Chapter 3: Hyaluronate-CD44 Interactions Can Induce Murine B-Cell Activation

Abstract

CD44 is a widely distributed cell surface glycoprotein whose principal ligand has been identified as hyaluronic acid (HA), a major component of the extracellular matrix (ECM). Recent studies have demonstrated that activation through CD44 leads to induction of effector function in T cells and macrophages. In the current study, we investigated whether HA or mAbs against CD44 would induce a proliferative response in mouse lymphocytes. Spleen cells from normal and nude but not severe combined immunodeficient (SCID) mice, exhibited strong proliferative responsiveness to stimulation with soluble HA or anti-CD44 mAbs. Furthermore, purified B cells but not T cells were found to respond to HA. HA was unable to stimulate T cells even in the presence of antigen presenting cells (APC) and was unable to act as a costimulus in the presence of mitogenic or submitogenic concentrations of anti-CD3 mAbs. In contrast, stimulation of B cells with HA *in vitro,* led to B cell differentiation as measured by production of IgM antibodies in addition to increased expression of CD44 and decreased levels of CD45R. The fact that the B cells were responding directly to HA through its binding to CD44 and not to any contaminants or endotoxins was demonstrated by the fact that $F(ab)'_2$ fragments of anti-CD44 mAbs or soluble CD44 fusion proteins could significantly inhibit the HA-induced proliferation of B cells. Also, HA-induced proliferation of B cells was not affected by addition of polymixin B and B cells from LPS-unresponsive C3H/HeJ strain responded strongly to stimulation with HA. Furthermore, HA but not chondroitin-sulfate, another major component of the ECM, induced B cell activation. It was also noted that injection of HA intraperitoneally, triggered splenic B cell proliferation *in vivo*. Together, the current study demonstrates that interaction between HA and CD44 can regulate

murine B cell effector functions and that such interactions may play a critical role during normal or autoimmune responsiveness of B cells.

Introduction:

CD44 is a cell surface glycoprotein expressed by various lymphoid and nonlymphoid tissues (Haynes et al., 1989; Flanagan et al., 1989). The CD44 molecule has been demonstrated to participate in lymphocyte adhesion to the matrix, lymph node homing and lymphopoeisis (Haynes et al., 1989; Miyake et al., 1990). Recent studies have demonstrated that the CD44 molecule may also participate in lymphocyte activation. Studies from our lab demonstrated that antibodies against CD44 can trigger the lytic activity of the cytotoxic T lymphocytes (CTL) as well as the double-negative T cells that accumulate in the MRL lpr/lpr mice (Seth et al., 1991; Hammond et al., 1993). Similarly, monoclonal antibodies (mAbs) directed against CD44 molecules have been shown to either upregulate (Huet et al., 1989; Shimizu et al., 1989) or downregulate (Rothman et al., 1991) anti-CD3 and anti-CD2 mAb induced proliferation of T cells. Furthermore, certain anti-CD44 mAbs have also been shown to induce proliferation of resting human T cells in the absence of costimulation with anti-CD3 or anti-CD2 mAbs (Galandrini et al., 1993; Pierres et al., 1992; Denning et al., 1990). All of these data together demonstrate that activation via CD44 can trigger effector functions in human T lymphocytes. In addition, antibodies against CD44 have also been shown to activate human monocytes and enhance the natural killer (NK) cell mediated cytotoxicity (Webb et al., 1990; Tan et al., 1993). Despite the recent demonstration that activation via CD44 can trigger effector function in T cells, NK cells and macrophages, whether a similar activation via CD44 would lead to B cell growth and differentiation has not been investigated previously.

Recently, hyaluronic acid (HA) has been reported to serve as an important ligand for CD44 (Lesley et al., 1993). HA is a major

glycosaminoglycan component of the extracellular matrix (ECM). Based on the amino acid sequence deduced from cDNA sequencing, the conserved amino terminal region of CD44 was shown to have sequence similarity to HA binding domains of cartilage link and proteoglycan core proteins (Idzerda et al., 1989). Furthermore, it was reported that various cell types form homoaggregates in the presence of HA (Lesley et al., 1990) and a recombinant fusion protein of CD44 was shown to bind to HA (Aruffo et al., 1990). Having established that HA is the principal ligand for CD44, several recent studies have addressed whether ligation of CD44 via HA would lead to activation of lymphocytes. HA was shown to bind to CD44 on activated T cells and was demonstrated to act as a costimulatory ligand for CD44 in the activation of human T cell effector functions (Galandrini et al., 1993). Recently, it was also demonstrated that HA stimulates the growth of $CD34^+$ umbilical cord blood cells to specifically differentiate into mature eosinophils (Hamann et al., 1995).

There is also growing evidence to suggest that interaction between HA and CD44 may regulate the functions of lymphoid and myeloid cells and that this interaction may play an important role in a variety of disease conditions. For example, large amounts of soluble CD44 were reported to be present in the sera of patients with advanced gastric and colon cancer Guo et al., 1994). Also, at the site of arthritic joints, an inverse relationship between soluble CD44 in the synovial fluid and the number of immigrating blood cells was reported (Tan et al., 1993).

In the current study, we investigated whether interaction between CD44 and HA would trigger the activation of murine lymphocytes and we made a surprising finding that murine B cells but not T cells would respond strongly and directly to stimulation with soluble HA as well as with mAbs against CD44. The activated B cells underwent both proliferation and differentiation. Our data suggest that interaction between CD44 and HA may play an important role at sites of inflammation and participate in the regulation of autoimmune response.

Materials and Methods

Mice:

Adult female C57BL/6 mice, C3H/HeJ, Nude mice (~ 8 months of age) and adult SCID mice were purchased from National Institutes of Health, Bethesda, MD.

Preparation of cells and Purification of Lymphocytes:

Mice were sacrificed and spleens and lymph nodes were removed and single cell suspension was prepared using a laboratory blender in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 10mM HEPES, 1 mM glutamine and 50 µg/ml gentamycin as described (Hammond et al., 1993). The FCS had less than 1 ng/ml of LPS and was purchased from SUMMIT Biotechnology (Ft. Collins, CO). The red blood cells were lysed using ammonium chloride and the cells were resuspended in complete medium after three washings. To purify the T cells, lymph node cells were passed over nylon wool columns and the nonadherent cells were then treated with anti-Ia antibody for 30 minutes on ice, washed twice and then treated with complement for 45 minutes at 37°C followed by washing three times (Kakkanaiah et al., 1992; Nagarkatti et al., 1989). To purify the B cells, the spleen cells were treated with a combination of anti-CD3, anti-CD4, anti-CD8, anti-αβTCR mAbs for 30 minutes on ice and then treated with complement for 45 minutes at 37°C and washed three times (Kakkanaiah et al., 1992). The purity of T and B cell preparations was checked by the ability of the cells to respond to specific mitogens as described in results.

Antibodies and Chemicals:

Monoclonal antibodies, were purified from the hybridomas as described recently (Seth et al., 1991; Hammond-McKibben et al., 1995). The following mAbs were used: anti-CD4 (GK 1.5), anti-CD8 (53-6.72), CD44 (KM201), anti-CD3 (145.2C11) and anti-αβ TCR (H57-597). The mAbs were purified and

concentrated from hybridoma culture supernatants or obtained as ascites by growing the hybridomas in nude mice. $F(ab)'_2$ fragments of anti-CD44 mAbs were prepared by treatment with pepsin and passing the fragments over a protein A column (Seth et al., 1991; Hammond-McKibben et al., 1995). The anti-CD45R Abs (B220) used in this study were purchased from Pharmingen (San Diego, CA). Hyaluronic acid (HA), chondroitin-sulfate and polymixin-B were purchased from Sigma Chemical Company (St. Louis, MO).

Soluble CD44 fusion protein:

The soluble CD44 receptor globulin (CD44 Rg) expressed plasmid was kindly provided by Dr. Ivan Stamenkovic (Massachusetts General Hospital, MA). The CD44 Rg was prepared by transfection of COS cells and was concentrated and purified, as described (Aruffo et al., 1990).

Proliferation Assays

Whole spleen cells, purified T cells or purified B cells were cultured in triplicate at a concentration of $6x10^5$ cells/well, in 96 well, flat bottom tissue culture plates in a final volume of 200 µl of complete medium per well. Medium alone (control), T cell mitogen (anti-CD3 mAbs) or B cell mitogens [LPS (100 µg/ml), anti-IgM antibodies (20 µg/ml)] were added to the wells and the cultures were incubated at 37°C for 48 hours. The cultures were pulsed with ^{3}H-thymidine (2 µCi) 6 hours before harvesting. The cells were harvested using a semiautomated cell harvester (Skatron Inc., Sterling, VA) and the labeled DNA was counted in a liquid scintillation counter.

B cell differentiation:

Splenic B cells (1×10^6) were cultured in 96 well tissue culture plates in the absence or presence of LPS (50 µg/ml), HA (0.5 mg/ml) or anti-CD44 mAbs (1:2 final dilution) in 0.2 ml of complete medium. After 4 - 5 days of culture, the supernatants were harvested, pooled and tested for the presence of IgM antibodies using ELISA.

Enzyme-Linked Immunosorbent Assay (ELISA):

IgM secreted in 4-5 day cultures were measured by an ELISA assay as described (Nagarkatti et al., 1989). Briefly, anti-IgM antibody was coated onto the microwells of an ELISA plate and the wells were blocked with a blocking solution consisting of PBS containing 1% egg albumin. The wells were washed and standard concentrations of purified IgM or a 1:10 dilution of the test supernatant was added to the wells in triplicate. The bound Ig was quantitated by the addition of 1:400 dilution of alkaline phosphatase-conjugated goat anti-mouse IgM and then developed with p-nitrophenyl phosphate. The optical densities were measured on an ELISA reader at a wavelength of 410 and the concentrations of IgM were calculated by linear regression analysis (Nagarkatti et al., 1989).

Studies on expression of CD44 and CD45R on B cells using immunofluorescence Analysis:

To investigate whether stimulation with HA would lead to increased expression of CD44 and decreased expression of CD45R, spleen cells and B cells enriched from normal C57BL/6 mice were cultured in the presence of medium or 0.1 mg/ml of HA for 24 or 120 hours. The cells were harvested and stained with FITC-anti-CD45R or PE-anti-CD44 antibodies for 30 minutes on ice and then washed twice. Next, ten thousand cells were analyzed by a flow cytometer (Epics V,

Model 752).

Statistical Analysis:

The statistical comparison between experimental and control groups was carried out using Studentìs t-test and $p < 0.05$ were considered to be significant.

Results

Stimulation with HA or mAb against CD44 triggers spleen cell proliferation *in vitro*

Inasmuch as, mAbs against CD44 have been shown to induce human T cell activation as well as proliferation (Galandrini et al., 1993; Pierres et al., 1992), we first addressed whether mAbs against murine CD44 or HA would induce proliferation of naive murine spleen cells. In these studies we used antibodies against CD44 obtained from hybridoma, KM201, because previous studies had demonstrated that these antibodies were specific for the hyaluronate binding site on CD44 (Miyake et al., 1990). To this effect, normal spleen cells from C57BL/6 mice were cultured in the presence of T or B cell mitogens, HA or mAbs against CD44, and the cell proliferation was measured after 48 hours of culture. As shown in Figure 3.1, stimulation with HA induced strong proliferation of spleen cells comparable to the proliferation induced by the T and B cell mitogens. Also, mAbs against CD44 (KM201) were able to induce significant proliferation of spleen cells, although this response was markedly lower than the response seen with HA or other mitogens. When spleen cells were cultured with varying concentrations of HA, there was a dose dependent increase in the proliferative responsiveness of spleen cells (Figure 3.2). Also, the response peaked at 48 hours (data not shown). Thus in all subsequent studies, 0.5 or 0.1 mg/ml of HA was used and the proliferative response was studied at 48 hours. Interestingly, when similar concentrations of chondroitin-sulfate, another major component of the ECM, were tested, no significant proliferative responsiveness was detected (Figure 3.2).

T cells fail to respond to stimulation with HA or mAb against CD44

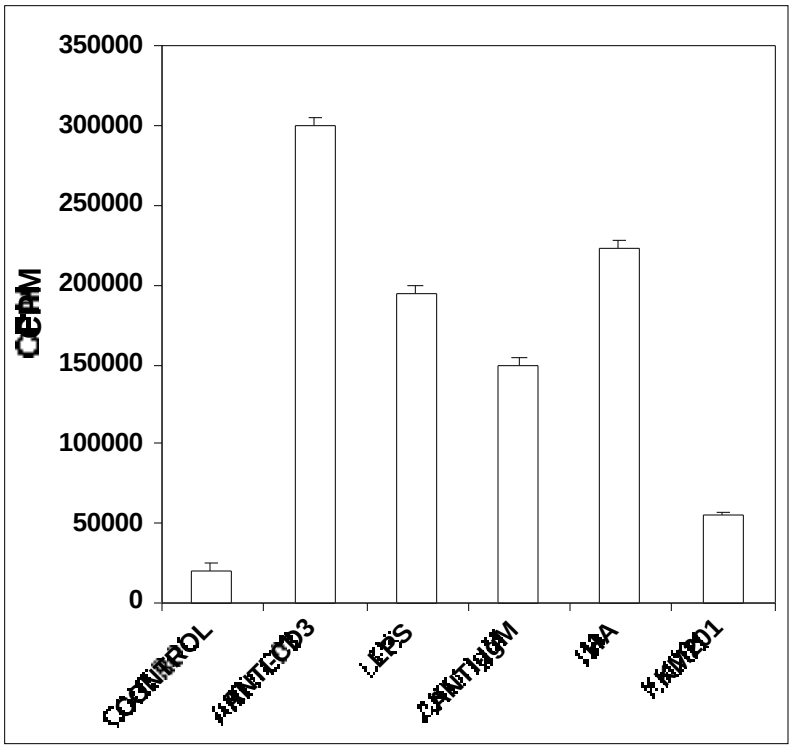

Fig. 3.1: Proliferative responsiveness of spleen cells to stimulation with HA and anti-CD44 antibodies. Spleen cells ($6x10^5$) from normal C57BL/6 mice were cultured in the absence (control) or presence of anti-CD3 (1:200 dilution), LPS (50 µg/ml), anti-IgM (20 µg/ml), HA (0.5 mg/ml) or anti-CD44 mAbs (KM201, 1:10 final dilution). After 42 hours of culture, the cells were pulsed with ^{3}H-thymidine and 6 hours later, the cells were harvested and ^{3}H-thymidine incorporation in the DNA was measured. The vertical bars represent mean counts per minute (CPM) of triplicate cultures ±S.E.

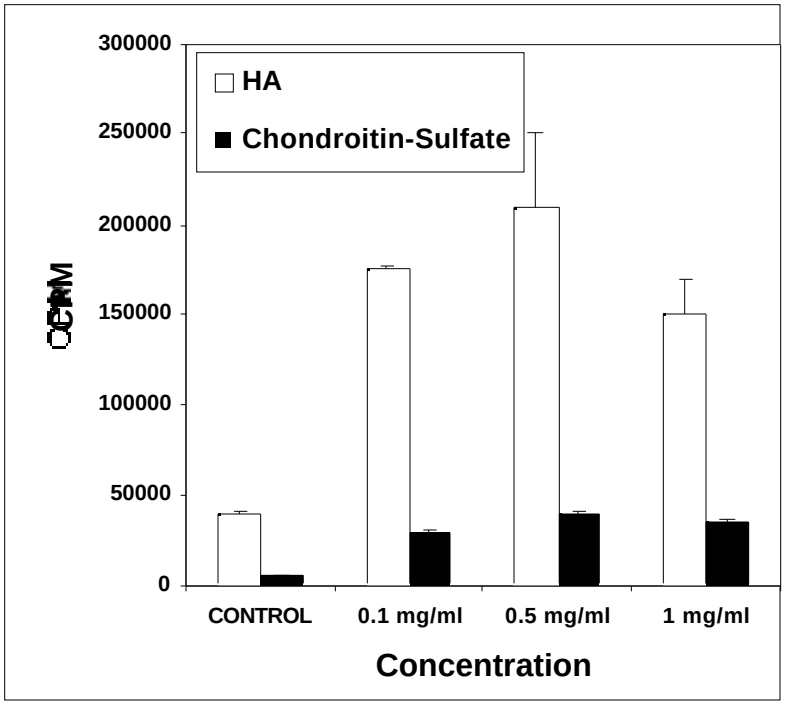

Fig. 3.2: Responsiveness of spleen cells to HA and chondroitin sulfate. Spleen cells ($6x10^5$) were cultured in the presence of increasing concentrations of HA or chondroitin sulfate (0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml) or medium alone (control). After 48 hours, cell proliferation was measured as described in figure 3.1.

We next addressed whether the HA or mAb against CD44 were inducing the proliferation of T cells or B cells found in the spleen cell population. To this effect, T cells were purified by depletion of B cells and tested for the ability to respond to various mitogens as described in Figure 3.1. The data shown in Figure 3.3 demonstrated that the T cells responded to stimulation with anti-CD3 mAbs but not to LPS, thereby suggesting that the responding population consisted of T lymphocytes and did not have any contaminating B lymphocytes. Interestingly, the T cells were unable to respond to stimulation with HA or KM201 antibodies directed against CD44. It should be noted that the purified T cells consisted of non-B accessory cells such as macrophages and dendritic cells which were responsible for giving rise to the proliferative response to soluble anti-CD3 mAbs. To address whether the T cells required the presence of accessory cells such as B lymphocytes to respond to HA, we cultured the purified T cells in the presence of irradiated spleen cells as a source of accessory cells and observed that despite the addition of accessory cells, the splenic T cells were unable to mount a significant response to stimulation with HA or KM201 mAbs (data not shown).

Inasmuch as, previous studies with human T cells have demonstrated that anti-CD44 mAbs could act as a costimulus (Huet et al., 1989; Shimizu et al., 1989), we tested whether HA would act as a costimulus and trigger the proliferation of T cells in the presence of mitogenic or submitogenic concentrations of anti-CD3 mAbs. The results from these studies suggested that HA was able to enhance the proliferation of T cells only modestly when stimulated with either mitogenic or submitogenic concentrations of anti-CD3 mAbs (data not shown). Also, HA was unable to augment the proliferation of murine T cells stimulated with PMA (data not shown). These data together suggested that HA was unable to act as a significant costimulus for murine T cells.

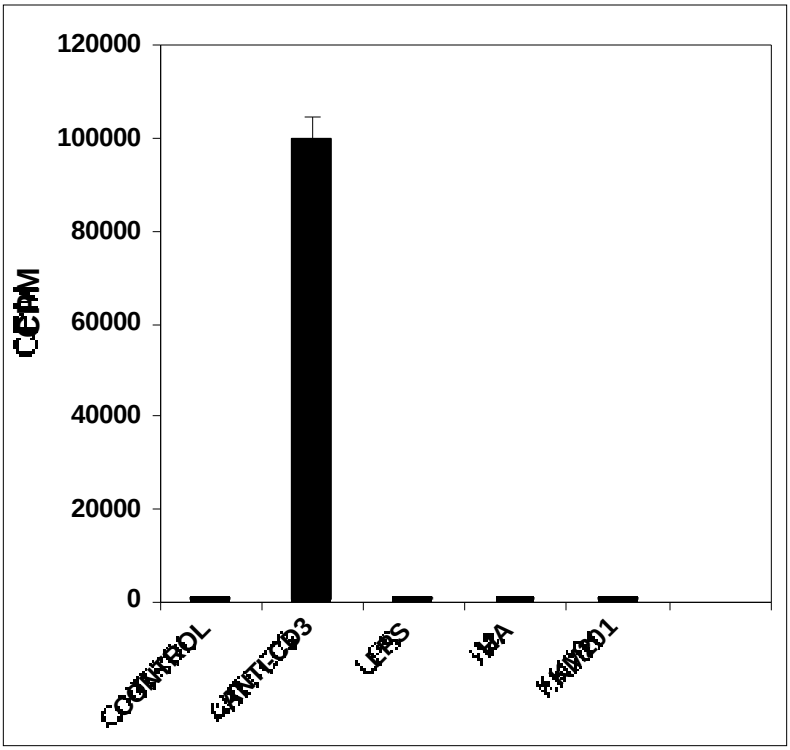

Fig. 3.3: T cells fail to respond to HA. Purified T cells (B cell depleted) from normal C57BL/6 mice were cultured in the presence of anti-CD3 (1:200), LPS (50µg/ml), HA (0.5 mg/ml), or KM201(1:2), or medium alone (control). Cell proliferation was measured as described previously in figure 3.1.

Proliferation of B lymphocytes in response to stimulation with soluble HA

We next addressed whether the B cells found in the spleen cell population were able to respond to HA. To this end, B lymphocytes were purified from the spleen cells by depleting T lymphocytes and were cultured in the presence of either B cell or T cell mitogens or with HA. The data shown in Figure 3.4 indicated that the purified B cell population responded to B cell mitogens but not to the T cell mitogens thereby confirming the purity of the B lymphocytes. Furthermore, the B cells responded strongly to stimulation with HA comparable to other B cell mitogens. These data therefore suggested that HA was stimulating the B but not the T lymphocytes and inducing proliferation. To further confirm that HA was activating B cells but not the T cells, similar experiments were carried out in nude and SCID mice. The data shown in Figure 3.5 demonstrated that nude mouse spleen cells responded strongly to stimulation with LPS and weakly to anti-CD3 mAbs. Furthermore, the nude mouse spleen cells also proliferated strongly in response to stimulation with HA (Figure 3.5). The weak responsiveness of nude spleen cells when compared to normal spleen cells (Fig 3.1), to stimulation with anti-CD3 mAbs may have resulted from a small number of T cells that appear in older nude mice. In contrast, spleen cells from SCID mice failed to respond to both T and B cell mitogens as well as to HA (Figure 3.5). These data together corroborated that HA was able to induce proliferation of B cells.

HA-induced proliferation of B cells is independent of endotoxins:

We also tested whether HA was able to serve as a costimulus to spleen cells cultured with mitogenic or submitogenic concentrations of LPS. The results from these studies revealed that addition of HA to spleen cell cultures in the presence of mitogenic or submitogenic concentrations of LPS, resulted only in an additive effect and failed to enhance the proliferative responsiveness of

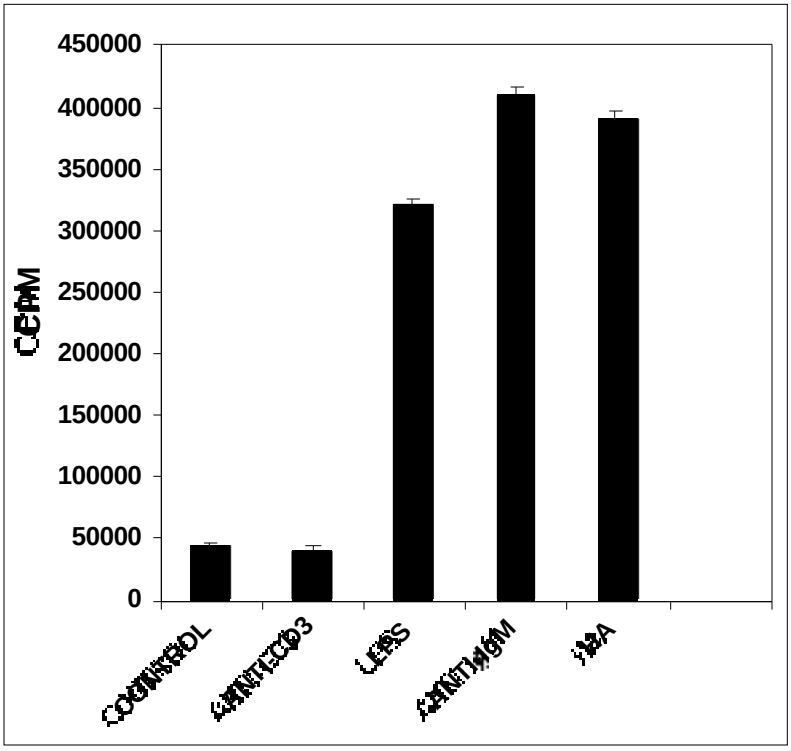

Fig. 3.4: Proliferation of B cells in response to stimulation with HA. B cells were purified from the spleen of normal C57BL/6 mice and cultured in the presence of T or B cell mitogens or with HA as described in Fig. 3.3. and cell proliferation was measured.

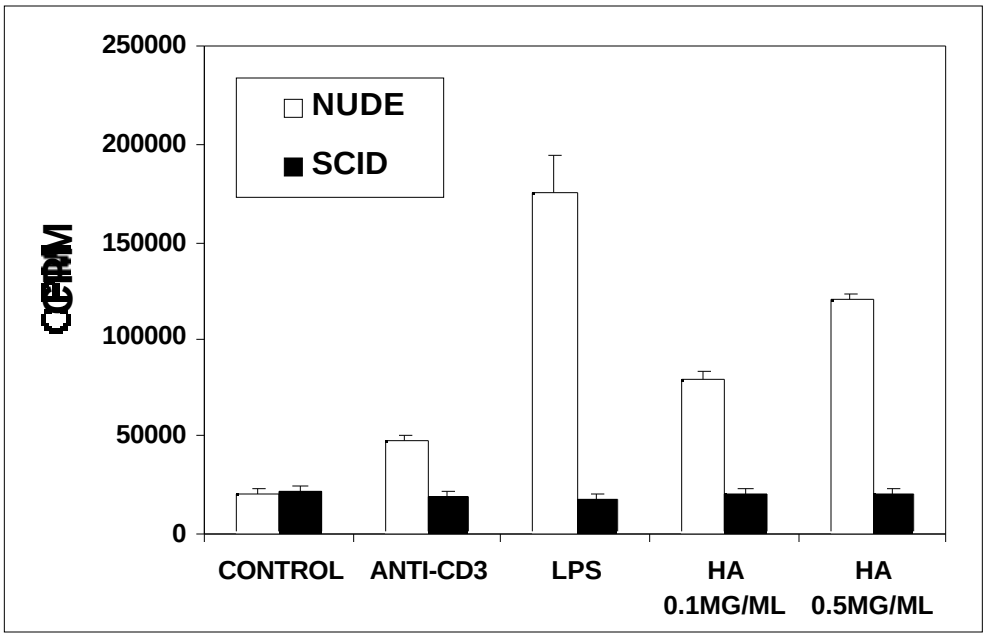

Fig. 3.5: Proliferation of spleen cells from immunodeficient mice in response to stimulation with HA. Spleen cells from nude or SCID mice were cultured in the presence of medium (control), T or B cell mitogens or with HA for 48 hours. Spleen cells from SCID mice were cultured in the presence of medium (control), T or B cell mitogens or with HA for 48 hours. The cell proliferation was measured as described in Fig. 3.1.

the splenic B cells (data not shown). These data also ruled out the possibility that HA may synergistically act with very low levels of endotoxins possibly found in fetal calf serum or HA containing medium. To further exclude the role of endotoxin in B cell proliferation, polymixin B was added to the cultures. It was observed that while polymixin B blocked the LPS-induced B cell proliferation, it failed to inhibit HA-induced B cell activation (Table I). Moreover, spleen cells from LPS-unresponsive C3H/HeJ strain responded strongly to stimulation with HA (Table I). Together, these data excluded the potential role played by endotoxin in HA-induced B cell proliferation.

Role of CD44 in B cell activation induced by HA:

To further corroborate that the proliferation of B lymphocytes was triggered directly by HA, via its binding to the CD44 molecule and to exclude for the possibility of any contaminants being able to induce B cell proliferation, we investigated the effects of addition of $F(ab)'_2$ fragments of antibodies against CD44 (KM201) or soluble CD44Rg to block the proliferation of spleen cells stimulated with HA. As seen from Figure 3.6(A), addition of soluble CD44 fusion protein to the cultures stimulated with HA, led to a dose dependent inhibition of proliferation triggered by HA. Similar concentrations of human IgG used as a control, failed to cause significant inhibition. Furthermore, similar addition of $F(ab)'_2$ fragments of KM201 mAbs also caused a dose-dependent inhibition of HA-induced proliferation (Fig 3.6B). These data conclusively demonstrated that the spleen cells were responding directly to stimulation with HA and furthermore that CD44 was serving as the receptor for HA, leading to proliferation of the spleen cells.

Stimulation with HA leads to B cell differentiation

Table I Demonstration of the fact that the cells responding to HA are B cells.

Mitogen	Cell proliferation (CPM ± S.E.)[a]			
	Expt. 1		Expt. 2	
	Absence of PB	Presence of PB[b]	C57BL/6	C3H/HeJ
None	3,041 ±225	3,370 ±865	5,552 ±223	10,823 ±466
HA	92,428 ±510	80,958 ±3945	80,362 ±1,80	111,222±4,505
LPS 1 µg/ml	137,833±7,909	5,283 ±204	100,080±837	22,569 ±1,318
LPS 5 µg/ml	144,597±6,893	18,491 ±2,377	115,214±8,048	37,718 ±639
LPS 10 µg/ml	156,766±3,383	42,527 ±5,751	124,387±2,694	43,423 ±975

[a] Spleen cells from C57BL/6 mice were cultured with various mitogens for 48 hours and cell proliferation was measured by ^{3}H-Thymidine uptake assay. The results are expressed as mean counts per minute (CPM) ± SE.

[b] Polymixin B(PB) was used at a concentration of 5 µg/ml.

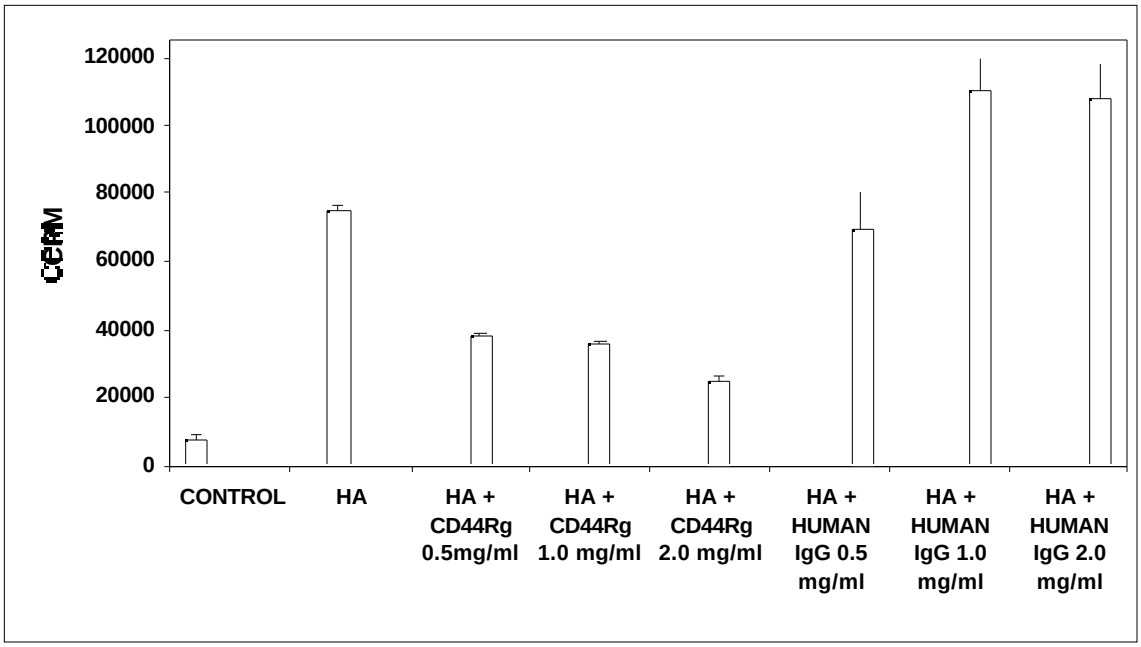

Fig. 3.6A: Addition of F(ab)$'_2$ fragments of anti-CD44 mAbs or soluble CD44 Rg blocks proliferation of spleen cells stimulated with HA. Spleen cells were incubated with medium (control) or stimulated with HA in the presence of soluble CD44 Rg or human IgG. Cell proliferation was measured as described in Fig. 3.1. Bars with an asterisk indicate statistically significant decrease when compared to the controls.

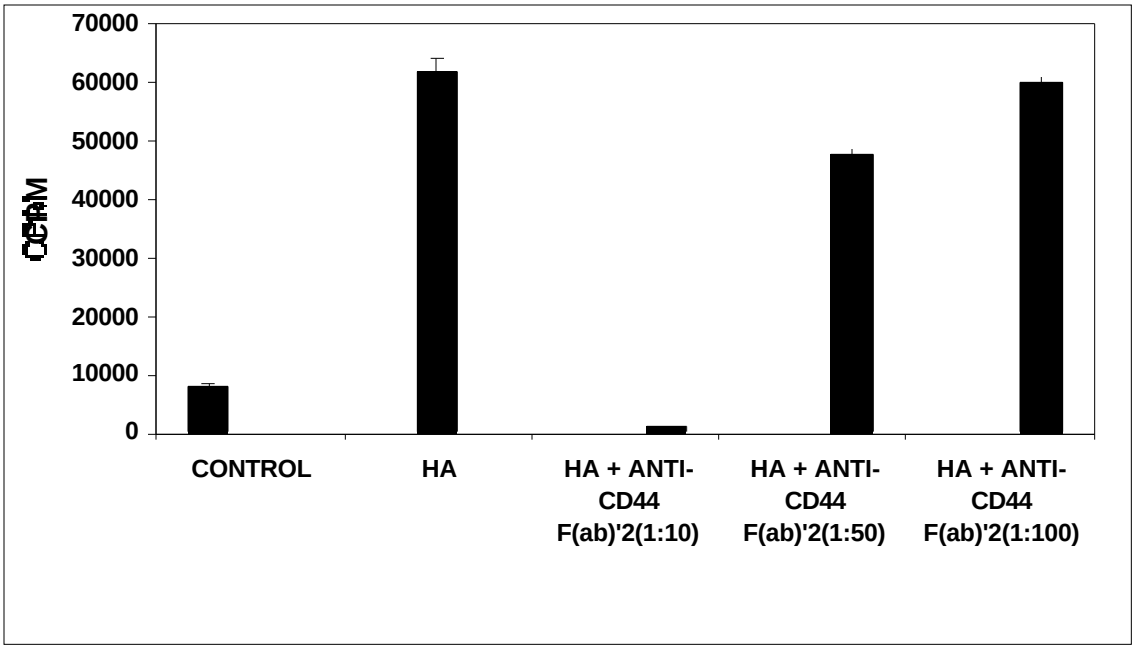

Fig. 3.6B: Addition of F(ab)'$_2$ fragments of anti-CD44 mAbs or soluble CD44 Rg blocks proliferation of spleen cells stimulated with HA. Spleen cells were incubated with medium (control) or stimulated with HA in the presence of F(ab)'$_2$ fragments of anti-CD44 mAbs alone (diluted 1:10, 1:50, 1:100 of hybridoma supernatants). Cell proliferation was measured as described in Fig. 3.1. Bars with an asterisk indicate statistically significant decrease when compared to the controls.

Whether B cells stimulated with HA would undergo differentiation and produce IgM antibodies was next investigated. To this effect, spleen cells were cultured in the presence of medium alone, LPS, HA or anti-CD44 mAbs. After culturing the cells for 4 days, the culture supernatants were harvested and the supernatants were analyzed for the concentration of IgM antibodies using an ELISA assay. The data depicted in Table II shows that stimulation of spleen cells with HA or KM201 mAbs triggered significant B cell differentiation and secretion of IgM antibodies.

It has also been shown that B cell differentiation induces upregulation of CD44 and down regulation of CD45R (Hodgkin et al., 1996). To this effect, spleen cells were cultured in the presence of medium alone or HA for 24 hours or 5 days. The cells were harvested and analyzed for the expression of CD44 and CD45R using flow cytometry. The data depicted in Fig 3.7 suggested that stimulation of spleen cells with HA triggered marked upregulation of CD44 and downregulation of CD45R on day 5 when compared to day 1 of culture, whereas cells cultured in medium alone failed to exhibit such changes (data not depicted). These data are consistent with recent studies which demonstrated that resting B cells that express lower levels of CD44 become strongly positive upon activation and retain high level expression for more than 8 divisions (Hodgkin et al., 1996). Secondly, CD45R (B220) which is expressed at high levels on resting and activated B cells, was downregulated upon B cell differentiation (Hodgkin et al., 1996), thereby suggesting that stimulation with HA induces B cell differentiation.

Administration of HA *in vivo* triggers spleen cell proliferation

Recent studies have demonstrated an increase in soluble CD44 or HA in disease conditions such as autoimmunity or tumor growth (Tan et al., 1993; Guo et al., 1994). We therefore tested whether administration of HA intraperitoneally

Table II B cell differentiation following stimulation with HA

Stimulation	Concentration of IgM (μg/ml)[a]
Medium	57.18±13.91
LPS	160.75±18.04
HA 0.1 mg/ml	100.85±15.4
HA 0.5 mg/ml	123.97±29.85
KM201	89.50±12.34

[a] Spleen cells from C57BL/6 mice were cultured with various mitogens and after 4 days the culture supernatants were harvested and the concentration of IgM was measured using an ELISA.

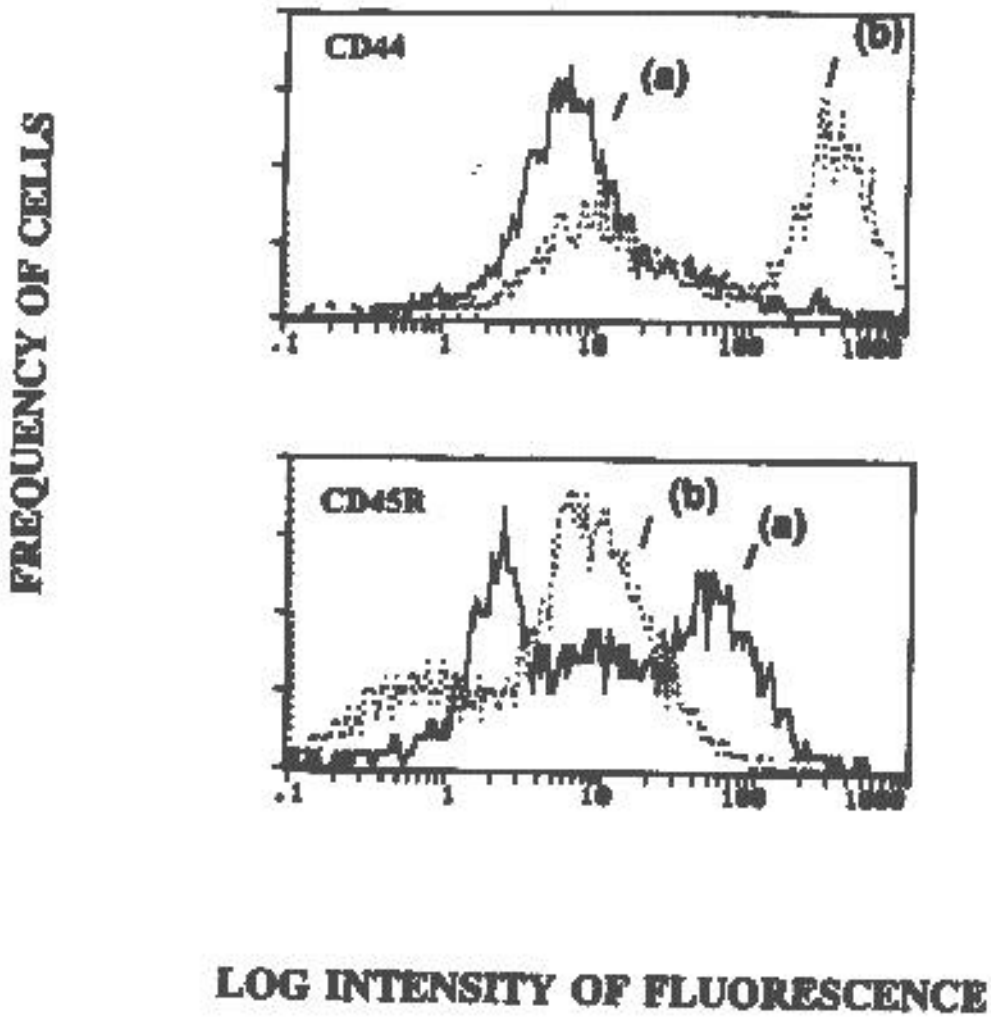

Fig. 3.7: Stimulation with HA triggers B cell differentiation. Splenic B cells or whole spleen cells were cultured in the presence of HA (0.1 mg/ml) for (a) 24 or (b) 120 hours. The cells were harvested and splenic B cells were stained for the expression of CD44 and whole spleen cells were stained for CD45R using flow cytometry. The two histograms were overlayed for comparison. The mean channel number suggestive of the intensity of fluorescence for the histograms was as follows: CD44(a) = 13.7, CD44(b) = 85.9; CD45R(a) = 13.9, CD45R(b) = 7.09.

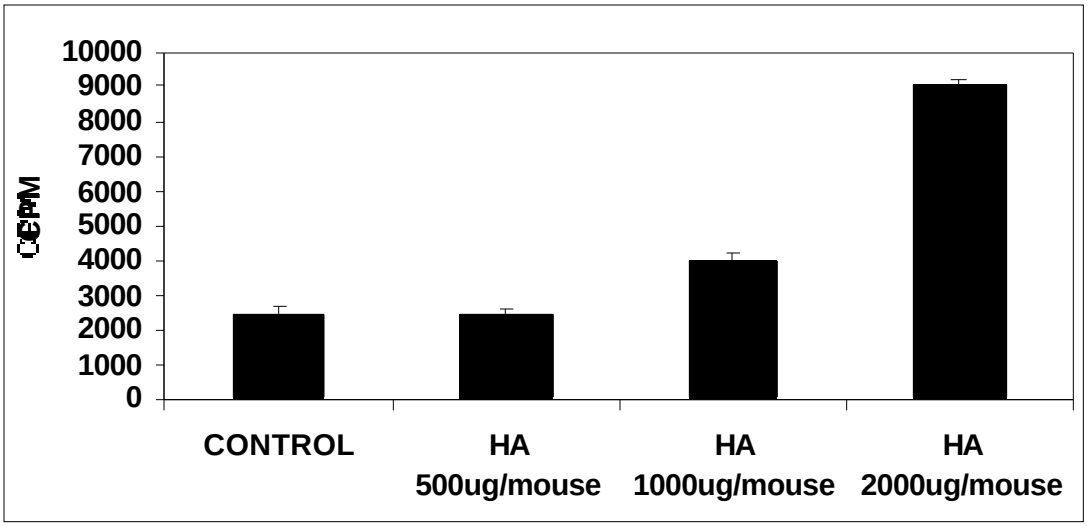

Fig. 3.8: Spleen cells exhibit a dose dependent spontaneous proliferative response to administration of HA *in vivo*. Normal C57BL/6 mice were injected with increasing concentrations of HA or PBS (control) and 48 hours later, the spleens were harvested. The number of cells undergoing spontaneous proliferation were quantitated by direct addition of ^{3}H-thymidine to the cultures and measuring the incorporation during the next 6 hours. Bars with an asterix denote statistically significant difference when compared to the control.

would induce proliferation of B lymphocytes *in vivo*. Normal C57BL/6 mice were immunized with increasing concentrations of HA and 48 hours later, the number of cells undergoing proliferation were quantitated by direct addition of ^{3}H-thymidine to the cultures and measuring the incorporation during the next 6 hours. As shown in Figure 3.8, injection of increasing concentrations of HA triggered a dose dependent increase in the spontaneous proliferative responsiveness of spleen cells. Furthermore, by depleting T or B cells it was observed that the proliferating cells were B cells but not the T cells (data not shown). These data suggested that administration of HA was able to induce significant proliferation of B lymphocytes *in vivo*.

Discussion

In the current study we demonstrated that mAbs against CD44 or HA which serves as an important ligand for CD44 can directly activate B lymphocytes from normal mice. Interestingly, the HA failed to activate T lymphocytes significantly despite the addition of accessory cells or submitogenic concentrations of anti-CD3 mAbs. B lymphocytes activated with HA expressed increased levels of CD44, decreased levels of CD45R and produced IgM antibodies thereby indicating that they were able to undergo differentiation into antibody-secreting cells. Several experiments demonstrated that the B lymphocytes were directly responding to HA and not to any contaminants found in the culture medium and that the proliferation of B lymphocytes was induced by the interaction between HA and the CD44 molecule. Furthermore, administration of HA intraperitoneally into mice triggered proliferation of B cells in the spleen demonstrating that HA can serve as an important activation signal *in vivo.*

Several recent studies have demonstrated that the CD44 molecule in addition to participating in lymphocyte homing or lymphocyte adhesion to the ECM, can also participate in signal transduction thereby regulating the

activation of lymphocytes and monocytes (Haynes et al., 1989; Seth et al., 1991; Hammond et al., 1993; Huet et al., 1989; Shimizu et al., 1989; Rothman et al., 1991; Galandrini et al., 1993; Pierres et al., 1992; Denning et al.,1990). Earlier studies from our lab demonstrated that activation of CTLs via CD44 would lead to induction of cytotoxicity of the target cells (Seth et al., 1991). Furthermore, activation via CD44 could also lead to the induction of cytotoxicity in DN T cells found in autoimmune susceptible MRL lpr/lpr mice (Hammond et al., 1993). We have also shown that the cytotoxicity induced by T cells can be inhibited in the presence of soluble HA thereby suggesting that HA may serve as an important molecule involved in the target cell recognition by the cytotoxic T lymphocytes (unpublished observation). Recent studies have demonstrated that antibodies against CD44 can either induce direct T cell proliferation and IL-2 production (Galandrini et al., 1993; Pierres et al., 1992) or that anti-CD44 antibodies can act as a costimulus enhancing the proliferation of T cells stimulated with mAbs against CD3 or CD2 molecules (Huet et al., 1989; Shimizu et al., 1989). In contrast to the human studies, in the current study we observed that mAb against CD44 or HA failed to induce significant T cell proliferation in the murine model. This observation is in contrast to our earlier finding that mAbs against CD44 can trigger the lytic machinery of the cytolytic T cells (Seth et al., 1991; Hammond et al., 1993). These data therefore suggest that while stimulation via CD44 can lead to signal transduction and possible granule exocytosis, it is not sufficient to induce IL-2 or other autocrine growth factor production which is essential for T cell proliferation. It is also possible that naive T cells which express lower densities of CD44 may not be able to bind to HA (Lesley et al., 1993) which may contribute to its inability to respond to stimulation with HA. In contrast to the murine T cells, the B lymphocytes were able to respond strongly to stimulation with anti-CD44 or HA by proliferation and differentiation. Previous studies from Miyake etal. have demonstrated that interaction between CD44 on

B lymphocyte precursors and an undefined ligand on bone marrow stromal cells is required for B cell lymphopoeisis in vitro (Miyake et al., 1990).

HA is one of the major glycosaminoglycans that forms the hydrated carbohydrate gel in which ECM glycoproteins are immersed (Shimizu and Shaw, 1991). HA consists of a regular repeating sequence of two nonsulfated monosaccharides, D-glucuronic acid and N-acetyl-D-glucosamine. Unlike the major glycosaminoglycans found in the ECM such as chondroitin sulfate and heparin sulfate, HA is not covalently linked to proteins. Lymphoid cell lines, B cell hybridomas and activated B cells have all been shown to bind to purified HA and this binding can be blocked by mAb against CD44 (Shimizu and Shaw, 1991). Several recent studies have suggested that the interaction between CD44 and HA may be critical for the differentiation of the progenitor cell population. Monoclonal antibodies against CD44 have been shown to inhibit B cell lymphopoeisis in long term bone marrow cultures (Miyake et al., 1990; Miyake et al., 1990). Furthermore, HA was shown to stimulate the growth of $CD34^{+}$ selected umbilical cord blood cells into specifically differentiated mature eosinophils (Hamann et al., 1995). Also, HA binding via specific interaction with CD44 was shown to act as a costimulus for human T cell effector functions (Galandrini et al., 1993) and HA was also demonstrated to induce signal transduction as measured by intracellular Ca^{++} mobilization in a murine T cell lymphoma line (Bourguignon et al., 1993). These studies together demonstrate that the interaction between HA and CD44 may play a crucial role in the differentiation and activation of a variety of cells. CD44-HA interactions may also be important in thymic differentiation because HA accounts for more than 40% of the total glycosaminoglycan produced by the thymic epithelial cells (Britz and Hart, 1983).

Previous studies have shown that normal B cells bind to HA to a lower degree than activated B cells, particularly those stimulated with IL-5 (Lesley et al., 1993; Hathcock et al., 1993). Also, there are strain differences in the

functional state of CD44 molecules expressed by unstimulated B cells which could influence HA binding (Hathcock et al., 1993). In the current study the HA may have directly activated the HA-binding $CD44^+$ B cells or alternatively HA may activate a few contaminating macrophages or T cells found in the purified B cell population and trigger cytokines (Denning et al., 1990; Webb et al., 1990) which in turn can induce increased HA-binding ability of B cells and facilitate B cell activation. In this context it was striking to note that B cells cultured with HA expressed marked enhancement in the expression of CD44. Alternatively, *in vitro* culture of cells could enhance the HA-binding ability, as shown recently with human peripheral blood monocytes (Levesque and Haynes, 1996).

Several lines of evidence suggested that the B lymphocytes were responding directly to HA via its binding to the CD44 molecule. The fact that the B cells were not responding to contaminants found in the HA preparation or endotoxin was demonstrated by several experiments. For example, $F(ab)'_2$ fragments of CD44 mAbs or soluble CD44 fusion protein were able to compete with soluble HA and inhibit the proliferation of B lymphocytes. Secondly, the possibility that HA may act synergistically with low levels of endotoxins that may be found in the cultures or that the B cells were responding to the endotoxins was ruled out by demonstrating that HA was not able to act as a costimulus to mitogenic or submitogenic concentrations of LPS. Furthermore, polymixin B failed to inhibit HA-induced B cell proliferation and B cells from C3H/HeJ mice responded strongly to stimulation with HA. These data together demonstrated that interaction between HA and the CD44 molecule was sufficient to trigger the activation of B lymphocytes.

Recent studies have demonstrated that the interaction between CD44 and HA may play an important role in a variety of disease conditions. For example, serum CD44 levels were reported to be significantly reduced in immunodeficient mice and elevated in tumor bearing mice (Katoh et al., 1994). Furthermore, mice undergoing graft versus host reaction as well as those

having autoimmune disease were shown to exhibit increased levels of CD44 in the serum (Katoh et al., 1994). Serum containing high concentrations of CD44 were able to block the binding of HA to CD44 bearing hybridoma cells (Katoh et al., 1994). Such data demonstrate that substantial quantities of CD44 are released into the circulation and this soluble CD44 may be able to interfere with the interaction between HA and CD44 expressed on lymphomyeloid cells. Increased levels of CD44 has been detected in patients with advanced gastric or colon cancer (Aruffo et al., 1990). Furthermore, it has also been demonstrated that in arthritic joints, there is an inverse relationship between soluble CD44 in the synovial fluid and the number of inflammatory blood cells found at that site (Elliott et al., 1993). Such studies therefore indicate that interaction between HA and the CD44 molecule may play an important role in a variety of disease conditions. The fact that administration of HA *in vivo* also leads to activation of B lymphocytes, as seen in the current studies, further confirms the important role played by CD44 and HA. Thus, use of soluble CD44 at localized inflammatory sites may provide important avenues for the manipulation of the immune system in autoimmune diseases.