Cross-linking the Murine Heat-stable Antigen Induces Apoptosis in B Cell Precursors and Suppresses the Anti-CD40-induced Proliferation of Mature Resting B Lymphocytes

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Summary
The murine heat-stable antigen (HSA) is a glycosyl-phosphatidylinositol-linked cell surface protein which has been implicated in cellular adhesion processes, the co-stimulation of CD4+ T cells, and B cell memory. We have recently demonstrated a significant reduction in pro-B and pre-B lymphocytes in transgenic mice that overexpress HSA. We now report that cross-linking HSA with the M1/69 monoclonal antibody induces the apoptosis of cultured B cell precursors in a stomal cell and cytokine-independent manner and that sensitivity to HSA-mediated cell death increases with developmental maturity. The cross-linking of HSA does not induce apoptosis in mature splenic B cells, but instead inhibits their ability to proliferate in response to anti-CD40 + IL-4. Taken together, these data implicate HSA as a potent negative regulator of B cell development and activation.

The murine heat-stable antigen (HSA)1 is a glycosyl-phosphatidylinositol (GPI)-linked protein (1) which has captured the interest of many laboratories for over a decade due to its unusual structure, its fluctuating pattern of expression on many hematopoietic lineages and its role in cellular adhesion, the co-stimulation of T lymphocytes and B cell memory. The molecular cloning (2) and biochemical analysis (2, 3) of HSA has revealed that it is composed of a 27-amino acid polypeptide core which contains several potential N- and O-linked glycosylation target sites. HSA displays considerable size heterogeneity among the hematopoietic lineages (35-70 kD) (2, 3) leading many to speculate that HSA is glycosylated in a cell type-specific manner (2, 3).

The expression of HSA is regulated during B lymphopoiesis. It is not detected on the earliest phenotypically defined pro-B cell found within the B220+Thy-1+ and Fraction A (B220+CD43+HSA-BP-1-) subpopulations, however, it is subsequently upregulated on B220+CD43+ lymphocytes undergoing D-J and V-DJ rearrangements and reaches its highest level of expression on large, cycling, IL-7-responsive pro-B cells (4). High levels of HSA expression are also found on pre-B and immature B cells within the bone marrow (4, 5) and on newly emerged B cells in the spleen (5), but further maturation is accompanied by the downregulation of HSA (5-7). Interestingly, low to absent HSA expression is thus far the only phenotypic marker which distinguishes populations enriched for memory versus primary B cell precursors (6-8).

The precise functional role of HSA remains unresolved. On mature peripheral B lymphocytes, HSA acts synergistically with B7 to co-stimulate CD4+ T cells (9). In the bone marrow, the regulated expression of HSA is critical for normal B lymphopoiesis, since transgenic mice that overexpress HSA within the B220+Thy-1+ and B220+CD43+ subpopulations display a two-fold depletion in the absolute number of cells from these compartments and a 20-fold reduction in the frequency of IL-7 responsive clonogenic progenitors (10). The defect in B lymphopoiesis was found to be inversely related to the level of HSA overexpression and intrinsic to the B cell compartment, since the phenotype was reproducible in BALB/c-nu/nu and SCID mice.

Several models could provide a mechanistic basis for the B cell phenotype found in these mice. HSA may be an adhesion molecule which is necessary for the homing and stabilization of B cell precursors to the bone marrow microenvironment, and thus its overexpression may inappropriately retain B cell precursors on the bone marrow stroma and impede further maturation. This model is supported by the findings that HSA may be a ligand for P-selectin (11). In addition, the HSA-specific monoclonal antibody 79 in...
hibits the aggregation of activated B lymphoblasts (12). Alternatively, HSA may modify the specificity and/or avidity of other adhesion molecules known to be important for B cell differentiation. Indeed, B cell precursors derived from HSA knockout mice display altered VLA-4-VCAM-1 and negligible VLA-4-fibronectin interactions in vitro (13). The observed depletion of B cells in mice which overexpress HSA could also be due to signals transduced either directly or indirectly via HSA which inhibit the expansion and/or survival of early B cell precursors. This model is supported by the ability of an HSA-specific monoclonal antibody to inhibit the anti-CD3-driven proliferative response of CD4+ T cells (14) and to induce Ca2+ fluxes in activated B lymphoblasts (12) and on neuronal cells when complexed with L1 (15). The modulation of HSA might control the responsiveness of B cell precursors to stromal cell adhesion molecules and cytokines, such as IL-7. This latter model is supported by the finding that the most profoundly affected cell population in the HSA transgenic mice are IL-7-responsive clonogenic progenitors.

To more directly evaluate the role of HSA in B cell development and to distinguish among the potential models which address its function, we have assessed the effects of the HSA-specific M1/69 monoclonal antibody on cultured bone marrow progenitors representative of various stages of B cell development and on mature resting B lymphocytes. We demonstrate that the cross-linking of HSA induces apoptotic cell death in early B cell precursors in a manner which is independent of the presence of stromal cells and/or cytokines. We further show that IL-7-responsive clonogenic progenitors and their progeny display the greatest sensitivity to induction of programmed cell death by the antibody despite not having any significant increase in cell surface HSA expression relative to their less mature counterparts. Finally, the modulation of HSA with M1/69 also inhibits the proliferative response of mature resting B lymphocytes to anti-CD40 + IL-4. These data suggest that HSA can negatively regulate early B cell differentiation by serving as an initiator of programmed cell death and can suppress B cell activation by inhibiting the anti-CD40-induced proliferative response of mature resting B cells.

Materials and Methods

Mice. 6-8-wk-old (C57BL/6J × C3H)F1 mice were bred and maintained in microisolators under pathogen-free conditions in the animal facility of the British Columbia Cancer Research Centre.

Antibodies Used in Proliferation Assays and B Cell Cultures. M1/69.16.11HL (rat IgG2b anti–mouse HSA), RG7/11.1 (mouse IgG2b anti–rat IgG2b Fc), FD441.8 (rat IgG2b anti–mouse LFA-1 β2 chain), R1-2 (rat IgG2b anti–mouse VLA-4 integrin receptor), J11d.2, and YE1/48.10 (16) (rat IgG2b anti–Ly-49A) hybridomas were obtained from the American Type Culture Collection (Rockville, MD). The antibodies were purified from hybridoma culture supernatants on protein G affinity columns and according to standard procedures (17). Purified 2C9 (hamster anti–mouse HSA) was generously provided by Dr. Yang Liu (New York University Medical Centre). The 1C10 hybridoma (rat IgG2a anti–mouse CD40) was graciously provided by Dr. Maureen Howard (DNAX, La Jolla, CA).

Flow Cytometry. M1.69.16.11HL was labeled with biotin–succinimidylester (Pierce Chem. Co., Rockford, IL), fluorescein isothiocyanate (FITC) (Sigma Chem. Co., St. Louis, MO), or cyanine 5–succinimidylester (xy-5) (FluoroLink, Pittsburgh, PA) by standard procedures (17). FITC- and phycoerythrin (PE)-labeled anti-B220, FITC- and biotinylated anti-CD43 (S7) and biotinylated anti-c-kit were purchased from PharMingen (San Diego, CA). FITC-labeled Thy.1.2 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). FITC-labeled anti-mouse IgM and PE-labeled anti-mouse IgD were obtained from Southern Biotechnology Associates (Birmingham, AL). R-PE- and FITC-conjugated streptavidin were purchased from Jackson Immunoresearch Laboratories (Westgrove, PA).

To phenotypically analyze B cell precursor populations, single cell suspensions of 1 × 106 cells were stained on ice for 30 min in 100 μl of Hanks' Balanced Salt Solution (HBSS) (StemCell Technologies, Vancouver, BC) supplemented with 5% fetal bovine calf serum (FCS). Cells were washed twice with HBSS containing 5% FCS and resuspended in the same buffer containing 1 μg/ml propidium iodide (PI). Flow cytometric analysis was performed using either a FACSort®, FACScan®, or FACStar®/PLUS flow cytometer equipped with PC LYSIS II® software (Hewlett Packard Co., Palo Alto, CA).

Cell Culture and Hemopoietic Assays

Pro-B Cell Assays. Pro-B lymphocyte cultures were initiated as described by Faust et al. (18). The S17 stromal cell line was maintained in RPMI 1640 supplemented with 5% FCS (no. 6350, lot 906; StemCell Technologies), 50 μM 2-mercaptoethanol, 10 mM sodium bicarbonate, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (complete RPMI) (all cell culture reagents used in this study unless otherwise specified are from StemCell Technologies). Stromal cells were plated into 6-well plates (Falcon 3046; Becton Dickinson and Co., Lincoln Park, NJ) at a density of 1 × 105 cells per 6-cm2 well and expanded to 80% confluency. Bone marrow was obtained by flushing the femurs of 6-8-wk-old mice with complete RPMI using a 22-gauge needle and 2 × 106 bone marrow cells/ml were plated onto pre-established stromal cell layers. Cultures were fed biweekly. Those and all other lymphoid and myeloid cultures described below were maintained in a humidified incubator at 37°C and 5% CO2. Cells were removed from the stroma as previously described (18). The cell surface phenotype and configuration of the immunoglobulin heavy and light chain loci of these cells were determined 3 wk after culture initiation. Consistent with previously published reports, the cultures contained up to 105 cells per 6-cm2 well and were reproducibly greater than 85% B220+ (see Fig. 1 A). The cells were heterogeneous with respect to their expression of the CD43, were BP-1 and expressed the same relative levels of HSA (Fig. 1 A). 11% of the B220+ population expressed surface μ chain, a finding which is supported by the presence of D-J and V-D-J rearrangements on the heavy chain loci as assessed by genomic PCR (data not shown). Light chain rearrangements on the κ loci were also detected (data not shown). Cells in the cultures were dependent on stromal cell contact for survival and displayed a 10-fold expansion in absolute cell number in the presence of S17 and IL-7, but failed to form colonies in the methylcellulose assay (data not shown). Taken together, these cells most closely parallel those found in Fraction B
of adult bone marrow (B220<sup>+</sup>CD43<sup>+</sup>HSA<sup>+</sup>BP-1<sup>-</sup>) as described by Hardy et al. (4), or in the pre-B I population described by Rolink and Melchers (19). This would be consistent with the findings that the inhibition of IL-7 activity in vivo (20, 21) and the generation of IL-7-deficient mice (22) arrests B cell development at the Fraction B stage. Our observation that Fraction B cells display heavy and light chain gene rearrangements is supported by the findings of others (23).

To assess the effects of anti-HSA antibodies on the survival and growth of cells derived from these cultures, the appropriate primary antibodies and corresponding controls were added at various concentrations and allowed to bind to 10<sup>6</sup> target cells for 15 min in the incubator. The number of viable cells at various time points after the addition of antibodies was assessed by counting the number of trypan blue negative cells in the culture. To confirm the data concerning the cell viability and to assess the expression of the B220 antigen, cells were stained with anti-B220 and PI as previously described. All assays were performed in triplicate. Proliferation assays were performed by culturing 2 × 10<sup>5</sup> pro-B cells per well in complete RPMI in 96-well round-bottom plates (Falcon 3077; Becton-Dickinson and Co.) for 24 h. The cells were pulsed with 1 μCi/well [3H]thymidine (Amersham) and were harvested 16 h later with an LKB 1295-601 cell harvester (Uppsala, Sweden). Proliferation was assessed by measuring isotope incorporation with a LKB 1205 Betaplate liquid scintillation counter (Uppsala, Sweden). All proliferation assays were performed in quadruplicate.

**Lymphoid Clonogenic Progenitor Assay.** Bone marrow lymphoid clonogenic progenitors were assayed in methylcellulose cultures containing Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM L-glutamine, 30% FCS no. 6350, 100 μM 2-mercaptoethanol, and 10 ng/ml human recombinant IL-7 derived from the supernatants of cos cells transfected with the human IL-7 gene. Bone marrow (5 × 10<sup>5</sup> cells) was plated into 1 ml cultures in 35-mm Greiner petri dishes (Belco Glass Inc., Vineland NJ) and cultured for 7 d. Colonies containing greater than 50 cells were enumerated 7 d after culture initiation. The cells which initiate methylcellulose colonies are found exclusively within the B220<sup>+</sup>CD43<sup>+</sup> population (M.S. Chappel, unpublished observation) and most closely correspond to Fractions C and C' (4) and the large pre-B II subpopulation (19).

The effects of various antibodies on the frequency of lymphoid colony initiation was assessed by pre-incubating bone marrow with the appropriate antibody at the indicated concentrations for 40 min on ice. Secondary antibodies were added where appropriate. Cells were washed and plated into methylcellulose cultures as described. All assays were performed in quadruplicate.

**Myeloid Clonogenic Progenitor Assay.** Bone marrow (2 × 10<sup>5</sup> cells) was assayed for myeloid clonogenic progenitors in 1 ml methylcellulose cultures containing IMDM supplemented with 30% FCS no. 6250, 2 mM L-glutamine, 3 U/ml erythropoietin, and 2% spleen cell conditioned medium. Cells were cultured for 14 d and colonies were enumerated and scored as being derived from erythroid (BFU-E) or granulocyte-macrophage (CFU-GM) progenitors according to established criteria.

Experiments to analyze the effects of various antibodies on the initiation of myeloid and erythroid colony formation were performed as described for the lymphoid clonogenic progenitor assay.

**IL-7-Responsive B Cell Precursor Culture.** IL-7-responsive B cell precursors were cultured as described by Griffiths et al. (24). Bone marrow cells were cultured at 2 × 10<sup>6</sup> cells/ml in IMDM supplemented with 20% FCS no. 6350, 50 μM 2-mercaptoethanol, 10 ng/ml human recombinant IL-7, 10 mM sodium bicarbonate, 10 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (complete IMDM). After 4 d in culture, the cells were fed with 1 vol of complete IMDM. The phenotype of these cells is depicted in Fig. 4 A. The cells in the culture were reproducibly greater than 95% B220<sup>+</sup> and heterogeneous with respect to CD43 expression. IgM was expressed on 22% of the B220<sup>+</sup> population, consistent with the presence of V-D-J rearrangements on the heavy chain loci of cells from these cultures. Rearrangements were also found on the k light chain genes. Although others have reported lymphoid colony formation by these cells (24), we have found that the proliferative potential of cells from these cultures is limited, since no colonies were evident when the cells were plated into methylcellulose cultures at plating densities of up to 10<sup>6</sup> cells per ml (data not shown). Taken together these cultures likely represent a mixture of Fraction C (B220<sup>+</sup>CD43<sup>+</sup>HSA<sup>+</sup>BP-1<sup>-</sup>), Fraction D (B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>+</sup>) and Fractions E and F (B220<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>) (4) or large and small pro-B II cells (19). The effects of anti-HSA antibodies on these cells were assessed as previously described for the pro-B lymphocyte population. Proliferation assays were performed as previously described using complete IMDM rather than complete RPMI.

**Anti-CD40-induced Proliferation of Mature Resting Splenic B Cells.** Spleen cells from 6-wk-old (C57BL/6J × C3H)F<sub>1</sub> mice were teased into complete RPMI. Red blood cells were lysed in 0.165 M ammonium chloride, pH 7.4. T cells were removed by two successive treatments with a 1:50 dilution of anti-mouse Thy-1.2 ascites (Cedarlane Laboratory, Ontario, Canada), 1:5 dilutions of GK 1.5 and 3.155 hybridoma supernatants (anti-mouse CD4 and CD8, respectively; both from the American Type Culture Collection) on ice for 30 min after by complement depletion at 37°C for 40 min using a 1:7 dilution of Low-1 ox-Rabbit complement (Cedarlane Laboratories, Ontario, Canada). Small, high buoyant density B lymphocytes were then purified on discontinuous percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Cells from the 1.085 g/ml/1.079 g/ml interface were harvested and washed in complete RPMI. Cells purified in this manner were >95% B220<sup>+</sup>, >97% Ig<sup>-</sup>, and >95% of these cells displayed small forward scatter. To assess the effects of anti-HSA antibodies on anti-CD40 driven proliferation of resting B cells, 1 × 10<sup>5</sup> purified cells in complete RPMI supplemented with 1% of hybridoma supernatant containing murine II-4 (generously provided by Dr. Yang Liu, New York University Medical Center) were plated into round-bottom 96-well tissue culture plates (Falcon 3077, Becton-Dickinson and Co.). Antibodies were added to a final concentration of 20 μg/ml. After 48 h in culture, the cells were pulsed for 6 h with 1 μCi/well [3H]thymidine (Amersham) and harvested as previously described.

**Preparation of M1/69 Fab Fragments.** M1/69 Fab was prepared as described (17).

**Apoptosis**

**Flow Cytometry.** Nuclear DNA fragmentation in apoptotic cells was assessed by measuring propidium iodide content of the cells as previously described (24). Cells (10<sup>6</sup>) were fixed for 20 min on ice in 75% ethanol. Cells were then washed in PBS and resuspended in PBS containing 20 μg/ml propidium iodide and 5 μg/ml RNase A. After RNA digestion at 37°C for 30 min, the cells were analyzed for DNA content by flow cytometry using a FACSort<sup>®</sup> flow cytometer equipped with a doublet discrimination module and PC LYSIS<sup>®</sup> software (Hewlett Packard Co., Palo Alto, CA).
Results

The effect of cross-linking HSA was evaluated in four developmentally distinct populations of murine B lymphocytes. Bone marrow cultures were used as a source of B cells representative of various stages of early B cell development. The following developmental stages were detected and assessed in these cultures: (a) stromal cell-dependent, IL-7-independent pro-B cells, (b) stromal cell-independent, IL-7-dependent clonogenic progenitors, and (c) the non-proliferating progeny of b. The most mature population studied was mature, resting splenic B lymphocytes.

Anti-HSA Antibodies Induce Rapid Cell Death in Cultured Pro-B Lymphocytes. To assess the effects of HSA modulation on the development of B cell precursors from stroma-supported, IL-7-deficient cultures, the HSA-specific M1/69 antibody (IgG2b) ± secondary antibody (RG7) was added to the media at culture initiation and was maintained at subsequent feedings for three weeks. In the presence of M1/69 alone (5 μg/ml), cell recovery was 44.3 ± 10.0% of untreated control cultures (n = 4, P <0.05) (Fig. 1 B). In the presence of a secondary antibody, there was a 400-fold mean reduction to 0.24 ± 0.11% of control, suggesting that the extent of HSA cross-linking is quantitatively related to the diminished survival and/or proliferation of the cells. As previously reported by others (26), the yield of precursors in the presence of an isotyped matched IgG2b anti-LFA-1 antibody (5 μg/ml), either alone or in the presence of cross-linker (2.5 μg/ml), was indistinguishable from that of the untreated cultures, despite its level of surface expression is comparable with that of HSA (data not shown). An irrelevant IgG2b antibody (anti-Ly49A) similarly had no effect on the yield of precursors (Fig. 1 B) and similar results were reproducibly obtained in the presence of heat-inactivated FCS (data not shown) ruling out significant effects mediated by the Fcγ receptor and/or complement activation.

We next sought to determine whether M1/69 exerted...
...it was thought to be a specific B cell precursor. Total bone marrow cells were incubated with M1/69 (5 μg/ml) ± secondary antibody (2.5 μg/ml) for 1 h on ice and washed extensively before plating on the S17 stromal cell layer. The cultures remained free of M1/69 at all subsequent time points. This single exposure of the cells to M1/69 before culture initiation failed to diminish the yield of cells at culture maturity (data not shown), suggesting the presence of a more primitive, M1/69-insensitive cell.

To assess the effect of the antibody on the culture-initiating cell, which is thought to be Thy-1<sup>+</sup>Lin<sup>-</sup>F<sup>-</sup>A<sup>-</sup>(27, 28), or whether the targets of the antibody were the more mature B220<sup>+</sup> B cell precursors. Total bone marrow cells were incubated with M1/69 (5 μg/ml) ± secondary antibody (2.5 μg/ml) for 1 h on ice and washed extensively before plating on the S17 stromal cell layer. The cultures remained free of M1/69 at all subsequent time points. This single exposure of the cells to M1/69 before culture initiation failed to diminish the yield of cells at culture maturity (data not shown). In contrast, B220<sup>+</sup> cells derived from 3-wk-old cultures displayed exquisite sensitivity to M1/69-mediated cell death (Fig. 2 A). Cell number was reduced to 38.79 ± 5.30% of control after a 24-h exposure to the antibody (5 μg/ml), and was further diminished to 1.18 ± 1.10% of control in the presence of a secondary antibody (2.5 μg/ml) (n = 4, P <0.05). Extensive washing of the stroma after a 24-h exposure to M1/69 + R G7 allowed the cultures to completely recover to numbers comparable to untreated cultures (data not shown), suggesting the presence of a more primitive, M1/69-insensitive cell.

To assess whether the effects of the antibody were independent of stromal cell contact and stromal cell-derived cytokines, cells were cultured at 10<sup>5</sup> cells/well in stromal cell-free cultures for 24 h with M1/69 (5 μg/ml) ± secondary antibody (2.5 μg/ml). M1/69 reduced the number of viable cells in the culture to an average of 44.39 ± 2.08% of control, with a further reduction to 11.31 ± 1.08% of control in the presence of secondary antibody (Fig. 2 B) (n = 4, P <0.05). Furthermore, M1/69 ± cross-linker similarly inhibited [3H]thymidine incorporation in a suspension culture over a 24-h period (Fig. 2 B). This effect is epistemic-independent, since the HSA-specific 20C9 antibody also reduced the number of viable cells in the culture to 55.83 ± 5.81% of control. Furthermore, this effect is dependent on the physical cross-linking of cell surface HSA, since the monovalent M1/69 Fab fragment (5 μg/ml) failed to diminish the number of B cell precursors over the duration of the experiment. Similarly, isotypically matched anti-LFA-1 and anti-VLA-4 controls had no effect on the yield of B cell progenitors. The inability of anti-VLA-4 to diminish the yield of cells in the absence of stromal cells (compare Fig. 1 B with 2 B) highlights its critical role in stromal cell adhesion processes as reported by others (26) and suggests that the mechanismic basis for the M169-mediated loss of precursors observed in the presence of stromal cells is distinct from that of anti-VLA-4. Finally, the cross-linking of M1/69 was also found to decrease the number of Balb/c B cell precursors derived from similar culture conditions, indicating that the effects of HSA modulation were strain-independent (data not shown).

M1/69 exerts its effects over a very short time course, since there was a 50% relative loss of viable cells within 5 h after the addition of M1/69 alone (5 μg/ml) to stroma-free suspension cultures (n = 2) (Fig. 2 C). Under the same conditions, the relative loss of viable cells was dose-dependent over a two log concentration range of M1/69 in a 24-h experiment, achieving a 50% relative reduction in the number of viable cells at a concentration of 5 μg/ml (n = 2) (Fig. 2 D).

We next wished to determine whether M1/69 inhibited the proliferation of B cell precursors, whether it directly induced cell death. The propidium iodide staining profile of cells derived from a representative dose response experiment (Fig. 3 A) revealed that untreated cultures consisted of 60% viable cells, whereas in the presence of 5 μg/ml M1/69 reduced viability to 25% of control. Cell viability was further reduced to 6% of control in the presence of 20 μg/ml M1/69. Consistent with the finding of extensive cell death, M1/69-treated cultures show a quantitative increase in DNA fragmentation with time, as measured by a sub-diploid quantity of DNA in propidium iodide-stained nuclei (Fig. 3 B). Apoptotic cell death was further confirmed electrophoretically by the presence of the character-
istic pattern of DNA fragmentation obtained from genomic DNA prepared from M1/69-treated cells after 4 h in culture (Fig. 3 C). Collectively, these data demonstrate that the engagement of HSA rapidly induces apoptotic cell death in early B cell precursors in a dose-dependent manner.

**HSA Cross-linking Prohibits Lymphoid Colony Initiation by IL-7-Responsive Clonogenic Progenitors.** To assess the effects of cross-linking HSA on more mature lymphoid clonogenic progenitors, adult bone marrow cells (3 × 10⁵ cells/ml) were incubated with 5 μg/ml M1/69 ± 2.5 μg/ml secondary antibody for 1 h on ice and were assayed for IL-7-responsive clonogenic progenitors in methylcellulose cultures. M1/69 reduced the frequency of clonogenic progenitors from 6.02 × 10⁻⁴ ± 4.41 × 10⁻⁵ to 2.21 × 10⁻⁴ ± 9.80 × 10⁻⁵, whereas the presence of secondary antibody further reduced colony formation to 1.53 × 10⁻⁵ ± 0.8 × 10⁻⁵ or 3% of control (n = 3) (data not shown). All isotype-matched controls and the monovalent M1/69 Fab fragment failed to diminish the frequency of colony formation. Dose response studies revealed that half maximal inhibition of colony formation occurs at an M1/69 concentration of 1.0 μg/ml (n = 2) and that 20C9 and J11/d similarly inhibit lymphoid colony initiation in a dose-dependent manner, indicating that the inhibitory effect of HSA engagement is epitope-independent (data not shown). Taken together, these data demonstrate that the specific modulation of HSA on lymphoid clonogenic progenitors negatively regulates their expansion and that this effect is dependent and proportional to the extent of cell surface HSA cross-linking.

The addition of IL-7 at concentrations of up to 100 ng/ml in the methylcellulose cultures failed to rescue lymphoid colony formation, suggesting that if the cross-linking of HSA negatively regulates cytokine responsiveness, this effect cannot be reversed with increased levels of IL-7.

**The Engagement of HSA Induces Cell Death in the Progeny of IL-7-Responsive Clonogenic Progenitors.** To assess the ef-
effects of the M1/69 antibody on the progeny of IL-7-responsive clonogenic progenitors, bone marrow was cultured in IL-7 for 5 d as previously described (24) and cells were then treated with anti-HSA antibodies. The mean viable cell recovery from M1/69-treated cultures was 1.93 ± 0.83% of control after 24 h (n = 3, P <0.05) (Fig. 4 B). The effects of HSA ligation were epitope independent and dependent on the physical cross-linking of the antigen (Fig. 4 B). All results were confirmed in a proliferation assay (Fig. 4 C). M1/69 exerts its effects rapidly, since there is a 50% loss of cells within 2.5 h (n = 2) (Fig. 4 D). This is twice as fast as that found for IL-7 unresponsive precursors, which experience the same reduction within 5 h. Consistent with the enhanced sensitivity of this cell population, half maximal inhibition of cell recovery occurred at an M1/69 concentration of 0.30 μg/ml (n = 2) (Fig. 4 E). This suggests that these cells are approximately threefold more sensitive to HSA modulation than are IL-7-responsive clonogenic progenitors and are 16-fold more sensitive than pro-B cells grown on S17 in the absence of IL-7 (Fig. 2 D).

Fig. 5 A demonstrates that there is a dramatic, dose-dependent loss of viable cells in M1/69-treated cultures, which was accompanied by extensive DNA fragmentation which increased over time following exposure to M1/69 (Fig. 5 B). The presence of apoptotic cell death was confirmed qualitatively by agarose gel electrophoresis of DNA obtained from control and M1/69-treated cells (Fig. 5 C). Taken together, these data suggest that the engagement of HSA on IL-7-expanded B cell precursors negatively regulates these cells by inducing rapid apoptotic cell death.

M1/69 Induces Apoptotic Cell Death Within the Bone Marrow Compartment in a Lymphoid-specific Manner. M1/69, in the presence and absence of secondary antibody had no effect on the frequency of GEMM, G/M, and BFU-E progenitors (three independent experiments in quadruplicate), despite their expression of liSA (16, 29, 30), suggesting that the direct targets of HSA-mediated cell death are B cell precursors (data not shown).

HSA Cross-linking Inhibits the Anti-CD40-induced Proliferation of Mature Resting Splenic B Cells. To determine if mature resting B cells, which also express HSA, are susceptible to HSA-mediated apoptosis, splenic B cells were purified, density fractionated and cultured in the presence and absence of the M1/69 + secondary antibody for 6 h. Apoptosis, as assessed by flow cytometry and DNA electrophoresis, was not detected in either resting B cells, or cells
activated for 48 h with anti-CD40, IL-4 anti-CD40 + IL-4, or LPS (data not shown). Nevertheless, the cross-linking of HSA on mature resting B cells inhibited their proliferative response to anti-CD40 by 50% (n = 3, P < 0.05) (Fig. 6). This was further exacerbated by the presence of IL-4, since cells stimulated with CD40 + IL-4 experienced an 80% mean reduction in thymidine incorporation as result of HSA cross-linking. In contrast with its effects on anti-CD40-induced proliferation, HSA cross-linking had no effect on LPS-mediated proliferation (data not shown). Taken together, this data suggests that HSA can provide a signal which suppresses the activation of mature B cells by a mechanism which is distinct from HSA-mediated apoptosis of early B cell precursors.

Discussion

Our findings have revealed two novel properties of HSA which may shed light on its potential roles in B cell development and activation. Cross-linking HSA on three functionally distinguishable precursor populations was found to significantly diminish their survival in a dose-dependent manner through the induction of apoptosis, and also markedly reduced the anti-CD40 proliferative responses of mature, resting splenic B cells. Taken together, these data support a role for HSA in the negative regulation of the survival of early B cell precursors and the proliferative response of mature, peripheral B cells.

Cultured B cell precursors displayed increasing sensitivity to HSA-mediated cell death with developmental maturity.
despite having the same relative levels of surface HSA expression. This finding could be associated with the stage-specific regulation of molecules known to be involved in regulating cell death, such as bcl-2 (31), bcl-x (32), bax (33), c-myb (34), p53 (35), as well as tyrosine kinases which have been shown to be activated in B cell precursors undergoing apoptosis (36). Indeed the progressive down regulation of bcl-2 in Fraction C and D populations (4, 24) support this hypothesis. Conversely, since Fraction C cells are rapidly proliferating in response to IL-7 (4, 19), the higher frequency of cells in S and G2 phases of the cell cycle could similarly render them more susceptible to M1/69-mediated cell death. The enhanced sensitivity of the IL-7 responsive populations could also point to a role for HSA in the conversion of a proliferative signal into an inhibitory one, a model which has been proposed by others who have shown that the induction of apoptosis by the cross-linking of CD38 on human B cell precursors (37) and CD43 on human bone marrow progenitors (38) is similarly dependent on the presence of and responsiveness to cytokines. Alternatively, developmentally regulated, differential glycosylation of HSA could account for stage-specific lateral interactions with molecules which participate with HSA in the induction of apoptosis.

The ability of M1/69 to induce apoptosis thus far appears to be restricted to the lymphoid compartment, since colony initiation by myeloid and erythroid progenitors was unaffected by the antibody, despite their expression of HSA (16, 29, 30). Interestingly, the ligation of HSA on all murine B cell lines tested thus far, including several Abelson-transformed B cell lines, WEHI 231, Bal 17, and A20 failed to inhibit proliferation or induce apoptosis (data not shown).

Two caveats argue against the simple model of HSA as an adhesion molecule. HSA expression increases as B cell precursors become less stromal cell-dependent (4) with stroma-independent precursors being the most sensitive to HSA-mediated apoptosis. Second, M1/69 Fab failed to induce cell death, suggesting that the steric blocking of intercellular interactions is not responsible for our observations. An alternative model is that HSA transduces signals either directly, or by virtue of its association with a complex of molecules, whose activity may also be regulated by HSA. The findings that M1/69 directly induces apoptosis in a cross-linking-dependent manner, that the cross-linking of HSA with mAb 79 induces a Ca²⁺ flux on its own (12) and when it is complexed with other molecules (15), and that other GPI-linked proteins such as Thy-1 (39), decay accelerating factor (40), CD59 (41), and the human homologue of HSA, CD24 (41), are found in close physical association with src tyrosine kinases also strengthens this hypothesis.

This work demonstrates that HSA cross-linking has significant and differential effects at several stages of B cell development. In a physiological context, the ligation of HSA in the bone marrow may participate in mechanisms that regulate the production of B cell precursors. Indeed, the cells which display the greatest sensitivity to HSA ligation in vitro are coincident with those lost in the developmental transition from large pre-B cells (Fractions C-C') to small pre-B cells (Fraction D) observed during B cell genesis in vivo (42, 43), thus, HSA may be involved in the elimination of precursors with aberrant or non-productive heavy and light chain gene rearrangements, or cells which display self specificity. Alternatively, HSA may be involved in the elimination of cells which remain inappropriately adhered to the stroma, hence, the ligation of HSA by continued attachment to the bone marrow matrix would rapidly induce cell death in cells which have acquired IL-7 responsiveness and enhanced sensitivity to HSA-mediated cell death.

The ligation of HSA on mature B cells may serve to limit the activation and expansion of non-specific bystander B cells in the propagation of the immune response. This model has similarly been proposed to explain the enhanced sensitivity of anti-CD40-activated mouse (44) and human (45, 46) B cells to Fas-mediated cell death in the absence of antigen receptor cross-linking in vitro. Intriguingly, a small population of cells appear to be resistant to the effects of HSA cross-linking, since thymidine incorporation of anti-CD40-activated cells remains unchanged at M1/69 concentrations of up to 100 µg/ml (data not shown). These cells may represent memory B cell precursors, which express very low levels of the HSA antigen (6, 7). Since relative levels of HSA expression on peripheral B lymphocytes distinguishes primary from memory precursors, signals transduced through HSA during early development may participate in the bifurcation of B cell lineages and account for the functional differences observed between B cell populations expressing relatively different levels of surface HSA (6, 7).

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