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

By Jayne Lesley, Nicole Howes, Astrid Perschl, and Robert Hyman

From the Department of Cancer Biology, The Salk Institute, San Diego, California 92186-5800

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 (H2b) and DBA/2 (H2a) mice were purchased from Harlan-Sprague Dawley Inc. (Indianapolis, IN). P815 (DBA/2 origin, H2d) mastocytoma cells were maintained in suspension culture in DME with 10% calf serum. C57BL/6 mice were injected i.v. or i.p. with 107 P815 cells or with 2 × 107 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,
Results and Discussion

Induction of HA-binding Cells in Spleen. C57BL/6 (H2b) mice were immunized with P815 (DBA/2 origin, H2d) mastocytoma cells to induce an allogeneic response. Hematopoietic and lymphoid tissues were assayed by flow cytometry for the presence of cells capable of binding FI-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 FI-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 2. Representative F1-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 F1-HA alone (top), or with F1-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 F1-HA in the presence of excess unlabeled HA (250 μg/ml).

In other experiments not shown, mice immunized by intraperitoneal injection of P815 (10⁷ 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 × 10⁷) 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 F1-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 F1-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-
Indeed, HA-binding cells were depleted from the spleen population by passage over nylon wool. HA-binding cells enriched by cell sorting, and reanimated 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 51Cr-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, 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 CD44hi, Mel-14−, 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.
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 im-
munologic response and that cells capable of CD44-mediated
HA-binding are actively participating in the response. How-
ever, the significance of HA binding by CD44 in an immune
response, and, indeed, the function of CD44 itself, remains
to be determined.

This work was supported by National Institute of Allergy and Infectious Diseases grant AI-31613, Na-
tional Cancer Institute grant CA-17733, National Cancer Institute Core grant CA-14195, and by the
Hansen Foundation.

Address correspondence to Dr. Jayne Lesley, Department of Cancer Biology, The Salk Institute, P.O. Box
85800, San Diego, CA 92186-5800.

Received for publication 1 November 1993 and in revised form 5 April 1994.

References

1. Lesley, J., R. Hyman, and P.W. Kincade. 1993. CD44 and its
interaction with ECM. Adv. Immunol. 54:271.

2. Haynes, R.F., M.J. Telen, L.P. Hale, and S.M. Denning. 1989.
CD44 — A molecule involved in leukocyte adherence and T cell
activation. Immunol. Today. 10:423.

3. Underhill, C. 1992. CD44: the hyaluronan receptor. J. Cell
Sci. 103:293.

4. Miyake, K., C.B. Underhill, J. Lesley, and P.W. Kincade. 1990.
Hyaluronate can function as a cell adhesion molecule and CD44
participates in hyaluronate recognition. J. Exp. Med. 172:69.

5. Aruffo, A., I. Stamenkovic, M. Melnick, C.B. Underhill, and
B. Seed. 1990. CD44 is the principal cell surface receptor for
hyaluronate. Cell. 61:1303.

6. Lesley, J., and R. Hyman. 1992. CD44 can be activated to
function as a hyaluronic acid receptor in normal murine T cells.
Eur. J. Immunol. 22:2719.

7. Lesley, J., Q. He, K. Miyake, A. Hamann, R. Hyman, and
P.W. Kincade. 1992. Requirements for hyaluronic acid binding
by CD44: a role for the cytoplasmic domain and activation
by antibody. J. Exp. Med. 175:257.

8. Murakami, S., K. Miyake, C.H. June, P.W. Kincade, and R.J.
Hodes. 1990. IL-5 induces a Pgp-1 (CD44) bright B cell sub-
population that is highly enriched in proliferative and Ig secre-
try activity and binds to hyaluronate. J. Immunol. 145:3618.

9. Murakami, S., K. Miyake, R. Abe, P.W. Kincade, and R.J.
Hodes. 1991. Characterization of auto-antibody secreting B
cells in mice undergoing stimulatory (chronic) GVH reactions:
Identification of a CD44 hi population that binds specifically
to hyaluronate. J. Immunol. 146:1422.

10. He, Q., J. Lesley, R. Hyman, K. Ishihara, and P.W. Kincade.
1992. Molecular isoforms of murine CD44 and evidence that
the membrane proximal domain is not critical for hyaluronate
recognition. J. Cell Biol. 119:1711.

11. Nottenburg, C., G. Rees, and T. St. John. 1989. Isolation of
mouse CD44 cDNA: structural features are distinct from the
primate cDNA. Proc. Natl. Acad. Sci. USA. 86:8521.

12. Sceatton, G.F., M.V. Bell, D. Jackson, F.B. Cornelius, U. Gerth,
and J. Bell. 1992. Genomic structure of DNA encoding the
lymphocyte homing receptor CD44 reveals at least 12 alterna-
tive spliced exons. Proc. Natl. Acad. Sci. USA. 89:12160.

13. Hofmann, M., W. Rudy, M. Zöller, C. Tölö, H. Ponta, P.
Herrlich, and U. Günthert. 1991. Human tumor cell lines ex-
press CD44 splice variants: the homologous sequences confer
metastatic behavior in rats. Cancer Res. 51:2592.

14. Arch, R., K. Wirth, M. Hofmann, H. Ponta, S. Matzku, P.
Herrlich, and M. Zöller. 1992. Participation in normal immune
responses of a metastasis-inducing splice variant of CD44.
Science (Wash. DC). 257:682.

15. Budd, R.C., J.C. Cerottini, and H.R. MacDonald. 1987. Phen-
otypic identification of memory cytolytic T lymphocytes in
a subset of Lyt-2+ cells. J. Immunol. 138:1069.

16. Budd, R.C., J.C. Cerottini, C. Horvath, C. Bron, T. Pedrazz-
ini, R.C. Howe, and H.R. MacDonald. 1987. Distinction of virgin and memory T lymphocytes. Stable acquisition of
the Pgp-1 glycoprotein concomitant with antigenic stimula-
tion. J. Immunol. 138:3120.

17. Butterfield, K., C.G. Pathman, and R.C. Budd. 1989. A subset
of memory CD4+ helper T lymphocytes identified by expres-
sion of Pgp-1. J. Exp. Med. 169:1461.

18. Mobley, J.L., and M.U. Dailey. 1992. Regulation of adhesion
molecule expression by CD8 T cells in vivo. Differential regu-
lation of gp90 Mel-14 (LECAM-1), Pgp-1, LFA-1, and VLA-4α½
during the differentiation of cytotoxic T lymphocytes induced
by allografts. J. Immunol. 148:2348.

19. Camp, R.L., A. Scheynius, C. Johansson, and E. Puré. 1993.
CD44 is necessary for optimal contact allergic responses but
is not required for normal leucocyte extravasation. J. Exp.
Med. 178:497.

20. Rodrigues, M., R.S. Nussenzweig, P. Romero, and F. Zavala.
1992. The in vivo cytotoxic activity of CD8+ T cell clones
correlates with their levels of expression of adhesion molecules.
J. Exp. Med. 175:895.