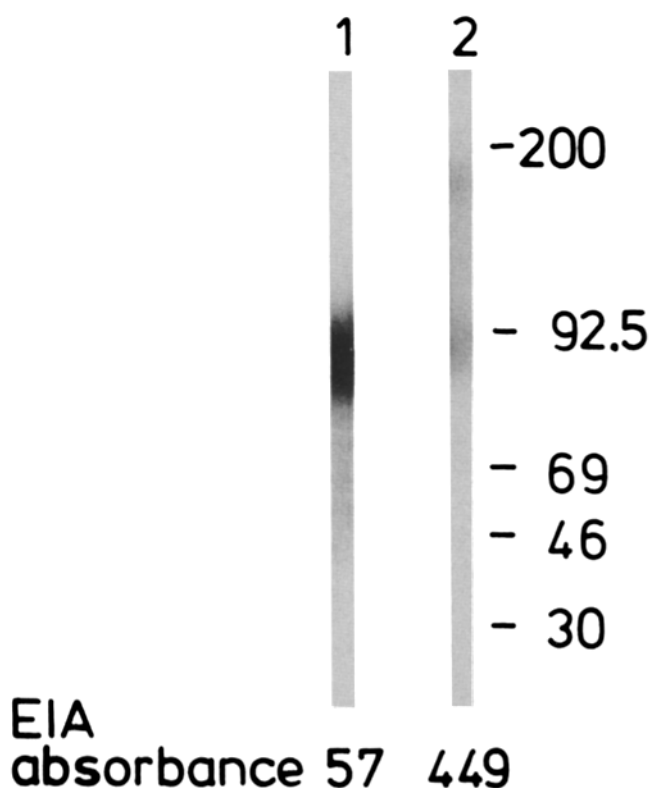
**Figure 4.** CD44 antigen binds to the heparin-binding domain of fibronectin. (A) Amido black staining of molecular weight markers (lane a), intact fibronectin (lane b), and fibronectin fragments after thermolysin treatment (lane c). Purified CD44 antigen was incubated on intact fibronectin (lanes d and e), on thermolysin-digested fibronectin (lanes f and g), and on molecular weight markers as control proteins (lanes h and i) after which the bound antigen has been detected either with monoclonal Hermes-3 antibody (lanes d, f, and h) or negative control antibody anti-CD3 (lanes e, g, and i). (B) Purified CD44 antigen was incubated on COOH-terminal heparin-binding fragments of fibronectin. Bound antigen was detected either with Hermes-3 antibody (lane a), or with the negative control antibody, anti-CD3 (lane b). Origin of the fragments was confirmed (lane c) by using mAb against COOH-terminal heparin-binding domain of fibronectin.

$\mu\text{g/ml}$  (Fig. 6). However, the ectodomain of a cell surface proteoglycan, syndecan, that binds to the COOH-terminal heparin-binding domain of fibronectin via its heparan sulfate side chains (Saunders and Bernfield, 1988) inhibited the binding more efficiently. Inhibition varied somewhat, depending on the experiment, from 85 to 98% at syndecan ectodomain concentration of 50  $\mu\text{g/ml}$  (Fig. 6). At a 0.5- $\mu\text{g/ml}$  concentration, no significant inhibition was seen. When the heparin-binding domain of fibronectin was used in this assay, inhibition by syndecan ectodomain was likewise almost complete (Table I). These data indicate that chondroitin sulfate side chains are involved in mediating the CD44 antigen binding to fibronectin and, moreover, that the binding site is the same as (or very near to) that of heparin and heparan sulfate. Soluble form of fibronectin did not significantly inhibit the binding (Fig. 6). Further, no binding inhibition was observed with mannan, which has been shown to inhibit lymphocyte binding to HEV in peripheral lymph nodes (Stoolman et al., 1984).

#### CD44 Mediates Lymphocyte Binding to Fibronectin

Involvement of CD44 in lymphocyte binding to fibronectin was tested using a cell line, KCA, that expresses VLA-5, known to mediate binding to the RGD-containing central cell-binding domain (Pytela et al., 1985), and VLA-4, known to mediate binding to CSI peptide (Wayner et al., 1989; Guan and Hynes, 1990). In contrast to low/moderate fluorescence intensity obtained with antibodies against alpha chains of VLA-4 and VLA-5, KCA cells were brightly positive with Hermes-3 mAb (Fig. 7). This cell line also expressed significant amount of the chondroitin sulfate-containing form of CD44 when tested in immunoblotting after SDS-PAGE (data not shown). In normal culture conditions, KCA cells form small clusters and grow in suspension. How-

ever, when plated on fibronectin-coated surfaces they adhered efficiently and showed fairly extensive spreading and formation of pseudopodia. They also adhered to peptides that mediate heparin binding as well as to CSI and RGDS. However, the peptides supported only minimal spreading. In mild washing conditions the number of cells bound per unit area (1.5  $\text{mm}^2$ ) was approximately the same to all these peptides used and to fibronectin. However, more vigorous washing conditions showed that binding to fibronectin was more efficient than to any of the peptides alone. Number of cells bound per unit area was practically unchanged in these washing conditions when fibronectin ( $443 \pm 7$  cells bound per unit area) was used for coating, whereas less cells adhered to peptides (PI = KNNQKSEPLIGRKKK,  $230 \pm 39$ ; PII = YEKPGSPPREVVPRPRPGV,  $152 \pm 14$ ; PIII = CSI,  $314 \pm 4$ , binding to RGDS was not studied systematically). Binding to BSA that was used as a negative control protein was 0.5% of that to fibronectin. Isolated CD44 inhibited binding of KCA cells both to fibronectin and heparin-binding peptides, but not to CSI peptide (Fig. 8). Binding to heparin-binding peptides PI and PII was inhibited 70% and 76%, respectively, when 10  $\mu\text{g/ml}$  of CD44 was used but binding to fibronectin was inhibited only 38%, indicating that also other cell surface proteins mediate KCA binding to fibronectin. This was confirmed by using a polyclonal antibody against  $\beta 1$  integrin that inhibited binding by 59% (Fig. 8). Anti- $\beta 1$  also inhibited binding of KCA to CSI but not completely. Partial inhibition by anti- $\beta 1$  together with low expression of  $\alpha 4$  suggest that binding of KCA to CSI may also involve molecules other than  $\alpha 4/\beta 1$ . Chondroitinase treatment of KCA as well as heparin present during the adherence assay also inhibited binding of these cells both to fibronectin and heparin-binding peptides but not to the CSI peptide (Fig. 8), further supporting the involvement of glycosaminoglycans in the binding of KCA to fibronectin. Inhibition percent-

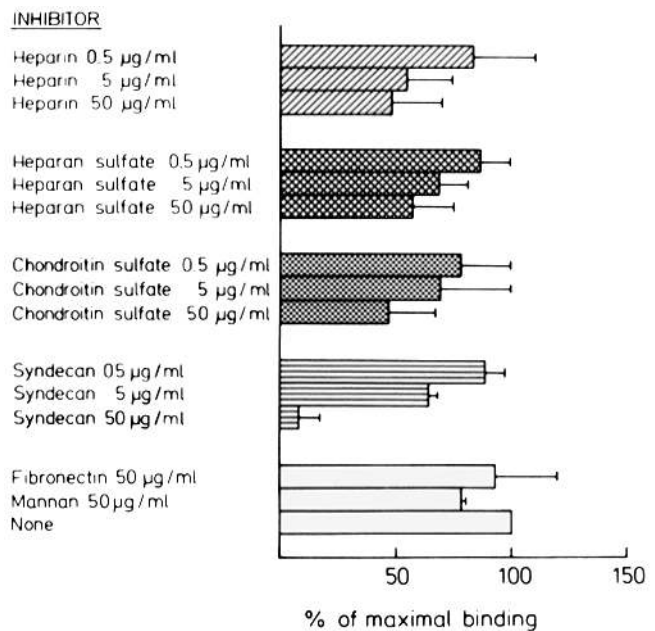


**Figure 5.** Ability of different forms of lymphocyte CD44 antigen to bind fibronectin. CD44 antigen was fractionated to yield different forms of the molecule. Lane 1 contains the 90-kD form of the antigen. Lane 2 contains the 180–200-kD chondroitin sulfate-containing form of the CD44 antigen and its spontaneous degradation product (90 kD). The fibronectin binding properties of the material on each lane was tested using an ELISA assay. The ELISA absorbance values (means of two analyses) are given below the corresponding lanes.

ages by soluble chondroitin sulfate were clearly lower than by heparin (Fig. 8). Good inhibitory activity of exogenous heparin, and poor inhibitory capacity of chondroitin sulfate as free polysaccharide has been reported earlier (Ruoslahti, 1988; McCarthy et al., 1990). mAbs Hermes-1 and Hermes-3 and polyclonal anti-CD44 did not have any effect on the binding. This further suggests that chondroitin sulfate side chains are critical in mediating binding of CD44 to fibronectin.

### Discussion

In this work, we have demonstrated that lymphocyte CD44 antigen involved in lymphocyte binding to HEV also interacts with extracellular matrix molecules. Moreover, these diverse abilities seem to be mediated through distinct structural domains of the molecule. These results, together with the recently observed identity between CD44, hyaluronate receptor (Aruffo et al., 1990), and collagen-binding extracellular matrix receptor type III (Carter and Wayner, 1988; Gallatin et al., 1989) indicate a broad role for CD44 molecules in cell adhesion. Similarly, other homing-associated molecules are multifunctional. LFA-1 has been described to have an accessory function in lymphocyte binding to HEV in addition to its participation in several adhesive interactions (Hamann et al., 1988; Pals et al., 1988). Yet,



**Figure 6.** Heparin, heparan sulfate, chondroitin sulfate, and syndecan inhibit CD44 antigen binding to fibronectin (10 µg/ml coating). Results are presented as percentages from control binding with standard deviations. Results from three experiments are pooled.

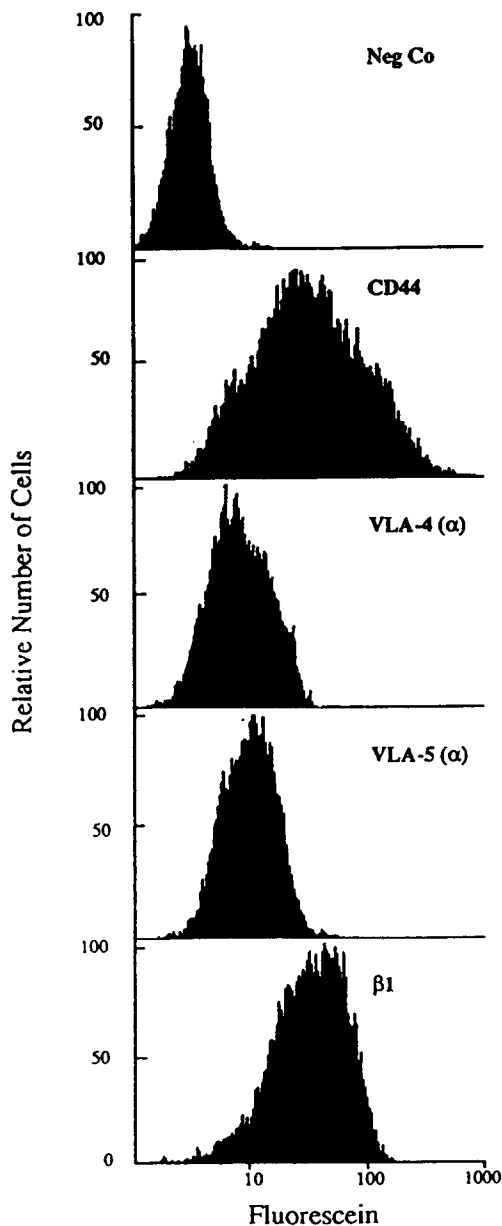
another member of the integrin family, LPAM-1/VLA-4, a mouse lymphocyte homing receptor to mucosal HEV, may have other functions as well (Holzmann et al., 1989). Recently, human VLA-4 has been shown to be involved in lymphocyte binding to mucosal HEV (Holzmann and Weissman, 1989); and it has also been shown to bind fibronectin (Wayner et al., 1989; Guan and Hynes, 1990; for review see Hemler et al., 1990). Apparent involvement of several structurally unrelated multifunctional adhesion molecules in lymphocyte binding to HEV and to extracellular matrix molecules demonstrates the complexity of mechanisms regulating lymphocyte recirculation, migration, and maturation.

### Specificity of the CD44 Antigen Binding to Fibronectin

A common functional feature of proteoglycans is the interaction with extracellular matrix molecules (Ruoslahti, 1988, 1989). Indeed, the interactions of proteoglycans with fibro-

**Table I.** Inhibition of Isolated CD44 Antigen Binding to the COOH-Terminal Heparin Binding Fragment of Fibronectin

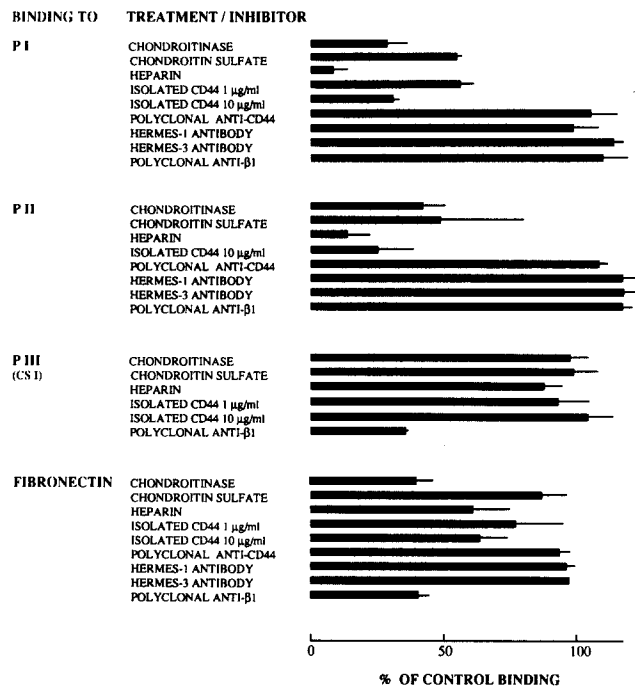
Inhibitor	Amount µg/ml	Percent of inhibition	
		Expt 1	Expt 2
Chondroitin sulfate	0.5	0	ND
	5	14	ND
	50	69	ND
Syndecan	0.5	0	0
	5	78	17
	50	100	97
RGDS	5	0	0
	50	0	0



**Figure 7.** Expression of VLA-4 $\alpha$ , VLA-5 $\alpha$ ,  $\beta$ 1, and CD44 (stained with mAb Hermes-3) on KCA cells used for inhibition studies. Indirect immunofluorescence was carried out as described in Materials and Methods. Negative control stainings were done with irrelevant mAb and normal rabbit serum as first stage reagents followed by the appropriate second stage reagents. Both negative controls gave similar results.

nectin involving heparan sulfate chains have been described in several studies (see Saunders and Bernfield, 1988, and references therein). Interactions of proteoglycans involving chondroitin sulfate chains have also been observed (Ruoslahti, 1988; Yamagata et al., 1986; Oldberg and Ruoslahti, 1982; Hedman et al., 1982; Smith, 1985). Isolated lymphocyte CD44 antigen bound reproducibly to the heparin-binding domain of fibronectin. This is in agreement with the finding that the chondroitin sulfate-containing form of the lymphocyte CD44 antigen showed significant binding to fibronectin.

Free-sulfated polysaccharides, such as heparan sulfate and



**Figure 8.** Inhibition of binding of a B lymphoblastoid cell line, KCA, to fibronectin and fibronectin peptides (PI, PII, and PIII). PI and PII are heparin binding peptides (PI = KNNQKSEPLIGR-KKT, PII = YEKPGSPPREVVPRPRGV) and PIII is CSI. Control binding for mAb inhibition was the binding of cells treated with class-matched irrelevant mAbs. Binding of cells treated with normal rabbit serum and normal mouse serum served as control binding for cells treated with polyclonal anti- $\beta$ 1 and anti-CD44, respectively. Control binding for heparin (500  $\mu$ g/ml), chondroitin sulfate (500  $\mu$ g/ml), and CD44 inhibition was counted from wells in which no inhibitor was present. Results are presented as percentages from control binding with their standard errors. Results from two experiments are pooled.

chondroitin sulfate, bind poorly to extracellular matrix. As a part of a proteoglycan in multivalent arrangement, however, they can interact with ligand molecules such as fibronectin (Ruoslahti, 1988). This explains the partial inhibition of binding of both isolated CD44 and KCA lymphoblastoid cells to fibronectin by free sulfated carbohydrates in our assays. On the other hand, CD44 antigen may have another binding site independent of chondroitin sulfate. This latter possibility is, perhaps, less likely, because polyclonal and mAbs against CD44 could not inhibit binding of KCA to fibronectin peptides. Moreover, syndecan, which binds to fibronectin via heparan sulfate side chains (Saunders and Bernfield, 1988), almost completely inhibited CD44 antigen binding. Isolated mouse and human syndecan have been reported to have relatively moderate affinity to fibronectin (Saunders and Bernfield, 1988; Elenius et al., 1990). If we assume that syndecan and CD44 are competing for the same binding site, the concentrations needed to inhibit the binding of the CD44 antigen suggest a relatively high affinity for CD44 antigen binding. However, affinities of purified antigens *in vitro* need not necessarily reflect the situation *in vivo*, where complicated interactions can take place. These inhibition studies, together with the binding studies using different forms of CD44 antigen (Fig. 5) suggest that CD44 antigen binds to fibronectin via its chondroitin sulfate side chains

and further, that the binding site is the same as that for heparin or heparan sulfate. This conclusion is also supported by the experiments where CD44 bound to the peptides earlier shown to mediate heparin binding to the COOH-terminal domain of fibronectin. Involvement of these peptides in promoting the binding of CD44 suggests that the binding may be mediated via a noncontiguous determinant in the COOH-terminal region of the molecule. The possibility, however, remains that CD44 also binds to the NH<sub>2</sub>-terminal heparin-binding domain. In that case, interaction with the NH<sub>2</sub>-terminal region is of low affinity, because syndecan that binds to the COOH-terminal region was able to inhibit the binding of CD44 almost completely.

Despite common properties, fibronectins from different sources exhibit molecular heterogeneity in terms of solubility and subunit structure because of alternative splicing (French-Constant et al., 1989). Thus, fibronectin in lymph nodes where it forms an intricate network throughout the tissue except in follicles (D'Ardenne et al., 1983), may have different functional properties than, for example, fibronectin found on the surfaces of vascular endothelial cells. In our assays, soluble fibronectin did not inhibit CD44 antigen binding to immobilized fibronectin indicating that some conformational changes occurring in anchored fibronectin molecule are required to support the binding of the CD44 antigen. On the basis of these results, it seems likely that in vivo recirculating lymphocytes do not use CD44 antigen to interact with fibronectin while still in the blood circulation. Rather, this interaction takes place inside the lymphatic tissues. In this regard, it is noteworthy that only a small subset of lymphocyte CD44 antigen isolated from PBL has covalently linked chondroitin sulfate side chains. It is not currently known whether certain peripheral blood lymphocytes have chondroitin sulfate-containing form of the CD44 antigen while other express only the 90-kD form, or whether each cell has both forms of the molecule. However, it is theoretically possible that lymphocytes with the chondroitin sulfate-containing form of the CD44 antigen represent a functionally unique subset of lymphocytes. Several cell types of hematopoietic origin have been shown to use VLA-5 or VLA-4 or both to bind fibronectin (Wayner et al., 1989; for review see Shimizu et al., 1990). In this work we have shown that a lymphoid cell line can also use CD44 molecule for binding to fibronectin. Perhaps, CD44 is not the primary adhesion receptor to fibronectin in a similar fashion as VLA-4 and VLA-5 but may serve as an additional binding site for lymphocytes at certain stages of their maturation and differentiation. Localization of CD44 to filopodia that are involved in cell-substratum contacts also supports the involvement of CD44 in binding to fibronectin (Brown et al., 1991; Jalkanen et al., 1991). Thus, lymphocyte binding may occur as a result of interactions between several surface molecules and different determinants of fibronectin. Several adhesion sites have already been shown to mediate adhesion of melanoma cells. Adhesion sites for melanoma cells include the same heparin-binding peptides as used in this study, RGD-containing region, and the CSI segment (McCarthy et al., 1988, 1990). It is obvious that involvement of several molecules increases the possibilities of regulation of cell adhesion to fibronectin.

### ***Binding Domains of the CD44 Antigen to HEV and Fibronectin Are Separate***

Our results clearly indicate that the binding domains of lymphocyte CD44 antigen to HEV and fibronectin reside on different parts of the molecule. The removal of chondroitin sulfate from lymphocytes or the addition of chondroitin sulfate into the HEV-binding assay, had no influence on lymphocyte binding to HEV. In contrast, Hermes-3 mAb inhibits lymphocyte binding to mucosal HEV (Jalkanen, S., et al., 1987) indicating that the region recognized by this antibody is involved in the interaction between lymphocytes and endothelium. Therefore, the glycosaminoglycan-containing region must be different from the HEV-recognition region. On the other hand, chondroitin sulfate chains are needed for fibronectin binding, and this binding is not inhibited by mAbs (Hermes series) against different epitopes of CD44. Furthermore, the polyclonal antibody against CD44 could not inhibit lymphocyte binding to fibronectin. This can be explained by the fact that the antibody does not contain any activity against native chondroitin sulfate side chains as a part of CD44. These results nicely demonstrate the separation of these two binding capabilities of the lymphocyte CD44 antigen.

The ability of CD44 antigen to interact with matrix components inside the lymphatic tissue may be essential for lymphocytes after leaving the vasculature. In fact, communication between lymphocytes and matrix may direct migration and localization inside the tissue. Tissue localization, on the other hand, may be intimately involved in the further differentiation and maturation of lymphocytes. Moreover, retention time inside the tissue might be determined by interactions between recirculating lymphocytes and matrix molecules. Furthermore, the ability to interact with endothelium, enter into, and attach to the tissue are characteristics that are important in determining the metastatic potential of lymphoid, as well as other malignancies. Since CD44 molecules are apparently involved in cell-endothelium and cell-matrix interaction, this class of molecules may eventually serve as useful prediction markers and, ultimately, elements critical to the understanding of tumor invasiveness.

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