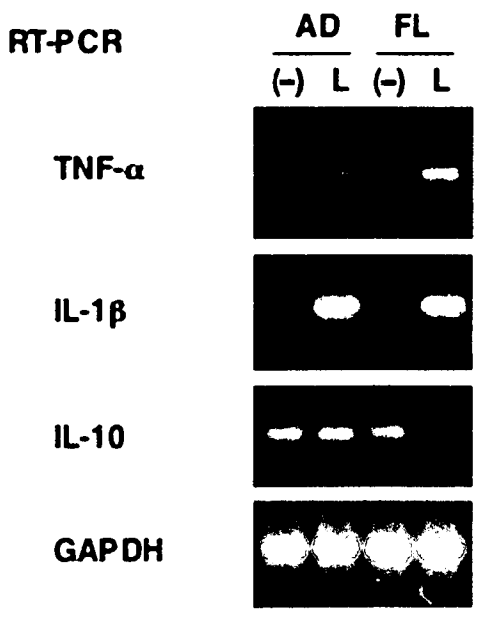


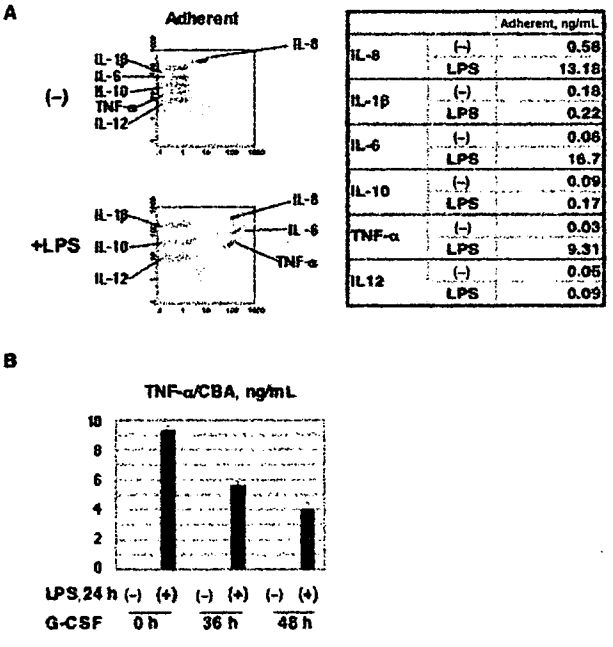
Figure 5. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of lipopolysaccharide (LPS)-stimulated cytokine production by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells as described for Figure 1, and then floating (FL) and adherent (AD) cell fractions were collected separately. After stimulation with (L) or without (-) LPS for 24 hours (as described for Figure 4), total RNA was extracted, and the indicated messenger RNA molecules were analyzed by the RT-PCR after complementary DNA synthesis. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. TNF-α indicates tumor necrosis factor α; IL-1β, interleukin 1β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells expressed myelomonocytic antigens, including CD31, CD33, and CD97. It is interesting that these 2 fractions exhibited different cytokine-secretion abilities. Figure 4 shows that both fractions spontaneously secreted IL-8 and IL-6, and they both secreted TNF-α upon stimulation with LPS. Only the adherent cell fraction secreted IL-10 after LPS stimulation, however. These characteristics suggest that the adherent cell fraction represents mature macrophages, whereas the floating cell fraction may be related to immature monocytes. Evidence that further cultivation of the floating cell fraction induced an adherent cell fraction (data not shown) supports this idea.

It is noteworthy that both cell fractions contained more IL-1β mRNA after LPS stimulation but that no IL-1β secretion at the protein level was detected in either fraction. The data indicate that monocyte-macrophage–lineage cells induced in vitro are capable of producing IL-1β upon stimulation with LPS but that the stimulation is insufficient to induce secretion of IL-1β.

On the other hand, the adherent cells exhibited a profile of cytokine secretion after 3 weeks of cultivation that was distinct from that obtained after 5 weeks. At 3 weeks, the adherent cell fractions displayed almost the same immunophenotype as monocyte-macrophage–lineage cells cultured for 5 weeks; however, the cells spontaneously secreted only low levels of IL-8, and not other cytokines. Although the cells secreted IL-6 and TNF-α after LPS stimulation, they did not secrete IL-10. Thus, our data indicate that different culture conditions induce different monocyte-macrophage–lineage subsets or monocyte-macrophage–lineage cells with different degrees of maturity.

Several studies have shown the induction of monocyte-macrophage–lineage cells by in vitro culture of cells from different cell sources. For example, Akagawa reported that M-CSF–induced monocyte-derived macrophages (M-Mphi) and GM-CSF–induced Mphi (GM-Mphi) differ in morphology, cell surface antigen expression, and function, including Fcγ receptor–mediated phagocytosis, hydrogen peroxide production and sensitivity, catalase activity, susceptibilities to human immunodeficiency virus type 1 and *Mycobacterium tuberculosis*, and suppressor activity [21]. She therefore concluded that the characteristics of GM-Mphi resemble those of human alveolar macrophages. Servet-Delprat et al also



		Adherent, ng/mL
IL-8	(-)	0.58
	LPS	13.18
IL-1β	(-)	0.18
	LPS	0.22
IL-6	(-)	0.06
	LPS	16.7
IL-10	(-)	0.09
	LPS	0.17
TNF-α	(-)	0.03
	LPS	9.31
IL12	(-)	0.05
	LPS	0.09

Figure 6. Effect of granulocyte colony-stimulating factor (G-CSF) on lipopolysaccharide (LPS)-stimulated tumor necrosis factor α (TNF-α) secretion by monocyte-macrophage cells induced from CD34⁺ bone marrow (BM) cells in vitro. A, Monocyte-macrophage–lineage cells were induced from human BM CD34⁺ cells after 3 weeks of cultivation in the presence of the mixture of cytokines described in "Materials and Methods." At the end of the culture period, cells were stimulated with LPS for 24 hours. Subsequent cytokine secretion was assessed as in Figure 4. B, Induced monocyte-macrophage cells pretreated and not pretreated with G-CSF were stimulated with (+) and without (-) LPS for 24 hours, and subsequent cytokine secretion was assessed as in (A). The experiment was performed in triplicate, and the data are presented as the mean + SD. The experiments were repeated 3 times, and reproducible results were obtained. Representative data are shown. IL-1β indicates interleukin 1β.

reported that a variety of monocyte-macrophage–lineage cells, including macrophages, osteoclasts, dendritic cells, and microglia, can be induced from murine BM cells by ex vivo culture with different combinations of cytokines [22]. These reports further support our hypothesis that different culture conditions can induce different subsets of monocyte-macrophage–lineage cells.

In conclusion, the results of this study indicate that monocyte-macrophage–lineage cells induced from CD34+ BM cells in vitro can be used for functional assays, at least in terms of cytokine secretion. Further investigation is clearly necessary, however; the establishment of culture conditions that enable the induction of different subsets of monocyte-macrophage–lineage cells should provide an ideal experimental model for the analysis of monocyte-macrophage–lineage cell function.

Acknowledgments

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Experimental Hematology

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Interleukin-7 contributes to human pro–B-cell development in a mouse stromal cell–dependent culture system

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Objective. **The role of interleukin (IL)-7 in human B lymphopoiesis is still controversial. We used an in vitro culture system to verify involvement of IL-7 in development of human pro-B cells from hematopoietic stem cells.**

Materials and Methods. **Human CD34$^+$ bone marrow cells were cultured for 4 weeks on MS-5 mouse stromal cells to induce pro-B cells. Expression of IL-7 receptor α or other B-cell differentiation marker genes on cultured human CD34$^+$bone marrow cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Colony assay of human CD34$^+$ bone marrow cells was also performed to determine the effect of IL-7 on colony-forming ability. Neutralizing antibody or reagent that eliminates the effect of IL-7 was added to the culture system, and the number of pro-B cells induced was estimated by flow cytometry.**

Results. **RT-PCR analysis revealed mRNA expression of IL-7 receptor α as well as B-cell differentiation marker genes in not only CD19$^+$ pro-B cells but also CD19$^-$ CD33$^-$ cells induced from CD34$^+$ bone marrow cells after cultivation for 4 weeks on MS-5 cells. Addition of anti-mouse IL-7 antibody, anti-human IL-7 receptor α antibody, or JAK3 kinase inhibitor reduced the number of pro-B cells induced, demonstrating that elimination of IL-7 reduces pro–B-cell development. Addition of anti-mouse IL-7 antibody emphasized the colony-forming ability of burst-forming unit erythroid cells.**

Conclusions. **IL-7 produced by MS-5 cells is required for human pro–B-cell development from CD34$^+$bone marrow cells in our culture system, and IL-7 appears to play a certain role in early human B lymphopoiesis.** © **2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Interleukin (IL)-7 is a cytokine that was first cloned from a murine bone marrow (BM) stromal cell line and is involved in the regulation of lymphopoiesis [1]. Several studies have shown that IL-7 is crucial to proliferation and development of murine B cells. For example, injection of mice with recombinant IL-7 has been shown to greatly increase the number of B cells [2], whereas injection of anti–IL-7 antibodies severely represses B-cell development [3,4]. Study of the effect of IL-7 on fractionated B-lineage cells from normal mouse BM in a stromal-cell–dependent culture system revealed that IL-7 is required for effective differentiation of pro-B cells into pre-B cells [5]. IL-7 is sufficient to induce differentiation of murine common lymphoid progenitors into pro-B cells in cultures under stromal-cell–free conditions [6].

The requirement for IL-7 in B-lymphocyte development in mice was further demonstrated by experiments in which components of the IL-7 signal transduction pathways were deleted by gene targeting [7–10]. Results showed that B-cell development is severely arrested at the common lymphoid progenitor stage in the BM of adult IL-7 receptor (R) α and common γ-chain–deficient mice, leading to a striking paucity of peripheral B cells.

In contrast to murine B-cell development, however, human B-cell development does not appear to require IL-7

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[11]. Unlike the mouse common γ knockouts, patients with human X-linked severe combined immunodeficiency, who lack a functional common γ chain, produce normal numbers of B cells [12]. Immunodeficiency patients with autosomal recessive mutations in either IL-7Rα chain or JAK3 tyrosine kinase, a downstream signaling molecule of IL-7R, also have normal numbers of peripheral B cells [13–15]. All of this evidence indicates that IL-7 is not always required for B-cell development in humans.

Nevertheless, some studies found that IL-7 affects human B-cell development in some way. For example, it was found that IL-7 transduces signals that lead to specific changes in gene expression during human B-cell development. IL-7 stimulation induces a specific increase in CD19 on the surface of human pro-B cells and decrease in RAG-1, RAG-2, and TdT messenger RNA levels [16]. Proliferation of $CD19^+CD34^+$ pro-B cells on human BM stromal cells is enhanced by inclusion of exogenous IL-7 in the culture [17]. Therefore, if not essential, IL-7 may play an integral role in some aspects of human B-cell development.

In an attempt to clarify the effect of IL-7 on human B-cell development, we used an in vitro culture system in which human hematopoietic stem cells are cocultured with murine BM stromal cells that induce pro–B-cell differentiation. In this article, we report finding that IL-7 is essential for the differentiation of human $CD34^+$ BM cells into pro-B cells in our culture system, and we discuss the possible role of IL-7 in early human B-cell development.

Materials and methods

Reagents

Monoclonal antibodies used were phycoerythrin (PE)-conjugated anti-CD33, from Becton Dickinson Biosciences (San Diego, CA, USA), and PE-cyanine (PC)-5–conjugated anti-CD19, from Beckman/Coulter Inc. (Westbrook, MA, USA). Goat polyclonal anti-mouse IL-7 antibody (Ab) and goat anti-human IL-7Rα Ab were obtained from R&D Systems (Abingdon, UK) and used in the cultures at concentrations of 1 to 5 μg/mL, as indicated. Recombinant human IL-2, -4, -7, -9, and -11 were obtained from PeproTech EC Ltd. (London, UK) and recombinant human IL-15, -21, and both human and mouse thymic stromal lymphopoietin (TSLP) were obtained from R&D Systems.

4-[(3′-Bromo-4′-hydroxyphenyl) amino]-6,7-dimethoxyquinazoline, a potent specific inhibitor of JAK3 kinase (IC_{50} = 5.6 μM) was obtained from Calbiochem-Novabiochem Co. (San Diego, CA, USA) and used in the cultures at a concentration of 5 μM. The specificity of this chemical compound as a JAK3 kinase inhibitor has been examined by Goodman et al. [18] and Sudbeck et al. [19]. They demonstrated that this compound exhibited detectable inhibitory activity only against recombinant JAK3, but not JAK1 or JAK2, in immune complex kinase assays and also inhibited IL-2–induced JAK3-dependent signal transducers and activators of transcription (STAT) activation, but not inhibited IL-3–induced JAK1/JAK2-dependent STAT activation in 32Dc11-IL2R cells. Unless otherwise indicated, all chemical reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cells, cultures, and colony assay

Human BM $CD34^+$ cells used were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). BM cells were isolated from human tissue after obtaining informed consent. A cloned murine BM stromal cell line, MS-5, was kindly provided by Dr. A. Manabe (St. Luke's International Hospital, Tokyo, Japan) and Dr. K. Mori (Nigata University, Nigata, Japan), and maintained in RPMI-1640 medium (Sigma-Aldrich Fine Chemical Co., St. Louis, MO, USA) supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich) at 37°C under a humidified 5% CO_2 atmosphere.

To induce pro-B cells, MS-5 cells were plated at a concentration of 1 × 10^5 cells in 12-well tissue plate (Asahi Techno Glass Co., Chiba, Japan) 1 day prior to seeding human BM $CD34^+$ cells. $CD34^+$ cells were plated 4 × 10^4 cells/well/2 mL onto the MS-5 cells in RPMI-1640 supplemented with 10% fetal calf serum and

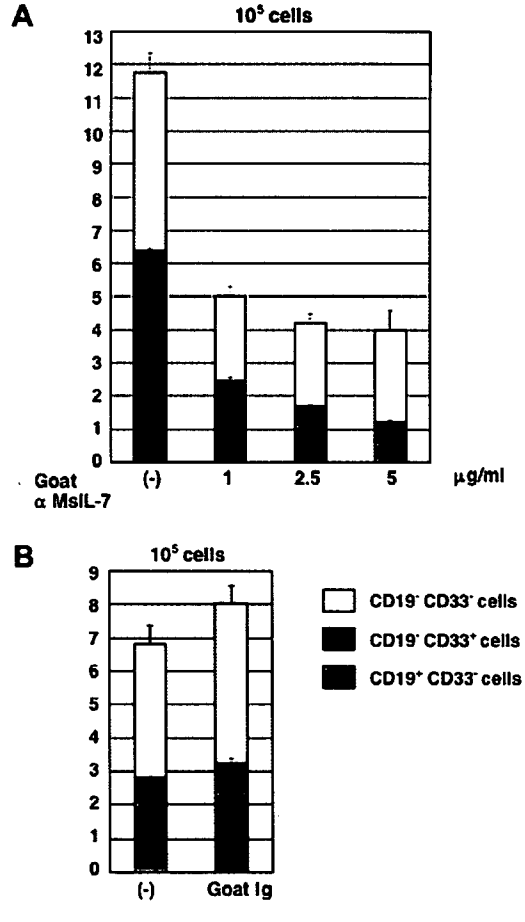

Figure 1. Effect of anti-mouse interleukin (IL)-7 antibody on human pro–B-cell development. (**A**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks in the presence or absence {(–)} of different concentrations of goat polyclonal anti-mouse IL-7 antibody. The subsequent $CD19^+CD33^-$ cell number (lower light gray column), $CD19^-CD33^+$ cell number (middle dark gray column), and $CD33^-CD19^-$ cell number (upper white column) of cultured $CD34^+$ cells were calculated by flow cytometry. (**B**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks in the presence or absence of goat immunoglobulin (goat Ig) as a negative control.

various combinations of cytokines or other reagents, as indicated in Figures 1,3,5,6,8. After cultivation for the periods indicated, the cells were harvested with 0.25% trypsin plus 0.02% ethylenediamine tetraacetic acid (IBL Co. Ltd., Gunma, Japan), the number of cells per well was counted, and cells were analyzed by flow cytometry.

For the colony assay, $CD34^+$ BM cells were cultured for 1 week on MS-5 cells in the presence or absence of goat anti-IL-7 Ab and the floating cell fraction was first collected with culture medium. The remaining adherent cell fraction with MS-5 cells were treated with trypsin, harvested, and plated in 6-well tissue culture plate (Asahi Techno Glass). After removing MS-5 cells by letting them attach to the bottom of the plate by 15-minute incubation, subsequent suspension cells were collected as adherent cell fraction. After counting the cell number by flow cytometry using Flow-Count (Beckman/Coulter), cells from each fraction were passaged into methylcellulose cultures containing the cocktail of cytokines (MethocultTM GF+H4435; Stem Cell Technologies Inc, Northampton, UK). Morphology and number of colonies comprising more than 50 cells was scored at 14 days. All experiments were performed in triplicate and the mean + SD of the values were shown in Figures 1,3,5,6,8.

Immunofluorescence study

Cells were stained with fluorescence-labeled monoclonal antibodies and analyzed by flow cytometry (EPICS-XL, Beckman/Coulter) as described previously [20]. Two-color immunofluorescence study was performed with a combination of PE and PC-5. Experiments were performed in triplicate, and the mean + SD of the cell counts were indicated in the Figures 1,3,5,6,8.

For cell sorting, human BM $CD34^+$ cells cocultured with MS-5 cells for 4 weeks were harvested and stained with PE-conjugated anti-CD33 monoclonal Ab and PC-5–conjugated anti-CD19 monoclonal Ab. $CD33^-CD19^-$, $CD33^+$ and $CD19^+$ cells were sorted in an EPICS-ALTRA cell sorter (Beckman/Coulter). Total RNA was extracted and used for reverse transcription polymerase chain reaction (RT-PCR).

RT-PCR

Total RNA was extracted from cultured cells, and cDNA was generated with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and a FirstStrand cDNA Synthesis Kit (Pharmacia Biotech, Uppsala, Sweden). cDNA synthesized from 150 ng total RNA was used as a template for one amplification. The sets of primers used in this study were listed in Table 1.

PCR was repeated for 30 to 35 cycles of heating at 94°C for 60 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 2 minutes; the products were then separated on a 1.5% agarose gel.

Results

MS-5 cells secrete IL-7

Murine stromal cell line MS-5 has been reported to possess the ability to support the differentiation of B-lineage cells and myeloid cells from human cord blood $CD34^+$ cells [21–25]. Consistent with previous observations, the human BM $CD34^+$ cells in our study generated $CD19^+CD33^-$ B cells and $CD19^-CD33^+$ myeloid cells after 4 weeks of

Table 1. List of primers used in this study

Name of gene	Primer sequence	Product size (bp)
Murine IL-7		
Forward	5′-TAAATCGTGCTGCTCGCAAGT-3′	
Reverse	5′-AGCAGTCAGCTGCATTTCTGTG-3′	392
Human IL-7R α		
Forward	5′-GTCACTCCAGAAAGCTTTGG-3′	
Reverse	5′- AGGAACTCTAGACTTCCCTTT-3′	
Human CD19		
Forward	5′-GTTCCGGTGGAATGTTTCGG-3′	386
Reverse	5′-AGATGAAGAATGCCCACAAGG-3′	576
Human TdT		
Forward	5′-ACACGAATGCAGAAAGCAGGA-3′	
Reverse	5′-AGGCAACCTGAGCTTTTCAAA-3′	315
Human PAX5		
Forward	5′-CCATCAAGTCCTGAAAAATC-3′	
Reverse	5′-CCCAAAGTGGTGGAAAAAAT-3′	319
Human Igα		
Forward	5′-TAGTCGACATGCCTGGGGGTCCAGGAGTCCTC-3′	
Reverse	5′-GATGTCCAGCTGGAGAAGCCGTGA-3′	681
Human GAPDH		598
Forward	5′-CCACCCATGGCAAATTCCATGGCA-3′	
Reverse	5′-TCTAGACGGCAGGTCAGGTCCACC-3′	
Murine actin		
Forward	5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′	
Reverse	5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′	661

GAPDH = glyceraldehyde phosphate dehydrogenase; IL = interleukin.

cocultivation with MS-5 cells (Fig. 1). Immunocytological analysis showed that the $CD19^+$ B cells in our culture system were surrogate light chain$^+$ μ^- pro-B cells [25]. Consistent with these observations, the human BM $CD34^+$ cells in our study generated $CD19^+$ B cells and $CD33^+$ myeloid cells after 4 weeks of cocultivation with MS-5 cells (Fig. 1). The detailed characterization of our culture system has been reported previously [25]. Starting with 4×10^4 $CD34^+$ cells, that containing <8% of $CD19^+CD34^+$, 0.4 to 1.3×10^6 mononuclear cells, 30.1% to 68.2% of which were $CD19^+CD34^-$ cells, were obtained (data not shown). Immunocytological analysis showed that most of these $CD19^+$ B cells expressed cytoplasmic-CD179a, a component of surrogate light chain known to be most specific molecular marker of precursor-B cells, whereas only a few percent of the $CD19^+$ cells were positive for surface and/or cytoplasmic-μ^- heavy chain. Considering the additional observations that CD10, CD24, and CD43 were expressed but CD20 were not in the $CD19^+$ cells, we concluded that most of the $CD19^+$ B cells obtained in our culture system were pro-B cells [25].

We investigated the expression of IL-7 by the MS-5 cells and IL-7Rα by cultured $CD34^+$ BM cells. RT-PCR analysis showed expression of murine IL-7 by MS-5 cells (Fig. 2A). In addition, expression of human IL-7Rα mRNA by the cultured human BM $CD34^+$ cells was observed (Fig. 2B).

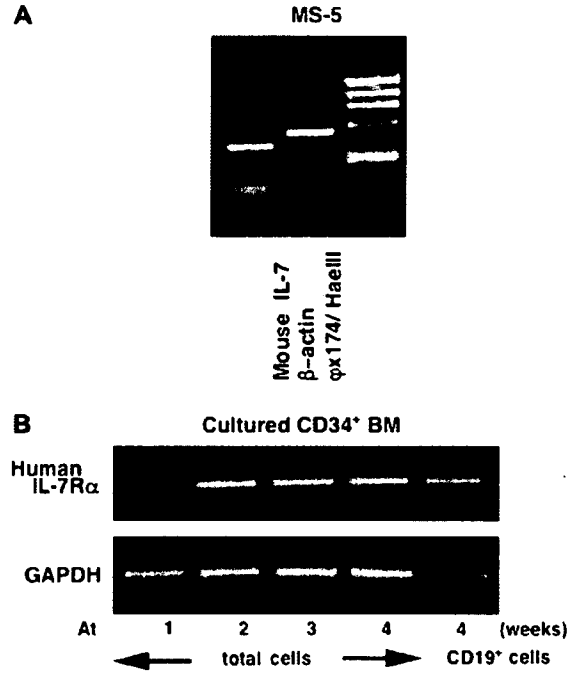

Figure 2. Expression of interleukin (IL)-7 by murine stromal MS-5 cells and of IL-7 receptor by cultured human bone marrow $CD34^+$ cells. (A) Expression of IL-7 by MS-5 cells was investigated by reverse transcription polymerase chain reaction (RT-PCR). Expression of mouse β-actin was also investigated as an internal control. The φχ174/*Hae*III molecular weight marker is shown on the right side. (B) Human bone marrow $CD34^+$ cells cultured on MS-5 cells for 1, 2, 3, and 4 weeks. At the end of each culture period, cultured human bone marrow cells was collected by gentry pipetting, and the expression of IL-7 receptor (R) α was investigated by RT-PCR. $CD19^+$ cells were sorted from 4-week cultured human bone marrow $CD34^+$ cells and similarly examined. Expression of human glyceraldehyde phosphate dehydrogenase was investigated as an internal control.

Elimination of IL-7 reduced pro–B-cell development

Because murine IL-7 is known to react with human IL-7R [26], the IL-7 secreted by MS-5 cells possibly affects cultured $CD34^+$ BM cells. We therefore investigated the effect of anti-mouse IL-7 antibodies, which neutralizes the effect of IL-7 on cultured $CD34^+$ BM cells. As shown in Figure 1, when anti-mouse IL-7 Ab was added, the $CD19^+CD33^-$ B-cell development was significantly reduced. In contrast, when goat immunoglobulin (Ig) G was similarly added, as a control experiment for Figure 1A, the $CD19^+CD33^-$ B-cell development was not reduced (Fig. 1B), indicating that the effect of anti-mouse IL-7 Ab is specific. The inhibitory effect of anti-mouse IL-7 Ab on pro–B-cell differentiation was found to be dose-dependent and time-dependent (Figs. 1 and 3). It is noteworthy that no significant change in $CD19^-CD33^+$ myeloid cell development was observed, whereas the subsequent cell number of $CD19^-CD33^-$ was also suppressed by addition of anti-mouse IL-7 Ab (Fig. 1).

Because we observed the inhibitory effect of anti-mouse IL-7 Ab on $CD19^-CD33^-$ cell fraction, we next investigated the expression of B-lineage marker genes to evaluate more detail characterization of these cells. As shown in Figure 4A, in addition to $CD19^+CD33^-$ pro–B cell, $CD19^-CD33^-$ cells but not $CD19^-CD33^+$cells also expressed IL-7Rα, after cultivation for 4 weeks. Expression of TdT was also detected in $CD19^-CD33^-$ cells. Although 30 cycles amplification failed in detection of PAX5 and Igα genes, 35 cycles amplification revealed the expression of these genes in $CD19^-CD33^-$ cells (Fig. 4B).

Effect of elimination of IL-7 on colony formation of $CD34^+$ BM cells

We also examined the effect of IL-7 elimination on colony formation ability of $CD34^+$ BM cells. The $CD34^+$ cells were cultured on MS-5 cells with and without anti-mouse IL-7 Ab for 1 week and examined by colony formation assay. As we reported previously [25], cultured $CD34^+$ cells on MS-5 cells can be classified into two subpopulations, namely, floating and adherent cell fraction. Interestingly, treatment with anti-mouse IL-7 Ab distinctively affected each cell fraction and the number of adherent cells was slightly decreased, whereas the floating cells were not reduced (Fig. 5A). Moreover, after treatment with anti-mouse IL-7 Ab, granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM) colony formation from floating cells was slightly reduced and burst-forming unit erythroid (BFU-E) colony formation

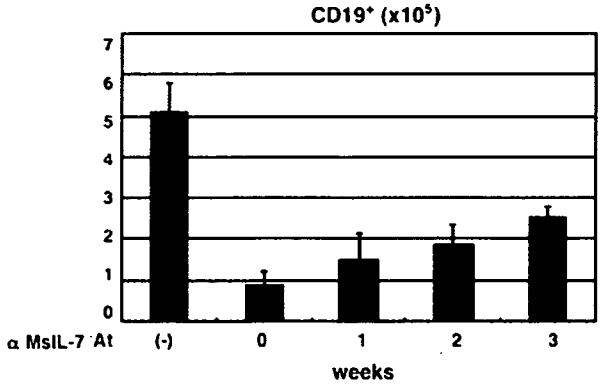

Figure 3. Time-dependency of anti-mouse interleukin (IL)-7 antibody–mediated inhibition of pro–B-cell development. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks. Goat polyclonal anti-mouse IL-7 antibody (2.5 μg/mL) was added at the start of culture (0), and after 1, 2, and 3 weeks of culture, and the number of $CD19^+$ cells was estimated by flow cytometry.

from adherent cells was significantly increased (Fig. 5B). Especially, subsequent BFU-E colony formation from total cells was also increased by anti-mouse IL-7 Ab treatment.

Effect of cytokines on anti–IL-7 Ab-mediated reduction in B-cell development

Since the reduction in $CD19^+$ B-cell development induced by anti-mouse IL-7 Ab was reversed by addition of recombinant human IL-7 to the coculture of $CD34^+$ BM cells and MS-5 cells (Fig. 6A), the effect of anti-mouse IL-7 Ab was concluded to be IL-7–specific. However, when we investigated the effect of exogenous recombinant human IL-7 alone, no significant increase in $CD19^+$ B-cell development was observed (Fig. 6A). Also, the proportion of different lineages cells was not affected by exogenous recombinant human IL-7 (data not shown). It is noteworthy that although exogenous recombinant human IL-7 did not change the number of pro-B cells, it increased the intensity of CD19 expression on $CD34^+$ BM cells (Fig. 7), while further differentiation of pro-B to pre-B cell was not observed (data not shown).

Next, we investigated the effect of exogenous recombinant human IL-2, IL-4, IL-9, IL-11, IL-15, and IL-21, which mediates signal transduction via common γ chain on the reduction in pro–B-cell development induced by anti-mouse IL-7 Ab, and no significant recovery in pro–B-cell development was observed (Fig. 6B). TSLP has been reported to mediate signal transduction via IL-7R and TSLPR heterodimer and have overlapping function with IL-7 [27,28]. Thus, we also investigated the effect of exogenous recombinant murine and human TSLP on reduction in pro–B-cell development induced by anti-mouse IL-7 Ab, whereas no significant recovery in pro–B-cell development was observed (Fig. 6C).

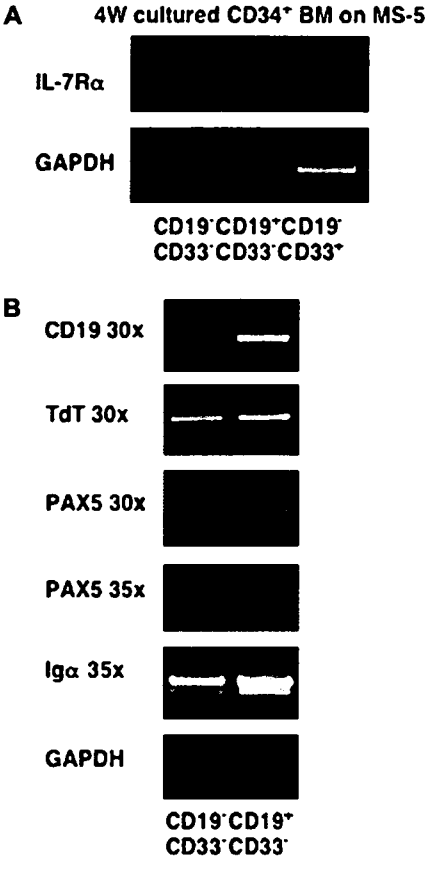

Figure 4. Expression of B-cell differentiation marker mRNAs by cultured human bone marrow $CD34^+$ cells. (**A**) Human bone marrow $CD34^+$ cells cultured on MS-5 cells for 4 weeks, $CD33^-CD19^-$, $CD33^+CD19^-$, and $CD33^-CD19^+$ cells were sorted, and expression of IL-7Rα was investigated by reverse transcription polymerase chain reaction with 30 cycles amplification. Expression of human glyceraldehyde phosphate dehydrogenase (GAPDH) was also investigated as an internal control. (**B**) $CD33^-CD19^-$ and $CD33^-CD19^+$ cells were sorted from 4-week cultured human bone marrow $CD34^+$ cells and expression of B-cell–differentiation marker genes as indicated were similarly examined as in (**A**) with either 30 or 35 cycles amplification. Expression of human GAPDH was investigated as an internal control.

Inhibition of IL-7 signaling reduced pro–B-cell development

Next, we investigated whether anti-human IL-7Rα Ab inhibits pro–B-cell development. As shown in Figure 8, addition of human IL-7Rα Ab that block the effect of IL-7 reduced the number of pro–B-cell development. Because IL-7R signaling transduces to JAK3, we investigated the effect of a JAK3 kinase inhibitor. As shown in Figure 8, the JAK3 kinase inhibitor significantly reduced pro–B-cell development.

Discussion

In this article, we demonstrated that IL-7 plays a certain role in development of human pro-B cells from

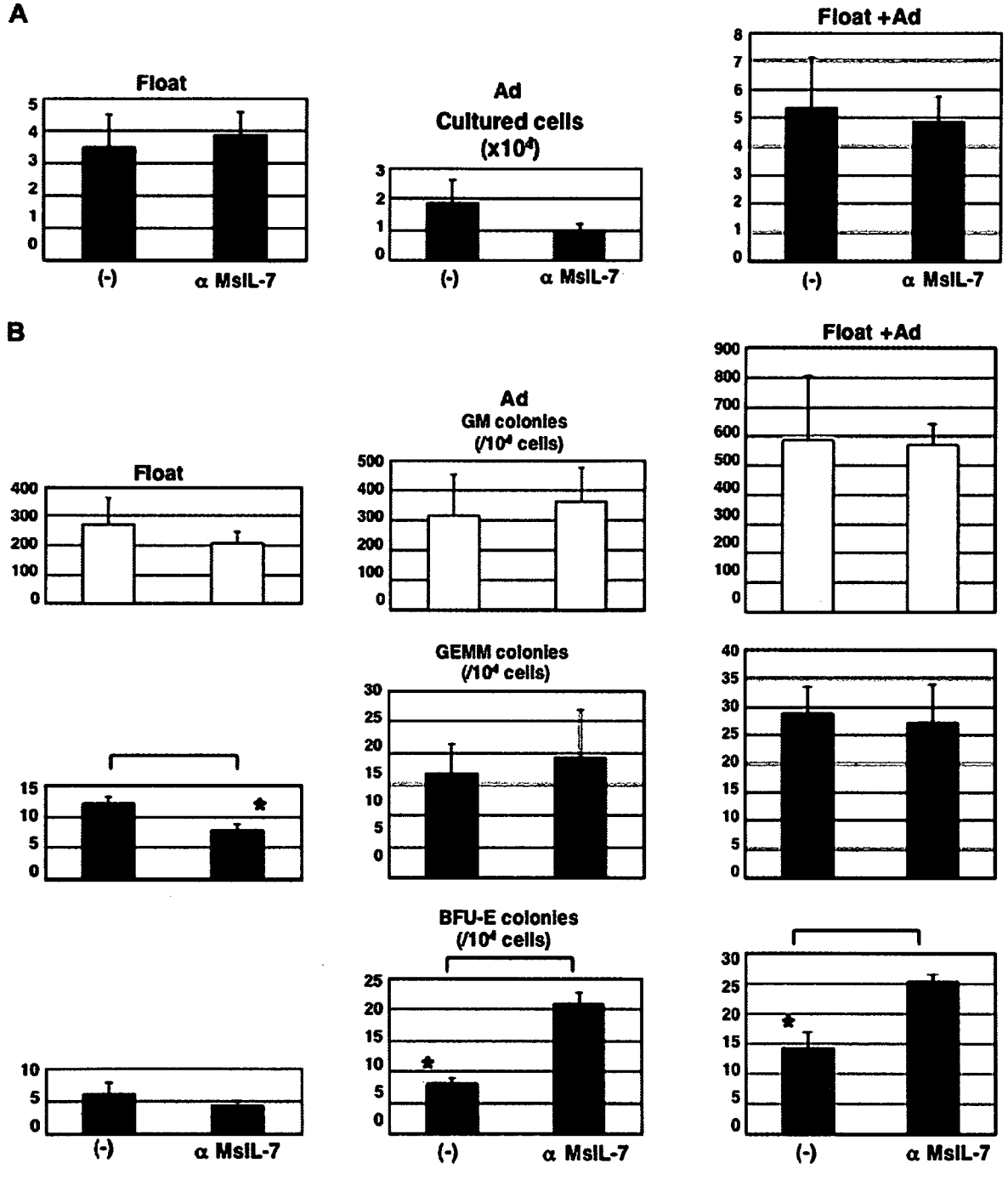

Figure 5. Effect of anti-mouse interleukin (IL)-7 antibody on colony formation ability of human bone marrow $CD34^+$ cells. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 1 week in the presence or absence {(–)} of goat polyclonal anti-mouse IL-7 antibody (2.5 μg/mL), and colony assay was performed with floating and adhesion cells separately as described in Materials and Methods. The number of granulocyte macrophage (GM), granulocyte-erythrocyte-macrophage-megakaryocyte (GEMM), and burst-forming unit erythroid (BFU-E) colonies per 10^4 cultured cells (**A**), and 1 weed-cultured cell number (**B**) was counted. *Statistically significant differences ($p < 0.05$).

hematopoietic stem cells in vitro. Results of the present study showed that MS-5 murine stromal cells produce IL-7 and that neutralization of the IL-7 they secrete with anti-mouse IL-7 Ab markedly reduced pro–B-cell development. As mentioned above, murine IL-7 is known to be capable of binding to the human IL-7R [26,29]. Although previous study of structure evaluation and enthalpy calculation performed on computer predicted that murine IL-7

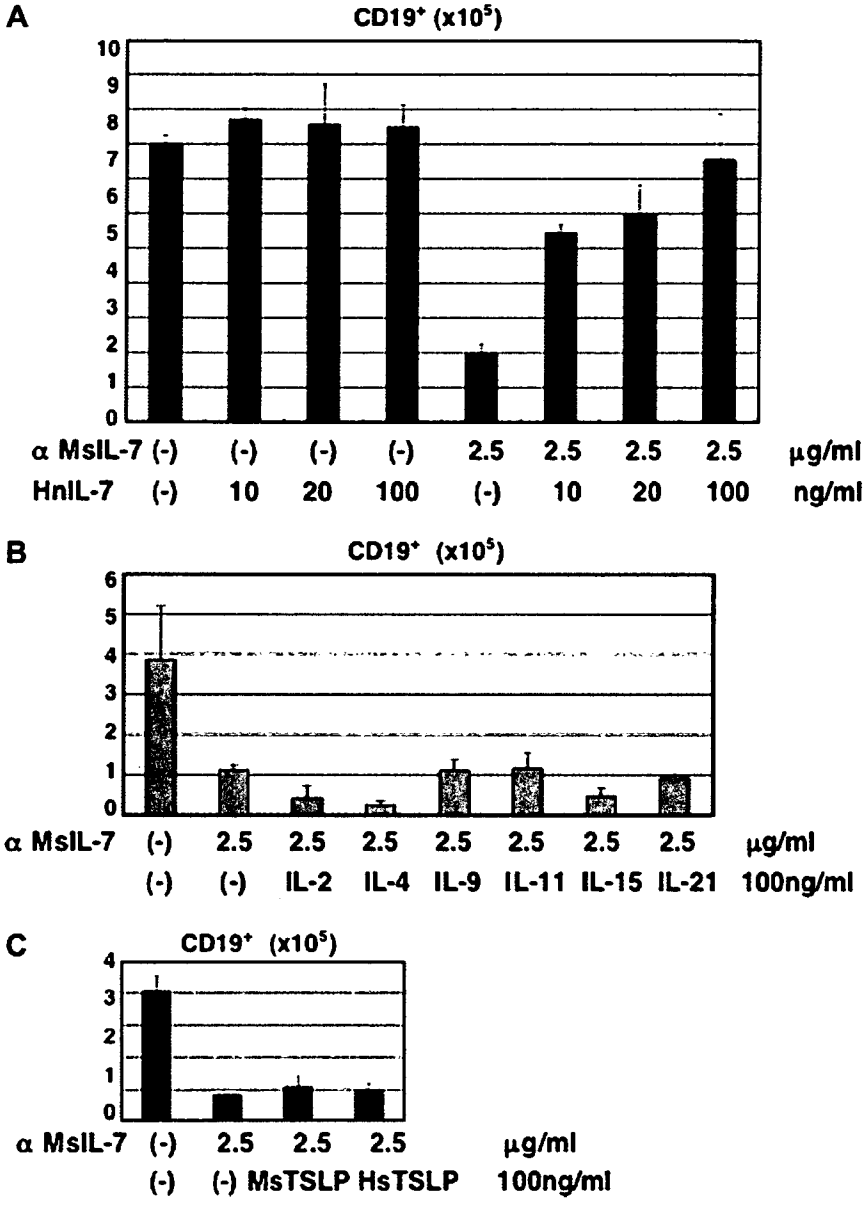

Figure 6. Effect of recombinant human interleukin (IL)-7 on anti-mouse IL-7 antibody–mediated inhibition of human pro–B-cell development. (**A**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL-7 (10 to 100 ng/mL, as indicated). The number of $CD19^+$ cells was counted, the same as described in Figure 5. (**B**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant human IL (100 ng/mL), as indicated. The number of $CD19^+$ cells was counted and presented as in Figure 5. (**C**) Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without the indicated combinations of goat anti-mouse IL-7 antibody (2.5 μg/mL) and recombinant murine or human thymic stromal lymphopoietin (TSLP) (100 ng/mL), as indicated. The number of $CD19^+$ cells was counted and presented as in Figure 5.

may display weaker binding with human IL-7R than human IL-7 [30], however, it has been reported that murine IL-7 still affect human $CD19^+$ cells and can induce downstream signaling of IL-7R [31]. Indeed, the anti-mouse IL-7 Ab–induced reduction in pro–B-cell development was reversed by the addition of recombinant human IL-7, suggesting specific inhibition of IL-7 function by anti–IL-7 Ab.

Inhibition of IL-7 binding to human IL-7R by anti-human IL-7Rα Ab also reduced pro–B-cell development, and a JAK3 kinase inhibitor that blocks signaling downstream of IL-7R showed a similar reduction in pro–B-cell development. As we presented, more significant inhibition of B lymphopoiesis was induced by addition of the JAK3 inhibitor. It may because the reason of that JAK3 mediates

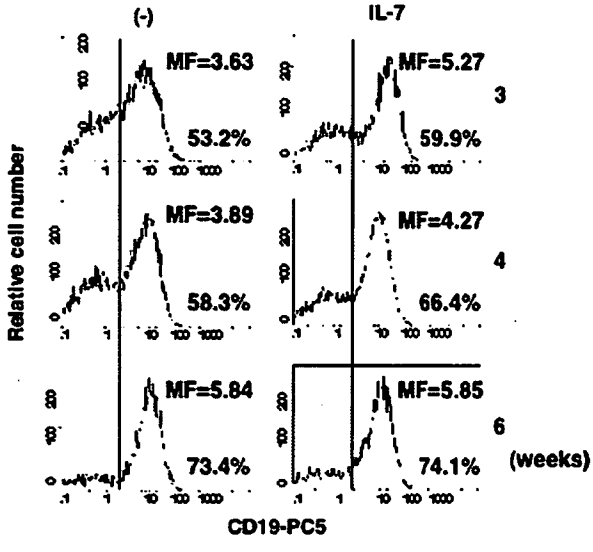

Figure 7. Effect of recombinant human interleukin (IL)-7 on human CD19 expression in pro-B cells. $CD34^+$ cells were cultured on MS-5 cells for 3, 4, and 6 weeks with or without 100 ng/mL recombinant human IL-7, and expression of CD19 was investigated by flow cytometry. The value of mean fluorescence intensity (MF) and positivity (%) of each histogram are indicated. Experiments were performed in triplicate, and similar results were obtained. X-axis, fluorescence intensity; Y-axis, relative cell number.

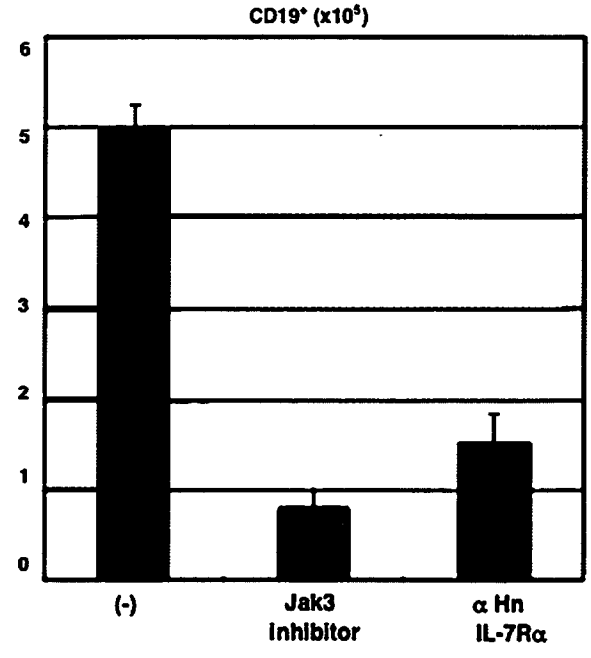

Figure 8. Effect of anti-human interleukin (IL)-7 receptor antibody and JAK3 kinase inhibitor on human pro–B-cell development. Human bone marrow $CD34^+$ cells were cultured on MS-5 cells for 4 weeks with or without goat polyclonal anti-human IL-7Rα antibody (2.5 μg/mL) or JAK3 kinase inhibitor (5 μM). The number of $CD19^+$ cells was counted the same as described in Figure 5.

signal transduction via the common γ chain of several lymphokines, including IL-2, IL-4, IL-9, IL-15, and IL-21, beside IL-7. All of the above findings clearly indicate that eliminating IL-7 function resulted in failure of pro–B-cell development in our culture system.

By contrast, addition of recombinant human IL-7 to the culture did not increase the number of pro-B cells, and thus the MS-5 cells possibly secrete IL-7 in sufficient amounts to support pro–B-cell development. Because the exogenous human IL-7 relatively increased CD19 expression on induced pro-B cells, excess IL-7 may accelerate pro–B-cell maturation, while further differentiation to pre-B cells was not occurred.

In the present study, we also presented that the elimination of IL-7 function results in the inhibition of cell growth in $CD19^-CD33^-$ cell fraction. Because we detected the gene expression of IL-7 Rα in the cell fraction of $CD19^-CD33^-$, but not $CD19^-CD33^+$, it is reasonable to consider that IL-7 can directly affect $CD19^-CD33^-$ cell fraction. The fact of the expression of B-lineage marker genes, such as PAX5 and Igα, should indicate that $CD19^-CD33^-$ cell fraction contain the B cell progenitors in which CD19 gene is not yet expressing. Consistently, Reynaud et al. reported that $IL\text{-}7R\alpha^+Ig\alpha^+CD19^-$ cells that produced by $CD34^+CD19^-CD10^-$ cord blood cells cultured in the presence of MS-5 with IL-2, IL-15, and stem cell factor cytokines, transcribed the B-lymphoid–specific genes E2A, EBF, TdT, Rag-1, had initiated DJH rearrangement [32]. Alternatively, IL-7 may affect not only lymphoid progenitor but also other lineage cells.

Interestingly, we observed that elimination of IL-7 function affects the colony-forming ability of cultured $CD34^+$ cells. The fact that the number of BFU-E colony remarkably increased by elimination of IL-7 function suggests the possibility that IL-7 promotes differentiation of hematopoietic progenitors into the B-lineage cells and thus lead a suppression of their differentiation into the erythroblast besides. The observation of Adolfsson et al. [33] that indicated the upregulated IL-7R gene expression in a population of $Lin\text{-}Sca1^+c\text{-}kit^+CD34^+Flt3^+$ lymphoid-myeloid stem cells in which the ability to adopt erythroid and megakaryocyte lineage fates have lost [33] should support our hypothesis. Moreover, decrease in the number of colony-forming unit-GEMM colony in adhesion cell fraction might suggest that IL-7 is taking part in the amplification of multipotent progenitor cells, though a more detailed investigation is necessary. It is also notable that floating and adhesion cell fractions seem to have different receptivity for the effect of IL-7 in our observation. Alternatively, IL-7 may influence the ability of adhesion of $CD34^+$ cell.

As shown above, IL-7 is required for human pro–B-cell development, at least in our culture system. In contrast to our observation, however, Pribyl et al. [11] showed that IL-7 is not necessary for human B-cell development in an in vitro study. They cocultured human $CD34^+$ hematopoietic stem cells and BM stromal cells from fetal BM for 3

weeks without exogenous cytokines and induced immature B cells expressing μ/λ or μ/κ surface Ig receptors. In their study enzyme-linked immunosorbent assay revealed secretion of about 1 to 2 pg/mL IL-7 by BM stromal cells, and addition of recombinant human IL-7 or anti-human IL-7 neutralizing Ab had no effect on the $CD19^+$ cell number. Consistent with this, congenital immunodeficiency patients who have mutations in common γ chain, IL-7Rα chain or JAK3 tyrosine kinase, have normal numbers of peripheral B cells [12–15].

Although the exact reason for the discrepancy is unknown, several explanations are possible. In contrast to our study, for example, they used human BM stromal cells, and the difference between the microenvironments produced by the human and murine stromal cells may have contributed to the difference in effect of IL-7 on human B-cell development. Another possibility is that, stimulation by another cytokine or a growth factor may compensate for the lack of IL-7 function in human B-cell development. In the mouse microenvironment, however, the factor may be absent or not have an IL-7 function–compensating effect. In this study, we have tried to identify the substitutional factor for IL-7, whereas IL-2, IL-4, IL-9, IL-11, IL-15, IL-21, and TSLP failed to compensate for the lack of IL-7 function. Therefore, another candidate(s) having substitutional effect for IL-7 need to be identified in the future experiments.

During revision, a similar observation to that presently reported has been published By Johnson et al. [31]. Using coculture system of $CD34^+$ cord blood cells and MS-5 cells supplemented with granulocyte-colony stimulating factor and stem cell factor to develop $CD19^+$ pro-B cells, they presented that murine and human IL-7 affect human pro-B cells and activate STAT5, resulting in proliferation. They also presented that neutralizing anti–murine IL-7 inhibited development of $CD19^+$ cells on their culture system. Our study further extends their observation and indicated that IL-7 is involved in the development of human pro-B cells from hematopoietic stem cells in vitro and affect $CD19^-CD33^-IL7R^+$ B-cell precursor fraction and hence influence on their colony-formation ability.

In view of the above findings, we concluded that the IL-7 is required for human pro–B-cell development from $CD34^+$ BM cells in our culture system and that IL-7 appears to play a certain role in early human B lymphopoiesis. Although further investigation needed to be done, our observations should contribute to a better understanding of the functional roles of IL-7 in the regulation of B lymphopoiesis.

Acknowledgments

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Preferential localization of SSEA-4 in interfaces between blastomeres of mouse preimplantaion embryos

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Abstract

The monoclonal antibody 6E2 raised against the embryonal carcinoma cell line NCR-G3 had been shown to also react with human germ cells. Thin-layer chromatography (TLC) immunostaining revealed that 6E2 specifically reacts with sialosylglobopentaosylceramide (sialylGb5), which carries an epitope of stage-specific embryonic antigen-4 (SSEA-4), known as an important cell surface marker of embryogenesis. The immunostaining of mouse preimplantation embryos without fixation showed that the binding of 6E2 caused the clustering and consequent accumulation of sialylGb5 at the interface between blastomeres. These results suggest that SSEA-4 actively moves on the cell surface and readily accumulates between blastomeres after binding of 6E2.

Keywords: SialylGb5; SSEA-4; Embryonic stem cells; Embryonal carcinoma cells; Preimplantation embryo; Immunostaining

Embryonal carcinoma (EC) cells isolated from teratocarcinomas have been shown to possess pluri- or multipotency in both mouse and human systems [1–3]. In mice, certain EC cells as well as embryonic stem (ES) cells have been considered to be developmentally equivalent to the inner cell mass of blastocysts [1]. These EC cells are useful for clarifying the molecular characteristics of early embryonic cells and thus many efforts have been made to establish EC cell lines and monoclonal antibodies (Mabs) that detect differentiation-related molecules on EC cells. As a consequence, a number of stage-specific markers for embryogenesis have been identified. Notably, it is important that this molecular information is adapted to research on ES cells or mouse preimplantation embryos. Stage-specific embryonic antigen (SSEA) -1, -3, and -4, as well as tumor rejection antigen (TRA) -1-60 and -1-81 [4], have been used as stage-specific markers for embryogenesis, though their functional significance in early development remains unclear. Interestingly, however, most of these antigens are carbohydrates themselves or closely related to the carbohydrates carried on glycosphingolipids (GSLs) and glycoproteins [5].

6E2 is a Mab established by immunizing with NCR-G3 cells, a previously established multipotent human EC cell

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line capable of differentiating into trophoblastic cell lineages other than somatic cells [3]. It has been revealed that 6E2 reacts with not only human ECs, including NCR-G2 and 3 cells, but also other germ cell tumors, as well as normal human germ cells such as spermatogonia and oocytes [6]. Although a previous study reported that 6E2 immunoprecipitates a cell surface protein having a molecular weight of approximately 80 kDa from ^{125}I-labeled NCR-G3 cells, the specific antigen recognized by 6E2 still remains unknown. To characterize the antigen specificity of 6E2, we examined the reactivity of the Mab with other cell lines using several distinct methods. In this paper, we present evidence that 6E2 recognizes SSEA-4 carried by sialylGb5. Using 6E2, we determined the localization of SSEA-4 in "living" mouse preimplantation embryos and observed its preferential localization in interface between blastomeres.

Materials and methods

Cells, antibodies, and animals. The human renal carcinoma cell line ACHN was purchased from American Type Culture Collection. The African green monkey kidney cell line Vero was a gift from Dr. T. Takeda of Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS). The human EC cell line NCR-G2 [3] was cultured in a 1:1 mixture of DMEM and Ham's F12 medium (DMEM/F12) (Invitrogen Gibco, Carlsbad, CA) supplemented with 10% FBS(JRH Bioscience), non-essential amino acid solution (NEAA) (Invitrogen Gibco), and Insulin-Transferin-Sodium Selenite media (Invitrogen Gibco). The cynomolgus monkey ES cell line CMK-6 [7] were provided by Dr. Yasushi Kondo of Mitsubishi Tanabe Pharma Corporation. ES cells were grown on mouse embryonic fibroblast feeder cells that were inactivated by gamma-irradiation in DMEM/F12 supplemented with 20% Knockout™ Serum Replacement, 2 mM Glutamax-I, 1% NEAA, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.1 mM 2-mercaptoethanol, 1% sodium pyruvate, and 5 ng/ml bFGF (all from Invitrogen GIBCO). The cultures were performed at 37 °C in a 5% CO_2 incubator. The human venous blood from a healthy consenting volunteer was drawn in a heparin-coated syringe. The blood was spun at 3000 rpm for 15 min and human red blood cells (hRBCs) were washed three times in phosphate buffered saline (PBS).

The conjugation of affinity-purified 6E2 (mouse IgG_3, κ) [6] to the fluorescence reagent was performed with an Alexa Fluor® 488 monoclonal antibody labeling kit (Molecular Probes, Eugene, OR.) according to the manufacturer's instructions. The anti-SSEA-4 Mabs used in this study were Raft.2 [8] and MC813-70 (R&D Systems, Inc Minneapolis, MN). Alexa Fluor® 488 goat anti-mouse IgG and Streptavidin Alexa Fluor® 568 were purchased from Molecular probes.

BDF_1 mice were purchased from Clea Japan (Tokyo, Japan).

TLC immunostaining of GSLs. TLC immunostaining of GSLs from cultured cells and hRBCs was performed as previously described [9]. Reference GSLs were purchased from Matlayer, Inc. (Pleasant Gap, PA). SialylGb5 was purified from ACHN cells by preparative TLC. Purified GM1 b was kindly provided by Dr. Nakamura of RIKEN, Saitama, Japan [10].

Flow cytometry. Cells were harvested and incubated with a primary antibody (1 μg/ml) for 1 h on ice, followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:50 and analyzed with an EPICS-XL flow cytometer (Beckman Coulter, Inc, Miami, FL).

Dot blot analysis. Purified sialylGb5 was serially diluted (0.1–60 ng) and vacuum blotted onto a PVDF membrane by using a 96-well format dot blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was immunostained with the Mab 6E2 or MC813-70 (0.5 μg/ml) according to a previously described procedure [9]. The antibodies that bound to the membranes were visualized with ECL-plus Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, UK) and scanned with a LAS-1000 luminescent imaging analyzer (Fujifilm, Tokyo, Japan). Scanned images were analyzed using the software Image Gauge with which the LAS-1000 was equipped.

Indirect immunostaining of cynomolgus monkey ES cells. Cells were grown on a glass-bottomed dish (IWAKI) for 3 days and then these cells were fixed for 30 min with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-200 in PBS for 20 min. Subsequently, the cells were washed three times with PBS for 5 min and blocked with 5% normal goat serum in PBS for 30 min. The fixed cells were incubated with anti-SSEA-4 antibodies or isotype-matched mouse IgG at a dilution of 1:300 for 2 h, followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse IgG at a dilution of 1:300 for 30 min. DAPI was used for counter staining of nuclei.

Immunostaining of mouse preimplantation embryos. Mouse preimplantation embryos were collected from superovulated mice. Seven-week-old BDF1 female mice were induced to superovulate with intraperitoneal injections of pregnant mare's serum gonadotropin (ASKA Pharmaceutical co., Ltd., Tokyo, Japan) (5 IU) and human chorionic gonadotropin (hCG) (ASKA Pharmaceutical co) (5 IU) 48 h apart and mated with individual BDF1 male mice after the hCG injection. The 2-cell, the 8-cell, and the morula stage embryos were flushed out from oviducts at 36, 60, and 72 h after the hCG injection, respectively. Animals were treated according to the institutional animal care and use guidelines of National Research Institute for Child Health and Development.

Embryos immediately after being collected and those prefixed with 2% paraformaldehyde in Hepes buffered saline were incubated in 30 μl drops of M16 medium containing 0.45 μg of Alexa Fluor® 488-conjugated 6E2 for 1 h or biotinylated MC813-70 for 1 h, treated with streptavidin Alexa Fluor® 568 diluted 1:300, and then they were washed three times in 30 μl drops of M16 medium. All staining steps were carried out at 37 °C in a CO_2 incubator for fresh embryos and at 4 °C for fixed embryos. The stained embryos were placed in drop of a M16 medium on glass-bottomed dishes (IWAKI, Tokyo, Japan), and were observed with a LSM510 Zeiss Confocal laser-scanning microscope (Carl Zeiss, Thornwood, NY) to obtain a field of view of the embryo only with a 40× objective lens.

Results and discussion

6E2 specifically binds to sialylGb5

In order to examine whether the 80 kDa membrane protein is recognized by 6E2, we performed a Western analysis of the cell lysates or their immunoprecipitates with 6E2. Since no significant signal was detected on the blot (data not shown), we examined TLC immunostaining of GSLs extracted from several 6E2-positive cell lines. ACHN cells showed the expression of comparable amounts of Gb3, Gb4, Gb5, and sialylGb5, whereas Vero cells and NCR-G2 cells expressed predominantly Gb3 (Fig. 1A). TLC immunostaining analysis revealed that 6E2 binds to a major slow-migrating GSL extracted from these three cell lines. The slow-migrating GSL was identified as sialylGb5, defined by the Mab Raft.2. We observed that 6E2 bound to sialylGb5 (LKE-antigen) of hRBCs [13] (Fig. 1B). Finally, we examined the reactivity of 6E2 with purified GSLs and found that the Mab reacts with purified sialylGb5, but not purified GM1 b (Fig. 1C). These results indicate that 6E2 specifically binds to sialylGb5 and thus is an anti-SSEA-4

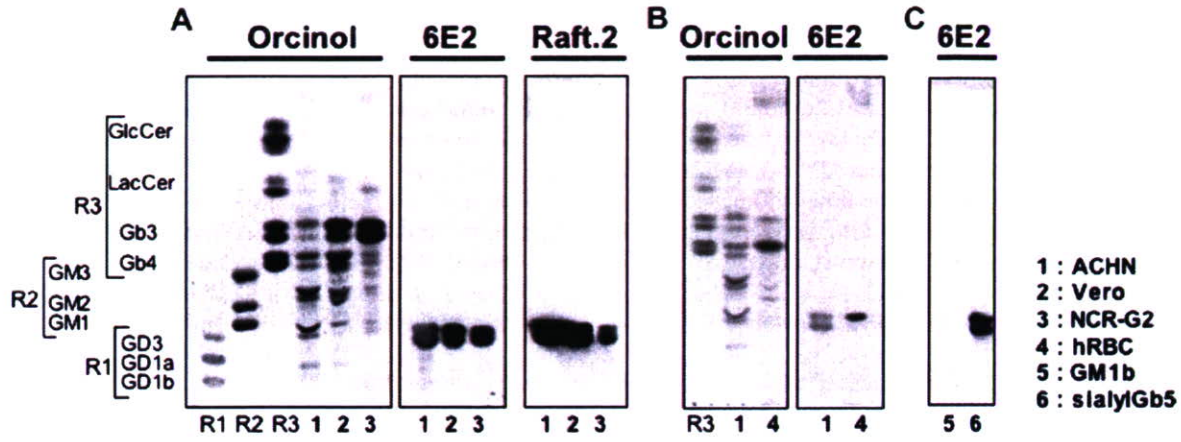

Fig. 1. TLC immunostaining of GSLs prepared from cultured cells and hRBCs. GSLs extracted from cultured cells and hRBCs or purified GSLs were separated by TLC in a solvent system of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, v/v/v). Plates were chemically stained with orcinol-sulfurnic acid or were immunostained with 6E2 and Raft.2. Lane 1, ACHN; Lane 2, Vero; Lane 3, NCR-G2; Lane 4, hRBCs; Lane 5, GM1b; Lane 6, sialylGb5. Reference markers used were disialosyl gangliosides of GD3, GD1a, and GD1b (R1), monosialosyl gangliosides of GM3, GM2, and GM1 (R2), and neutral GSLs of GlcCer, LacCer, Gb3, and Gb4 (R3). The nomenclature for GSLs follows the recommendations [11] of the IUB, and the ganglioside nomenclature of Svennerholm [12] was used.

Mab. The 80 kDa protein might be associated with sialylGb5 in NCR-G3 cells and thus co-immunoprecipitated by 6E2.

Comparison of reactivity to sialyl Gb5 between 6E2 and MC813-70

MC813-70 established by immunizing with human EC cell lines has been most widely used as an anti-SSEA-4 antibody (mouse IgG_3, κ) [14]. Therefore we compared the reactivities of the Mabs 6E2 and MC813-70 by flow cytometry and dot-blot immunostaining. The fluorescence intensity obtained with 6E2 was stronger than that with MC813-70 in each cell line and hRBCs (Fig. 2A). A recent flow cytometric study showed that MC813-70 strongly stains hRBCs, but other anti-sialyGb5 Mabs do not [15]. However, our data indicate that 6E2 is more reactive than MC813-70. Next we compared the reactivity of the two

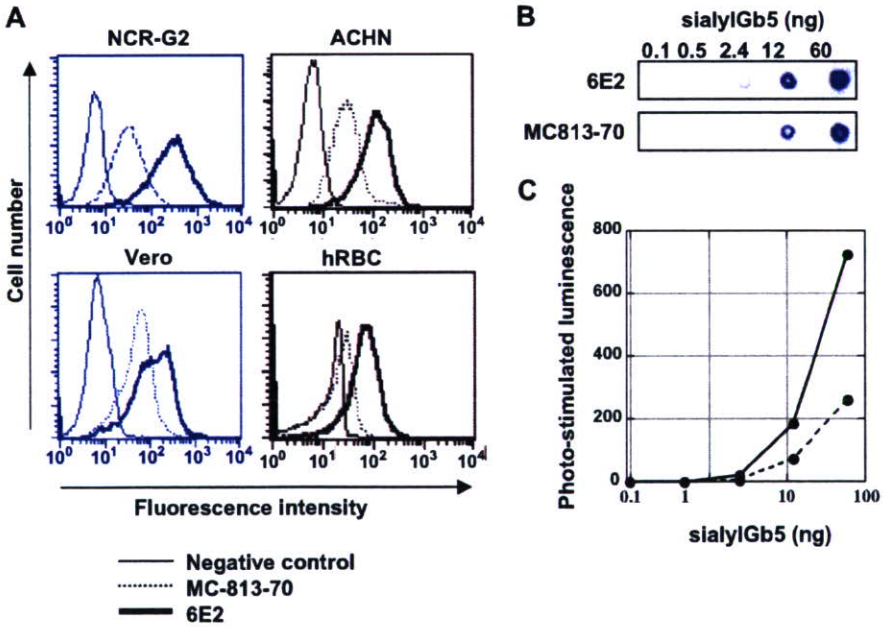

Fig. 2. Reactivity of 6E2 and MC813-70 with sialylGb5. (A) Flow cytometric analysis of SSEA-4-positive cells with 6E2. NCR-G2 cells, ACHN cells, Vero cells, and hRBCs were stained with 6E2 (bold line) or MC813-70 (dotted line) and with a FITC-conjugated secondary antibody and analyzed by flow cytometry. (B) An image of the dot-blot immunostaining of sialylGb5 obtained with a LAS-1000 luminescent imaging analyzer. (C) Measurement of antibodies bound (6E2: solid line, MC813-70: broken line).

Mabs with that of sialylGb5 by dot-blot immunostaining. Serially diluted sialylGb5 was dot-blotted onto a PVDF membrane, and the membrane was immunostained with the two Mabs. Both 6E2 and MC813-70 bound to more than 12 ng of sialylGb5, but the signals induced by 6E2 were stronger than those induced by MC813-70 (Fig. 2B,C). Thus, in addition to the flow cytometric analysis, the reactivity of 6E2 with sialylGb5 was stronger than that of MC813-70 by dot-blot immunostaining.

SSEA-4 Immunostaining of cynomolgus monkey ES cells

To confirm whether Mab 6E2 reacts with SSEA-4 on monkey ES cells, we performed an indirect immunofluorescence staining of cynomolgus monkey ES cells with Mab 6E2 and MC813-70. Mab 6E2 reacted with monkey ES cells (Fig. 3A) as well as MC-813-70 did (Fig. 3B). No difference in staining patterns of SSEA-4 between the two Mabs was observed. Mab 6E2 certainly stained SSEA-4 on monkey ES cells.

SSEA-4 immunostaining of "living" mouse preimplantation embryos without fixation

During early embryogenesis in mice, SSEA-4 had been reported to be expressed in fertilized eggs with levels gradually increasing to the morula stage and then decreasing [5]. Thus we examined the expression and distribution of SSEA-4 in preimplantation mouse embryos by immunostaining with both 6E2 and MC813-70. Both Mabs evenly stained the whole surface membranes of fixed mouse embryos, and no difference in staining pattern between the two was observed (data not shown). In order to perform a time-course of SSEA-4 distribution in a viable state, we performed immunostaining of preimplantation embryos without fixation.

3D-images of the 6E2 staining pattern obtained by confocal laser scanning microscopic observation clearly showed the localization of SSEA-4 on mouse preimplantation embryos. Two-cell embryos showed patches of SSEA-4 over the whole surface membrane with some accumulation at the interface between blastomeres (Fig. 4A). In 8-cell embryos, the amount accumulated at interfaces was further increased, as if planer membranes separate each blastomere, and some large patches were internalized but others were left on the surface membranes (Fig. 4B). The amount of SSEA-4 concentrated at the interfaces in morula was not as significant as in 8-cell embryos but still clearly observed and some patches were internalized (Fig. 4C).

2D-images of embryos stained with 6E2 showed a marked accumulation of SSEA-4 at the interfaces between blastomeres (Fig. 4D–F). These results suggest that sialylGb5 actively moves during development and tends to accumulate where blastomeres come into contact with each other.

Interestingly, however, the staining pattern of SSEA-4 using MC813-70 was different from that using 6E2. MC813-70 evenly stained the surface and the interface between blastomeres of 2-cell embryos with patches (Fig. 4G), and the amount of SSEA-4 at interfaces was not significant (Fig. 4J). In 8-cell embryos, there were patches of SSEA-4 in the central area of the outer surface of each blastomere (Fig. 4H, indicated by arrows), but the 2D-image showed that clustering also occurred at surfaces facing blastocoels (Fig. 4K, indicated by arrowheads). In morula embryos, SSEA-4 was distributed on the surface in patches and was enriched at the boundaries between blastomeres on the outer surface (Fig. 4I,L).

It remains unclear why the pattern of staining of mouse preimplantation embryos differs between 6E2 and MC813-70. The composition of fatty acids in GSLs influences the binding of antibodies [16,17] or bacterial toxins [18]. SialylGb5 recognized by the two MAbs might differ in composition of fatty acids, resulting in different immunostaining patterns. It was reported that the clustering of sialylGb5 by a Mab induces the activation of sialylGb5-associated kinases in raft microdomains of human mammary carcinoma cells, leading to downstream signaling [19,20]. The clustering of sialyGb5 by 6E2 on preimplantation mouse embryos may also induce the activation of some kinases, followed by downstream signaling. Recently, Comisky et al. suggested that lipid rafts and their associated molecules are spatiotemporally positioned to play a critical role in preimplantation developmental events [21]. The patches or clusters of sialylGb5 shown in our study suggest the presence of lipid rafts containing sialylGb5 on mouse embryos.

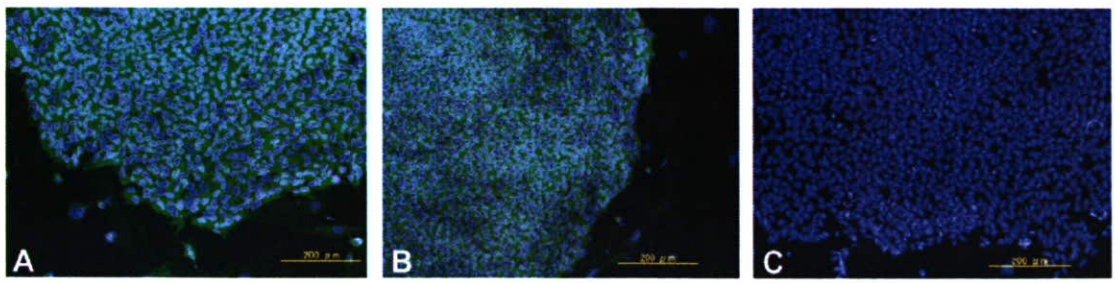

Fig. 3. Indirect immunostaining of cynomolgus monkey ES cell line CMK-6 with 6E2 and MC813-70. The CMK-6 cells were stained with 6E2 (A), MC813-70 (B), or isotype-matched mouse IgG (C), and visualized with secondary antibodies (green), followed by counterstaining of nuclei with DAPI (blue). Scale bars = 200 μm.

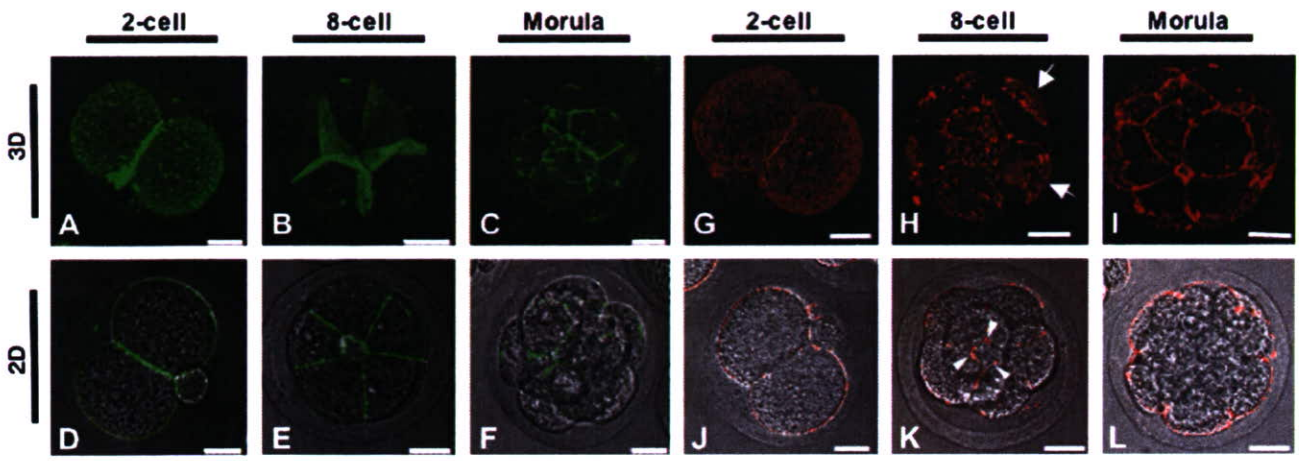

Fig. 4. Immunostaining of SSEA-4 on mouse preimplantation embryos with 6E2 and MC813-70. The embryos at the 2-cell (A, D, G, J), the 8-cell (B, E, H, K), and the morula (C, F, I, L) stages were stained with 6E2 (green) or MC813-70 (red). The panels designated 3D (A, B, C, G, H, I) are three-dimensional images reconstructed by stacking optical slice images using LSM software and the panels designated 2D (D, E, F, J, K, L) are an overlay of a fluorescent image and a differential interference contrast micrograph. Scale bars = 20 μm.

6E2 has high affinity for sialylGb5 and can be effectively conjugated with fluorescence reagents, leading to excellent staining of SSEA-4 in the surface membrane of "living" mouse preimplantation embryos. 6E2 should be of use for research into lipid rafts in early development and of great advantage for the characterization of ES cells and EC cells.

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SHORT COMMUNICATION

The detergent-insoluble microdomains, rafts, can be used as an effective immunogen

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Abstract Detergent-insoluble microdomains, or rafts, act as a platform to transduce signals from the extracellular space into the cytoplasm. In the process of developing monoclonal antibodies against raft molecules for the purpose of studying the molecular mechanism of raft-mediated signaling, we observed the uniqueness and certain advantages of immunization with rafts. Simple subcutaneous injection of mice with a phosphate-buffered saline (PBS) suspension of rafts without mixing with Freund's adjuvant made it possible to increase the titer of antiserum reacting with raft components. Interestingly, injection of rafts prepared from certain specific cell lines induced monoglycolipid-specific antibodies. Furthermore, antibodies were produced by raft-immunization of even syngeneic mice. Our findings suggest that this phenomenon does not represent a breakdown of immunological self-tolerance, but typical immune reactions accompanying the class switch from IgM antibodies to IgG antibodies.

Keywords Raft · Antibody · Immune Response · Monoglycolipid-specific · Syngeneic antigen

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Introduction

There is evidence that detergent-insoluble microdomains, or rafts, are important in signal transduction, because a variety of signaling molecules, such as Src-family kinases, heterotrimeric G proteins, and GPI-anchored proteins, are concentrated in rafts. We have previously shown that the binding of Shiga-toxin (Stx) to the globotriaosylceramide (Gb3[1]) in rafts temporally activates the Src-family kinase Yes in human renal cancer cell line ACHN [1]. In order to study the downstream signaling mechanism after Stx binding to Gb3, we attempted to develop monoclonal antibodies against components of rafts prepared from ACHN cells and established several clones [2].

In the process we observed the uniqueness of immunization using raft suspensions. Before immunizing animals in an attempt to induce antibody production, antigen solutions or cell suspensions are generally mixed with Freund's adjuvant to obtain an oil emulsion, whereas we succeeded in raising antibody titer by the raft immunization method without mixing them with adjuvants. Interestingly, two thirds of the clones obtained reacted with lipid components of the raft, and further analysis showed that all of the lipid-reactive clones recognized monosialosylgalactosylglobosides (sialylGb5).

To ascertain whether raft immunization always induces monospecific antibodies that recognize a certain glycolipid, we immunized mice with rafts prepared from several cell lines and examined the glycolipid antigens recognized by the antibodies induced. In this paper we report that injection with

[1] Glycosphingolipids are abbreviated according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature. http://www.chem.qmul.ac.uk/iupac/misc/glylp.html.

rafts prepared from certain specific cell lines can induce the production of monoglycolipid-specific antibodies and that raft immunization can induce antibody production even in syngeneic mice.

Materials and methods

Cell culture and antibodies Human renal cancer cell line ACHN, human T-cell leukemia cell lines Jurkat and MOLT-4, and mouse myeloma cell line P3U1 were purchased from the American Type Culture Collection, and the African green monkey kidney cell line Vero was a gift of Dr. T. Takeda of the Department of Infectious Diseases Research, National Children's Medical Research Center, Tokyo, Japan. Anaplastic large cell lymphoma Karpas 299 cells [3] were gifted by Dr. K. Kikuchi of Sapporo Medical University, School of Medicine, Sapporo, Japan. Human pre-B ALL cell line NALM-6, mouse T lymphoma cell line EL4, mouse melanoma cell line B16F1, and mouse leukemia cell line RL2 were obtained from the Institute of Development, Aging and Cancer of Tohoku University, Sendai, Japan. The ACHN cells, Vero cells, and B16F1 cells were cultured in Dulbecco's modified Eagle's medium (Sigma Chem., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Cansera International Inc., CCT, Canada). All other cell lines were cultured in RPMI 1640 supplemented with 10% FBS. The NZB/WF1 serum was a kind gift of Dr. S. Kon of the Institute of Genetic Medicine, Hokkaido University, Sapporo.

Raft preparation Rafts were prepared as described previously [2]. Briefly, packed cells were homogenized in 1% Triton lysis buffer (1% Triton X-100, 25 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl) by 20 strokes with a hand-driven Teflon glass homogenizer. Cell lysates, sucrose concentration of which was adjusted to 40% with 85% sucrose solution, were placed on the bottom of an ultracentrifuge tube, and a 5/30% discontinuous sucrose gradient was formed over the sample. After centrifugation at 39,000 rpm for 18 h at 4°C in a Beckman SW 40Ti rotor, rafts were recovered as visible bands at the interface between 5 and 30% sucrose solution. After several washes with PBS, raft suspensions in PBS were stored at −30°C until used.

Immunization of mice Rafts prepared from 1.2×10^6~1.5×10^8 cells or 10^7 cells irradiated at 10 Gy were suspended in 100 μl of PBS. They were subcutaneously injected into mice in triplicate, followed by three booster shots at 1-week intervals. Five days after the final injection, a peripheral blood specimen was collected from the mice, and the level of antibodies against rafts was evaluated.

TLC immunostaining Lipids were prepared from packed cells as previously described [4] and separated on a Silica gel 60-precoated HPTLC aluminium sheet (Merck, Darmstadt, Germany) with a solvent system consisting of chloroform/methanol/water containing 0.2% $CaCl_2$ (5:4:1, *v/v*). After drying, the TLC plates were coated with 0.1% polyisobutylmethacrylate (Sigma-Aldrich, Milwaukee, WI) in cyclohexane and blocked with 1% bovine serum albumin (BSA) in PBS. The plates were probed with anti-sera (diluted to 1:500 in 1% BSA in PBS) for 1 h at room temperature. After three washes with PBS for 5 min each, horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulins G+M (DAKO, A/S, Denmark) at a 1:2,000 dilution ratio were used as the second antibody. The antibodies that bound to the plates were visualized with enhanced chemiluminescence reagent Super Signal (Pierce, Rockford, IL) and detected with a luminescent imaging analyzer, LAS-1000 (Fuji Film, Tokyo, Japan). To compare the amounts of antibodies in the sera, the intensity of chemiluminescence was measured with Image Gauge analysis software equipped to LAS-1000 and shown as Photo Stimulated Luminescence (PSL).

Dot-blot immunostaining assay The ACHN rafts were dot-blotted on a PVDF membrane (Millipore Corp., Bedford, MA) and immunostained as described previously [2] with a slight modification. The dots were probed with antisera (diluted to 1 in 500 with 1% BSA in PBS) for 1 h at room temperature. After four washes with PBS containing 0.025% Tween 20 (PBS-Tween), the membranes were treated with HRP-conjugated rabbit anti-mouse IgG antibodies specific to Fcγ fragment and HRP-conjugated goat anti-mouse IgM antibodies specific to μ chain (Jackson Immuno Research Laboratories, West Grove, PA) to detect IgG and IgM, respectively. The antibodies that bound to the membrane were visualized with enhanced chemiluminescence (ECL Western blotting system; Amersham Pharmacia Biotech. UK Ltd., Buckinghamshire) and detected by a luminescent imaging analyzer as mentioned above.

Flow cytometry Cells were harvested from culture plates, and after incubating with the antisera (diluted to 1:100 in RPMI medium containing 5% FBS and 0.1% NaN_3) for 1 h on ice, they were treated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Jackson Immuno Research Laboratories) at a 1:50 dilution ratio and analyzed by flow cytometry (EPICS-XL, Beckman-Coulter, Fullerton, CA).

Measurement of anti-ss and -dsDNA antibodies in sera by ELISA The ELISA was performed as described by Iizuka *et al.* [5] with a slight modification by using calf thymus ssDNA (Sigma) and salmon sperm dsDNA (Sigma). For