Brief Definitive Report

Hyaluronan Binding Function of CD44 Is Transiently Activated on T Cells during an In Vivo Immune Response

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Summary

Though CD44 functions as a cell surface receptor for hyaluronan (HA) in some cell lines, most normal hematopoietic cells expressing CD44 do not bind HA. Certain CD44-specific monoclonal antibodies (mAbs) can rapidly induce CD44-mediated HA binding in normal murine T cells. This observation suggests that in vivo mechanisms may exist for activating the HA receptor function of CD44 on normal T cells. Here, it is shown that up to one third of splenic T cells are capable of CD44-mediated binding of fluorescein-conjugated HA (Fl-HA) during an in vivo allogeneic response. HA binding activity peaks at 7–8 d postinjection and declines rapidly. These rapid kinetics could be the result of transient activation of CD44 function and/or differentiation or expansion of short-lived population(s) that have constitutive HA-binding function. Both CD4 and CD8 T cells are included in the HA binding population which is strongly CD44 positive. After separation of HA-binding cells from nonbinding cells by cell sorting, it is shown that almost all cytotoxic effector cells are found in the HA-binding population. However, there is no evidence that CD44-mediated HA recognition is directly involved in the killing of target cells, since cytotoxicity could not be inhibited by CD44-specific mAbs that inhibit HA binding or by soluble HA. PCR amplification of cDNA reverse transcribed from RNA of sorted HA-binding cells indicated no evidence for CD44 isoforms other than the standard (hematopoietic) form. Though CD44 expression is known to be elevated upon T cell activation, and, as shown here, HA-binding function is induced in a portion of CD44-expressing T cells including cytotoxic effector cells, the role of CD44 and HA-recognition in immune responses is not known.

The cell surface glycoprotein CD44 is an adhesion receptor for extracellular matrix molecules that has been implicated in lymphocyte recirculation, cell migration, T cell signaling, cell–cell interactions, and metastasis (1–3). Though a number of ligands have been proposed for CD44, its interaction with the glycosaminoglycan hyaluronan (HA) is the most thoroughly documented (1, 3–5). Nevertheless, many normal cells that express CD44 do not bind HA (1, 6, 7). Thus, whether HA-recognition is involved in the biological functions proposed for CD44 is not yet established.

It has been suggested that CD44, like a number of other adhesion receptors of hematopoietic cells, may be inactive in resting cells and may require specific activation events to induce its ligand-recognition function (6, 7). Though HA binding cells are not detected in resting populations of normal murine hematopoietic cells, Murakami et al. (8, 9) have shown that a population of B cells capable of binding HA can be induced by graft vs. host reaction in vivo or by culture for several days in IL-5. Also, a CD44-specific mAb (IRAWB14) has been described that can rapidly activate HA binding by CD44 on some T cells (6, 7), suggesting that physiological mechanisms may exist for activation of CD44 function in T cells. In the present study, we examined whether CD44-mediated HA binding was induced in T cells after immunological stimulation in vivo. A strong allogeneic response was used as a model in order to induce sufficient numbers of cells to be detected by flow cytometry.

Materials and Methods

Animals and Immunization. 4–6-wk-old C57BL/6 ($H2^b$) and DBA/2 ($H2^d$) mice were purchased from Harlan-Sprague Dawley Inc. (Indianapolis, IN). P815 (DBA/2 origin, $H2^d$) mastocytoma cells were maintained in suspension culture in DME with 10% calf serum. C57BL/6 mice were injected i.v. or i.p. with 10^7 P815 cells or with 2×10^7 DBA/2 spleen cells. Spleen, bone marrow, peripheral lymph nodes (brachial and inguinal), and mesenteric lymph nodes were harvested at various times after immunization and single cell suspensions prepared.

Flow Cytometry and Cell Sorting. Cells were labeled with fluorescein-conjugated hyaluronan (Fl-HA) as previously described (6,

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7). Controls included Fl-HA staining blocked with excess unlabeled HA (250 μg/ml) or with the CD44-specific mAb KM201 (4) which inhibits HA binding to CD44 (see Fig. 2). For two-color studies, biotin-conjugated mAbs specific for other lymphocyte cell surface molecules were detected with phycoerythrin-streptavidin (Biomeda Corp., Foster City, CA) as described previously (6). These biotinylated mAb included 57.597 for TCR-α/β, 53.6.72 for CD8, GK1.5 for CD4, Mel-14 for L-selectin, RA3.2C2 and RA3.6B2 for B220 (B cell–specific CD45), RB6.8C5 for the myeloid lineage, and M1/70 for Mac-1.

Cells stained for one- (fluorescein) or two- (fluorescein and phycoerythrin) color fluorescence were run on a FACScan+® flow cytometer (Becton Dickinson & Co., Mountain View, CA). Cell sorting was carried out on a FACStar+® (Becton Dickinson & Co.) using total spleen cells stained with Fl-HA. Sort windows for Fl-HA positive and negative cells were chosen on the basis of control staining with Fl-HA in the presence of 250 mg/ml of unlabeled HA. A window composed at 5–10% of the total cells, between the Fl-HA positive and negative populations, was discarded.

Cell-mediated Cytotoxic Assays. Target P815 cells were labeled with ^{51}Cr and distributed into 96-well V-bottom microtiter plates (Dynatec Laboratories, Inc., Chantilly, VA) at 2×10^4 cells/well. Stained and sorted spleen cell populations were added to wells at the ratios indicated in Fig. 4. Plates were incubated for 3–4 h, the cells centrifuged into a pellet, and 100 μl of supernatant removed and counted. In other experiments, total (unsorted) spleen cells (at ratios of 1:10, 1:50, 1:200) were added to ^{51}Cr-labeled P815 cells in the presence of mAb (1:6 dilution of hybridoma supernatant) or unlabeled HA (335 mg/ml). Each ratio for each condition was assayed in triplicate. Percent cytotoxicity was calculated as follows: Percent cytotoxicity = (experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximal ^{51}Cr release − spontaneous ^{51}Cr release) × 100. Spontaneous release was determined by culturing ^{51}Cr-labeled P815 cells in the absence of C57BL/6 spleen cells. Maximal release was determined by adding 1% NP-40 in 0.1 N NaOH to wells containing ^{51}Cr-labeled target cells.

Reverse Transcription and PCR Amplification (RT-PCR) of RNA from Sorted Spleen Cells. Total cellular RNA from sorted HA-binding (1.2×10^6) and nonbinding (1.6×10^6) cells was isolated using an RNA isolation kit (Stratagene, La Jolla, CA). 5×10^6 AKR1 cells transfected with murine M1 and M2 isoforms of CD44 (containing variant exons v9-10 and v8-10, respectively), as described in (10), were treated identically. 30% of total cellular RNA from the sorted cells and 10% from control cells were used as templates for the synthesis of cDNA. First strand syntheses were carried out for 2 h at 37°C using 200 U reverse transcriptase (Superscript; Life Technologies, Grand Island, NY) with the following primer: 5′ GTTCACCAAATGCACCATTTCCTG 3′. The cDNA products (two-fifths of the total for sorted cells, three-tenths for control cells) were then used as template for PCR using 2.5 U Taq polymerase (Stratagene) with the aforementioned 3′ primer located 288 bp downstream of the insertion site (insertion site corresponds to position 704 in reference 11) and the 5′ primer located 74 bp upstream of the insertion site with the sequence 5′ TACCTTCCTACTGAACAGCCCTACTGGGAAGAT 3′. After purification the PCR products were run on an agarose gel (one-tenth of PCR product in all cases), transferred to a Nylon membrane (Hybond-N; Amersham Corp.; Arlington Heights, IL), and hybridized with a radiolabeled CD44-specific probe (nucleotides 700–1183). The blot was exposed to x-ray film for 2 d. The film was scanned on a Scan Jet IIcx/T (Hewlett-Packard Co., Palo Alto, CA). Canvas 3.5.1 software was used for contrast adjustment and labeling the image, and the printer was a Phaser II SDX (Tektronix, Wilsonville, OR).

Results and Discussion

Induction of HA-binding Cells in Spleen. C57BL/6 ($H2^b$) mice were immunized with P815 (DBA/2 origin, $H2^d$) mastocytoma cells to induce an allogeneic response. Hematopoietic and lymphoid tissues were assayed by flow cytometry for the presence of cells capable of binding Fl-HA. Unimmunized mice had no detectable HA-binding cells. Mice immunized intravenously had detectable levels of HA-binding cells in the spleen between days 5 and 10 post injection (Fig. 1). In these same mice, no HA-binding cells were detected in lymph nodes or bone marrow. Examples of HA-binding in total spleen cell populations from mice 6, 7, and 8 d after intravenous injection of P815 cells are shown in Fig. 2 (*first row*). Specificity and CD44 dependence of the HA binding activity were verified by inhibition of Fl-HA binding with excess unlabeled HA (Fig. 2, *dotted insets*) or with CD44-specific mAb KM201 (Fig. 2, *second row*), which has been shown to block the HA-binding function of CD44 (4). HA binding was very heterogeneous, and dull cells often overlapped the background staining measured in the presence of unlabeled HA (see Fig. 2). Also, as can be seen from Fig. 1, the proportion of HA binding cells in immunized spleens varied greatly among individual mice. HA-binding cells detectable in the spleen declined after 9 d. The transient appearance of HA binding activity could be due to transient activation of CD44/HA-binding function and/or to differentiation or expansion of short-lived population(s) of constitutive HA-binding cells.

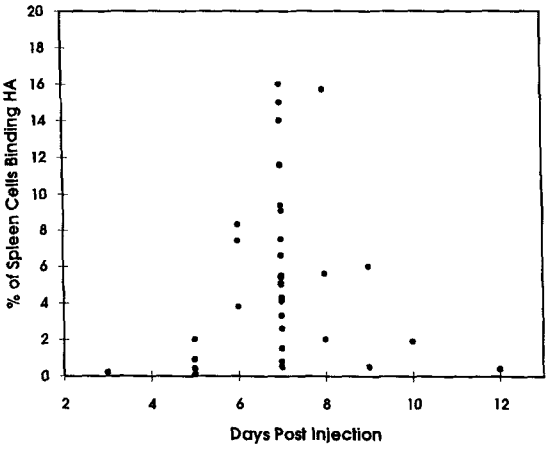

Figure 1. Percentage of HA binding cells in the spleens of C57 BL/6($H2^b$) mice undergoing an allogeneic immune response to P815 ($H2^d$) cells. Total nucleated cells from the spleens of individual mice injected with 10^7 P815 cells i.v. were prepared at the indicated times post injection, stained with Fl-HA, and analyzed by flow cytometry. The percent of total spleen cells binding HA was determined by comparison with control cells stained with Fl-HA in the presence of excess unlabeled HA as follows: a cursor was set so that <2% of control cells were positive (with a staining intensity beyond cursor); this background percent was then subtracted from the percentage of positive cells observed in the absence of unlabeled HA using the same cursor setting. In unimmunized mice, there was a <1% difference between cells stained with Fl-HA in the presence or absence of unlabeled HA.

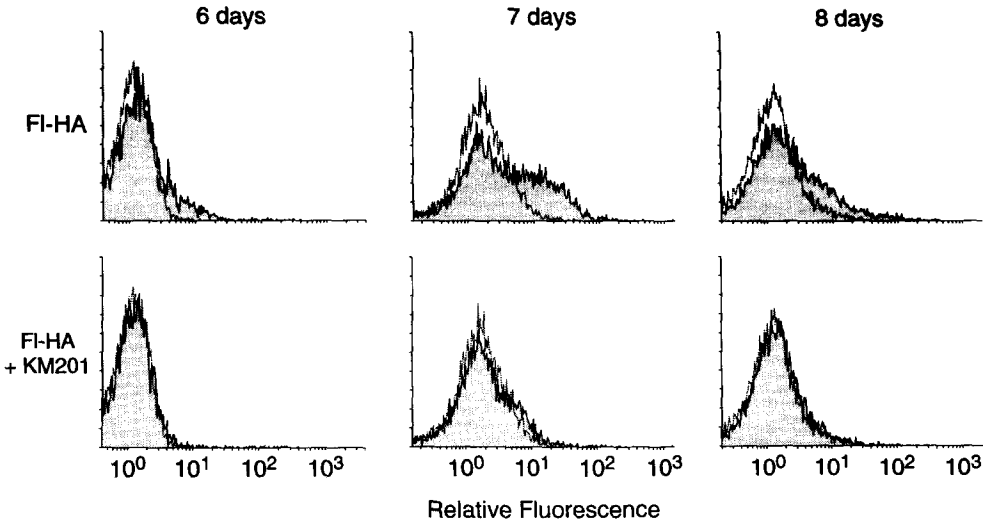

Figure 2. Representative Fl-HA staining of total spleen cells from P815 immunized C57BL/6 mice. At the indicated days post injection, total spleen cells were prepared and stained with Fl-HA alone (*top*), or with Fl-HA in the presence of the CD44-specific mAb KM201 (*bottom*), which inhibits HA binding to CD44 (references 4, 7). The dotted line in both rows indicates the staining with Fl-HA in the presence of excess unlabeled HA (250 μg/ml).

In other experiments not shown, mice immunized by intraperitoneal injection of P815 (10^7 cells) also had detectable levels of HA-binding cells in the spleen, but the percentage of HA-binding cells was generally lower than with i.v. injection and peaked later, at about 10 d after injection. HA-binding cells were not detected in the peritoneal cavity at 8, 10, or 12 d, or in the lymph nodes of these intraperitoneally injected mice. Mice immunized intravenously with DBA/2 spleen cells (2×10^7) instead of P815 mastocytoma cells had lower, but detectable, numbers of HA binding cells in the spleen at 7 d after immunization.

Phenotype of HA-binding Cells in the Spleen. Two-color flow cytometry was used to assess the phenotype of the HA-binding cells in spleen. Because the Fl-HA staining was heterogeneous, the number of positive cells was often low, and the positive cells were not clearly separated from the background, it was difficult to determine more than a general picture of the phenotype of HA-binding cells based on data from numerous experiments. Most HA-binding cells were T cells (67–90% in 12 experiments) by virtue of their staining with mAbs against TCR-α/β (Fig. 3 *C*). In the maximum responses observed, HA-binding cells represented up to one-third of the splenic T cells. The HA^+ cells were among the brightest $CD44^+$ cells. They included $CD8^+$ and $CD4^+$ cells in proportion to the distribution of these cells in the total spleen T cell population (Fig. 3, *E* and *F*). The HA-binding cells that were not T cells included B cells and small numbers of myeloid cells (not shown).

Attempts to enrich for HA^+ cells other than by cell sorting were unsuccessful. Panning on HA immobilized on plastic followed by recovery of cells with hyaluronidase gave low recoveries and did not significantly enrich for HA^+ cells, indicating that many Fl-HA binding cells did not adhere, unlike the HA-binding B cells described by Murakami et al. (6, 7). Procedures to enrich for T cells such as removal of B cells by panning on anti-Ig–coated plates or by nylon wool columns also did not enrich for HA-binding cells. In-

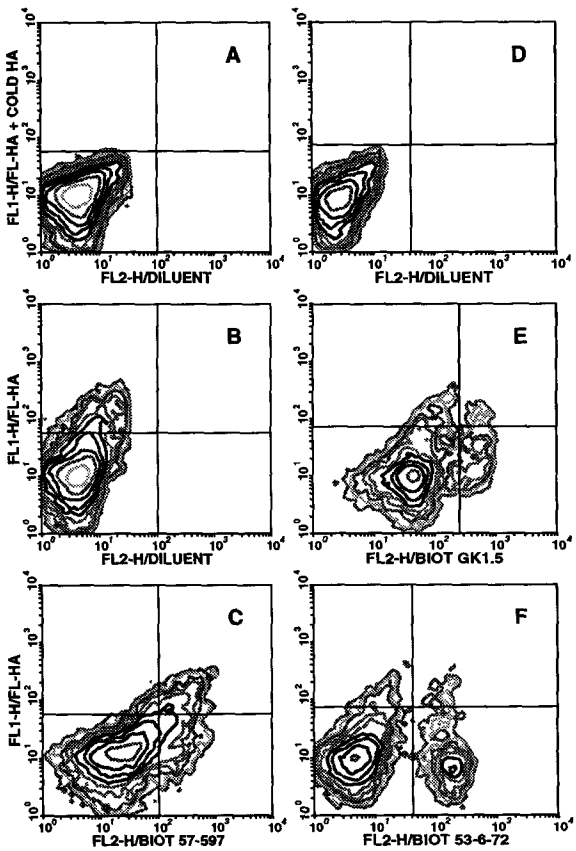

Figure 3. Two-color flow cytometry analysis of HA-binding cells of P815 immunized C57BL/6 mice at 7 d post injection. Total nucleated spleen cells were stained with Fl-HA and biotin-conjugated mAb specific for lymphocyte surface molecules as follows: mAb 57-597 specific for TCR-α/β (*C*); mAb GK1.5 specific for CD4 (*E*); mAb 53-6-72 specific for CD8 (*F*). Biotin-conjugated mAb were detected with phycoerythrin-streptavidin. Controls included cells stained with Fl-HA in the presence of unlabeled HA (250 μg/ml) and with phycoerythrin-streptavidin (*A* and *D*); and cells stained with Fl-HA and phycoerythrin-streptavidin (*B*). *A–C* are from one experiment and *D–F* are from another experiment.

deed, HA-binding cells were depleted from the spleen population by passage over nylon wool. HA-binding cells enriched by cell sorting, and restained with biotinylated mAb against other cell surface markers, were all strongly CD44 positive and otherwise gave the same general staining pattern described above for two-color analysis of total spleen (data not shown).

Cytotoxic Function of HA-binding Cells. HA^+ and HA^- populations were isolated from Fl-HA stained spleen by cell sorting as described in Materials and Methods. The positive and negative populations and control stained and sorted cells (all live cells within the forward scatter range used for the positive and negative populations) were assayed for cytotoxic activity using ^{51}Cr-labeled P815 targets. As can be seen in Fig. 4, almost all cytotoxic activity recovered after sorting was found in the HA-binding fraction of the spleen, which was enriched in cytotoxic activity compared with stained only or control-sorted cells. Thus, the HA-binding cells induced by immunization included the cytotoxic cell population. Since not all of the HA-binding cells are cytotoxic effectors, other functional T cells may be represented in the HA-binding population.

The question as to whether CD44 binding to HA is directly involved in the cytotoxic function of effector cells was addressed by the inclusion of soluble HA and mAb specific for CD44 in the cytotoxic assay. None of the mAbs tested, including several that block CD44-mediated HA binding, inhibited the cytotoxicity of effector cells. Excess HA (335 μg/ml) was slightly inhibitory at low effector/target cell ratios, but this might be due to physical interference with cell–cell contacts because of the increased viscosity of the medium.

Variant CD44 Isoforms. Alternative splicing in the extracellular domain of CD44 can give rise to isoforms of CD44 with higher molecular weights than the hematopoietic or "standard" isoform expressed on all hematopoietic cells (1, 12). Though these variant isoforms are only rarely expressed on normal resting cell types, they are often found on tumor cells and, in certain situations, may contribute to metastasis (13). Using a mAb specific for alternate exons of higher M_r variant isoforms of rat CD44, Arch et al. (14) detected transient expression of variant CD44 isoforms on cells from lymphatic tissues of rats undergoing allogeneic responses. Since no mAbs are yet available to detect variant isoforms of murine CD44, we used reverse transcription of RNA from sorted HA-binding and nonbinding cells, followed by PCR amplification, to determine the CD44 isoform(s) expressed in these populations. As shown by Southern blot of the RT-PCR products in Fig. 5, only RNA of the standard isoform of CD44 was detected in both HA^+ (Fig. 5, lane *A*) and HA^- (Fig. 5, lane *B*) populations. To verify that the procedure would detect larger isoforms of CD44, AKR1 lymphoma cells transfected with CD44 isoforms containing variant exons (described in reference 10) were processed in parallel (Fig. 5, lanes *C* and *D*).

CD44 in Immune Responses. It has been known for some time that CD44 is upregulated upon T cell activation and remains elevated in memory T cells (15–17), but the possible role of CD44 in T cell function is not known. A number of reports have presented evidence that CD44 can deliver costimulatory signals to T cells upon ligation (see references 1 and 2). In these in vitro studies, stimulation through CD44 was mediated by CD44-specific mAbs, as the natural ligand for CD44 was not known. A number of more recent studies have implicated CD44 in immunological responses in vivo: (*a*) Mobley and Dailey (18) found a unique population of $CD44^{hi}$, Mel-14^{lo} $CD8^+$ T cells at the site of graft rejection and as a very rare population in draining lymph nodes; (*b*) Camp et al. (19) showed that mAb-induced stripping of CD44 from the surface of lymphocytes retarded a delayed hypersensitivity response (measured by ear swelling) for 24 h; and (*c*) Rodrigues et al. (20) found that the ability of malaria-reactive cloned cytotoxic cell lines to protect malaria-infected mice correlated with high CD44 expression. These observations suggest that CD44 is participating in immunologic reactions, but the mechanisms are unclear. CD44 may be promoting cell migration into the site of inflammation, as suggested by Camp et al. (19), or enhancing cell–cell contact between effector and target cells, as suggested by Rodrigues

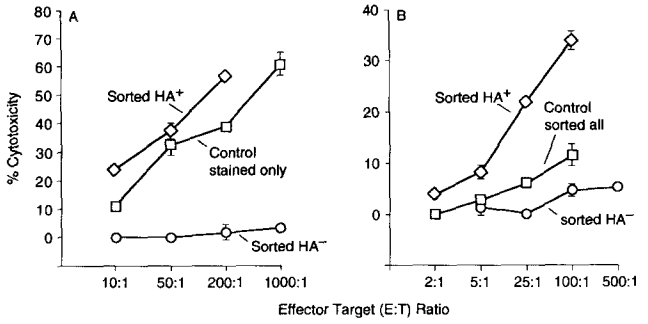

Figure 4. Cytotoxicity of spleen cells from C57BL/6 mice 7 d after immunization separated by cell sorting into HA-binding (HA^+) and nonbinding (HA^-) populations. Fl-HA stained total spleen cells were separated by cell sorting on a FACStar+® as described in Materials and Methods. Sorted and control populations were assayed for cytotoxicity at the indicated ratios of spleen cells (effector) to ^{51}Cr-labeled P815 cells (target) as described in Materials and Methods. *A* and *B* are two independent experiments. In *A*, the control population was stained with Fl-HA as for sorting, but was not sorted. In *B*, the control population was stained with Fl-HA and sorted using the same scatter and dead cell gates used for the experimental populations, but with no windows set on fluorescein fluorescence.

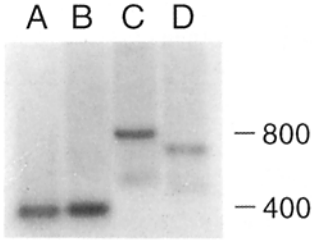

Figure 5. RT-PCR products from sorted HA-binding and nonbinding spleen cells. RNA, cDNA and Southern blot were prepared as described in Materials and Methods. Cells used for RNA were: HA-binding (lane *A*) and nonbinding (lane *B*) spleen cells from 7-d immune mice, isolated by cell sorting; and AKR1 cells transfected with CD44 isoforms containing variant exons v8-10 (lane *C*) and v9-10 (lane *D*), described in He et al. (10) as M2 and M1, respectively. The legend indicates bp, and the expected size of standard CD44 isoform is ~400 bp.

et al. (20), as well as possibly contributing to cell activation signals. In any case, the natural ligand interacting with CD44 in these systems has not been established.

The studies described here demonstrate that the hyaluronan binding function of CD44 is activated during an in vivo immunologic response and that cells capable of CD44-mediated HA-binding are actively participating in the response. However, the significance of HA binding by CD44 in an immune response, and, indeed, the function of CD44 itself, remains to be determined.

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