Brief Definitive Report

INTERLEUKIN 7 PRODUCTION AND FUNCTION IN STROMAL CELL-DEPENDENT B CELL DEVELOPMENT

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It is well established that the stromal cell plays a key role in the intramarrow differentiation of hemopoietic cells (1). In fact, it was shown that the cell layer consisting of a single stromal cell clone is sufficient for supporting the B cell development from a single hemopoietic precursor (2). Recently, as the first novel molecule isolated from the stromal cell clone, the complementary DNA of pre-B cell proliferation factor, lymphopoietin 1 (LP-1)/IL-7, was cloned (3). Previous studies, however, suggested strongly that the stromal cell-dependent B cell development is a process requiring a set of molecules expressed on stromal cells (4–6); meaning the actual role of IL-7 can properly be assessed only in the environment where it is working. Here, we selected two stromal cell clones, ST2 (6, 7) and PA6 (8) (the former can support B lymphoiesis while the latter can not), and demonstrated that IL-7 is an essential factor that determines the stromal cell's ability to support B lymphopoiesis.

Materials and Methods

Cell Lines and Cell Culture. Stromal cell clones PA6 and ST2 and stromal cell-dependent B cell clone DW34 were described in our previous reports (6–8). The medium used for culturing bone marrow cells on the stromal cell layers was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS (lot no. 1115741; HyClone Laboratories, Logan, UT), 5×10^{-5} M 2-ME, 50 μg/ml streptomycin, and 50 U/ml penicillin (Meiji Seika Co. Ltd., Tokyo, Japan). The procedure for culturing bone marrow cells on the stromal cell layer and cell analysis by flow cytometer was described previously (6, 7).

IL-7 Gene and rIL-7. Poly(A)$^+$ RNA was isolated from ST2, which was incubated with 1 μg/ml LPS, *Escherichia coli* 055:B5 (Difco Laboratories, Detroit, MI) and 1 ng/ml 4-phorbol-12-myristate-13-acetate (TPA; Sigma Chemical Co., St. Louis, MO) for 3 d, and used for the construction of a cDNA expression library according to Aruffo and Seed (9). The library was screened by a colony filter hybridization procedure with synthetic mouse IL-7 probes prepared according to the published sequence (3). Sequences of the probes are 5′ AGCTTTTC-TAAATCGTGCTCGCAAGTTGAAGCAATTTCTTAAAATGAATATCAGTGAAG 3′ and

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5′ AATTCTTCACTGATATTCATTTTAAGAAATTGCTTCAACTTGCGAGCAGCAC-GATTTAGAAA 3′. The Pst I/Xmn I fragment from the IL-7 cDNA clone, CDMmIL7, was subcloned into the expression vector, pCDL-SRα (10), and the resulting clone was designated pSRα mIL-7. rIL-7 was obtained as a culture supernatant of COS 1 cells that were transfected with pSRα mIL7 by a calcium phosphate method 3 d previously. The supernatant was used at a dilution of 5%.

Northern Blot Analysis. Total cellular RNA was isolated from various cells by the guanidinium/CsCl method and poly(A)$^+$ RNA was purified by oligo-dT-cellulose column chromatography. 2 μg of each poly(A)$^+$ RNA sample was subjected to electrophoresis on a 1% agarose/formaldehyde gel, transferred to a nylon membrane (Gene Screen, New England Nuclear, Boston, MA), and hybridized with the IL-7 probe. The same filter was rehybridized with the IL-6 probe (11) (a kind gift of Dr. J. van Snick, Ludwig Institute for Cancer Research, Bruxelles, Belgium) or the Cμ probe as controls.

Results and Discussion

To obtain the cDNA clone encoding the IL-7 gene, we prepared a cDNA library of ST2, which was stimulated with LPS and TPA by using the CDM8 expression vector (9). The library was screened by the oligonucleotide probe prepared according to the sequence of the IL-7 coding region reported by Namen et al. (3). Four clones out of 160,000 recombinant colonies were isolated and the longest clone, CDMmIL7, having a 2.6-kb insert (Fig. 1), was further characterized. This clone was identical with that reported by Namen et al. (3), except for its 1-kb longer 3′ noncoding region, and corresponded to the dominantly expressed mRNA species in the stromal cell clones and organs, including thymus, spleen, and kidney. This confirmed that multiple IL-7 mRNA species are functional products arisen from the use of an alternative splicing site and/or polyadenylation site. Next we recloned the IL-7 coding region into the expression vector pCDL-SRα (10), and the resulting clone, designated pSRα mIL7, was transfected into COS-1 cells. The supernatant of the cells was assayed for IL-7 activity by using stromal cell-dependent pre-B cell line DW34 (6) and also by normal bone marrow cells. The supernatant effectively induced the proliferation of DW34 cells and stimulated normal bone marrow cells to form colo-

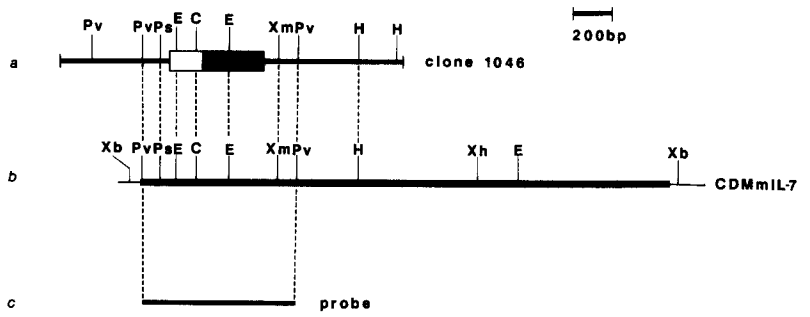

FIGURE 1. Restriction map and derivation of the probe of the mouse IL-7 cDNA. (*a*) Clone 1046 represents the murine IL-7 cDNA clone isolated by Namen et al. (3). The open box and the filled box illustrate the leader sequence and mature IL-7, respectively. Oligonucleotide probes for the screening of ST2 stromal cell line cDNA library were synthesized based on the nucleotide sequence of clone 1046. (*b*) CDMmIL7 respresents the cDNA clone isolated from the ST2 cDNA library. The restriction map around the IL-7 coding sequence is identical to that of clone 1046. CDMmIL7 contains an extended 3′ noncoding region that is lacking in clone 1046. (*c*) For Northern blot analysis of IL-7 transcripts, the Pvu II restriction fragment of CDMmIL7 was used as the probe. Pv, Pvu II, Ps, Pst I, E, Eco RI, C, Cla I, Xm, Xmn I, H, Hind III, Xh, Xho I, Xb, Xba I.

nies that consisted of B220$^+$ lymphoid cells (data not shown). Thus, we concluded that the factor produced by pSRα mIL-7 is IL-7.

Using the Pvu II/Pvu II fragment shown in Fig. 1 as the IL-7 probe, we carried out Northern blot analysis of IL-7 and IL-6 mRNA from two stromal cell clones, ST2 and PA6 (Fig. 2). Both of the stromal cell clones did not express IL-7 mRNA constitutively (Fig. 2, lanes *1* and *5*). When ST2 was stimulated with LPS/TPA or human rIL-1α, however, IL-7 mRNA became detectable (Fig. 2, lanes *2* and *3*). On the other hand, IL-7 mRNA remained undetectable in PA6 after the same stimuli, while IL-6 mRNA was detectable on the same filter (Fig. 2, lanes *6* and *7*). This result indicates that the producibility of IL-7 correlates well with the stromal cell ability to support B lymphopoiesis. Moreover, it should be noted that even in ST2, which supports B lymphopoiesis, IL-7 synthesis is inducible rather than constitutive.

This observation lead us to the question of why the IL-7-dependent B cell line DW34 can grow on ST2, which does not produce IL-7 constitutively. The most straightforward explanation is that DW34 itself can activate ST2 to produce IL-7. To test this possibility, we cultured 2 × 10^6 DW34 cells on the ST2 layer for 3 d, separated DW34 cells from the adherent ST2 layer by gentle pipetting, prepared poly(A)$^+$ RNA from each fraction, and measured the level of IL-7 mRNA. As shown in Fig. 2, DW34 did induce the expression of IL-7 mRNA in ST2 more effectively than IL-1 (lane *4*), while DW34 in the same culture could not produce IL-7 mRNA (lane *8*). Because 25% of the mRNA from the adherent ST2 layer was derived from contaminated DW34 cells, as estimated by the DW34-specific Cμ message (data not shown), the level of IL-7 mRNA actually induced by DW34 may be higher than

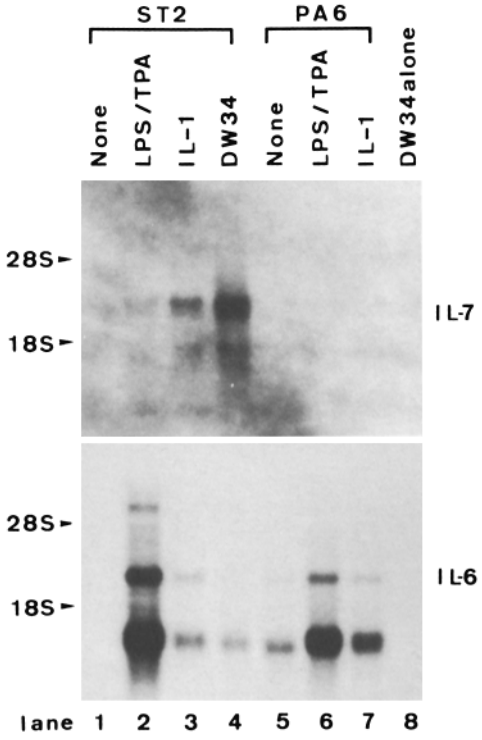

FIGURE 2. Analysis of IL7 mRNA, expressed in stromal cell clones cultured under various conditions. ST2 or PA6 was grown to the confluency in a T75 culture flask. The confluent stromal cell layer was then cultured for three more days in the absence (lanes *1* and *5*) or presence of 1 μg/ml LPS plus 5 ng/ml TPA (lanes *2* and *6*), or 1 U/ml of human rIL 1α (lanes *3* and *7*). The ST2 layer was also stimulated with 2 × 10^6 of DW34 cells/flask (lane *4*). After 3 d of coculture, DW34 cells were removed from the ST2 layer by gentle pipetting and were collected separately from the adherent cell population (lane *8*). Approximately 25% of mRNA from the adherent ST2 layer was derived from DW34 cells. This estimation was carried out by hybridizing the same filter with the Cμ probe, which is specific to the B cell (data not shown). 2 μg of poly(A)$^+$ mRNA was subjected to electrophoresis.

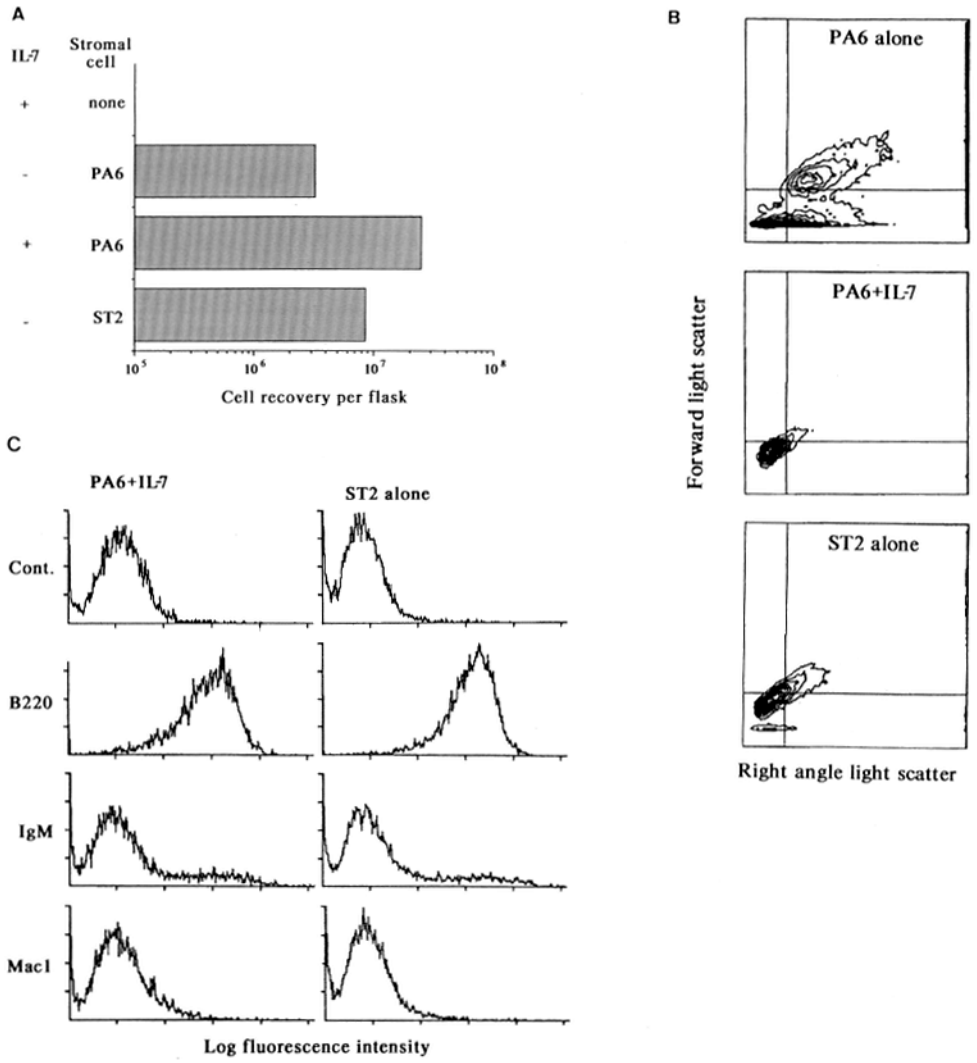

FIGURE 3. Induction of mature B cells by IL-7 on the non-B supportive stromal cell clone PA6. 2×10^6 normal BALB/c bone marrow cells were cultured on the PA6 layer. 2 wk later, the total cells were harvested and 10^6 of them were cultured successively in various conditions: (*a*) IL-7 alone (5% supernatant of COS 1 cells transfected with pSRα mIL7); (*b*) on the PA6 layer in the presence of 5% the supernatant of COS 1 cell transfected with pCDL-SRα (vector without IL-7 cDNA insert); (*c*) on the PA6 layer in the presence of IL-7; or (*d*) on the ST2 layer (*A*) Cell recovery 3 wk after the initiation of the secondary culture. (*B*) Light scatter analysis of the cells generated in the cultures. Majority of the lymphocytes distribute in the left-lower square (lymphocyte gate) in this analysis. Due to the low cell recovery in the culture with IL-7 alone, this culture was not further analyzed. (*C*) Surface phenotype of the lymphocytes generated in the culture with the PA6 layer in the presence of IL-7 or with the ST2 layer. RA3-6B2 (anti-B220; reference 12), Bet 1 (anti-Igh6.5; reference 14) and Mac 1 (anti-C3b1 receptor; reference 15) rat mAbs were used for staining.

estimated here. This result indicates that stromal cell–hemopoietic cell interaction is not an unidirectional one but that the mutual interaction is, in fact, taking place as a regulatory event during hemopoiesis.

If IL-7 is actually the only factor determining the ability of stromal cell to support

B lymphopoiesis, addition of IL-7 to the PA6 layer should render this in vitro environment B lymphopoietic. For a suitable cell population to test this possibility, we chose the bone marrow cells precultured on the PA6 layer for 2 wk. This cell population can not give rise to mature B cells unless it is transferred on the B lymphopoietic stromal cell layer, ST2(6). This cell population was transferred and cultured for 3 wk in each condition as follows: IL-7 alone, PA6 and control supernatant, PA6 and IL-7, or ST2 alone. IL-7 alone could not induce the proliferation of the bone marrow cells precultured on the PA6 layer (Fig. 3 *A*). No lymphoid cells appeared in the culture with PA6, instead, myeloid cell proliferation was maintained (Fig. 3 *B*). When the same cells were cultured on the PA6 layer in the presence of IL-7, the proliferation of the cells was enhanced, and $B220^+$ cells (12), including mature $sIgM^+$ cells, were generated (Fig. 3, *B* and *C*). The same cell population also generated the mature B lymphocytes on the ST2 layer (Fig. 3, *B* and *C*).

These results demonstrate clearly that IL-7 is actually an absolute requisite for the stromal cell-dependent process of B cell development, and it is predicted that any stromal cell clones incapable of producing IL-7 fail to support B lymphopoiesis. It has already been shown that IL-7, per se, has an activity to induce the proliferation of pre-B cells in normal bone marrow (3, 13). Therefore, there does exist a stage of pre-B cell that can proliferate in response to IL-7 without the aid of other signals. Our result that IL-7 alone failed to induce the proliferation of the bone marrow cells precultured on the PA6 layer, however, indicates that there should be an earlier stage of B cell that requires both IL-7 and other stromal cell signal(s) for the proliferation. We propose that stromal cell-dependent B cell development is subdivided into three stages: the first pro-B stage, which is independent from IL-7, the second early pre-B stage, which requires both IL-7 and other stromal cell signal(s), and the third mature pre-B stage, which can proliferate in response to IL-7 alone.

It is of particular interest to note that addition of IL-7 is sufficient for inducing $sIgM^+$ B cells from the bone marrow cells cultured on IL-7 (−) PA6 stromal cells. Our recent results further demonstrated that individually harvested IL-7-reactive colonies generated from normal bone marrow cells in semi-solid methyl-cellulose medium contained 5–20% $sIgM^+$ cells (Suda et al., submitted for publication). These results suggest that once B progenitors reach the IL-7-reactive stage on the stromal cell, further differentiation will occur spontaneously through the cell proliferation.

In conclusion, IL-7 is an essential molecule to the stromal cell-dependent process of B cell development. The present study suggests that there are other stromal cell molecule(s) yet to be identified in the earlier stage of B cell differentiation. Several kinds of stromal cell clones differing in their function together with recombinant factors will make a helpful system for elucidating these molecules one by one.

Summary

The role of IL-7 in the stromal cell-dependent B cell development was investigated using two stromal cell clones, ST2 and PA6; the former supports B lymphopoiesis while the latter can not. We demonstrate here that: (*a*) the ability of the stromal cell clone to produce IL-7 correlates well with the stromal cell activity to support B lymphopoiesis; (*b*) IL-7 production by ST2 is inducible rather than constitutive; (*c*) the IL-7-dependent B cell itself is a potent inducer of IL-7 production by ST2; (*d*) addition of rIL-7 to the PA6 layer renders this in vitro environment B lymphopoietic;

and (*e*) the differentiation from early B progenitor to pre-B cell requires both IL-7 and other stromal cell molecule(s) yet to be identified.

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