Requirements for Hyaluronic Acid Binding by CD44: A Role for the Cytoplasmic Domain and Activation by Antibody

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Summary

The CD44-negative T lymphoma AKR1 (CD44.2 genotype) was transfected with a CD44.1 cDNA. The intact cDNA conferred on the transfected cells the ability to bind hyaluronic acid (HA) both from solution and immobilized on culture plates. It also conferred a CD44-dependent and hyaluronidase-sensitive increase in adhesion to a lymph node endothelial cell line. A mutant cDNA which codes for a CD44 molecule lacking most of the cytoplasmic domain of CD44 was also transfected into AKR1, and cell sorting was used to select transfectants expressing levels of cell surface CD44 expression comparable with the line transfected with the wild-type CD44 cDNA. The cells transfected with the mutant construct bound fluoresceinated HA from solution very poorly, but did adhere to immobilized HA, though less well than cells transfected with the wild-type construct. This result indicates that the cytoplasmic domain of CD44 is necessary for binding of HA from solution but is not required for binding to immobilized HA, although it may contribute to adhesion following ligand recognition. A monoclonal antibody (mAb), IRAWB 14, which reacts with CD44 on all CD44+ cells dramatically induced HA binding by some CD44+ cell lines that did not constitutively bind HA. The transfectant expressing a CD44 molecule with a truncated cytoplasmic domain could be induced by this antibody to bind fluoresceinated-HA from solution. Splenic T cells did not bind fluoresceinated HA constitutively. In the presence of the IRAWB 14 mAb, virtually all CD44+ splenic T cells bound HA. Induction was immediate and occurred equally well at room temperature and at 4°C, indicating that the new HA-binding activity was due to preexistent CD44 molecules. These results are compatible with an antibody-induced activation of CD44 by either a conformational change in the CD44 molecule or a change in the distribution of CD44 molecules on the cell surface.

It has recently been established that the murine Pgp-1 alloantigen, Ly-24 alloantigen, and hyaluronic acid receptor, as well as the human CD44 antigen, Hermes antigens, ECM-III receptor, p85, and In (Lu)-related p80 are all identical or closely related molecules (1-6). We will refer here to the molecules bearing these determinants as CD44. This broadly distributed membrane glycoprotein has been implicated in several important hematopoietic cell functions including lymphocyte and progenitor cell homing, lymphopoiesis and T cell activation (7-14), as well as fibroblast adhesion and migration (15). It now seems likely that the diverse activities attributed to CD44 are subsumed by its function as a cell adhesion molecule.

Sequencing of cDNAs of human and mouse CD44 revealed a conserved amino terminal region with sequence similarity to hyaluronic acid-binding domains of cartilage link and proteoglycan core proteins (16-21). This suggested a possible function in binding hyaluronic acid (HA).1 CD44-dependent HA binding activity has been demonstrated for several CD44+ hematopoietic cell lines (22, 23), for a recombinant fusion protein of human CD44 (24), and for a B cell line transfected with the hematopoietic form of human CD44 (25). CD44 has also been reported to bind extracellular matrix (ECM) proteins, (collagen types I and VI, and fibronectin) (26) and a ligand on high endothelial venules (HEV)-vascular addressin (27).

Although CD44-dependent HA-binding activity can be

1 Abbreviations used in this paper: ECM, extracellular matrix; F1-HA, fluorescein-conjugated hyaluronic acid; HA, hyaluronic acid; HEV, high endothelial venules; TME, transformed mouse endothelium; w.t., wild-type.
demonstrated with CD44-expressing cell lines, there are many examples of CD44* cells that do not bind HA. In particular, HA binding activity has not been readily demonstrable among the CD44* normal hematopoietic cells in the mouse (22, 23, 28). Thus, CD44 expression alone does not confer HA binding activity (29).

Molecular cloning and biochemical studies suggest that there may be as many as four protein isoforms of CD44. The major species on hematopoietic cells, CD44H, has a core transmembrane polypeptide of 37 kD and typically shows an Mr of about 90 kD (19–21). A second major form of CD44, CD44E, characteristic of epithelial cells, contains an insert (132 aa in the human) in the extracellular portion of the molecule near the transmembrane region (25, 30, 31). Transfection of CD44E cDNA into a CD44-negative B cell line did not confer hyaluronic acid-dependent binding of the transfectants to lymph node HEV cultures (23). Both the shorter (hematopoietic) and larger (epithelial) core proteins are extensively and diversely modified by glycosylation (1, 2, 4, 25, 30, 32), which accounts in part for the many different forms found in different cell lines. A minor isoform of CD44H with a truncated cytoplasmic tail may exist (33), and there are indications that CD44 may exist as a soluble protein (34).

The structural requirements for HA recognition by CD44 are not well-defined, and it is not known why many CD44* cells do not bind HA. Here, we demonstrate that CD44H confers HA binding activity when transfected into a T lymphoma line lacking CD44 expression and that the cytoplasmic domain contributes to binding of HA from solution. We also describe an antibody to a particular CD44 epitope that dramatically increases the ability of CD44-bearing cells to recognize HA. We discuss the implications of these results for understanding the mechanism of HA binding by CD44.

Materials and Methods

Cells and mAbs. The murine T lymphomas AKR1 (35), BW5147 (36), and the CD44* variant of SAKRTLS 12 (29) were maintained in DMEM with 10% horse serum. AKR1 is negative for CD44 expression (7) and for HA binding (22). The LN endothelial cell line TME-3H3 (37) was maintained on tissue culture dishes in DMEM with 10% FCS.

T cell–enriched spleen cells were prepared from the spleens of AKR/Cum mice (Cumberland View Farms, Clinton, TN) by spinning on petri dishes coated with rabbit anti–rat Ig (38).

mAbs, used as tissue culture supernatants were: rat anti–mouse CD44 IM7 (7), KM201 (11), and IRAWB 14 (this paper); CD44 allele-specific antibodies (39), RAMB44 specific for CD44.1, and C71/26 specific for CD44.2; and anti-V-CAM-1 antibody M/K-2 (40, 41). IRAWB 14 mAb was obtained as previously described (7) by fusion of SI94 myeloma cells with spleen cells of a Sprague–Dawley rat which had been immunized with an HA-binding variant line of SAKRTLS 12 (29) induced with PMA (22, 29) to further increase HA binding activity. The hybridoma was chosen on the basis of screening for effects on the binding of fluorescein-labeled HA to BW5147 cells (see Fig. 3).

Construction of Plasmids for Expression of CD44. A cDNA clone MHR6 (provided by E. Butcher, Stanford University School of Medicine, Stanford, CA) encoding full length murine CD44.1 was modified as follows to permit the stable expression of wild-type CD44. The entire insert was cut out by EcoRI digestion (20) and subcloned into double-stranded M13mp18, to yield a single strand template. A 25-mer oligonucleotide 5' GTC CAT GGC GAA TAC TTC GTC CGG CAT G 3' was used to insert an EcoRI site 3 nucleotide residues before the initiation codon ATG by in vitro oligonucleotide-directed mutagenesis. The mutated insert was digested with EcoRI and subcloned into vector pBG367 (42) yielding plasmid pMCD44/BG367. A Sall/XhoI fragment from pMCD44/BG367 which contains 3' termination sequences necessary for expression was subcloned into vector pTFCMo (43), resulting in the expression plasmid pMCD44.1/TFCMo. Expression is driven by a long term repeat (LTR) promoter and contains the neo gene to allow selection in G418 (Gibco Laboratories, Grand Island, NY).

For the construction of a plasmid for expression of CD44.1 with a truncated cytoplasmic domain, the mutated insert was subjected to another cycle of in vitro oligonucleotide-directed mutagenesis with the use of a 25-mer oligonucleotide 5' TTC TTC TTC CCT TTA CAC CTT CTC C Y, which inserted a stop codon TAA at the position of Gly278. This insert was subcloned into pBG367 and pTFCMo as above, yielding the expression vector pMCD44ΔCY/TFCMo coding for a CD44.1 molecule containing a deletion of all but the first six residues of the cytoplasmic domain. Both inserts were checked by sequencing, and no mutations other than the desired ones were found.

Transfection by Electroporation. Cells were transfected by electroporation as described previously (44). Briefly, AKR1 cells were suspended at 1.0 × 10^7/ml in ice-cold phosphate-buffered saline (Mg^2+ and Ca^2+ free). 0.5 ml of the cell suspension was transferred into a precooled electroporation cuvette and 80 μg of purified plasmid DNA was added. The cuvette was then pulsed using the Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA) with 550 V and 25 μF of capacitance. G418 was added at 1 mg/ml after 48 h of growth and drug-resistant colonies appeared within 2 wk.

Flow Microfluorometry and Cell Sorting. Fluorescein-conjugated HA (Fl-HA) was prepared as described (45) using rooster comb HA (Sigma Chemical Co., St. Louis, MO). Purified IM7 (7) mAb was conjugated with fluorescein using FITC-celite (22). Labeling of cells for flow cytometry, data collection on the Salk Institute's modified Los Alamos flow microfluorometer, and processing of the data were as previously described (46). Geometric mean fluorescence of unstained cells was assigned a value of 1.0 U and positive fluorescence was expressed relative to this. Blocking and enhancement of Fl-HA binding was performed by adding hybridoma supernatant (1:20 final dilution) or unlabeled HA (100 μg/ml final concentration) to cells and shaking for 30 s on a micro shaker just before adding the Fl-HA.

To select transfectants expressing CD44 with a deletion of the cytoplasmic domain at levels equivalent to the level of CD44 expressed by transfectants for the wild-type gene, cells were stained with fluorescein-conjugated IM7 mAb and sorted for the most fluorescent cells (29). After three cycles of enrichment sorting, the cells were cloned by limit dilution, and clones were screened for expression of CD44 comparable to that of AKR1 cells transduced with the wild-type CD44 construct.

Cell Adhesion Assays. ^51Cr labeling of lymphoma cells and adhesion to HA-coated plates were performed as described by Miyake et al. (23). For adhesion to TME-3H3 cells (37), the adherent cells were harvested with trypsin, washed, and plated at 1–2 × 10^5 per well in 24-well plates (Corning cell wells, #25820; Corning Glass Works, Corning, NY) the day before the assay. Just before addition of ^51Cr-labeled lymphoma cells, the adherent TME-3H3 cells were rinsed once with warm DMEM with 5% newborn calf serum
(Hyclone Laboratories, Logan, UT). Incubations and washes were the same as for adhesion to HA-coated wells (23).

For antibody blocking of adhesion, a 1:20 dilution of culture supernatant containing mAb was mixed with the $^{51}$Cr-labeled lymphoma cells just before their addition to the assay plate.

Northern Blotting, Cell Surface Labeling, and Immunoprecipitation.

Northern blotting was performed as described in reference 41. Lactoperoxidase-catalyzed iodination, immunoprecipitation (using a rabbit anti–rat Ig serum) and SDS-polyacrylamide gel electrophoresis were as described previously (47).

**Results**

**CD44 Expression by Transfectants.** Expression of the transfected CD44 genes was confirmed by Northern blotting, immunoprecipitation of surface $^{125}$I-iodinated proteins, and flow cytometry. As shown in Fig. 1 A, AKR1 cells transfected with the wild-type CD44 cDNA construct (AKR1.CD44 $^{+}$ w.t.) bound high levels of CD44-specific antibody (fluorescein-conjugated IM7) with over 80% of the cells averaging 40 times the background staining. A small negative population of variable size was consistently seen and could not be eliminated entirely by recloning or culture in G418.

AKR1 cells transfected with a cDNA construct coding for a CD44 molecule with a truncated cytoplasmic domain (see Materials and Methods) originally expressed lower levels of CD44 than AKR1.CD44 $^{+}$ w.t. This difference in expression made it difficult to compare the HA-binding activity of these two transfected cell lines. We used enrichment sorting (see Materials and Methods) to isolate a derivative, AKR.CD44 $^{+}$ ACY, that expressed CD44 at levels equivalent to the transfected for the wild type construct (Fig. 1, G).

Fig. 2 A shows Northern blots of the CD44 transcripts in the transfected AKR1 cells. A control B cell line, BM2 (23), has three prominent transcripts at 4.6, 3.3, and 1.8 kb. Transfected cells have a single transcript of 1.8 kb, which is absent in the parental line.

Immunoprecipitation of $^{125}$I-CD44 from the AKR1.CD44 $^{+}$ w.t. and AKR1.CD44 $^{+}$ ACY cell lines is shown in Fig. 2 B, using mAb specific for the alternative alleles of CD44 (39) to confirm that the cells are expressing the transfected, and not the endogenous allele. The T cell lymphoma, BW5147, expresses the CD44.2 allele characteristic of the AKR/J strain of mouse from which both the BW5147 and AKR1 cell lines were derived (lanes 1 and 2). AKR1 cells transfected with wild-type CD44.1 cDNA, CD44 § w.t., express CD44.1, but not CD44.2, and the labeled protein has the same $M_s$ as CD44 from BW5147 (lanes 3 and 4). The AKR1 cells transfected with the mutant CD44.1 construct, CD44 ACY, also express CD44.1, but on a molecule that is about 10 kD smaller than the CD44 $^{+}$ w.t. molecule (lanes 5 and 6).

**Binding of Fl-HA from Solution.** Binding of Fl-HA to transfectants (Fig. 1, B and H) was assayed by flow cytometry as described previously (22). Most AKR1.CD44 $^{+}$ w.t. cells bound high levels of Fl-HA (about 30 x background). With the AKR1.CD44 $^{+}$ ACY transfectant, most cells showed no

Figure 1. CD44 expression and hyaluronic acid binding activity of AKR1 cells transfected with CD44 cDNA constructs. AKR1 cells transfected with a w.t. CD44 cDNA construct (A–F) and a mutant cDNA construct that resulted in a truncated cytoplasmic domain (G–L), were stained with fluorescein-conjugated mAb IM7.8.1, specific for CD44 (A and G), or fluorescein-conjugated hyaluronic acid (Fl-HA) (B–F and H–L). Fl-HA-labeled cells were pretreated with KM201 mAb (C and J) or IRAWB 14 mAb (E, F, K, and L) and/or unlabeled hyaluronic acid (D, F, J, and L) (see Materials and Methods). The dotted-line insert in each panel is the fluorescence profile of unstained cells, which was assigned a relative fluorescence value of 1.0 U.
Figure 2. Northern blot of CD44 mRNA and immunoprecipitation of surface labeled CD44 in AKR1 cells transfected with CD44 cDNA constructs. (A) CD44 mRNA accumulation by AKR1 cell lines transfected with CD44 constructs determined by Northern blotting. BM2 is a CD44+ control cell line (23). Numerals indicate approximate size of transcripts in kb. (B) Cell surface-iodinated BW5147 (lanes 1 and 2), AKR1.CD44+ w.t. (lanes 3 and 4) and AKR1.CD44+ ΔCY (lane 5 and 6) cells were solublized in NP40, and CD44 was immunoprecipitated with mAb RAMB44 (lanes 1, 3, and 5) specific for CD44.1 or mAb C71/26 (lanes 2, 4, and 6) specific for CD44.2. Immunoprecipitates were electrophoresed on a 7.5% polyacrylamide-SDS gel.

Binding to Immobilized HA. Binding of transfected cells to HA immobilized on plastic was performed as previously described (23). As shown in Table 1, the parental line AKR1 did not adhere to HA-coated plates. The AKR1.CD44+ w.t. transfectant bound well to the dishes (40% as shown in Table 1, 42.0 ± 4.6% in four experiments). The AKR1.CD44+ ΔCY line also bound to HA coated dishes, but binding was reduced compared to AKR1.CD44+ w.t., although both cell lines express comparable levels of CD44. Binding by the transfected cell line expressing CD44 with a truncated cytoplasmic domain was consistently observed to be about half or less than half the level of binding by the cell line transfected with the wild-type CD44 construct. CD44 levels were analyzed by flow cytometry at the time of each adhesion assay and were always approximately the same (40-50x background) for the two lines.

Binding to TME-3H3 Cells. An SV40 transformed, LN-derived endothelial cell line TME-3H3 (37) was used as a target for cell adhesion assays. As shown in Table 2, AKR1 parental cells adhered to this cell line and transfection of wild-type CD44 into the AKR1 cell line resulted in a two-fold increase in adhesion to TME-3H3 cells. This increased binding was inhabitable by KM201 antibody, indicating that it was due to CD44. An antibody specific for a V-CAM-1-like determinant of stromal cells (41) did not inhibit. Binding of the parental line, AKR1, was not inhibited by either KM201 or the V-CAM-1-specific antibody. This result suggests that the basal binding of the parental AKR1 cell line to 3H3 cells is not mediated by either CD44 or VLA-4, the counter-receptor of VCAM-1 (40). In other experiments (not shown) antibodies against VLA-4, Thy-1, LFA-1, and ICAM-1 failed to inhibit the basal binding of the AKR1 cell line.

TME-3H3 cells were treated with hyaluronidase to determine if the CD44-dependent component of the binding was due to recognition of HA (Table 3). Hyaluronidase reduced the binding of the CD44 transfected cells and the residual binding was not further reduced by KM201. This suggested that HA was the ligand mediating the binding activity due to CD44 (KM201 inhabitable), while the residual binding (non-CD44) was not directed to HA.

The transfectant expressing CD44 with a truncated cytoplasmic domain also bound to 3H3 cells, though less well than had the transfectant expressing wild-type CD44. Its binding was also reduced by hyaluronidase treatment of the endothelial cell monolayer to the same level as that seen when binding of this transfectant to untreated TME-3H3 cells was assayed in the presence of KM201 antibody (Table 3).

Antibody Enhancement of Hyaluronate Binding. In an effort to identify cell surface determinants specifically involved in the binding of hyaluronic acid, mAbs were made against an HA-binding mutant of the AKR/J lymphoma SAKRTLS 12 induced with PMA to increase HA binding activity (29). Antibodies were screened for ability to influence binding of Fl-HA to BW5147, which already bound HA significantly. One of the antibodies, IRAWB 14, markedly increased the binding above background but a variable minor population bound up to about 10 times above background. In both lines, the Fl-HA binding detected was inhibited by unlabeled HA (Fig. 1, D and J) and by the CD44-specific antibody KM201 (Fig. 1, C and L), which has previously been shown to inhibit binding of cells to HA-coated plastic dishes (see references 23 and Table 1), establishing the specificity and CD44-dependence of the HA binding. The parental line AKR1, bound neither CD44-specific antibody nor HA (reference 22 and data not shown).
Table 1. Adhesion to HA Immobilized on Plastic*

| Cell line      | Antibody†  | Input cpm§ | Mean cpm\(^1\) adhering | Adhering\(^4\) |
|----------------|------------|------------|--------------------------|---------------|
| AKR1 parent    | -          | 69,544     | 1,018 ± 594              | 1.5% ± 0.8    |
| + KM201        |            |            | 424 ± 10                 | 0.6%          |
| AKR1.CD44+w.t  | -          | 82,454     | 33,105 ± 808             | 40.1% ± 1.0   |
| + KM201        |            |            | 337 ± 28                 | 0.4%          |
| AKR1.CD44+ACY  | -          | 56,722     | 10,158 ± 709             | 17.9% ± 1.2   |
| + KM201        |            |            | 203 ± 7                  | 0.4%          |

* Adhesion assays performed as described in Materials and Methods and Reference 23.
† Antibody was a 1:20 dilution of hybridoma supernatant, added to the \(^{31}\)Cr-labeled cells just prior to their addition to culture wells.
§ Input cpm listed here = input cpm at the start of incubation = spontaneous release.
\(^1\) Mean and standard error of triplicate samples.
\(^4\) % Adhering = cpm adhering/input cpm (as defined in §).

binding of Fl-HA to BW5147 cells (Fig. 3, compare B and E). This antibody also induced Fl-HA binding by several T cell lines which expressed CD44 but did not bind HA, such as EL4 and SAKRTLS 12 (22), and the CD44\(^{nl}\) variant of SAKRTLS 12 (29), which binds Fl-HA poorly (Fig. 3, H and K). On the basis of immunoprecipitation and the pattern of binding on CD44\(^{+}\) and CD44\(^{-}\) cell lines, it was determined that the IRAWB 14 antibody recognizes a CD44 determinant which is present on all CD44\(^{+}\) cells, including those which do not bind HA (data not shown).

When the IRAWB 14 antibody was added to AKR1.CD44+ ACY cells just before incubation with Fl-HA, either at room temperature or at 4°C, binding of Fl-HA increased from background levels to 40-50 times background (Fig. 1, K). Thus, the IRAWB 14 antibody rapidly converted CD44 molecules with a deletion of the cytoplasmic domain into a form able to bind HA. Antibody was present during the 15-20-min incubation allowed for the binding of Fl-HA, so the activation occurred within this period. The new binding activity was specific, since it was blocked with

Table 2. CD44-mediated Adhesion to TME-3H3 Endothelial Cell Line*

| Cell line      | Antibody†  | % of Input cpm adhering§ |
|----------------|------------|--------------------------|
| AKR1 parent    | -          | 43.5 ± 1.5               |
| + KM201        | (anti-CD44)41.0 ± 2.1 6|
| + M/K-2        | (anti-V-CAM-1)53.6 ± 2.40|
| AKR1.CD44+w.t. | -          | 89.9 ± 1.9               |
| + KM201        | 43.3 ± 0.1 52|
| + M/K-2        | 92.2 ± 2.5 0|

* 3H3 endothelial cells (reference 39) were plated at 2 x 10\(^4\) cells/well in 24-well tissue culture plates the day before the assay was performed. Wells were rinsed once with DMEM containing 5% newborn calf serum before assay.
† Antibody was a 1:20 dilution of hybridoma supernatant added to the \(^{31}\)Cr-labeled lymphoma cells just before their addition to culture wells.
§ Mean and standard error of triplicate samples. See legend for Table 1.

Table 3. Hyaluronate Dependence of CD44-mediated Adhesion to TME-3H3 Cells*

| Cell line +/- antibody | Untreated Hyaluronidase-treated TME-3H3 cells§ |
|-----------------------|-----------------------------------------------|
| AKR1.CD44+ w.t., alone | 82.5 ± 2.0                                  |
| AKR1.CD44+ w.t. + KM201 | 29.7 ± 1.5                                  |
| AKR1.CD44+ ACY, alone  | 27.6 ± 2.4                                  |
| AKR1.CD44+ ACY + KM201 | 20.5 ± 1.5                                  |

* See figure legend for Table 2.
§ Mean and standard error of triplicate samples. See legend for Table 1.
§ 3H3 cells plated the day before assay were rinsed twice in warm DMEM without serum. Hyaluronidase (1 mg/ml in DMEM) was added to each well (0.5 ml) and the plate incubated at 37°C for 30 min. After incubation, the hyaluronidase was removed and the wells were rinsed twice with DMEM containing 5% newborn calf serum at 37°C.
unlabeled HA (Fig. 1, L). Slight (two- to threefold) increases in binding of Fl-HA to the wild type transfectant were also seen (Fig. 1, E).

The ability of IRAWB 14 antibody to induce HA binding in normal hematopoietic cells was tested on splenic T cells from AKR/Cum mice (Fig. 4). About 45% of the cells were CD44+ on the basis of binding of fluoresceinated-IM7 antibody (first panel, Fig. 4), but binding of Fl-HA was negative (second panel, Fig. 4). The few scattered events above background in this panel were not competed by unlabeled HA (not shown) and so were not specific. In the presence of IRAWB 14 antibody, Fl-HA binding was induced in 41% of the cells (third panel, Fig. 4), equivalent to the number of CD44+ cells. The binding of Fl-HA induced by IRAWB 14 antibody was competed by unlabeled HA (fourth panel, Fig. 4).

When total bone marrow was incubated with IRAWB 14 and stained for Fl-HA binding, most cells were negative, though most were strongly CD44+ (data not shown). Thus, not all CD44+ cells can be induced to bind HA by this antibody.

**Discussion**

AKR1 cells, transfected with a CD44 cDNA construct coding for a wild-type CD44 molecule, express hyaluronate binding activity both for HA in solution and HA immobilized on plastic culture wells. This result indicates that CD44 is able to confer HA binding activity, and if any additional factors are needed for HA binding, they are provided by the AKR1 cell. Thus, in agreement with Aruffo et al. (24) and our previous data (22, 23), the hematopoietic form of CD44 is a cell surface receptor for HA. Our experiments show that the cytoplasmic domain contributes to this function and that HA recognition can be dramatically influenced by mAb to particular epitopes.

Increased binding to a lymph node-derived endothelial cell line, TME-3H3 (37), was shown by the AKR1.CD44+ w.t. transfectant, compared with the AKR1 parental cells (Table 2). This increased binding was attributable to CD44-hyaluronate interactions, since it could be inhibited by the antibody KM201 specific for the hyaluronate binding site on CD44 (11, 23), and by hyaluronidase treatment of the endothelial cells (Table 3). This result indicates that CD44 can contribute to lymphocyte adhesion to certain endothelial cells via its hyaluronate binding activity, in agreement with the result of Stamenkovic et al. (25) using cells transfected with the hematopoietic form of human CD44. The results also reemphasize that adhesion to endothelial cells may be mediated by more than one mechanism (48-50), since the CD44-negative parental line did adhere to the endothelial cells, though less well than did the transfected cells, and since CD44-specific antibody and/or hyaluronidase only partially inhibited binding of the transfectants. Elsewhere, we show that recognition between other lymphocyte-endothelial cell line combinations is predominantly mediated by VLA-4 and VCAM-1 (37, 41).

The AKR1 transfectant expressing a CD44 molecule with a truncated cytoplasmic domain, AKR1CD44+ ΔCY, did
not bind Fl-HA from solution, but could be induced to do so very efficiently by pretreatment with the CD44-specific antibody, IRAWB 14. This observation suggests that the mutant CD44 molecule must be activated in some way in order to bind HA. Activation, as used here, refers to the conversion of preexisting CD44 molecules from a non-HA-binding state to a form able to bind HA. We suggest two possible, not necessarily exclusive, mechanisms of antibody-induced activation: a conformational change in the CD44 molecule; or a change in distribution of the CD44 molecules on the cell surface. A change in distribution might involve clustering of CD44 molecules into a specific configuration or association of CD44 with other cell surface molecules. Either mechanism may, or may not, require intracellular signalling. The cytoplasmic domain of the wild-type molecule could mediate either a conformational or distributional change, perhaps through interaction with cytoplasmic elements. On the other hand, antibody-induced activation may mediate HA binding by a different mechanism than that mediated by the cytoplasmic domain of the wild-type CD44 molecule.

AKR1.CD44 + ΔCY cells had some affinity for immobilized HA without antibody activation, although less than cells transfected with the wild-type CD44 molecule. Immobilized HA, unlike the soluble form, may itself provide an activation stimulus (similar to that provided by the IRAWB 14 antibody) to effect either a conformational or distributional change in the tailless receptors. Alternatively, immobilized HA may represent a ligand that can be recognized without activation. The relative inefficiency of binding to immobilized HA by the CD44 + ΔCY cell line lacking the cytoplasmic domain of CD44 may suggest a requirement for cytoplasmic interactions to stabilize adhesion.

The IRAWB 14 antibody activates HA binding on several CD44 expressing T cell lymphomas that do not constitutively bind HA from solution (e.g., Fig. 3). In these lines, failure to bind HA is not due to the absence of a cytoplasmic domain: SDS-gel electrophoresis of immunoprecipitates and northern blot analysis indicates no difference in the migration of CD44 proteins or spectrum of CD44 mRNAs in these lines compared to related lines which bind HA (29). The ability of these CD44 + HA-receptor negative cells to bind HA upon brief exposure to IRAWB 14 antibody (at 4°C) establishes that the CD44 on their cell surface is capable of forming HA receptors without metabolic alteration. Like the cells transfected with a CD44 construct containing a mutation affecting the cytoplasmic domain, the CD44Δ variant of SAKR1TS 12, shown in Fig. 3, is able to bind to immobilized HA without antibody activation (unpublished results) but requires activation to bind HA from solution. Thus, the same mechanisms for antibody or immobilized ligand-mediated recognition as have been suggested above might be postulated for these cells. However, the failure to constitutively bind HA must have a different basis than the absence of an intracellular domain.

A portion of splenic T cells express CD44 but fail to bind HA. As in the case of the lymphomas discussed above, IRAWB 14 antibody induced binding of Fl-HA in these normal lymphocytes (Fig. 4). Other normal hematopoietic cells, such as bone marrow myeloid cells, were not induced by IRAWB 14 to bind Fl-HA. Further studies will be required to deter-
mine the susceptibility of different hematopoietic cell populations to induction of HA binding and the physiological factors involved in such induction.

The failure of many CD44+ cells to bind HA, and the ability of a CD44-specific antibody to activate HA binding, suggests that CD44 is a member of the growing list of cell adhesion molecules that require activation to bind ligand most efficiently (51–56). This list includes many of the integrins: platelet GP IIb-IIIa (α11β3) (52); LFA-1 (51); Mac-1/CR3 (53–54); β1-class integrins on developing neurons (55); and Lam-1 (56), a member of the Lec-Cam (lectin-like) family. Best studied to date is the platelet integrin GP IIb-IIIa, which shows many parallels with the HA-binding requirements of the CD44-expressing cells described here. Cells expressing this integrin constitutively bind immobilized fibrinogen, but require activation, by antibody or phorbol ester, to bind fibrinogen from solution. In this case, antibody activation is believed to be due to a conformational change, since antibody can activate detergent-solubilized integrin and monovalent Fab fragments of antibody can activate (52). Further, there is evidence that ligand can induce an activating conformational change (57). For several integrins (GP IIb-IIIa, Mac-1, LFA-1, and p150, 95) activation exposes new epitopes that are recognized by activation-specific antibodies (52, 54, 58, 59). On the other hand, localized redistribution of the CR3 integrin, postulated to result in high affinity adhesion patches, has been demonstrated on neutrophils stimulated to bind C5b by phorbol ester (60). Alteration or total deletion of the cytoplasmic tails of cadherins, integrins, and other cell interaction molecules has been shown to dramatically reduce ligand binding ability (61–64).

Most hematopoietic cells in the mouse express CD44 at some level (7), yet HA binding is not detectable (22, 23, 28), or is only stimulated under particular conditions (28, 65, and this paper). Does this general failure to demonstrate HA binding by normal hematopoietic cells indicate that CD44 has functions other than or in addition to HA binding in vivo and/or that HA binding is only rarely or transiently activated? It is reasonable to suggest that a cell-adhesion mechanism involving such abundant and broadly distributed molecules as HA and CD44 could not be constitutively active if it is to provide any selectivity of interaction, and that specific activation signals would be required to elicit function at the appropriate times and locations. The intracellular mechanisms by which different cell types regulate CD44 activity and the physiological factors involved in triggering activation to allow binding of HA remain to be defined.

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