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Identification of an IL-7-Associated Pre-Pro-B Cell Growth-Stimulating Factor (PPBSF). I. Production of the Non-IL-7 Component by Bone Marrow Stromal Cells from IL-7 Gene-Deleted Mice

Sean D. McKenna, Fangqi Chen, Laijun Lai, and Irving Goldschneider

Mouse bone marrow (BM) stromal cell conditioned medium (CM) from our long-term lymphoid culture system selectively induces the in vitro proliferation and presumptive differentiation of pre-pro-B cells (B220<sup>+</sup>, HSA<sup>+</sup>, TdT<sup>−</sup> or TdT<sup>−</sup>, cμ<sup>−</sup>) from adult rat, mouse, and human BM. However, the responsible growth factor(s) has not yet been identified. Inasmuch as IL-7 is one of the cytokines most closely associated with early B-lineage development, we utilized BM adherent cells and stromal cell lines from IL-7 gene-deleted (−/−) mice in combination with rIL-7 and anti-IL-7 mAb to investigate its possible regulatory role in our culture system. The results show that, although rIL-7 and IL-7 (−/−) CM each can maintain the viability of freshly harvested pre-pro-B cells in vitro, neither induces them to proliferate and/or differentiate, even in the presence of recombinant stem cell factor (rSCF) and/or recombinant insulin-like growth factor (rIGF). The results also show that anti-IL-7 mAb fails to neutralize the pre-pro-B cell growth-stimulating activity in IL-7 (+/+) CM. Yet rIL-7 enables IL-7 (−/−) CM to induce proliferation of pre-pro-B cells, and to “prime” them to respond directly to monomeric IL-7. Furthermore, anti-IL-7 mAb adsorbs the pre-pro-B cell growth-stimulating activity from both IL-7 (+/+) CM and rIL-7-supplemented IL-7 (−/−) CM; but rIL-7 does not restore this activity. Lastly, both pre-pro-B cell growth-stimulatory activity and IL-7 are quantitatively recovered by ultrafiltration in the 50 to 100 kDa, rather than the 10 to 50 kDa, apparent molecular mass fraction. These results suggest that the pre-pro-B cell growth-stimulating activity in our culture system is the property of a self-associating complex of IL-7 and a second BM stromal cell-derived cofactor.


Interleukin 7 (IL-7) was first identified as a bone marrow (BM) stromal cell-derived cytokine capable of stimulating the proliferation of murine B cell precursors in vitro (1). However, IL-7 does not appear to be able to support the long-term maintenance of B-lineage cells unless the lymphoid precursors also receive contact-mediated signals from BM stromal cells (2, 3). Thus, while it has been proposed that IL-7 is capable of acting on primitive B220<sup>−</sup> B cell progenitors in the presence of stem cell factor (SCF) (4), most investigators have concluded that the principle B-lineage targets for IL-7 are B220<sup>+</sup> pro-B cells and pre-B cells, but not pre-pro-B cells (2, 3, 5–10). Indeed, it has been reported that 1) the in vivo administration of neutralizing anti-IL-7 Abs to mice eliminates B-lineage subsets as early as the pro-B, but not the pre-pro-B, cell stage (11); 2) a similar maturational arrest occurs in mice having disrupted IL-7 receptor α-chain genes (IL-7Rα−/−) (12); 3) both pre-pro-B cells and pro-B cells are well represented in BM of IL-7 gene-deleted (IL-7−/−) mice (13); and 4) human B-lineage cells can be generated from fetal precursors in an IL-7-independent manner (14). Although not excluding a role for IL-7 under physiologic conditions, these results suggest that IL-7 is not essential for regulating the earliest stages of B-lineage development.

We have described a long-term xenogeneic lymphoid cell culture system that selectively generates large numbers of pre-pro-B cells (B220<sup>+</sup>, HSA<sup>−</sup>, TdT<sup>−</sup> or TdT<sup>−</sup>, cμ<sup>−</sup>) and pro-B cells (B220<sup>+</sup>, HSA<sup>−</sup>, TdT<sup>+</sup> or TdT<sup>−</sup>, cμ<sup>−</sup>) from rat, mouse, and human BM in the presence of mouse BM adherent cells (15–19). Unlike more traditional pre-B cell-type cultures (20), pre-pro-B cells and pro-B cells are selectively generated from adult BM in our culture system, even when the lymphoid progenitors are separated from the adherent cell layer by a microporous membrane insert or cultured in stromal cell conditioned medium (CM) (21). Moreover, neither cμ<sup>+</sup> pre-B cells nor slgM<sup>+</sup> B cells are produced in significant numbers, even after the addition of rIL-7 (22). Hence, stage-specific growth factors different from those observed in pre-B cell-type cultures appear to be responsible for the long-term generation of primitive B-lineage cells in our culture system.

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5 Abbreviations used in this paper: BM, bone marrow; SCF, stem cell factor; IGF, insulin-like growth factor; PPBSF, pre-pro-B cell growth-stimulating factor; PE, phycoerythrin; TRITC, tetramethylrhodamine isothiocyanate; TRST, Tris-buffered saline/ Tween 20.

6 Rat B220<sup>+</sup>, HSA<sup>−</sup>, TdT<sup>−</sup>, cμ<sup>−</sup> lymphoid cells originally were designated early pro-B cells (21). However, we refer to them here as pre-pro-B cells for consistency with the developmental classification of murine B-lineage cells (3).
In the present study, the results of in vitro Ab neutralization, immunoadsorption, and cytokine reconstitution experiments, combined with preliminary m.w. determination, suggest that the pre-pro-B cell growth-stimulating factor (PPBSF) in CM from our culture system (21) is a bimolecular complex of IL-7 and an as yet unidentified cofactor, the latter of which is produced by BM stromal cells from IL-7 (-/-) mice. These results are confirmed and extended by Western immunoblotting in a companion paper (23). Unlike IL-7, PPBSF is resistant to neutralization with anti-IL-7 mAb and does not induce proliferation of pre-B cells or pre-B cells. Rather, PPBSF selectively stimulates proliferation and (presumably) differentiation of pre-pro-B cells, which normally are adherent to BM stromal cells (24, 25).

Materials and Methods

Animals

Male 4- to 6-week-old IL-7 gene-deleted (IL-7 (-/-)) and nondeleted (IL-7 (+/+)) mice (13), bred from (129 × B6F1) stock generously provided by Drs. Richard Murray and Ursula von Freeden-Jeffry (DNAX Research Institute of Cellular and Molecular Biology, Palo Alto, CA), were used as donors of BM-adherent cells and stromal cell lines. Male 4- to 6-week-old Lewis strain rats, bred from stock originally obtained from the National Cancer Institute, National Institutes of Health, were used as donors of BM lymphoid precursor cells. Animals were maintained on standard chow and water ad libitum in the Center for Laboratory Animal Care, the University of Connecticut Health Center.

Cytokines and Abs

Recombinant IL-7, SCF, IGF-1, and neutralizing mAbs cross-reactive with human and mouse IL-7 were purchased from Genzyme Corporation (Cambridge, MA). Mouse IgG, isotype control was obtained from Sigma Immunochemicals, St. Louis, MO. Murine mAb to the HIS40 (anti-IgM) (26), anti-BrdU/nuclease reaction mixture for 60 min, and dehydrated with absolute ethanol with 5% glacial acetic acid, received 5 to 10 μl of adsorbate or eluate lyophilized, and the supernatant was removed. This process was repeated 3 times. Nonspecific binding was controlled by incubation CM with 100 samples of serially diluted rIL-7 were fractionated in a companion paper (23). 0.5% BSA/TBST at 37°C for 1 h and washing thrice with TBST, the nonspecific binding was controlled by incubation CM with un conjugated Protein A-beads and beads conjugated with a mouse IgG, isotype control. Adsorbed CM was then filter sterilized, and assayed for residual IL-7 by semi quantitative dot blot analysis and thymocyte proliferation analysis, respectively. The bound Ag was recovered from the beads by elution with 0.1 M NaHCO3 buffer (pH 9.3) containing 0.5 M NaCl, and the eluate was dialyzed for 16 h in PBS (pH 7.2) at 4°C.

Immunoadsorption of CM with anti-IL-7 mAb

Anti-IL-7 mAb (mouse IgG) was conjugated to Protein A-Sepharose by incubating 15 μl of Ab with 80 μl of packed beads for 4 h. The beads were extensively washed with PBS to remove unbound Ab. Immunoadsorption was accomplished by incubating 100× concentrated CM with Ab-conjugated Protein A-Sepharose beads (1 ml CM/80 μl packed beads) in a rotating mixer for 2 h at 4°C. The beads were pelleted in a microfuge (8,000 r.p.m.) and the supernatant was removed. This process was repeated 3 times. Nonspecific binding was controlled by incubating CM with unconjugated Protein A-beads and beads conjugated with a mouse IgG, isotype control. Adsorbed CM was then filter sterilized, and assayed for residual IL-7 by semi quantitative dot blot analysis and thymocyte proliferation analysis, respectively. The bound Ag was recovered from the beads by elution with 0.1 M NaHCO3 buffer (pH 9.3) containing 0.5 M NaCl, and the eluate was dialyzed for 16 h in PBS (pH 7.2) at 4°C.

Dot immunoblotting of CM for IL-7

Immobil-P membranes (Millipore, Bedford, MA), presoaked in Tris-Glycine Buffer with 20% ethanol, received 5 to 10 μl of adsorbate or eluate per sample of CM by micropipet. Standards of serially diluted rIL-7 were included to determine relative concentrations of Ag. After blocking with 0.5% BSA/TBST at 37°C for 1 h and washing thrice with TBST, the membrane was incubated for 3 h at 37°C with a 1:1000 dilution of anti-IL-7 mAb in 0.1% BSA/TBST, washed, incubated for 1.5 h at 37°C with

Immunofluorescence

Purified FITC-conjugated goat IgG (28). Cytosmears prepared from these cells for BrdU cell proliferation-labeling reagent (Amersham Interna tional) in a final concentration of 1:1000. Cytosmears prepared from these cells were fixed in cold absolute ethanol with 5% glacial acetic acid, received 5 to 10 μl of adsorbate or eluate per sample of CM by micropipet. Standards of serially diluted rIL-7 were included to determine relative concentrations of Ag. After blocking with 0.5% BSA/TBST at 37°C for 1 h and washing thrice with TBST, the membrane was incubated for 3 h at 37°C with a 1:1000 dilution of anti-IL-7 mAb in 0.1% BSA/TBST, washed, incubated for 1.5 h at 37°C with

Lymphoid culture system

Rat BM pre-pro-B cells and pro-B cells were generated in our culture system as previously described (16). Briefly, single cell suspensions of mouse BM (8 × 10^6 cells) were added to 2 ml RPMI 1640 containing 20% lot-selected, defatted BSA (HyClone, Logan, UT) in 55-mm diameter culture plate wells and incubated at 37°C in 5% CO₂. After 10 days, the confluent adherent cell layers were washed and seeded with 5 × 10^5 freshly harvested rat BM cells/ml. In some experiments, the rat BM cells were seeded into microporous membrane culture inserts (4 μm pore size; Transwell-3408, Costar, Cambridge, MA) placed over (but not in contact with) the mouse BM-adherent cell layers. In experiments in which the cultures were treated with Ab, cells were fixed with 4% paraformaldehyde and stained with rat BM cells. Total cells from the culture inserts and nonadherent lymphoid cells from the standard cultures were recovered in serum-free medium on day 10 for cytologic and phenotypic analysis or for transfer to secondary cultures (21).

Establishment of mouse BM stromal cell lines

Day 10 primary BM adherent cell layers from IL-7 (+/+) or IL-7 (-/-) mice grown in RPMI 1640 with 20% FBS were detached with 0.05% trypsin 0.53% EDTA-4Na (Life Technologies, Grand Island, NY) and dissociated by gentle pipetting. The suspended cells were plated in a 25-cm² flask and grown to confluency. The cells were repeatedly passaged at 3- to 4-day intervals for approximately 2 mo to generate morphologically homogenous stromal cell lines, as described (21).

Conditioned medium (CM)

Washed confluent mouse BM-adherent cell layers or stromal cell lines therefrom were used to condition medium for 10 days (21). The CM for cell stimulation was filtered to remove any cells, concentrated twofold by ultrafiltration in Centricon-10 Concentrator units (Amicon, Danvers, MA), dialyzed for 16 h in serum-free normal medium at 4°C, and stored at −70°C. For cell stimulation, CM was diluted to twofold its original concentration with medium containing 20% FBS; for immunoadsorption, 10× concentrated CM in serum-free normal medium was used; for semiquantitative dot blot analysis for IL-7, serum-free CM was collected after 4 days incubation and concentrated 10-fold.

Fractionation of CM using size exclusion membranes

Serum-free CM was concentrated 20-fold in a series of Amicon filters with graded MW cutoffs as follows: CM was concentrated in a stirred cell filtration unit using a YM-100 55-mm presoaked membrane. Retentate was kept as the >100-kDa molecular mass fraction and filtrate was sequentially concentrated using an XM-50 membrane (to obtain a 50- to 100-kDa molecular mass fraction), Centricon-30 (30- to 50-kDa molecular mass fraction), and Centricon-10 (10- to 30-kDa molecular mass fraction).

Immunoadsorption of CM with anti-IL-7 mAb

Anti-IL-7 mAb (mouse IgG) was conjugated to Protein A-Sepharose by incubating 15 μl of Ab with 80 μl of packed beads for 4 h. The beads were extensively washed with PBS to remove unbound Ab. Immunoadsorption was accomplished by incubating 100× concentrated CM with Ab-conjugated Protein A-Sepharose beads (1 ml CM/80 μl packed beads) in a rotating mixer for 2 h at 4°C. The beads were pelleted in a microfuge (8,000 r.p.m.) and the supernatant was removed. This process was repeated 3 times. Nonspecific binding was controlled by incubating CM with unconjugated Protein A-beads and beads conjugated with a mouse IgG, isotype control. Adsorbed CM was then filter sterilized, and assayed for residual IL-7 by semi quantitative dot blot analysis and thymocyte proliferation analysis, respectively. The bound Ag was recovered from the beads by elution with 0.1 M NaHCO3 buffer (pH 9.3) containing 0.5 M NaCl, and the eluate was dialyzed for 16 h in PBS (pH 7.2) at 4°C.

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a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG, washed, and developed with Protoblot AP substrate (Promega Corp.).

Thymidine incorporation
To evaluate cell proliferation induced by CM, 1 × 10^5 freshly harvested rat thymocytes or day 10 culture-generated rat BM lymphoid cells were pulsed with 1 μCi/well of [3H]Thdr (New England Nuclear, Boston, MA) 12 h before harvesting. Incorporation of [3H]Thdr was determined by liquid scintillation spectroscopy.

Results
IL-7 is required to generate pre-pro-B cells and pro-B cells in vitro
To examine the possible role of IL-7 in our culture system, rat BM cells were incubated for 10 days on BM-adherent cell layers from IL-7 (+/+)+ and IL-7 (−/−)+ mice. Results in Figure 1 show that IL-7 (+/+)+ BM-adherent cells preferentially stimulated the expansion of pre-pro-B cells and pro-B cells, whereas IL-7 (−/−)+ BM-adherent cells maintained input numbers only of pro-B cells. However, rIL-7 (5 ng/ml) not only enhanced lymphopoiesis in cultures containing IL-7 (+/+)+ BM-adherent cells, but also enabled cultures containing IL-7 (−/−)+ BM-adherent cells to expand the pool of pre-pro-B cells and to generate pro-B cells. In contrast, rIL-7 alone was able to maintain input numbers only of pro-B cells, even at higher concentrations of rIL-7 (10–100 ng/ml) alone or in the presence of rSCF (10–500 ng/ml) and/or rIGF (4–80 ng/ml) (data not shown).

To quantify and further define the effects of the soluble products of mouse BM-adherent cells on the maintenance and/or expansion of lymphoid precursor cell activity, rat BM cells were incubated for 4 days in IL-7 (+/+)+ or IL-7 (−/−)+ CM; and the surviving cells were serially twofold diluted, passaged onto BM-adherent cell layers in standard pro-B-type cultures for an additional 10 days. The mean numbers of lymphoid cells in these secondary (2°) cultures were then compared with those generated in standard primary (1°) cultures of serially twofold diluted freshly harvested rat BM cells. Results represent the means of duplicate cultures. No lymphoid cells were generated in secondary cultures after primary incubation of BM in normal culture medium.

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To quantify and further define the effects of the soluble products of mouse BM-adherent cells on the maintenance and/or expansion of lymphoid precursor cell activity, rat BM cells were incubated for 4 days in IL-7 (+/+)+ or IL-7 (−/−)+ CM; and the surviving cells were serially twofold diluted, passaged onto BM-adherent cells from IL-7 (+/+)+ mice, and cultured for an additional 10 days. Results in Figure 2 show that rIL-7 in normal medium (B) and IL-7 (−/−)+ CM (C) each maintained input levels of lymphoid precursor activity (A), whereas a combination of rIL-7 and IL-7 (−/−)+ CM (D) or IL-7 (+/+)+ CM alone (E) expanded this activity four- to eightfold above input levels. Even greater precursor activity was detected among the cells from primary cultures that contained both rIL-7 and IL-7 (+/+)+ CM (F). These observations were repeated with CM produced by established lines of BM stromal cells from IL-7 (+/+)+ and IL-7 (−/−)+ mice (data not shown, but see Ref. 21).

Anti-IL-7 mAb adsorbs, but does not neutralize, the pre-pro-B cell growth-stimulating activity in IL-7 (+/+)+ CM
As shown in Figures 3 and 4, respectively, almost all of the growth-stimulating activity for pre-pro-B cells was removed from 10-fold concentrated IL-7 (+/+)+ CM by adsorption with anti-IL-7 mAb bound to Protein A-Sepharose, and this activity was quantitatively recovered by elution under high salt concentration. Nonetheless, the pre-pro-B cell growth-stimulating activity of the anti-IL-7 mAb-adsorbed CM was not reconstituted by addition of rIL-7 (Fig. 3). The latter result was not due to the action of residual anti-IL-7 mAb, inasmuch as rIL-7 fully reconstituted the thymocyte growth-stimulating activity of the adsorbed CM (data not shown). Hence, adsorption of IL-7 (+/+)+ CM with anti-IL-7 mAb appears to simultaneously remove IL-7 and a physically associated cofactor, which together constitute a pre-pro-B cell growth-stimulating factor (PPBSF).
medium plus rIL-7 were obtained after treatment with culture-generated rat BM lymphoid cells were incubated in (culture-generated rat BM lymphoid cells were incubated in)

PPBSF activity in IL-7 (represent mean c.p.m./well (

105 day 10

105 freshly harvested rat BM cells/ml and incubated for 10 days. Results represent mean numbers of lymphoid cells in triplicate wells. NM, normal culture medium.

Results in Figure 5 show that the pro-pro-B cell growth-stimulating activity in IL-7 (+/+) CM was not neutralized by doses of anti-IL-7 mAb up to 5 times greater than that required to completely inhibit the activity of 5 ng/ml rIL-7 added to either normal medium or IL-7 (+/+) CM. Yet, as shown in Figure 6, lymphopoiesis could be inhibited by addition of anti-IL-7 mAb to cultures of rat BM cells placed directly on, or separated by, a microporous membrane culture insert from IL-7 (+/+) mouse BM-adherent cells. These observations suggest that, under these circumstances, IL-7 on the stromal cell surface (or recently released into the medium) is neutralized prior to the formation of the PPBSF complex.

rIL-7 forms a biologically active molecular complex with a cofactor in IL-7 (−/−) CM

To test our working hypothesis that PPBSF is a self-associating molecular complex of IL-7 and a second, stromal-cell derived, growth factor, rIL-7 was added to IL-7 (−/−) CM before and/or after adsorption with anti-IL-7 mAb. The respective growth-stimulatory activities of the CM for thymocytes (Fig. 7) and pre-pro-B cells (Fig. 8) were then determined.

As shown in Figure 7, A and B, the ability of rIL-7 to stimulate thymocyte proliferation when added to normal medium or IL-7 (+/+) CM was neutralized by anti-IL-7 mAb. In contrast, the ability of rIL-7 to stimulate thymocyte proliferation when added to IL-7 (−/−) CM was not neutralized by the subsequent addition of anti-IL-7 mAb. Furthermore, as shown in Figure 7C, addition of rIL-7 to IL-7 (+/+) CM and IL-7 (−/−) CM after adsorption with anti-IL-7 mAb, enabled both to stimulate thymocyte proliferation. However, only the activity in the IL-7 (+/+) CM could be neutralized with anti-IL-7 mAb. Conversely, addition of rIL-7 to IL-7 (−/−) CM prior to adsorption with anti-IL-7 mAb enabled rIL-7 to restore the thymocyte-stimulatory activity in a neutralizable manner.

Similarly, results in Figure 8A show that immunoadsorbed IL-7 (−/−) CM to which rIL-7 had not been added initially was able to stimulate proliferation of pre-pro-B cells in a non-neutralizable manner.

FIGURE 3. Adsorption with anti-IL-7 mAb prevents IL-7 (+/+) CM from supporting lymphopoiesis in pro-B-type cultures of rat BM. Tenfold concentrated IL-7 (+/+) CM was adsorbed with anti-IL-7 mAb on Protein A-Sepharose beads, after which aliquots were reconstituted with rIL-7 (5 ng/ml). Triplicate wells were incubated with 5 × 105 freshly harvested rat BM cells/ml and incubated for 4 days. Results indicate mean numbers of total B-lineage cells (± SD) in 3 experiments. *Denotes value significantly greater (p < 0.05) than that for adsorbed CM, with or without rIL-7. No significant reduction in lymphopoietic activity was observed by sham-adsorption of CM with Protein A-Sepharose beads alone or conjugated with IgG2b isotype control mAb.

FIGURE 4. The eluted Ag from anti-IL-7 mAb-adsorbed IL-7 (+/+) CM supports lymphopoiesis in pro-B-type cultures of rat BM cells. Ten milliliters of 10-fold concentrated IL-7 (+/+) CM were adsorbed with anti-IL-7 mAb on Protein-A-Sepharose beads, and the bound Ag was eluted by 1 ml 0.1 M NaHCO3 buffer as described in Materials and Methods. Wells containing twofold concentrated IL-7 (+/+) CM plus rIL-7 (5 ng/ml) were incubated with 5 × 105 freshly harvested rat BM cells/ml and incubated for 10 days. Results represent mean numbers of lymphoid cells in triplicate wells. NM, normal culture medium.
manner after subsequent addition of rIL-7. However, rIL-7 was unable to restore PPBSF activity to immunoadsorbed IL-7 (\(2/2\)) CM to which rIL-7 had been added initially (Fig. 8B). As anticipated (see Figs. 3 and 4), PPBSF activity was quantitatively recovered in the eluate (data not shown).

Hence, rIL-7 appears to complex spontaneously with a soluble factor in IL-7 (\(2/2\)) CM to form a functional PPBSF, similar to that which normally exists in IL-7 (\(1/1\)) CM. In both instances, PPBSF has thymocyte and pre-pro-B cell growth-stimulating activities, and is bound, but not neutralized, by anti-IL-7 mAb.

**Apparent molecular mass of PPBSF**

The approximate molecular mass of PPBSF in IL-7 (\(1/1\)) CM was determined by ultrafiltration using a series of membranes with graded molecular mass exclusion sizes. These fractions were then tested for their ability to support the growth of pre-pro-B cells and pro-B cells in vitro. Virtually all of the PPBSF activity in the CM was recovered in the 50- to 100-kDa apparent molecular mass fractions (Table I). Similarly, all detectable IL-7 (nominal molecular mass 25 kDa) was recovered in this fraction, as determined by semiquantitative dot blot analysis (data not shown). These results further support the notion that PPBSF is a molecular complex of IL-7 and an associated cofactor.

**PPBSF “primes” pre-pro-B cells to respond to rIL-7 alone**

To more precisely define the role of PPBSF in the development of early B-lineage cells, freshly harvested rat BM cells were incubated for 4 days in IL-7 (\(1/1\)) CM or IL-7 (\(2/2\)) CM plus rIL-7, after which the cells were transferred into normal medium plus rIL-7 for an additional 4 days. Results in Figure 9 demonstrate that rIL-7 was able to stimulate the expansion of both pre-pro-B cells and pro-B cells in secondary cultures of BM lymphoid cells that had first been exposed to PPBSF; and simultaneous analysis of BrdU incorporation and antigenic phenotype in these secondary cells revealed that rIL-7 could indeed “prime” BM cells to respond to rIL-7 alone.

**FIGURE 6.** Anti-IL-7 mAb prevents BM-adherent cells from IL-7 (\(+/-\)) mice from supporting lymphopoiesis in pro-B-type cultures of rat BM. Triplicate wells were inoculated with \(5 \times 10^5\) freshly harvested rat BM cells placed directly on (A) or in microporous membrane culture inserts in the presence of (B) IL-7 (\(+/-\)) BM adherent cells. An amount equal to 10 \(\mu\)g/ml anti-IL-7 mAb or an unrelated mouse IgG2b control mAb was added to alternate wells. Lymphoid cells were harvested and phenotyped on day 10. Results indicate the means (\(\pm\) SD) of total B-lineage cells/well in three experiments.

**FIGURE 7.** Anti-IL-7 mAb adsorbs, but does not neutralize, rIL-7 that has been added to IL-7 (\(-/-\)) CM. Rat thymocytes (\(1 \times 10^7\)/well) were incubated for 48 h in the presence or absence of rIL-7 (5 ng/ml) and/or anti-IL-7 mAb (10 \(\mu\)g/ml) added to (A) normal medium; (B) IL-7 (\(+/-\)) or IL-7 (\(-/-\)) CM; or (C) IL-7 mAb-adsorbed IL-7 (\(+/-\)) or IL-7 (\(-/-\)) CM. As indicated in (C), rIL-7 (5ng/ml) was added to some aliquots of CM before adsorption with anti-IL-7 mAb. Results represent mean c.p.m./well (\(\pm\) SD) of incorporated \(^{3}H\)Tdr after a 12-h pulse. *p < 0.05 as compared with respective values for normal medium. **p < 0.05 between pairs of media with or without anti-IL-7 mAb. No significant reduction in thymocyte stimulation was observed by sham-adsorption of CM with Protein-A-Sepharose beads alone or conjugated with IgG2b isotype control mAb.

**FIGURE 8.** Anti-IL-7 mAb adsorbs the PPBSF activity from IL-7 (\(-/-\)) CM to which rIL-7 has been added. IL-7 (\(-/-\)) CM was adsorbed with anti-IL-7 mAb before and/or after the addition of rIL-7 (5 ng/ml), as indicated. The adsorbed CM were then inoculated with \(5 \times 10^5\) freshly harvested rat BM cells/ml and incubated for 10 days before harvest. Results represent mean number of lymphoid cells (\(\pm\) SD) of triplicate wells. *Denotes value significantly greater (\(p < 0.05\)) than that for corresponding CM adsorbed in the presence of rIL-7.
filters with graded m.w. cutoffs. An amount equal to 5 \times 10^3 added to IL-7 (curred. However, PPBSF activity was not detected when rIL-7 was reconstituting IL-7-deficient CM with rIL-7. This in fact existed independently of IL-7, it should be detectable functionally of mouse or human presumptive pre-pro-B cells in vitro or in other than (or in addition to) IL-7 are required to stimulate expansion of pro-B cells and pre-pro-B cells were proliferating (data not shown). This is in contrast to results obtained with the primary cultures, in which only pre-pro-B cells proliferated (also see ref. (21).

**Discussion**

The present results demonstrate that IL-7 can maintain the viability of pre-pro-B cells in vitro but suggest that an additional BM stromal cell-derived cofactor, acting in concert with IL-7, is required to stimulate their proliferation and to “prime” them to respond to IL-7 alone. These observations are consistent with those of other investigators (6, 8–11, 31–35) who have postulated that factors other than (or in addition to) IL-7 are required to stimulate expansion of mouse or human presumptive pre-pro-B cells in vitro or in vivo. Hence, we reasoned that, if the cofactor in our culture system existed independently of IL-7, it should be detectable functionally by reconstituting IL-7-deficient CM with rIL-7. This in fact occurred. However, PPBSF activity was not detected when rIL-7 was added to IL-7 (-/-) CM that had first been adsorbed with anti-IL-7 mAb in the presence of rIL-7; neither was it detected when rIL-7 was added to IL-7 (+/+ ) CM that had been adsorbed with anti-IL-7 mAb. Thus, by analogy with other lymphoid growth factors (e.g., 36–38), PPBSF appeared to be a molecular complex of IL-7 and a second, independently regulated, growth-factor. Under these circumstances, adsorption with anti-IL-7 mAb would eliminate PPBSF activity from IL-7 (+/+ ) CM and rIL-7-supplemented IL-7 (-/-) CM by simultaneously removing both IL-7 and the antigenically unrelated cofactor. This hypothesis was corroborated by 1) quantitative recovery of PPBSF activity from the anti-IL-7 mAb immunoadsorbent beads; 2) recovery of both PPBSF activity and IL-7 in the 50- to 100-kDa apparent molecular mass ultrafiltrate fraction; and 3) failure of anti-IL-7 mAb to neutralize the IL-7 activity in CM.

Although it might be argued that sufficient IL-7 is produced by the rat BM cell inoculum itself to mask the neutralizing effect of anti-IL-7 mAb on PPBSF, this does not appear to be the case. Anti-IL-7 mAb-adsorbed IL-7 (+/+ ) CM fails to support BM lymphopoiesis when reconstituted with rIL-7, and anti-IL-7 mAb neutralizes the activity of rIL-7 added to IL-7 (+/+ ) CM. Also, rat BM-adherent cells do not support pro-B cell development in our culture system unless supplemented with rIL-7 (16, 21; and our unpublished observations).

Additional evidence that PPBSF is a covalently linked, IL-7-associated heterodimer is provided in a companion manuscript (23). Therefore, only the developmental implications of the present observations are discussed here. Specifically, the question arises as to why a specialized form of IL-7 selectively supports the growth of pre-pro-B cells in vitro. Two possibilities are manifest based on properties inherent to pre-pro-B cells. The first relates to the need for cognate interactions between pre-pro-B cells and BM stromal cells for optimal lymphopoiesis; the second reflects the need of pre-pro-B cells to self-replicate to maintain the precursor cell pool (2, 3, 21, 24, 25, 39). The ability of PPBSF to satisfy both requirements may help to explain why our culture system is able to generate pre-pro-B cells indefinitely (16, 21, 24). Thus, the fact that neat CM is only about 10% as effective as are BM stromal cells in supporting the growth of pre-pro-B cells (21, 24) suggests, as one possibility, that PPBSF functions primarily as a membrane (or extracellular matrix)-bound complex. Similarly, continued expansion of the pool of pre-pro-B cells in the absence of pluripotent stem cells suggests that PPBSF can induce proliferation without differentiation (24).

It is more difficult at this point to assess the role of PPBSF in inducing differentiation of pre-pro-B cells, even though most pro-B cells in our culture system appear to originate from such precursors (21, 24, 25, 40). At a minimum, PPBSF must be indirectly involved, since neither IL-7 nor the PPBSF co-factor induces the appearance or survival of pro-B cells. However, it is possible that PPBSF merely “primes” pre-pro-B cells to differentiate (as well as to proliferate) in response to subsequent stimulation with trace amounts of monomeric IL-7. The answer to this question should be forthcoming from ongoing experiments using purified PPBSF and “early” pre-pro-B cells (B220+, HSA-, TdT-; cμ-).

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**Table I. Molecular weight determination of pre-pro-B cell growth-stimulatory activity in conditioned medium**

<table>
<thead>
<tr>
<th>Ultrafiltrate Fractions</th>
<th>No. Pre-pro-B/Pro-B Cells Per Well (× 10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonconditioned medium</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Conditioned medium</td>
<td></td>
</tr>
<tr>
<td>Unfractionated</td>
<td>12.2</td>
</tr>
<tr>
<td>&gt;10 kDa</td>
<td>9.2</td>
</tr>
<tr>
<td>&gt;30 kDa</td>
<td>4.2</td>
</tr>
<tr>
<td>&gt;50 kDa</td>
<td>8.9</td>
</tr>
<tr>
<td>&gt;100 kDa</td>
<td>0.5</td>
</tr>
<tr>
<td>10–30 kDa</td>
<td>0.1</td>
</tr>
<tr>
<td>30–50 kDa</td>
<td>0.1</td>
</tr>
<tr>
<td>50–100 kDa</td>
<td>7.0</td>
</tr>
</tbody>
</table>

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**Figure 9.** rIL-7 supports continued lymphopoiesis in pro-B-type cultures after rat BM cells have been “primed” by incubation with PPBSF. Freshly harvested rat BM cells were incubated for 4 days in IL-7 (+/+) or IL-7 (-/-) CM in the presence or absence of rIL-7 (5 ng/ml). The cells from each well of these primary (1°) cultures were then transferred into secondary (2°) cultures containing rIL-7 in normal culture medium (NM), and incubated for an additional 4 days. Results represent the mean number of B-lineage cells/well in primary and secondary cultures from three experiments. *p < 0.05 compared with respective values for total B-lineage cells in NM + rIL-7. **p < 0.05 for difference of values for total B-lineage cells between paired primary and secondary cultures.
Regardless of the result, a plausible scenario that would permit different forms of IL-7 to provide developmental continuity between pre-pro-B cells and pro-B cells envisions stage-specific differences in the expression of the IL-7R. Given that IL-7Ry chain gene-deleted mice generate both pre-pro-B cells and pro-B cells (41, 42), whereas IL-7Rα-chain gene-deleted mice appear to generate pre-pro-B cells only (12), it is possible that PBBSF is designed to transmit a proliferative signal involving low affinity (or otherwise altered) forms of the IL-7R. Conversely, monomeric IL-7 may require high affinity IL-7R for efficient signal transduction (43–46). Hence, PBBSF may selectively regulate the G1/S transition of pre-pro-B cells (21), much as monomeric IL-7 selectively regulates the G1/S transition of pro-B cells (47).

The postulated sequential expression of low and high affinity IL-7R during early B-lineage development is analogous to events observed during early thymocyte development (48, 49). It is supported by the reported difficulty in detecting expression of high affinity IL-7R on pre-pro-B cells, but not pro-B cells (50). It is also consistent with the failure of an excess of IL-7 (51, 52) and a decrease of high affinity IL-7R (12, 53), respectively, to increase or decrease the generation of pro-B cells in vivo. Induction of pre-pro-B cells only (12), it is possible that PBBSF is developmentally regulated the G1/S transition of pre-pro-B cells. It might also be argued that PBBSF can identify these issues.

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