Induction of Recombination Activating Gene Expression in a Human Lymphoid Progenitor Cell Line: Requirement of Two Separate Signals From Stromal Cells and Cytokines

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The activation and expression of recombination activating genes (RAGs) plays critical roles in V(D)J gene recombination machinery and lymphocyte development. We showed that RAG gene expression was induced in freshly isolated human bone marrow cells and a human lymphoid progenitor cell line, FL8.2.4.4, by coculture on a monolayer of a murine bone marrow-derived stromal cell line, PA6, in the presence of a mixture of recombinant cytokines. The RAG transcripts were detected 12 hours after initiation of culture, and the increased level was sustained at 24 hours. Among recombinant cytokines, interleukin-3 (IL-3), IL-6, and IL-7, but not IL-2, IL-4, stem cell factor (SCF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) could induce RAG-1 activation in FL8.2.4.4 cells, and a significant synergistic effect between IL-3, IL-6, and IL-7 was observed. Using a double chamber culture technique, it was shown that a cognate interaction between FL8.2.4.4 cells and PA6 stromal cells was a prerequisite for RAG-1 activation. Furthermore, RAG-1 transcripts were induced in FL8.2.4.4 cells when they were cocultured on paraformaldehyde-fixed PA6 stromal cells in the presence of cytokines. These results suggest that two separate signals are both required for induction of RAG-1 activation in lymphoid progenitors; one from the cell surface molecule(s) on stromal cells, and the other from the recombinant cytokine(s). Finally, we showed that expression of RAG mRNA in FL8.2.4.4 cells was concomitant with induction of recombinase activity. This system may provide useful means for further understanding the mechanisms controlling RAG activation and lymphocyte development in the human system.

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and fetal BM.23 We also showed that the biphenotypic acute lymphocytic leukemia (ALL)-derived leukemic lymphocyte precursors, with TdT+ CD2+CD10+CD19+CD34+ immunophenotype, differentiated into the pre-B cells after stimulation with PMA.23 To further delineate the feature of human lymphoid progenitors, we established and characterized the Epstein-Barr virus (EBV)-transformed cell lines from mononuclear cells in the human fetal liver at 8 weeks gestation. It was found that the established fetal liver cell lines retained germline configuration of Ig and TCR genes and expressed early lymphoid surface markers of CD2 and CD19, but not surface or intracytoplasmic IgIs or TCRs. The cell lines did not express mRNA for Rag-1 and Rag-2.24 From these findings, we have presumed that the established cell lines are lymphoid progenitor cells that have not yet been committed to either T or B lymphoid lineage and are thus useful for the study of the differentiation programs of human lymphoid progenitors.

In the present study, we have investigated the effects of recombinant cytokines and stromal cells on activation of RAGs in a human lymphoid progenitor cell line, FL8.2.4.4. Our data clearly show that the human lymphoid progenitor cell line requires two independent signals for Rag gene activation; one from cell surface molecule(s) on BM-derived stromal cells, and the other from cytokine(s) such as IL-3, IL-6, or IL-7.

MATERIALS AND METHODS

Recombinant cytokines. All cytokines used in this study were recombinant (r) cytokines. rIL-2 was kindly provided by Shionogi Pharmaceutical Co (Osaka, Japan); rIL-3, recombinant stem cell factor (rSCF) and recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) by Kirin Brewery Co (Tokyo, Japan); rLIF by Ono Pharmaceutical Co (Osaka, Japan); rIL-6 from Ajinomoto Co (Tokyo, Japan). rIL-7 was purchased from Pepro Tech Inc (Rocky Hill, NJ).

Cells and cell culture. Heparinized BM samples were obtained at surgical osteotomy from patients (12 to 30 years old) with osteopetrosis. BM mononuclear cells (BM-MNC) were separated by the standard Ficoll-Hypaque gradient method. The viability was greater than 90% as determined by trypan blue exclusion.

FL8.2.4.4 (a human lymphoid progenitor cell line) was established in this laboratory.25 RPMI1188 and Daudi (human B cell line), Nalm6 (human pre-B cell line), and U937 (human monocyte cell line) were obtained from Dr P. Ralph (Cetus Corp, Berkeley, CA). A description of these cell lines is provided by Minowada.25 PA6 (a murine BM-derived stromal cell line)26 and 3T3 (a murine fibroblast cell line) were obtained from Dr S-I. Nishikawa (Kyoto University, Kyoto, Japan). Cell lines were maintained in a culture medium (RPMI1640 containing 5% heat inactivated fetal calf serum (FCS), 50 μg/mL 2-ME, 100 U/mL penicillin, 100 μg/mL streptomycin) at 37°C in a humidified CO2 incubator. Only logarithmically growing cells were used for experiments.

For coculture of cells with the stromal cell line (PA6), PA6 monolayer was prepared by seeding PA6 cells (2 x 10⁶/well) in a 24 well-plate ( Falcon) 2 days before the experiments. BM-MNC (1 x 10⁶/well) from patients with the disease BM-MNC (1 x 10⁶/well) or FL 8.2.4.4 cells (5 x 10⁶/well) were cultured on PA6 monolayer in the presence of recombinant cytokines for various periods. After coculture, cells including PA6 were harvested and RNA was prepared for reverse transcriptase-polymerase chain reaction (RT-PCR) assay as described below. In some experiments where indicated, cognate interaction between FL8.2.4.4 cells and the monolayer was prevented by a 0.45 μm membrane of Millicell-CM (Millipore, Bedford, MA). In other experiments where indicated, FL8.2.4.4 cells were cocultured on fixed-Pa6 stromal cell line. For cell fixation, cells were treated with 0.1% paraformaldehyde for 5 minutes and washed three times with medium before the experiments.

RT-PCR and Southern blot analysis. The expression of human RAG-1 mRNA was assessed by the RT-PCR method as described previously.27 A total of 2 μg of total RNA was reverse transcribed with 100 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase in the presence of 50 pmol of random primer (Takara Shuzo, Kyoto, Japan), 10 U of RNAsin (Promega, Madison, WI), 1 mmol/L dithiothreitol and 10 pmol of each deoxynucleotide (Takara Shuzo) in 10 μL for 1 hour at 37°C. Five microliters of the reverse transcripts were amplified by PCR with 1.25 U of Taq DNA polymerase (Takara Shuzo) in a 50-μL reaction containing 50 μL of each primer (see below), and 20 pmol of each deoxynucleotide. Primers used were: RAG-1 sense primer, 5′GAGAGCAGAGAACACCTTT3′ (1-20 nt of the published cDNA); RAG-1 antisense primer, 5′CTTTTCAAGGATCICACC3′ (213-232 nt of the cDNA); RAG-2 sense primer, 5′GACATGCTTACAGTCAGTAA3′ (90-109 nt of the published cDNA); RAG-2 antisense primer, 5′ATGATGATTTAGTGAC3′ (344-363 nt of the published cDNA); hypoxanthine phosphoribosyltransferase (HPRT) sense primer, 5′CTCCCGCTTCTCTGTGCTC′ (12-31 nt of the published cDNA); HPRT antisense primer, 5′TTCGACCTTGACCACTTT3′ (550-569 nt of the cDNA). With these primers, PCR products were not detectable with RNA derived from murine PA6 cells. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer/Cetus) for 30 cycles (denaturation for 1 minute at 94°C; annealing for 2 minutes at 55°C; extension for 1 minute at 72°C). PCR products were separated on 2% agarose gels, transferred to Gene Screen Plus nylon membranes (DuPont, Boston, MA) and hybridized with a 32P-labeled oligonucleotide complementary to sequences within the region flanked by the PCR amplification primers. The membrane was washed to a final stringency of 2X SSC and 0.1% sodium dodecyl sulfate (SDS) at 65°C. Autoradiography was performed using Fuji RX film (Fuji Photo Film, Tokyo, Japan) at room temperature for 1 hour. Oligonucleotide probes were as follows: RAG-1, 5′AGGACGCTCCTGTCGAAAG (90-99 nt of the cDNA); RAG-2, 5′TGCTACCTCCTCTCTTGC3′ (191-210 nt of the cDNA); HPRT, 5′TTCGACCTTGACCACTTT3′ (550-569 nt of the cDNA). The quantity of radioactive binding to specific PCR products was determined using an AMBIAS radioanalytic imaging system (Automated Microbiology System Inc, San Diego, CA) or BAS2000 Bio-Imaging Analyzer (Fuji Photo Film Co, Tokyo, Japan). The radioactivities of RAG-1, RAG-2 and HPRT transcripts detected were linearly related to the amount of RNA used for cDNA synthesis (Fig 1 and data not shown), showing that this PCR-assisted method is useful for quantitation analysis of RAG-1, RAG-2, and HPRT gene expression. The amount of the transcripts in FL8.2.4.4 cells was calculated by the standard curve that had been obtained using RNA from Nalm6 cells as shown in Fig 1.

Recombinase activity assay and restriction enzyme analysis. Cells (2 x 10⁶) were suspended in 0.7 mL of chilled phosphate-buffered saline (PBS) with 30 μg of the vector DNA, MHSDR1/2/2ST-132 (generously provided by Dr M. Abe, Radiation Effect Research Foundation, Nagasaki, Japan). Electroporation was performed at 270 to 300 V and 960 μL using a Gene Pulser (Bio Rad, Richmond, CA). Transfected cells were placed on a monolayer of PA6 in a 10-cm diameter dish (Falcon) and cultured in 15 mL of culture medium containing rIL-3 (100 ng/mL), rIL-6 (20 ng/mL), and rIL-7 (10 ng/mL). After a 60-hour incubation, cells were collected and washed with PBS, and the plasmid DNA was recovered by the alkaline lysis procedure.28 Recovered plasmid DNA was intro-
duced into competent Escherichia coli strain (DH10B) by electroporation and ampicillin resistant (Amp')- and ampicillin/chloramphenicol-resistant (Amp'Cam') DH10B were selected.

For restriction enzyme analysis, plasmid DNA recovered from Amp'Cam' colonies, was digested with EcoRI and ApaI (Nippon Gene, Toyama, Japan). A reaction volume of 5 μL, containing 5 U of enzyme and 1 μg of recovered plasmid DNA, was incubated for 6 to 14 hours at 37°C.

RESULTS

Induction of RAG-1 and RAG-2 mRNA expression by coculture and recombinant cytokines. To examine whether RAG expression is induced in lymphoid progenitors by coculture on stromal cell lines and recombinant cytokines, freshly isolated human BM-MNC from normal donors were cultured on PA6 stromal cells in the presence of a mixture of cytokines. To examine whether RAG expression is induced in lymphoid progenitors by coculture on stromal cell lines and recombinant cytokines, freshly isolated human BM-MNC from normal donors were cultured on PA6 stromal cells in the presence of a mixture of cytokines (rIL-2, rIL-3, rIL-4, rIL-7, rSCF, rGM-CSF), for 24 hours, and the levels of RAG-1 and RAG-2 mRNA were determined (Fig 2). Both RAG-1 and RAG-2 transcripts were detected in freshly isolated BM-MNC before culture. The levels of RAG-1 and RAG-2 mRNA increased about fivefold to sevenfold in 24 hours of culture in the presence of PA6 and the cytokines (Fig 2), but they did not increase when BM-MNC were cocultured with 3T3 fibroblasts in the presence of the cytokines (data not shown). Thus, results in normal lymphoid progenitors are consistent with those in FL8.2.4.4 cells. The precise study as to which compartments of the cells in normal BM-MNC are induced to express RAG mRNA, or which signals are required for the induction of RAG expression in those cells was completely hampered by the limited number of normal BM-MNC. To overcome this problem, we further analyzed the mechanisms of RAG activation using an EBV-transformed lymphoid cell line derived from human fetal liver.

In the previous study, we reported the successful establishment and cloning of EBV-transformed cell lines derived from the human fetal liver. The established cloned cell lines were thought to belong to the category of lymphoid progenitors because (1) they expressed early lymphoid surface markers of both T and B cells (CD2 and CD19, respectively), (2) they retained germline configuration of Ig and TCR genes, and (3) they did not express RAG-1 mRNA. To determine whether RAG expression is induced in the lymphoid progenitor cell lines by coculture on stromal cell lines and/or recombinant cytokines, one of the established lymphoid progenitor cell lines, FL8.2.4.4, were cocultured for 24 hours on a murine BM-derived stromal cell line, PA6, or a fibroblast cell line, 3T3, with or without a mixture of human recombinant. As shown in Fig 3, the RAG-1 and RAG-2 transcripts were induced in FL8.2.4.4 when FL8.2.4.4 were cocultured on PA6 in the presence of a mixture of cytokines. The RAG transcripts were not detected when cells were cultured alone, or with a mixture of cytokines in the absence of PA6, or cocultured on PA6 in the absence of a mixture of cytokines. No RAG transcripts were found when FL8.2.4.4 were cocultured on the 3T3 fibroblast cell line with the cytokines. These data show that RAG-1 and RAG-2 of FL8.2.4.4 are activated and transcribed by signal(s) derived from a stromal cell line and the cytokines. The induction of mRNA expression of RAG-1 and RAG-2 was also found in other lymphoid progenitor lines like FL8.2.2.1, FL8.2.4.10, FL8.2.4.11 [25], but not in cell lines such as RPMI1788 and Daudi (mature B-cell lines) or U937 (histiocytic leukemic cell line), all of which had been cocultured on PA6 for 24 hours in the presence of a mixture of cytokines (data not shown).
The kinetics of the induction of RAG-1 mRNA was investigated by coculturing FL8.2.4.4 cells on PA6 in the presence of a mixture of cytokines for various durations (Fig 4). RAG-1 mRNA were induced 12 hours after initiation of culture, and the elevated mRNA level was sustained at 24 hours of culture. No RAG-1 transcripts were detected during the 24 hour incubation of FL8.2.4.4 cells that had been cultured on PA6 in the absence of cytokines.

Effect of individual cytokine on RAG activation in FL8.2.4.4 cells. To determine the effect of the individual recombinant cytokine on the induction of RAG-1 mRNA in FL8.2.4.4 cells, FL8.2.4.4 cells were cocultured on PA6 cells in the presence of varying concentrations of a gallery of distinct recombinant cytokines; rIL-2, rIL-3, rIL-4, rIL-6, rIL-7, rSCF, and rGM-CSF. As shown in Fig 5A, RAG-1 transcripts were detected when FL8.2.4.4 cells were cultured on PA6 cells in the presence of rIL-3, rIL-6, or rIL-7, but not in the presence of rIL-2, rIL-4, rSCF, or rGM-CSF. The effect of rIL-3, rIL-6, and rIL-7 on the induction of RAG-1 in FL8.2.4.4 was dose-dependent (Fig 5B).

To determine whether there is, if any, a synergistic effect between these three RAG-1 mRNA-inducing cytokines, FL8.2.4.4 cells were cocultured on PA6 for 24 hours in the presence of combinations of the suboptimal concentrations of each cytokine (Fig 6). The level of RAG-1 mRNA in FL8.2.4.4 cells rose 10-fold to 20-fold when the suboptimal concentrations of rIL-3, rIL-6, and rIL-7 together were added to culture. The combinations of each two cytokines (rIL-3 + rIL-6, rIL-3 + rIL-7, or rIL-6 + rIL-7) showed very little effect on RAG-1 induction. These data show that among cytokines tested in this study, rIL-3, rIL-6, and rIL-7 can induce RAG-1 mRNA in FL8.2.4.4 cells, and that there is a significant synergistic effect between these three cytokines on RAG-1 activation in FL8.2.4.4.

Cognate interaction with the stromal cell line is essential for RAG-1 activation in FL8.2.4.4 cells. To investigate whether a cognate (direct) interaction between FL8.2.4.4 cells and the stromal cell line is necessary for the induction of RAG-1 mRNA, FL8.2.4.4 cells were cultured on PA6 cells (cognate interaction) or cultured separately with PA6 cells on a membrane filter that blocks the direct interaction with PA6 (noncognate interaction) in the presence or absence of each cytokine combination (rIL-3, rIL-6, or rIL-7). RAG-1 mRNA was detected only when FL8.2.4.4 cells were cocultured on PA6 cells in the presence of cytokines. No RAG-1 mRNA was detected when FL8.2.4.4 cells were cultured on the membrane filter with or without cytokines (Fig 7). The result shows that a cognate interaction between FL8.2.4.4 cells and the stromal cell line is essential for RAG activation in FL8.2.4.4 cells.

Induction of RAG-1 mRNA in FL8.2.4.4 cells by coculture on fixed PA6. To investigate whether cytokines or any unknown soluble factors released from the murine stromal cell line, PA6, affect the induction of RAG-1 mRNA in FL8.2.4.4 cells, we used chemically fixed PA6 cells. The fixation of human T cells or macrophages by 1% paraformaldehyde was reported to be capable of preserving surface molecules, but inhibiting any metabolic activity or production of soluble factors. As shown in Fig 8, RAG-1 transcripts were induced in FL8.2.4.4 cells when FL8.2.4.4 cells were cocultured on paraformaldehyde-fixed PA6 in the presence of a mixture of cytokines (rIL-3, rIL-6, and rIL-7). The result indicates that soluble factors produced by PA6 are not required for the RAG activation in FL8.2.4.4 cells.

Induction of recombinase activity in FL8.2.4.4 by coculture on PA6 in the presence of cytokines. We further investigated the levels of recombinase activity in FL8.2.4.4 before or after coculture on PA6 in the presence of cytokines. The strategy used here to measure recombinase activity employs a recombination substrate, MHSDR(1/2)ST-13, which con-
Fig 3. PCR detection of RAG-1 and RAG-2 transcripts in FL8.2.4.4 cells. FL8.2.4.4 cells (5 × 10⁶) were cultured with or without a mixture of recombinant cytokines; rIL-2 (100 U/mL), rIL-3 (100 ng/mL), rIL-4 (10 U/mL), rIL-6 (20 ng/mL), rIL-7 (10 ng/mL), rSCF (100 ng/mL), rGM-CSF (100 ng/mL) (lanes 1 to 4), or cocultured on PA6 with or without the mixture of cytokines (lanes 5 to 8), or cocultured on 3T3 with the mixture of cytokines (lanes 9 and 10). After a 16-hour incubation, RNA was prepared from FL8.2.4.4 cells and RT-PCR for RAG-1, RAG-2, and HPRT was performed in the presence or absence of RT. PCR products were visualized by Southern blot hybridization with the specific probes as described in Materials and Methods. The RAG-1, RAG-2, and HPRT products are indicated by arrows. A representative result of three different experiments with similar findings is shown.

Fig 4. The kinetics of RAG-1 mRNA expression in FL8.2.4.4 cells. FL8.2.4.4 cells (5 × 10⁶) were cocultured on PA6 for various periods (2 to 24 hours) in the presence or absence of a mixture of recombinant cytokines; rIL-2 (100 U/mL), rIL-3 (100 ng/mL), rIL-4 (10 U/mL), rIL-6 (20 ng/mL), rIL-7 (10 ng/mL), rSCF (100 ng/mL), rGM-CSF (100 ng/mL). After incubation, RNA was prepared from FL8.2.4.4 cells and RT-PCR for RAG-1 and HPRT in the presence or absence of RT was performed. PCR products were visualized as described in the legend to Fig 1. The RAG-1 and HPRT products are indicated by arrows. Time 0 shows FL8.2.4.4 cells before incubation. A representative result of three separate experiments with similar findings is shown.

Thus, the ratio of double resistant (Amp'Cam') colonies to Amp' colonies reflects the fraction of DNA that is rearranged at the heptamer-nonamer joining signal. We further tested whether a precise signal junction (heptamer to heptamer fusion) is formed in the recombinants by a restriction enzyme analysis using ApolI of plasmid DNAs recovered from the double resistant colonies (Fig 9B and C).
The recombination substrate was transfected into FL8.2.4.4 cells by electroporation. One half of transfected cells were cocultured on PA6 in the presence of cytokines and the remainder were cultured alone. After a 60-hour incubation, the plasmid DNA in FL8.2.4.4 cells was recovered, introduced into E. coli, and the colonies were selected by Amp or by Amp and Cam. The plasmid DNAs recovered from the double resistant colonies were digested with ApaL1 to identify the proper signal joining. As shown in Table 1 and Fig 8D, when FL8.2.4.4 cells were cultured alone, no double resistant colonies that contain ApaL1 site (Amp’Cam’ApaL1+) were obtained. When FL8.2.4.4 cells were cocultured on PA6 in the presence of cytokines, 10 Amp’Cam’ApaL1+ colonies were obtained. As a positive control cell line with high recombinase activity, Nalm6 cell line that had been shown to express high levels of RAG mRNA was used. As many as 488 Amp’Cam’ApaL1+ colonies were detected after the recombination substrate was transfected into Nalm6 cells. In the present experiments, the level of recombinase activity in Nalm6 was about 100-fold compared with that seen in FL8.2.4.4 cells that had been cocultured on PA6 in the presence of cytokines. This difference of the levels of recombinase activity may reflect the levels of RAG-1 mRNA detected in these two cell lines, because Nalm6 expressed more than 100-fold RAG-1 transcripts compared with the stimulated FL8.2.4.4 cells when calculated by the standard curve obtained using Nalm6 RNA as described in the Materials and Methods. The results show that coculture of FL8.2.4.4 cells on PA6 in the presence of cytokines induces not only the expression of RAG mRNA, but also the recombinase activity in FL8.2.4.4.

Fig 5. Effect of individual cytokines on RAG-1 mRNA induction in FL8.2.4.4 cells. (A) FL8.2.4.4 cells (5 x 10^5) were cocultured on PA6 with each individual cytokine; rIL-2 (100 U/mL), rIL-3 (100 ng/mL), rIL-4 (10 U/mL), rIL-6 (20 ng/mL), rIL-7 (10 ng/mL), rSCF (100 ng/mL), rGM-CSF (10 ng/mL). After a 16-hour incubation, RNA was prepared from FL8.2.4.4 cells and RT-PCR for RAG-1 and HPRT was performed in the presence or absence of RT. PCR products were visualized as described in the legend to Fig 1. PCR products of RAG-1 and HPRT are indicated by arrows. A representative result of three different experiments with similar results is shown. (B) The standardized mRNA levels for RAG-1 in FL8.2.4.4 cells. Radioactivities of each band presented in (A), as well as bands obtained from other experiments using the lower concentrations of individual cytokines, were measured by an image scanner and standardized with that of HPRT. The mRNA level in FL8.2.4.4 cells cultured without cytokines is set at 1.0; RAG-1 transcripts, 57 ? 5.6 cpm; HPRT transcripts, 1218.0 ? 258.8 cpm. A representative result of five different experiments with similar results is shown.

Fig 6. Synergistic effect of rIL-3, rIL-6, and rIL-7 on RAG-1 mRNA induction in FL8.2.4.4. (A) FL8.2.4.4 cells (5 x 10^5) were cocultured on PA6 with the suboptimal concentration of each individual cytokine or combinations of the suboptimal concentration of the cytokines; rIL-3 (10 ng/mL), rIL-6 (2 ng/mL), rIL-7 (1 ng/mL). After a 16-hour culture, RNA was prepared from FL8.2.4.4 cells and RT-PCR for RAG-1 and HPRT was performed in the presence or absence of RT. PCR products were visualized as described in the legend to Fig 1. PCR products of RAG-1 and HPRT are indicated by arrows. A representative result of five different experiments with similar data is shown. (B) The standardized mRNA levels for RAG-1 in FL8.2.4.4. Radioactivity of each band shown in (A) was measured by an image scanner and standardized with that of HPRT. The mRNA level in FL8.2.4.4 cells cultured without cytokines is set at 1.0; RAG-1 transcripts, 160.6 ? 21.5 cpm; HPRT transcripts, 2131.5 ? 215.5 cpm. Data represent the mean (± SD) of five independent experiments.
Fig 7. Requirement of cognate interaction between FL8.2.4.4 cells and stromal cell line for RAG-1 induction. (A) FL8.2.4.4 cells (5 x 10^6) were cocultured on PA6 (cognate interaction) or cultured separately with PA6 on an inner chamber of the double chamber plates (noncognate interaction) in the presence or absence of a mixture of rIL-3 (100 ng/mL), rIL-6 (20 ng/mL), and rIL-7 (10 ng/mL). (B) After a 16-hour incubation, RNA was prepared from FL8.2.4.4 cells and RT-PCR for RAG-1 and HPRT was performed in the presence or absence of RT. PCR products were visualized as described in the legend to Fig 1. PCR products of RAG-1 and HPRT are indicated by arrows. A representative result of three different experiments with similar results is shown.

DISCUSSION

RAGs play an indispensable role in VDJ recombination machinery and hence in lymphocyte development. In the present study, we sought to identify signals that are required for RAG activation in lymphoid progenitors using a cloned human EBV-transformed lymphoid progenitor-like cell line, FL8.2.4.4, which had been established from human fetal liver at 8 weeks gestation. Our present data clearly show that RAG gene activation is induced in FL8.2.4.4 cells by two independent signals; one from cytokines and the other from the surface molecule(s) on a BM-derived stromal cell line.

Various cytokines, including GM-CSF, IL-3, IL-4, IL-6, IL-7, IL-11, IL-12, or SCF, have been shown to be involved. Among them, rIL-3, rIL-6, and rIL-7 were found to exhibit direct and synergistic effects on induction of RAG-1 gene expression in FL8.2.4.4 cells in the stromal cell-coculture system. rIL-2, rIL-4, rSCF, or rGM-CSF by itself did not show any activity to induce RAG-1 gene expression in FL8.2.4.4 cells. IL-3 is known as a mast cell growth factor, but it also induces growth and differentiation of hematopoietic precursors. Similarly, IL-6 is a well-known growth and differentiation factor for both B and T lymphocytes, but it exerts its effect on the hematopoietic precursors to drive them to proliferate and differentiate. Our data suggest that IL-3 and IL-6 acts, as a
cofactor, on a hematopoietic stem cell or a lymphoid-restricted progenitor cell to induce RAG-1 activation and V(D)J gene rearrangement. IL-7 serves as a growth factor in B-cell development. Thus, it is somewhat surprising that our data in Fig 4 showed that IL-7 in combination with BM stromal cells induced RAG activation. Our data are in accordance with those recently described by Muegge et al. and Appasamy et al. They have shown that IL-7 induces or sustains RAG gene expression and induces V(DJ) recombination of TCRγ or TCRβ gene segments in fetal liver cells or fetal thymocytes in mice. Thus, it is likely that IL-7, which is abundantly expressed in fetal liver or thymus, may induce RAG gene activation, driving V(D)J gene segments to be rearranged during very early T-cell development. Recently, Billips et al. have shown that in the absence of stromal cells, IL-7

Table 1. Recombinase Activity in FL8.2.4.4 Cells With or Without Induction

<table>
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<th>Cells</th>
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<th>Amp'</th>
<th>Amp'Cam'</th>
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FL8.2.4.4 and Nalm6 were transfected with a recombination substrate (MHSDR1/2ST-13) by electroporation. One half of FL8.2.4.4 were cocultured on PA6 in the presence of a mixture of IL-3 (100 ng/mL), IL-6 (20 ng/mL), and IL-7 (10 ng/mL) (induction +). The remainder of FL8.2.4.4 and Nalm6 were cultured in medium alone (induction -). After a 60-hour culture, cells were harvested and plasmid DNAs were recovered by an alkaline lysis method. One fifth of the recovered plasmid DNAs were transfected to E coli (DH10B) and selected by ampicillin and ampicillin/chloramphenicol. Nalm6 was used for the positive control of cell line with recombinase activity. The plasmid DNAs of double resistant (Amp'Cam') colonies (50 from Nalm6 and 51 from FL8.2.4.4) were digested with ApaLI, and the sizes of DNA fragments were analyzed by agarose gel electrophoresis as shown in Fig 9.
downmodulates RAG expression of CD34+CD19+ population of human pro-B cells in vitro and CD19-ligation counteracts the IL-7 effect. Therefore, it is conceivable that the signal(s) from stromal cells may be a prerequisite for IL-7-induced RAG expression in human lymphoid progenitors.

We have not determined the effects of IL-11 and IL-12 on RAG-1 activation in FL8.2.4.4 cells in this stromal cell-culture system, simply because those recombinant products were not available. It may be possible that cytokines or cytokine combinations of IL-11 and IL-12 can induce RAG-1 activation in FL8.2.4.4 cells, as they have direct and synergistic effects on generation of lymphoid cells from lymphohematopoietic progenitors in the semi-solid culture system. In the present study, we have shown that cognate interaction between FL8.2.4.4 cells and the PA6 cells is a prerequisite for RAG-1 activation (Fig 6). The molecule(s) on the stromal cells that activate RAG-1 in FL8.2.4.4 cells remains undetermined. In human B lymphopoiesis, it was shown that in vitro growth of B-cell precursors requires the presence of BM stromal cells and cytokines, and the adhesive interaction between B-cell precursors and BM stromal cells is mediated primarily by very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1)/CD106, respectively. In murine B-cell development, it was shown that CD44 plays an essential role in B-cell differentiation in BM. However, our preliminary experiments indicate that anti-CD44 antibody does not inhibit RAG-1 activation in FL8.2.4.4 cells in this BM stromal-dependent culture system (Tagoh et al, personal communication). In murine B lymphopoiesis, Nishikawa et al showed that BM stromal cells and IL-7 was prerequisite for in vitro growth of B-cell precursors and that signal(s) from BM stromal cells could be substituted by SCF. With this in mind, in our system, SCF did not induce RAG-1 expression in the presence of IL-7 (Fig 1). The result indicates that unknown surface molecule(s) other than SCF on PA6 stromal cells are responsible for RAG-1 activation in FL8.2.4.4 cells. Generation of monoclonal antibodies against either FL8.2.4.4 cells or PA6 cells that inhibit the RAG-1 activation should provide a powerful strategy for isolation and molecular characterization of the novel molecule(s) responsible for the RAG-1 activation. Trials for producing such monoclonal antibodies are being performed in our laboratory.

How are RAG activation and transcription induced in FL8.2.4.4 cells after the cognate interaction with PA6 in combination with cytokine stimulation? The binding of ligands to the specific receptors may induce the activation of a variety of signal transduction pathways, as well as an assembly of multiprotein complexes capable of inducing RAG gene transcription. The latter transcription factors may function by binding to promoter or enhancer or both elements. In view of this, we recently cloned and characterized human RAG-1 gene promoter to further understand the regulatory mechanism of RAG-1 expression. It is noteworthy that a binding motif for NF-IL6, which regulates IL-6 and IL-6-inducible gene expression, is present at 5' upstream region of the major transcription start site of human RAG-1 gene (Kurioka et al, manuscript submitted). This motif might be involved in IL-6-induced RAG transcription in FL8.2.4.4 cells. This is merely a speculation and should be investigated at the molecular level.

In the present study, using the MHSDR(1/2)ST-13 recombination substrate, we investigated the recombinase activity in unstimulated or stimulated FL8.2.4.4 cells. By coculture on the PA6 stromal cells in the presence of cytokines, the rearrangement of the introduced recombination substrate that made proper signal joints was induced in FL8.2.4.4 cells. However, no proper joints were produced in unstimulated FL8.2.4.4 cells (Table 1 and Fig 8). The results show that the recombinase activity was induced with signals from stromal cells and cytokines. In this study, recombination frequencies were quite low even in Nalm6 cells, and this may, in part, be due to the inclusion of Amp' colonies derived from plasmid DNAs that attached to cell surface membranes and did not enter the cells. It is noteworthy that aberrant recombination occurred frequently in both unstimulated and stimulated FL8.2.4.4 cells, producing an improper signal joint. V(D)J recombination can be devided into two stages: first, double-strand breaks are produced at coding and signal borders. It has been shown that RAG-1 and RAG-2 proteins are sufficient to produce double-strand breaks in vitro. Then coding ends are joined together. In severe combined immunodeficiency (SCID) mice that have a defect in DNA-dependent protein kinase, lymphoid progenitor cells show improper signal joints, disturbing proper rearrangement of Ig or TCR genes. It is conceivable that FL8.2.4.4 cells might lack some component(s) of recombinase machinery that are required for proper and sufficient recombination activity.

Does DJ or V(D)J gene recombination of endogenous IgH genes or TCRβ genes in FL8.2.4.4 cells occur in this coculture system? At the present time, we do not have any clear evidence indicating endogenous DJ or V(D)J recombination of IgH or TCRP loci in FL8.2.4.4 cells by coculture on stromal cells in the presence of cytokines (Tagoh et al, personal communication). There are two conceivable explanations for this result. One is that the frequency of the recombination event was too low to detect the recombination pattern with conventional Southern blot analysis. Alternatively, as implicated by Alt et al, if IgH or TCRβ loci of FL8.2.4.4 cells do not have accessibility to the recombinase due to either chromatin structure or the degree of methylation, FL8.2.4.4 cells may then fail to undergo detectable rearrangements of their endogenous IgH, or TCRβ loci, despite their ability to rearrange introduced recombination substrate.

Lymphoid precursors in normal BM cells were also induced to express RAG transcripts on PA6 stroma cells in the presence of IL-3, IL-6, and IL-7 (Fig 3), indicating that RAG induction in FL8.2.4.4 cells in this coculture system may represent some stage of lymphocyte development. Thus, this system may provide useful means for further understanding the regulation of RAG gene expression during human lymphocyte development.

ACKNOWLEDGMENT

We are grateful to Dr S-I. Nishikawa, Kyoto University, for providing the PA6 and 3T3 cell lines; Dr M. Abe, Radiation Effect
Research Foundation, for the gift of recombinase substrate plasmid DNA and encouragement of this study, Drs H. Matsuno and M. Murashima, Toyama Medical and Pharmaceutical University, for supplying the BM preparations. We thank K. Ikawa for her technical assistance and M. Kyohara for preparing the manuscript.

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Induction of recombination activating gene expression in a human lymphoid progenitor cell line: requirement of two separate signals from stromal cells and cytokines

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