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Heparan Sulfate and Heparin Enhance ERK Phosphorylation and Mediate preBCR-Dependent Events during B Lymphopoiesis

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As B lineage cells develop, they interact with cells, proteins, and extracellular matrix components of the surrounding microenvironment. In vitro, one critical checkpoint for developing cells occurs as they lose responsiveness to IL-7. These cells require contact with either stromal cells or other B lineage cells to mature. Our results demonstrate that heparan sulfate and heparin are able to promote this transition when added exogenously to the culture system or when heparan sulfate-bearing cell lines are cocultured with primary B cell progenitors. Addition of heparan sulfate or heparin to LPS-stimulated cultures of primary B cell progenitors resulted in more IgM secreted compared with untreated cultures. Heparan sulfate has been reported to be a ligand for the pre-B cell receptor (preBCR). Extending this observation, we found that treatment of preBCR+ cells with heparan sulfate before anti-μ stimulation leads to increased phosphorylation of ERK1/2. Consequently, preBCR+ cells proliferate more in the presence of IL-7 and heparan sulfate, whereas preBCR− cells are unaffected, suggesting that in these experiments, heparan sulfate is not directly affecting IL-7 activity. Heparin treatment of cultures induces many of the same biological effects as treatment with heparan sulfate, including elevated pERK levels in preBCR+ cells. However, heparin reduces the proliferation of cells expressing only the preBCR (opposed to both the preBCR and BCR) possibly due to internalization of the preBCR. Heparan sulfates are present on stromal cells and B lineage cells present in hematopoietic tissues and may provide stimulation to preB cells testing the signaling capacity of the preBCR. The Journal of Immunology, 2008, 180: 2839–2847.

The development of B lineage cells depends on the integration of signals from several biochemical pathways. Prominent among these are pathways linked to the pre-B cell receptor (preBCR) and the IL-7R, however, contributions from accessory molecules such as CD19, CD22, CD45, and others can alter the signaling milieu and influence the development of B lineage cells (1, 2). The central importance of the preBCR has been recognized for many years and is most easily demonstrated in experimental systems that are either deficient in one or more components of the preBCR or in systems that artificially mimic preBCR-dependent signaling (3–5).

Despite these observations, the precise mechanism by which the preBCR influences B cell development remains elusive and a number of models have been proposed (6–8). At one extreme, it is thought that simple assembly of preBCR components is sufficient to generate critical signals that promote development. Others postulate that receptor engagement is needed to initiate signal transduction and a number of ligands have been proposed (9). In support of this, Jäck and colleagues (10, 11) developed a soluble preBCR-like molecule and found that it could bind to an unidentified 90–135 kDa protein in stromal cell lysates. This interaction requires the unique tail of λ5, which interacts with heparan sulfate chains associated with this protein.

We have previously described a culture system that allows for the maturation of B cell progenitors from lineage uncommitted cells through to the IgM-secreting mature B cell stage (12–14). Consequently, this culture system supports any required signaling by the preBCR or other receptors necessary to mediate this maturation. Ig-secreting B lineage cells can be generated in vitro from B220− bone marrow progenitors by culturing cells under a series of different conditions. Early phases require IL-7 for proliferation, survival, and maturation of progenitor cells; however, to reach the Ig-secreting stage in vitro, cells must be transferred to cultures containing LPS. This transition phase is not well understood, but has classically required a coculture period between stromal cells and B lineage progenitors (15). This coculture permits B lineage cells to mature to the LPS-responsive stage and, ultimately, secrete Ig. In the absence of this contact-dependent stage, cells do not respond to LPS and do not secrete Ig. Stromal cells produce many cytokines, growth factors, and adhesion molecules, but it is unclear which specific molecules are necessary to mediate the maturation of B lineage cells at this stage.

One model suggests that an important stimulus for developing B lineage cells is the loss of IL-7 availability. This has been reported to induce maturation of pre-B cells to the sIgM+ stage; however, we have previously shown this not to be true (16). The loss of IL-7 does not change the number of cells reaching the sIgM+ stage; it merely changes the proportion of cells surviving in culture, to favor more sIgM+ cells at the expense of sIgM− pro-B and pre-B cells. Although possibly acting upon similar cells, the...
Heparan sulfate and heparin mediate preBCR-dependent events

Contact-dependent interactions necessary to mediate the maturation of progenitors to the LPS-responsive stage are measured by Ig secretion, the productive result of LPS-stimulation. These experiments do not require IL-7, nor are they hindered by its presence.

Previous experiments (15) demonstrated that stromal cells could be replaced at this transition if IL-7-expanded B lineage cells were cultured in conditions that promoted cell-cell contact (homotypic interactions). Progenitors cultured in either condition become responsive to LPS, which subsequently promotes further development and activation of the cells to secrete IgM. Although much of the current literature focuses on the interface between stromal cells and preB cells, there is less known about potential mediators that promote development in the absence of stromal cells, such as in our system. For example, we have found that the addition of anti-μ Fab to these cultures prevented maturation (15). We could not detect signaling events initiated within the B cell progenitors exposed to the anti-μ Fab and hypothesized that this reagent may block the interaction between the preBCR and an external ligand, or it may disrupt the formation of oligomeric complexes of the preBCR. These observations prompted us to design experiments to test molecules that may mediate cell-cell interactions during B lymphopoiesis.

Materials and Methods

Cell culture

B lineage progenitors were harvested from bone marrow (bothibia and femurs) using a 26⁄2 gauge needle and isolated in MACS buffer (PBS, 1% FCS, 1 mM EDTA, 0.25% BSA) on ice. B220+ cells were isolated from BM directly using the anti-B220 (clone RA3-6B2) Ab coupled to magnetic beads (Miltenyi Biotec) at 5 x 10^6 total cells. Cells were positively selected on a VarioMACS magnet with an LS adaptor. Typically, 8–14 x 10^6 B220+ cells were recovered.

Cells were cultured in OptiMEM (Life Technologies) supplemented with 10% FCS (FCS, lot selected - Life Technologies), 50 μM 2-ME, 2.4g/L NaHCO3, and 100 μg/ml penicillin-streptomycin or kanamycin. B220+ cells were cultured at 10^6/ml in 2 ml of medium containing IL-7 and cultured in a humidified atmosphere at 37°C and 5% CO2 for four days. The resulting population is designated as B220+ d4IL-7. IL-7 was obtained from a supernatant of the stable-transfected J558 cell line (Dr. Ana Cunama, Institute Pasteur, Paris, France) and used at dilution of 1/400, previously calibrated to be equivalent to 5 ng/ml, or a titration where indicated.

To assess mitogen-responsiveness, B220+ d4IL-7 cells were washed three times to remove residual IL-7 and subsequently cultured in 96-well plates (Costar/Fisher) containing 200 μl medium and 15 μg/ml LPS (isolated from Salmonella typhosa; Sigma-Aldrich catalog no. L6386). S17 stromal cells were irradiated with 2000 rads and cultured at 1000 cells/well where indicated. Alternatively, 5000 cells of indicated cell line were added to wells at the initiation of culture. Cultures were incubated in a humidified atmosphere at 37°C and 5% CO2 for 7 days without feeding. Subsequently, 50 μl of supernatant was sampled for ELISA.

In some experiments, chondroitin sulfate (from porcine rib cartilage; Sigma-Aldrich catalog no. C-7571), heparin (from porcine intestinal mucosa; Sigma-Aldrich catalog no. H-3149), or heparan sulfate (from bovine kidney; Seikagaku/Associates of Cape Cod catalog no. 400700) was included in the cultures at the indicated concentrations.

Cell lines

The following cell lines were used in these studies: S17 (BM stroma), B62, B62c, B62.1 (IL-7-dependent B lineage lines), 45193C (IL-7-dependent B lineage cell isolated from CD45+/- mouse), R5b (IL-7-dependent B lineage cell isolated from Rag2-/- mouse), C55 (erythroid cell line), EL4 (thymoma), A20 (B lymphoblast), 70Z/3 (pre-B cell line), HSB5 (IL-7-dependent B lineage cell isolated from Rag2-/-, μH-transgenic), IB4 (pro-B cell), MC9 (mast cell line), and SCID T (pro-T cell line).

IL-7-dependent cell lines were generated by limiting dilution of B220+ cells cultured in the presence of IL-7 for 6–8 wk as described previously (17). These cells were maintained in culture in OptiMEM medium containing 5% FCS in the absence of IL-7. Adherent cell lines were passaged by trypsinization for 5 min at 37°C, followed by one wash to remove excess trypsin.

Heparan sulfate digestion

Cells were treated with heparan sulfate (from flavobacterium heparinum; Seikagaku/Associates of Cape Cod catalog no. 100703) at 0.1μM/ml/10^6 cells at 37°C for 60 min. Subsequently, cells were washed one time and cultured in the presence of 0.02 μM heparan sulfate. An additional 0.02μM heparan sulfate was added every 24 h for the first 3 days of culture. This treatment was sufficient to reduce the epitope detected by the mAb clone 10E4.

FACS analysis

Cells were washed in FACS buffer (PBS, 3% FCS, and 0.01% sodium azide) and resuspended in 96-well, round-bottom assay plates at 10^5–10^6 cells in 15 μl. Labeling was done at 4°C for 15 min in a total volume of 65 μl containing optimized dilutions of the following Abs (clone): IgM (33.60; made in-house), B220 (RA3-6B2; made in-house); CD2 (BD Biosciences), CD19 (MB1-9; eBiosciences), CD43 (S7-5; BD Biosciences), CD117/c-kit (2B8; eBiosciences), IgG (18G1; BD Biosciences or polyclonal goat anti-IgG; Southern Biotechnology), CD23 (clone B3B4; BD Biosciences), IgD (10.4; made in-house), CD86 (M5/114; BD Biosciences), CD21 (7G6; BD Biosciences), CD22 (Cy34.1; BD Biosciences), B1 (6C3; BD Biosciences), J5 (FS1; made in-house), HSA (M1/69; BD Biosciences), CD5 (53-7; BD Biosciences), B220 (RA3-6B2; made in-house), CD2 (BD Biosciences), CD45/LCA (30F11; BD Biosciences), preBCR (SL165; BD Biosciences), CD44 (KM114; BD Biosciences), syndecan 1 (Seikagaku), CD48 (HM48-1; BD Biosciences), TLR4 (MT510; eBiosciences), GR-1 (RB6-8C5; BD Biosciences), CD18 (M18/2; made in-house), CD102 (3C4; BD Biosciences), syndecan 4 (KY-8; BD Biosciences), Heparan sulfate (10E4; Seikagaku), and anti-goat Fc (Jackson ImmunoResearch Laboratories).

Cells were washed twice using 200 μl and incubated with secondary reagents if needed: streptavidin-perCP or anti-rat IgG (both from BD Biosciences). In some experiments, propidium iodide was added before analysis to label dead cells.

FACS analysis was performed using a FACScalibur or FACSscan (BD Biosciences). Acquisition and analysis were performed using CellQuest software version 3.1.

Cell stimulation and Western blotting

Cells were rested in OptiMEM medium plus 0.5% FCS in the absence of IL-7 for 2–3 h at 37°C. At the indicated time, cells were stimulated with 100 μg/ml heparan, heparan sulfate, or 5 μg/ml anti-CD19. At time –2 min, cells were stimulated with goat anti-mouse μ Fab1), at 25 μg/ml. Cells were pelleted at time 0, and lysed at 5 x 10^5/ml in 1% NP40, 150 mM NaCl, 20 mM Tris-HCI (pH 7.4), 10 mM NaF, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 1 mM PMSF, 5 μg/ml aprotinin, and leupeptin (Roche) on ice for 30 min. The detergent insoluble fraction was removed by centrifugation at 10 min at 4°C.

A total of 10–15 μl of the protein lysates were mixed with 4X NuPage sample buffer (Invitrogen/Helixx Technologies) and 0.7M 2-ME. This was resolved on a 4–12% Bis-Tris gradient gel (Invitrogen/Helixx Technologies) and transferred to a polyvinylidine difluoride membrane in 20 mM Tris, 150 mM glycine, and 20% methanol. Membranes were blocked in 5% milk/TBS plus 0.2% Tween 20 (TBST) for ≥1 h. Detection of phosphorylated ERK (New England Biolabs) was performed, according to the manufacturer’s instructions. For loading controls, membranes were stripped using the Re-Blot Plus recycling kit (Chemicon International), according to the manufacturer’s instructions. Re-probing for nonphosphorylated ERK (New England Biolabs) was performed, as above. Membranes were washed five times for 5 min each in TBST and probed with a secondary Ab (goat anti-rabbit IgG conjugated to HRP; New England Biolabs) in 5% milk/TBST for 1 h. ECL was performed using ECL substrates (Amersham Pharmacia Biotech) as described by the manufacturer.

ELISA

ELISA plates (Costar/Fisher catalog no. 3590) were coated with 5 μg/ml goat anti-mouse IgM or IgG (Jackson ImmunoResearch Laboratories) in 0.05% Tris-HCl, pH 9.8, 0.15M NaCl for 2–18 h. Plates were washed with distilled water 8 times and patted dry. PBS plus 5% FCS was used to block plates for 30 min and then washed before the addition of 50 μl of culture supernatant. Supernatants were added undiluted, at 1/10, and 1/100 serial dilutions. A standard curve was established using purified murine IgM 500 ng/ml and 11, 2-fold serial dilutions. Plates were incubated for 4–8 h and washing. Plates were developed by incubation with anti-mouse IgG conjugated to HRP for 1 h at room temperature. After washing, 50 μL of substrate (0.5 mg/ml 2,2,3,3-tetramethylbenzthiazoline-6-sulfonic acid, 0.05M phosphate citrate buffer, and 0.03% sodium perborate
Results

Contact between primary B cell progenitors and certain cell lines promotes maturation

B lineage progenitors can be generated from B220<sup>+</sup> bone marrow cells cultured in the presence of IL-7 for 4 days (as described previously in Ref. 16, B220<sup>+</sup>d4IL-7 cells). These cells remain unresponsive to LPS stimulation unless they undergo a period of coculture with stromal cells or are cultured under conditions that promote contact between the B lineage cells themselves. These assays monitor the emergence of LPS-responsive B cells by measuring the production of IgM found in culture supernatants. Although evidence suggests that μ is required for the transition to the LPS-responsive stage, it is unclear what other molecules may function during the contact-dependent events at this stage (15). To help establish the molecular basis for this potent maturation signal, we cocultured a variety of cell lines with primary B cell progenitors (B220<sup>+</sup>d4IL-7 cells) to determine which ones were able to promote maturation to the LPS-responsive stage.

We began our screen using the B lineage cell line B62c. In many ways, this line is similar to the primary B cell progenitors that undergo stromal cell or pre-B-pre-B-mediated maturation (17). The number of primary B220<sup>+</sup>d4IL-7 cells used in this culture was substantially lower than the number of B62c cells thus ensuring that nearly all cell-cell interactions occurred between the B220<sup>+</sup>d4IL-7 cells and the B62c cells. We found that the primary cells were able to secrete IgM in the presence of B62c, indicating the B62c cells possessed the required attributes to stimulate maturation of the primary B cell progenitors. (Fig. 1A). We failed to detect IgM in cultures of LPS-stimulated B62c in the absence of B220<sup>+</sup>d4IL-7 cells. These data indicate that although B62c cells contributed signals that promoted the maturation of primary B cell progenitors, the cell line itself was unable to respond to LPS stimulation and secrete Ig.

Using this assay as a screening tool, we tested a panel of cell lines (Fig. 1B) including IL-7-dependent B lineage cell lines (B62, B62c, B62.1, R5b, 45193C, HC3b, and μ23), transformed B lineage lines (IIIB4, 70Z/3, A20), a T lineage cell line (SCID T), an erythroid line (CB5), a mast cell (MC9), and inert, 10-μm polystyrene beads. The presence of B62, B62c, B62.1, R5b, 45193C, or 70Z/3 led to significantly increased amounts of IgM detectable in culture supernatants from B220<sup>+</sup>d4IL-7 cells compared with when B220<sup>+</sup>d4IL-7 cells were cultured in the absence of these cell lines (p value of <0.05). Similar to B62c, none of these cell lines produced detectable levels of IgM when cultured in the absence of B220<sup>+</sup>d4IL-7 cells (data not shown), confirming that B220<sup>+</sup>d4IL-7 cells are the sole source of IgM detected. In fact, some of the cell lines are incapable of producing Ig because of inherent mutations (R5b is RAG2<sup>−/−</sup>; HC3b is RAG2<sup>−/−</sup>, IgHC transgenic; μ23 is μMT<sup>−/−</sup>). In contrast, the inclusion of a number of different cell lines (CB5, MC9, A20, and SCID T) or polystyrene beads failed to promote B cell maturation as indicated by a lack of IgM in the culture supernatants.

Having established that some cell lines, but not others, have the capacity to promote the maturation of B220<sup>+</sup>d4IL-7 cells, we next investigated whether this is the result of a soluble factor or required cell contact-dependent interactions. To distinguish between these possibilities, we separated B220<sup>+</sup>d4IL-7 cells from the cell lines using a transwell system. The transwell membrane has (Sigma-Aldrich)) was added and the absorbance was read at 405/630 nm on an OptiMax microplate reader (Molecular Devices). Values were subtracted for a plate blank and included in the analysis only if they fell within the linear range of the standard curve. Data are presented using GraphPad Prism (GraphPad Software).

![FIGURE 1.](image-url)
0.2-μm pores, which allow for the exchange of nutrients, gases, peptides, and proteins but not cells. When the cell lines are isolated from B220<sup>+</sup> d<sub>aIL-7</sub> cells using the transwell, the resulting IgM detectable in culture supernatants is similar to that found when B220<sup>+</sup> d<sub>aIL-7</sub> cells are cultured in the absence of any cell line (Fig. 1C). Taken together, these data suggest that certain cell lines provide a contact-dependent interaction to B220<sup>+</sup> d<sub>aIL-7</sub> cells that supports their maturation to the LPS-responsive stage.

**Supportive cell lines express heparan sulfate**

Having defined two groups of cell lines based on their ability (or inability) to support contact-mediated maturation of B220<sup>+</sup> d<sub>aIL-7</sub> cells, we next examined the surface protein expression of these lines using FACS analysis to identify any similarities or differences. Table I summarizes these data. We observed that although only B lineage cells supported the maturation of B220<sup>+</sup> d<sub>aIL-7</sub> cells, not all B lineage cell lines were capable of doing so. For example, A20 is an IgG<sup>+</sup> B cell lymphoma that does not support B220<sup>+</sup> d<sub>aIL-7</sub> cell maturation. Analyzing the phenotypes of the supportive group of cell lines, we observed that they differ in expression of CD45/B220, HSA, preBCR (using the SL-165 Ab), sIgM, sIgκ, CD19, CD44, CD2, CD22, syndecan-1, and CD48. In contrast, they all express CD102, CD43, BP-1, and syndecan-4. All cell lines in the supportive group, with the exception of R5b, also express heparan sulfate as detected by the 10E4 mAb. The structure of heparan sulfate is highly variable and the epitope recognized by the 10E4 clone requires a disulfated GlcNSer sequence (18). Therefore, it is possible that R5b may express a form of heparan sulfate not recognized by 10E4. Notably, this cell line expresses syndecan-4, which is known to be heavily heparan sulfated (19).

Recently, heparan sulfate was identified as a putative ligand for the preBCR using a soluble preBCR-like molecule (10). We, therefore, tested whether heparan sulfate or a related molecule, heparin, added exogenously to the culture system could influence B220<sup>+</sup> d<sub>aIL-7</sub> cells stimulated with LPS. In fact, there is a dose-dependent increase in IgM secretion when heparin or heparan sulfate is titrated into the culture (Fig. 2). This is not the case with another extracellular matrix family member, chondroitin sulfate, suggesting that these results are not solely dependent on the presence of charged molecules. Similarly, Chen and colleagues (20) found that intraperitoneal injections of heparin into mice increased the numbers of plaque-forming cells, suggesting that heparin may play a role as B lineage cells mature and ultimately secrete Ig.

**Heparitinase treatment of cultures leads to maturation of B cell progenitors**

Heparan sulfates are linear polysaccharide chains consisting of repeating disaccharide units of glucuronic acid and N-acetyl glucosamine. Similarly, heparin consists of repeating units of 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine. Both molecules exhibit exceptional structural diversity due to substitutions of other saccharide residues and differences in sulfation patterns (18). Therefore, it is possible that commercially prepared heparan sulfates and heparin may differ in biological outcomes when compared with native heparan sulfates expressed on the surfaces of the cells that comprise the hemopoietic microenvironment. To address this issue, we treated supportive cell lines with heparitinase and compared their ability to support the maturation of B220<sup>+</sup> d<sub>aIL-7</sub> cells (21, 22).

The effectiveness of the heparitinase treatment can be assessed by flow cytometry using the 10E4 mAb. Fig. 3A illustrates that heparan sulfate can be digested from the surface of B62.1 cells using heparitinase, however, expression returns to normal levels

**Table 1. Summary of FACS analysis of cell lines**

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<tr>
<th>CD45/LCA</th>
<th>B220</th>
<th>HSA</th>
<th>PreBCR</th>
<th>sIgM</th>
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a Cell lines were analyzed for the expression of various markers. ND, FACS not performed.
within 4 h if the enzyme is washed away. We found that an initial digest using 0.1 mU heparitinase, followed by cultures containing an additional 0.02 mU heparitinase supplemented every 24 h, is sufficient to reduce the 10E4 epitope of heparan sulfate levels for \( \leq 72 \) h. Longer time points were not assessed. Furthermore, continued exposure to heparitinase had no effect on cell number (data not shown) or viability as assessed by exclusion of propidium iodide (Fig. 3A).

To test the role heparan sulfate may play in mediating the contact-dependent interactions that support B cell progenitor maturation to the LPS-responsive stage, we compared the amount of IgM detected from supernatants of \( B220^{d4IL-7} \) cells in the absence of the cell lines also resulted in an increased amount of IgM secreted. Considering that these cultures are not washed during the 7-day incubation period, heparitinase treatment may result in an increase in free heparan sulfate chains present in culture, effectively mimicking the situation in Fig. 2 where heparan sulfate chains were added exogenously to the culture. Alternatively, heparitinase treatment of these cultures may result in the exposure of new carbohydrate epitopes on heparan sulfated-proteins that influence \( B220^{d4IL-7} \) cells, leading to more IgM detected.

The influence of heparin, heparan sulfate, and heparitinase in cultures containing IL-7

Experiments presented so far have been performed in the absence of IL-7 and focused on the transition of progenitor cells to the LPS-responsive stage and ultimately, Ig-secretion. However, because the starting population of cells from these experiments (\( B220^{d4IL-7} \) cells) is taken from cultures containing IL-7, we next examined whether there were any effects of heparan sulfate or heparin in these cultures. Previous studies have found a link between heparan sulfate/heparin and IL-7 and have suggested that heparan sulfate may augment IL-7-induced proliferation of early B cell progenitors. This may occur by promoting presentation of IL-7 to B lineage cells or act as a hybrid molecule with IL-7 to stimulate pre-pro-B cells (23, 24).

FIGURE 2. Soluble heparin and heparan sulfate cause an increase in the amount of IgM secreted from LPS-stimulated B lineage cultures. A total of 200 \( B220^{d4IL-7} \) cells were cultured in 96-well plates containing LPS and a titration of heparin, heparan sulfate, or chondroitin sulfate. The emergence of mitogen responsive B cells was monitored 7 days later by ELISA measuring secreted IgM. Results are expressed as the mean \( \pm \) SE of four independent experiments.

FIGURE 3. Heparitinase treatment of cultures results in more IgM secreted and more CD21+sIgM+ cells arising. A, \( B62c \) cells were stained for the expression of heparan sulfate using the 10E4 mAb. Some \( B62c \) were treated with 0.1 mU of heparitinase and stained for the expression of heparan sulfate or returned to culture containing IL-7 \( \pm 0.02 \) mU heparitinase. Cells were harvested at the indicated times, stained, and analyzed for the expression of heparan sulfate. Histograms represent live-gated cells and are representative of two independent experiments with two different cell lines. On day 3, cells were also labeled with propidium iodide (PI) to examine live/dead cells. Filled histogram represents negative staining. Open histograms days 2 and 3 represent heparitinase treated cells. On day 1, cells were treated with heparitinase, then allowed to recover at 37°C for 4 h (open histogram, right panel) or examined immediately following treatment (left panel). Data are representative of five experiments with the exception of the 4 h time point \(( n = 1 \) ). B, Cell lines (\( B62c, B62.1, \) or \( R5b) \) were treated using 0.1 mU heparitinase 60 min at 37°C or left untreated. These were subsequently used in a coculture system containing 200 \( B220^{BMIL-7} \) cells and 5000 filler cells, LPS \( \pm 0.02 \) mU heparitinase. None represents \( B220^{BMIL-7} \) cells only. The emergence of mitogen responsive B cells was monitored 7 days later by ELISA measuring secreted IgM. Points represent the mean of 4–5 wells from individual experiments. Bar shown is the mean average of all experiments. Values of \( p \) were calculated using the paired \( t \) test comparing the amount of IgM secreted in the presence or absence of heparitinase.
We have previously shown that CD2−sIgM− cells require IL-7 for continued growth and survival (16) and that a fraction of the CD2−sIgM− cells are fated to become CD2+sIgM−, regardless of the presence or absence of IL-7 in the culture system. To test the effects of heparan sulfate, heparin, or heparitinase, B220+ cells were harvested from BM and cultured in the presence of IL-7. Heparitinase treatment in the presence of IL-7 caused no detectable change in the ratio of CD2+sIgM− cells to CD2−sIgM− cells, or in the number of cells surviving after 4 days (Fig. 4). Heparin however, caused a shift in the proportion of cells present. A total of 57% of cells were CD2−sIgM− after the culture period in the presence of IL-7 and heparin, vs a total of 46% in wells containing IL-7 alone. This appeared to be a result of a loss of CD2−sIgM− cells in wells containing heparin, suggesting that cells at this stage either may be particularly sensitive to heparin treatment or, alternatively, heparin may cause CD2− cells to mature to the nonproliferating CD2+ stage, thus reducing the overall number of cells in the culture over time. Heparan sulfate did not significantly alter the proportion of cells in culture but did result in an elevated number of cells present. These results may support previous findings that heparan sulfate can augment IL-7-induced proliferation of cells, however, given our results that heparan sulfate can influence the transition of progenitors to the LPS-responsive stage (in the absence of IL-7), we examined the biochemical outcomes of stimulating cells with heparan sulfate or heparin.

**Heparin and heparan sulfate increase signaling via the preBCR**

The transition of cells from the CD2−sIgM− to the CD2+sIgM− stage approximates the transition from large preB cells to small preB cells (16) and current models suggest this results from successful signaling generated by the preBCR on large preB cells. Also, Jack and colleagues (10, 11) have demonstrated that stromal cell-associated heparan sulfates bind specifically to the A5 tail of the preBCR, but not to the mature BCR. Given the current data that modifications of heparan sulfate, either by addition of exogenous molecules or digestion of endogenous molecules, has profound effects on the maturation of progenitor cells, we examined biochemical responses of the preBCR with varying conditions.

Using the cell line B62c, which was previously shown to express μ and A5, but not κ or λ, basal levels of pERK representing constitutive activity of the preBCR were undetectable by Western blot. However, when cells were exposed to cross-linking anti-μ Abs for 2 min, pERK can be detected (Figs. 4 and 5, A). Heparan sulfate or heparin alone did not induce pERK signaling (data not shown); however, pretreating cells with heparin followed by anti-μ generated increased levels of pERK compared with anti-μ alone. This suggests that heparin is either recruiting coreceptors that can reduce the signaling threshold of the preBCR, or that heparin is modifying the cell surface milieu and allowing the anti-μ Ab to be more effective.

These findings prompted us to compare the results when cells are pretreated with an activating, anti-CD19 Ab because CD19 has been reported to establish signaling thresholds of the preBCR (25–28). Activating the CD19 pathway before anti-μ stimulation resulted in increased pERK levels, similar to when cells were pretreated with heparin.

To determine whether these results depend on the presence of the preBCR, we used the cell line R5b that was generated from a long-term culture of Rag2−/− BM cells, and thus does not express the preBCR. Shown in Fig. 5C, R5b cells do not generate pERK in response to heparan sulfate or heparin stimulation. In contrast, both B62c (preBCR+) and B62.1 (preBCR+/BCR−) generate elevated levels of pERK when heparan sulfate or heparin are used to pretreat cells in conjunction with anti-μ. These results are consistent with similar, independently generated cell lines (data not shown).

**Heparan sulfate and heparin affect the proliferation of preBCR+ cells**

Given that heparan sulfate and heparin are able to increase levels of pERK after anti-μ stimulation, and given the importance of pERK with respect to integrating signals from the IL-7R and the preBCR (29), we examined the ability of cells to proliferate in limiting concentrations of IL-7. Previously, we reported that preBCR+ cells have reduced proliferation in ‘low’ picogram concentrations of IL-7 compared with preBCR− cells. To test the effects of heparan sulfate and heparin on proliferation in IL-7-containing cultures, we measured [3H]thymidine incorporation of R5b, B62c, and B62.1 cells under various conditions. R5b cells require high concentrations of IL-7 to proliferate likely due to the absence of a preBCR (29). Heparin and heparan sulfate do not alter the proliferation of these cells suggesting that there are no nonspecific interactions that affect proliferation. One model suggests that heparan sulfate may bind IL-7 and increase its effective biological activity, (23) however these current results demonstrate that, at least under these specific conditions, heparan sulfate does not yield increased proliferation of cells in the presence of IL-7.

In contrast to these findings, heparan sulfate increases the ability of preBCR+ B62c cells to proliferate in cultures containing IL-7, ~2-fold (Fig. 6B). Our group has previously reported that proliferation of preBCR− cells is influenced by signals generated from the IL-7R and the preBCR. Based on this model, these data support...
the findings shown in Fig. 5 that heparan sulfate leads to increased preBCR signaling.

Strikingly, heparin stimulation results in different results. At high concentrations of IL-7, cells proliferate to approximately the same levels whether or not heparin is included in the culture. However, at low concentrations of IL-7, cells cultured in the presence of heparin and IL-7 incorporate approximately one tenth as much \([\text{H}]\text{thymidine}\) as cells cultured with IL-7 alone. This supports the data presented in Fig. 4 that the presence of heparin in cultures of B220^-dil-7 cells and IL-7 results in more CD2^- cells maturing to the CD2^+ stage, resulting in a reduced proliferation of the culture as a whole.

When the cell line B62.1, which has both the preBCR and the BCR, was cultured in the presence of heparan sulfate and IL-7, more thymidine incorporation was observed when compared with conditions containing IL-7 alone. This is consistent with the results
observed with preBCR<sup>+</sup> B62c. However, in contrast to B62c, heparin resulted in consistently increased thymidine incorporation of B62.1 regardless of IL-7 concentration, suggesting that the presence of the BCR on B62.1 cells greatly influences the outcome of heparin activity on these cells. These results are consistent with similar, independently generated cell lines (data not shown).

**Discussion**

The complex process of B lineage cell development is controlled by many factors produced from the surrounding microenvironment. These factors can include cytokines, extracellular matrix molecules, chemokines, adhesion molecules, and cell surface proteins that act in concert to regulate B lineage cells. In vitro assays have provided invaluable insights into the biology of many of these factors and have helped to identify the minimum necessary components that B lineage cells require during their development. These include cytokines IL-11, MGF, and IL-7, however following these cytokine-dependent phases, B cell progenitors require contact-dependent interactions to mature to the LPS-responsive stages (12). Many experimental systems depend on the interactions between stromal cells and B lineage cells, however we have previously recognized that B lineage cells can direct their own maturation if cultured in conditions that promote contact between cells (15). One important molecule mediating this transition is the preBCR. Anti-μ Fab added to cultures prevented the maturation of B220<sup>+</sup> B62c cells to the LPS-responsive phase, but did not appear to induce signaling. One possibility is that the anti-μ Fab could bind to the preBCR and by masking binding sites or steric hindrance, prevent the necessary contact-dependent interactions (30).

To screen for other molecules that are important during this cellular transition, we developed an assay whereby cell lines are cultured in excess compared with B cell progenitors, ensuring that most cell-cell contacts occur between cell lines and primary cells, or between the cell lines themselves. None of the cell lines were capable of producing IgM in these assays. Results demonstrated that some B lineage cell lines could be as effective as stromal cells in supporting the maturation of primary B220<sup>+</sup>-dT<sup>+</sup> cells to the IgM-secreting stage. Although stromal cells are popularly included in in vitro assays of B lineage cell development, these results suggest that with the exception of cytokines, B lineage cells express all of the necessary components to direct their maturation.

All of the cell lines that could support primary cell maturation were B lineage cells, and they expressed a number of common proteins. By FACS analysis, BP-1, syndecan-4, and heparan sulfate were uniquely expressed by cells that could support maturation, but not by other cell lines. BP-1 is a cell surface aminopeptidase that is expressed during the IL-7-responsive stages. Syndecan-4 is a proteoglycan, decorated with heparan sulfate side chains, that has been shown to be involved in cell-cell binding and interactions with the extracellular matrix (19).

Previous reports suggest a link between heparin/heparan sulfate and B lineage progenitors. One study found that soluble heparin can cause the internalization of the preBCR (20). In addition, Jack and colleagues have identified that stromal cell-associated heparan sulfate may act as a ligand for the preBCR (10). Another study found that heparin treatment of Whittlock-Witte cultures prevents the formation of lymphocytes (23). However, when the addition of heparin was delayed, the authors report normal to elevated numbers of B lineage cells.

Our results demonstrate that heparan sulfate-bearing B lineage cells can increase the amount of IgM secreted by LPS-stimulated B220<sup>+</sup>B62.1 cells; this is further enhanced by treating cultures with heparitinase. Heparin and heparan sulfate are linear chains consisting of repeating disaccharide subunits of uronic acid and D-glucosamine with many substitutions by N-sulfate, O-sulfate, or N-acetyl groups. Heparitinase cleavage of heparan sulfate reveals a desaturated uronase epitope recognized by the 3G10 mAb and destroys an N-sulfated glucosamine recognized by the 10E4 Ab. This may release small oligosaccharides that are able to stimulate B lineage development, similar to conditions where heparan sulfate chains were added exogenously to the culture system. Considering the widespread distribution of heparan sulfates and their exceptional structural diversity, it is possible that specific heparan sulfate conformers are more or less able to influence B lineage development. Whether these conformers are uniquely expressed in lymphopoietic tissue remains to be determined.

Heparin and heparan sulfate appear to influence the preB cell stage. The results obtained from Jack and colleagues (10, 11) are dependent upon the unique tail of A5 binding to heparan sulfate. In addition, our results reveal that heparin or heparan sulfate potentiates signaling by the preBCR and may result in the cellular outcomes observed. In fact, heparin or heparan sulfate in combination with anti-μ treatment of cells causes a similar response by pERK compared with anti-CD19 and anti-μ. Current models suggest CD19 lowers the threshold of activation of the preBCR and BCR, and establishes critical signaling thresholds (25–28). In its absence, there is an impaired response to B cell transition and a severe reduction in mature B cells. CD19<sup>−/−</sup> B lineage cells have a marked reduction in phospho-ERK and phospho-BTK levels following stimulation with anti-IgM Abs (27, 31). One possibility is that B cell-associated heparan sulfate may form a bridge stabilizing CD19-preBCR interactions, and thus lower the threshold of activation of the preBCR.

Previously, co-operation between the preBCR and the IL-7R resulting in ERK phosphorylation was shown to be critical for preBCR<sup>+</sup> cells to proliferate in low concentrations of IL-7. Herein, we demonstrate that when heparan sulfate is added exogenously to cultures of preBCR<sup>+</sup> cells and IL-7, more proliferation is observed. This suggests a strong link between the ability of heparan sulfate to stimulate pERK and the resultant increased proliferation of these cells. This does not occur with preBCR<sup>+</sup> cells demonstrating that the observed effects are not due to an interaction with heparan sulfate and IL-7 directly as has been proposed for other model systems (23).

Including heparin in these experiments revealed different outcomes when compared with heparan sulfate. Like heparan sulfate, heparin treatment of preBCR<sup>+</sup> cells leads to elevated levels of pERK. However, unlike heparan sulfate, heparin resulted in a reduced proliferation of cells in response to IL-7. Similarly, using B220<sup>+</sup>BM cells, heparin resulted in a reduced number of CD2<sup>−</sup> cells, whereas heparan sulfate resulted in an increased number of CD2<sup>−</sup> cells. One possible explanation for this discrepancy may stem from the observation that heparin treatment of preBCR<sup>+</sup> 70Z/3 cells causes the preBCR to be internalized, but not the BCR on more mature cells (20). Our results demonstrated that heparin decreased the ability of B62c cells (μ<sup>+</sup> and λ<sup>5</sup>−) to proliferate, but did not have the same effect on B62.1 cells (μ<sup>+</sup>, λ<sup>5</sup>−, and κ<sup>+</sup>). If heparin caused preBCR structures on B62.1 to be internalized, these cells may be protected from the decreased proliferation observed in B62c by remaining BCR structures that are resistant to heparin-induced internalization.

Ultimately, heparan sulfates are expressed as a normal constituent of hemopoietic tissues, both by stromal cells and by developing B lineage cells. Mounting evidence has highlighted the potential importance of these molecules during B lymphopoiesis. The results presented herein substantiate previous findings that heparan
sulfates bind the preBCR by documenting the biological and biochemical events resulting from manipulating heparan sulfate/heparin as B lineage cells progress. Heparan sulfates may bind the preBCR and lower the activation threshold needed for signal propagation, resulting in enhanced pERK, proliferation, and eventually maturation to LPS-responsive stages.

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