Human Pre-B Cells Differentiate Into Ig-Secreting Plasma Cells in the Presence of Interleukin-4 and Activated CD4+ T Cells or Their Membranes

By Juha Punnonen, Gregorio Aversa, and Jan E. de Vries

Studies on human B-cell development have been hampered by the lack of reproducible culture techniques to induce pre-B cells to differentiate into Ig-secreting plasma cells. Here, we describe that highly purified surface (s) μ−, cytoplasmic (c) μ+, CD10+, CD19+ human pre-B cells derived from fetal bone marrow (BM) differentiate with high frequencies into Ig-secreting plasma cells, when cocultured with activated, cloned CD4+ T cells and with interleukin-4 (IL-4). Production of IgM, total IgG, IgG4, and IgE in pre-B-cell cultures was detected, indicating that the cells also underwent Ig isotype switching. Pre-B−cell differentiation occurred in the absence of BM stromal cells, IL-7, and stem cell factor (SCF). However, IL-7 significantly enhanced the levels of Ig produced, whereas SCF was ineffective. Neutralizing anti-IL-4 monoclonal antibodies (MoAbs) completely inhibited pre-B−cell differentiation showing the specificity of the reaction. Intact CD4+ T-cell clones could be replaced by membrane preparations of these cells, indicating that the costimulatory signals provided by the activated CD4+ T cells are contact-mediated.

In contrast, anti-CD40 MoAbs failed to provide the costimulatory signal required for pre-B−cell differentiation, which may be related to the very low expression of CD40 on fetal BM B cells. Activated CD4+ T cells and IL-4 also induced sμ expression and Ig synthesis in cultures initiated with pre-B cells that had been preincubated in medium for 2 days, and from which spontaneously emerging sμ+ B cells were removed by using a fluorescence-activated cell sorter. These results support the notion that the Ig synthesis observed in pre-B−cell cultures was not caused by outgrowth and differentiation of cells that spontaneously matured into sμ+ B cells. In addition, IL-4 and CD4+ T cells strongly enhanced CD40 and HLA-DR expression on the majority of cultured pre-B cells, further indicating that CD4+ T cells and IL-4 activate bona fide pre-B cells. Taken together, these data indicate that activated CD4+ T cells and IL-4 can provide all the necessary signals required for human pre-B cells to differentiate into Ig-secreting plasma cells.

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MATERIALS AND METHODS

Reagents. Recombinant IL-4 was provided by Schering-Plough Research (Bloomfield, NJ). IL-7 was purchased from R&D Systems Inc (Minneapolis, MN). Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated monoclonal antibodies (MoAbs) specific for CD10, CD19, CD20, CD34, and HLA-DR and control antibodies with irrelevant specificities were obtained from Becton Dickinson (Mountain View, CA). FITC-conjugated anti-CD19 MoAb (IgG1+) was purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands). The purified anti-CD40 MoAb 89 (IgG1) was a gift of Dr J. Banchereau (Schering-Plough France, Dardilly, France). The neutralizing anti-II-4 (25D2) and anti-II-7 (BVD10) MoAbs were kindly provided by Dr J. Abrams (DNAX, Palo Alto, CA).

Cell preparations. Human fetal bones were obtained from fetuses varying from 15 to 24 weeks of gestation. Fetal tissues were obtained with informed consent from Advanced Bioscience Resources Inc (Alameda, CA) in compliance with regulations issued by the state and by the federal government. BM cells were obtained by flushing RPMI-1640 (JRH Biosciences, Lenexa, Kansas) into the intramedullary cavities of the long bones. The cells were washed twice. Red blood cells were lysed by osmotic shock using sterile water or Tris-buffered 0.83% NH4Cl (pH 7.2), after which the cells were washed twice. The cells were finally counted and resuspended in Yssel’s medium supplemented with 10% fetal calf serum (FCS). The CD4+ T-cell clone B21 was cultured as described previously. The cells were obtained 2 to 3 days after they had been activated by the feeder-cell mixture and PHA, and viable T cells were isolated after centrifugation over Histopaque-1077 (Sigma, St Louis, MO).

Cell surface analysis and cell sorting experiments. Cell surface analysis was performed by using a FACScan flow cytometer (Becton Dickinson). After the cells were washed twice, FITC- and PE-conjugated MoAbs were added at saturating concentrations and incubated at 4°C for 30 minutes. FITC- and PE-conjugated MoAbs with irrelevant specificities were used as negative controls. The cells were washed twice with phosphate-buffered saline, and cells with light-scatter characteristics of lymphocytes were analyzed. B-cell precursors were sorted by using two-color immunofluorescence and a FACStar Plus (Becton Dickinson). The purities of the sorted populations were 98% to 100%.

Preparation T-cell membranes. The membranes of a CD4+ T-cell clone were obtained as described previously. Briefly, the CD4+ T-cell clone B21 was harvested 12 days after activation with feeder-cell mixture and PHA. These resting T cells were washed and subsequently restimulated with 10 μg/mL of Concanavalin A (ConA) for 8 hours at 37°C. During the last 30 minutes of the ConA stimulation, 100 μg/mL of α-methyl-D-mannoside (Sigma) was added. From these ConA-activated T cells, membranes were prepared using the method described by Maeda et al and Brian. The membranes were stored under liquid nitrogen until use.

Culture conditions. B-cell precursors were cultured in triplicate in round-bottomed 96-well plates (Linbro, McLean, VA) in a humidified atmosphere containing 5% CO2 in 0.2 mL. Yssel’s medium supplemented with 10% FCS. The cells were cultured at 5,000 cells/well. IL-4 was added at the onset of the cell cultures at concentrations of 400 U/mL. In coculture experiments, the activated human CD4+ T-cell clone B21 and IL-4 differentiated into Ig-secreting cells, highly purified, sorted pre-B cells were cultured at decreasing cell numbers per well, and Ig production was measured after a culture period of 2 weeks. The experiments were performed as described previously for mature B cells by culturing decreasing concentrations of sorted pre-B cells (from 5,000 to 1 cell/well) in the presence of a fixed concentration of CD4+ T cells (5,000/well).

An increasing number of replicates were used when the number of cells/well decreased, and the frequency was determined according to Tasswell by plotting the percentage of Ig-negative wells against the number of pre-B cells in the culture. The frequencies of pre-B cells that can be induced to differentiate into Ig-secreting cells were determined from the 37% intercept.

RNA isolation and detection of stem cell factor (SCF) transcript by polymerase chain reaction (PCR). The cloned CD4+ T cells were cultured with or without IL-4, CD19+ pre-B cells as described above in the presence or absence of IL-4 (400 U/mL). Total RNA was isolated after 2 days of culture using the guanidium thiocyanate-CsCl procedure. cDNA was synthesized using total RNA as template, random hexanucleotides as primers, and avian myeloblastosis virus-reverse transcriptase as described. Amplification of the cDNA was performed using 40 cycles of PCR. Primers for detection of SCF transcripts were 5'-CGA TCT GCA GGC CCTT TTC TTA TGA AGA AGA CAC AA-3', 5'-GCT GAG ATT CTA GGC TGG AGT CTC CAG GGG GAT TT-3', and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts as a control were 5'-CCA TGG AGA AGG CTG GGG S, 5'-CAA ACT TGT CAT TGG ACT CTC CAG GGG GAT TT-3'. Products of PCR reactions were electrophoresed through 1.2% agarose and transferred by capillary blotting to GeneScreen nylon membranes (New England Nuclear Research Products, Boston, MA). The oligonucleotides used to analyze the amplified cDNAs were 5'-GAG TCG GTG AGG AAA GAA AAC A TC TCA A-3' and 5'-CTA AGC AGT TGG TGG TGC A-3' for SCF and GAPDH, respectively.

RESULTS

Phenotypical analysis of fetal BM B cells. Pre-B cells are defined as pre-B or B-lineage cells, and all CD19+ cells express CD19, and all CD19+ cells express CD10. Pre-B cells were obtained as described previously. Pre-B cells were isolated from fetal BM cells. Pre-B cells were obtained by sorting from fetal BM. Gating for pre-B cells was always performed conservatively to exclude contaminating cells expressing minimal levels of CD19. Reanalysis of the sorted pre-B-cell populations indicated that these cells were 98% to 100% CD19+.

Pre-B cells differentiate into Ig-secreting plasma cells when cultured with activated CD4+ T cells and IL-4. Sorted pre-B cells cultured in the presence of the activated CD4+ T-cell clone B21 and IL-4 differentiated into Ig-secreting cells. IgM, total IgG, and IgE synthesis was observed indicating that the cells also underwent Ig isotype switch.
Fig 1. Phenotype of fetal BM B cells. The cells with light-scatter characteristics of lymphocytes were analyzed by FACScan flow cytometer. Pre-B cells were obtained using cell sorting by FACStar Plus. The bottom panel on the left shows the gate used to sort CD19+, sμ− pre-B cells, and the bottom panel on the right shows the reanalysis of the sorted cells (purity > 99%).

Type switching. Induction of Ig production was not a specific property of the clone B21, because two other CD4+ T-cell clones were also found to have Ig-secretory stimulatory activity (data not shown). The levels of Ig produced in the pre-B–cell cultures were comparable with those observed in cultures of fetal splenic or liver mononuclear cells. No IgA synthesis was obtained in these cultures, which is in agreement with previous observations indicating that IL-4, under these culture conditions, inhibits IgA synthesis.

To determine the frequencies of pre-B cells that can differentiate into Ig-secreting cells limiting dilution experiments were performed as described. IgM and IgG production in pre-B–cell cultures was detected in every well (n = 12) containing as few as 90 CD19+, sμ− cells/well. The frequencies of pre-B cells that can be induced to differentiate into Ig-secreting cells were determined from the 37% intercept and were found to vary between 1:24 and 1:40 (data not shown). Taking into account that the proportion of contaminating non-B-lineage cells among the pre-B-cell preparations was 0% to 2%, and that contaminating sμ+ cells were undetectable (<0.5%) by FACS analysis (Fig 1), we conclude that the possibility of contaminating sμ+ B cells as the cause of Ig synthesis in our pre-B–cell cultures is highly unlikely. Kinetic studies indicated a relatively rapid onset of Ig synthesis in pre-B–cell cultures. Detectable levels of IgM and IgG in the culture supernatants appeared at day 7, whereas IgE was detected at day 12 (Fig 3).

Pre-B–cell differentiation does not require IL-7 or SCF. IL-7 acts as a growth factor for B-cell precursors in both mice and humans. However, IL-7 was not required for pre-B–cell differentiation in the present culture system. Anti-IL-7 MoAbs failed to inhibit Ig synthesis induced in pre-B–cell cultures (Fig 4), whereas they strongly inhibited IL-7–induced T-cell proliferation (data not shown). In contrast, neutralizing anti-IL-4 MoAb virtually completely prevented pre-B–cell differentiation induced by IL-4 and the CD4+ T-cell clone, showing the specificity of the reaction (Fig 4). SCF has been shown to promote proliferation of early lymphoid-cell progenitors. However, human pre-B–cell differentiation under the present culture...
conditions appears to be independent of SCF, because no SCF production by the T-cell clone or by the pre-B cells was observed using PCR analysis (data not shown).

The costimulatory signals provided by the T cells are in a membrane-bound form. To investigate whether the costimulatory helper-T-cell signal was mediated by a soluble factor, we studied the ability of supernatants of the T-cell clone to induce pre-B-cell differentiation in the presence of exogenous IL-4. Supernatants of the CD4+ T-cell clones, activated similarly as in the experiments above, consistently failed to induce pre-B-cell differentiation (data not shown). Addition of IL-7 into these cultures was ineffective as well. These results suggested that the costimulatory signals provided by the T cells were in a membrane-bound form. This was indeed the case, because membrane preparations of activated, cloned CD4+ T cells were also capable of inducing IgM and IgG synthesis by pre-B cells in the presence of IL-4 (Fig 5). The signal mediated by the membrane preparations was usually less effective than that mediated by intact T cells, and the levels of IgE produced in these cultures were minimal. However, this is consistent with previous studies indicating that membrane preparations of CD4+ T cells are less efficient than those for intact CD4+ T cells in inducing Ig synthesis by normal, adult B cells.33 The ligand for CD40 (CD40L), which is expressed on CD4+ T cells after activation, can induce proliferation and differentiation of normal B cells in the presence of IL-4.44-47 This effect of CD40L on normal B cells can be mimicked by anti-CD40 MoAbs.30,48-50 However, anti-CD40 MoAbs consistently failed to induce pre-B-cell differentiation in the presence of IL-4 (Fig 5). The results were similar irrespective of whether the anti-CD40 MoAbs were in solution or cross-linked to L-cells transfected with FcγRII/CD32 (data not shown).

B-cell progenitors can be induced to differentiate into Ig-secreting cells only after they have reached the pre-B-cell stage. The population of s- CD19+ fetal BM cells were virtually cp- (refs 14 and 21, and our unpublished observations), indicating that these cells represent pro-B cells.14 In 5 separate experiments, sorted CD34+, CD19+ cells failed to differentiate into Ig-secreting cells us-

Fig 2. Induction of Ig synthesis in pre-B-cell cultures. Highly purified CD19+, s- pre-B cells were cultured at 5,000 cells/well in the presence or absence of IL-4 (400 U/mL) and the CD4+ T-cell clone B21 (5,000 cells/well). After a culture period of 14 days, Ig levels in the culture supernatants were measured by ELISA. The values represent the mean ± SEM of Ig levels obtained in six separate experiments, each performed in triplicate.

Fig 3. Kinetics of pre-B-cell differentiation. Highly purified CD19+, s- pre-B cells were cultured at 5,000 cells/well in the presence of IL-4 (400 U/mL) and the CD4+ T-cell clone B21 (5,000 cells/well). After culture periods indicated in the figure, supernatants were harvested and Ig levels were measured. The values represent the mean ± SEM of Ig levels obtained in triplicate cultures in a representative of three experiments.

Fig 4. Effects of neutralizing anti-IL-4 and anti-IL-7 MoAbs on pre-B-cell differentiation. Highly purified CD19+, s- pre-B cells were cultured at 5,000 cells/well with IL-4 (400 U/mL) and the CD4+ T-cell clone B21 (5,000 cells/well) in the presence or absence of neutralizing anti-IL-4 and anti-IL-7 MoAbs (1 μg/mL each) as indicated. The values represent the mean ± SEM of triplicate cultures of one of three experiments.
HUMAN PRE-B CELL DIFFERENTIATION

A)  

<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
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<tbody>
<tr>
<td>IgM synthesis (ng/ml)</td>
<td>10</td>
<td>5</td>
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</table>

B)  

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>anti-CD40/IL-4</th>
<th>T cell membranes/IL-4</th>
</tr>
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<tbody>
<tr>
<td>IgG synthesis (ng/ml)</td>
<td>20</td>
<td>10</td>
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Fig 5. Effects of membrane preparations of activated CD4+ T cells, anti-CD40 MoAbs, and IL-4 on pre-B→cell differentiation. Highly purified CD19+ , sμ- pre-B cells were cultured at 5,000 cells/well, and IL-4 (400 U/mL) and anti-CD40 MoAbs (10 μg/mL) or membranes of CD4+ T-cell clone were used to stimulate the cells as indicated. Ig levels in the culture supernatants were measured after a culture period of 14 days. The values represent the mean ± SEM of triplicate cultures obtained in two experiments.

In the present study we describe a short-term culture system in which human sμ-, cμ+, CD10+, CD19+ pre-B cells differentiate into Ig-secreting plasma cells. Pre-B→cell differentiation occurred relatively rapidly, because Ig synthesis was already observed after 7 days of culture. Pre-B→cell differentiation occurred in the absence of BM stromal cells, IL-7, or SCF, but it was dependent on IL-4 and a contact-mediated signal provided by CD4+ T cells.

The gating to sort pre-B cells was always performed conservatively to prevent any contamination of B cells expressing low levels of sμ, and several lines of evidence indicate that our sorted pre-B→cell populations were indeed devoid of sμ+ B cells. sμ- cells were undetectable (~0.5%) among sorted sμ+, CD19+ cells by FACS analysis. Moreover, limiting dilution experiments showed that the frequencies of pre-B cells that can be induced to secrete Ig varied between 1:24 and 1:40. Furthermore, in contrast to sμ+, CD10+ immature fetal BM B cells, sμ+ pre-B→cell populations failed to differentiate into Ig-secreting cells in the presence of anti-CD40 MoAbs of CD4+ T cells obtained 4 to 6 days after activation with feeder-cell mixture and PHA (Fig 5), indicating that the sorted pre-B cells were also functionally devoid of sμ+ B cells. In addition, because of a lower proportion of CD34+, CD19+ cells in total fetal BM, the purity of sorted CD34+,
Fig 6. Effects of CD4⁺ T cells and IL-4 on phenotype of pre-B cells sorted after preincubation of 2 days in vitro. Fetal BM cells were first cultured for 2 days in the presence of CD4⁺ T-cell clone B21 (pre-B cell/T cell ratio 1:1) and IL-4 (400 U/mL). Thereafter, cells that had matured into sμ⁺ B cells were depleted by FACS sorting, and sμ⁻, CD19⁺ pre-B cells were collected and cultured for additional 2 days in the presence or absence of B21 cells and IL-4. Then, the cells were harvested, washed, and double-labeled with CD19-PE and FITC-conjugated MoAbs indicated in the histograms. CD19⁺ cells were gated, and FITC-staining on the cells was analyzed using FACScan flow cytometer. Open and stippled histograms indicate analysis of cells cultured in the presence or absence of B21 cells and IL-4, respectively, in (A), (B), and (C). (D) and (E) indicate individual stainings for sμ⁻ of cells cultured in the presence (E) or absence (D) of B21 cells and IL-4.

CD19⁺ B-cell precursors was usually slightly lower than that of sorted sμ⁻, CD19⁺ pre-B cells (data not shown). However, the sorted populations of CD34⁺, CD19⁺ B-cell precursors could not be induced to produce Ig under the present culture conditions. Therefore, we conclude that the possibility of contaminating sμ⁺ B cells as the cause of Ig synthesis observed in pre-B-cell cultures is highly unlikely.

Our results also indicate that CD4⁺ T cells and IL-4 directly activate pre-B cells to express high levels of CD40 and HLA-DR and enhance differentiation of pre-B cells into sμ⁺ B cells. Enhancement of CD40 and HLA-DR expression was detected on the majority of the sorted pre-B cells, whereas only 2% to 3% of these pre-B cells spontaneously expressed sμ⁺, excluding the possibility that they were the few contaminating sμ⁺ B cells that were induced to express higher levels of CD40 and HLA-DR. These data, together with the observation that significant levels of Ig were produced in cultures initiated with pre-B cells sorted after 2 days of preincubation during which most of the spontaneous differentiation of pre-B cells occurred, support the conclusion that CD4⁺ T cells and IL-4 indeed activate bona fide pre-B cells and induce them to differentiate into Ig-secreting plasma cells.

CD34⁺, CD19⁺ cells are TdT⁺ and μ⁻ (refs 14 and 21, and our unpublished observations), which is characteristic for pro-B cells. TdT plays a role in the early events of Ig-gene rearrangements, indicating that these CD34⁺ pro-B cells are undergoing Ig-gene rearrangements. The observation that μ⁻, TdT⁺, CD34⁺, CD19⁺ pro-B cells cannot be induced to undergo differentiation into Ig-secreting cells under the present culture conditions indicates that nonrearranged B-cell progenitors require additional signals to differentiate into functional, mature B cells. Although these signals have yet to be defined, they appear to be provided by BM cells, because depletion of CD34⁺ cells from Percoll-enriched BM preparations was reported to reduce the number of cells that give rise to plaque-forming colonies.29 These results also imply that B-cell progenitors under the present culture conditions can differentiate into Ig-secreting cells only after they have reached the pre-B-cell stage in B-cell ontogeny.

Neutralizing anti-IL-4 MoAbs completely prevented Ig synthesis in pre-B-cell cultures induced by IL-4 in the presence of CD4⁺ T-cell clone, showing the specificity of the response. The presence of IL-4 receptors on early-B-cell precursors has been shown, but studies on the effects of
Table 1. Induction of Isotype Switching and Ig Production by Pre-B Cells Sorted After 2 Days of Culture In Vitro

<table>
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<tr>
<th>Stimulus</th>
<th>Isotype Produced (ng/mL)</th>
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<tr>
<td></td>
<td>IgM</td>
</tr>
<tr>
<td>Exp 1</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>&lt;1</td>
</tr>
<tr>
<td>B21 + IL-4</td>
<td>8±2</td>
</tr>
<tr>
<td>B21 + IL-4 + IL-7</td>
<td>28±9</td>
</tr>
<tr>
<td>Exp 2</td>
<td></td>
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<tr>
<td>Medium</td>
<td>&lt;1</td>
</tr>
<tr>
<td>B21 + IL-4</td>
<td>3±1</td>
</tr>
<tr>
<td>B21 + IL-4 + IL-7</td>
<td>12±4</td>
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<tr>
<td>Exp 3</td>
<td></td>
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<tr>
<td>Medium</td>
<td>&lt;1</td>
</tr>
<tr>
<td>B21 + IL-4</td>
<td>3±1</td>
</tr>
<tr>
<td>B21 + IL-4 + IL-7</td>
<td>82±56</td>
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<tr>
<td>Exp 4</td>
<td></td>
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<tr>
<td>Medium</td>
<td>&lt;1</td>
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<tr>
<td>B21 + IL-4</td>
<td>6±2</td>
</tr>
<tr>
<td>B21 + IL-4 + IL-7</td>
<td>16±4</td>
</tr>
</tbody>
</table>

Sorted sp+CD19+ pre-B cells or total fetal BM cells were first cultured for 2 days as follows: Exp 1, sorted sp+CD19+ pre-B cells cultured in medium only; Exp 2, sorted sp+CD19+ pre-B cells cultured in the presence of activated CD4+ T-cell clone B21 and IL-4 (400 U/mL); Exp 3 and 4, total fetal BM cells cultured in the presence of B21 cells and IL-4. Thereafter, cells that had differentiated into sp+B cells were depleted by FACSSorting and sp+,CD19+ pre-B cells were collected and cultured for an additional 13 days in the presence or absence of B21 cells, IL-7 (100 U/mL), and/or IL-4 (400 U/mL). Ig levels in the culture supernatants were measured at the end of the cultures by ELISA. The data represent mean ± SEM of triplicate cultures.

Abbreviation: Exp, experiment.

IL-4 on these cells has resulted in somewhat contradictory results. IL-4 was shown to induce growth and differentiation of human pre-B cells, whereas other studies showed no effect or inhibition of pre-B-cell proliferation. However, IL-4 may have different effects on different subpopulations of B-cell precursors, and it was suggested that the effects of IL-4 on pre-B cells depend on the combinations of different stimuli acting on a given cell. Nevertheless, the presence of both IL-4 and activated CD4+ T cells was an absolute requirement for pre-B-cell differentiation in the present culture system. IL-7 has been shown to induce proliferation of both murine and human B-cell precursors; it enhances tyrosine phosphorylation of several phosphoproteins and stimulates inositol phospholipid turnover and DNA synthesis in human B-cell precursors. On the other hand, inhibitory effects of IL-7 on the growth of B-lineage colonies from human BM have been reported as well. The present data indicate that the presence of IL-7 is not necessary for successful differentiation of human sp+, CD19+, CD10+, CD19+ pre-B cells, because, in contrast to anti-IL-4 MoAbs, anti-IL-7 MoAbs did not inhibit pre-B-cell differentiation. However, IL-7 enhanced Ig synthesis by pre-B cells cultured in the presence of activated CD4+ T cells and IL-4. Because IL-7 also induces activation and proliferation of T cells, it is possible that the increased levels of Ig produced were caused indirectly by enhancement of the costimulatory signals provided by the T cells. This notion is compatible with the observation that IL-7 enhances Ig synthesis by CD2+ thymic B cells, but not in the presence of CD4+ T cells. However, in contrast to thymic B cells, IL-7 induced proliferation of sp+, CD19+ pre-B cells (Punnonen et al, unpublished observation), which is in agreement with previous studies suggesting that direct growth-promoting effect of IL-7 on pre-B cells may also contribute to the IL-7-mediated increase in Ig synthesis in pre-B-cell cultures. The relative contribution of the indirect effects of IL-7 on the helper-T cells and the direct effects on the pre-B cells to the enhancement of Ig synthesis under the present culture conditions remains to be determined.

The activation state of the T-cell clone was essential for induction of pre-B-cell differentiation. The cloned T cells used in the present study were obtained 2 to 3 days after activation with the feeder-cell mixture and PHA. Previous studies have shown that T-cell clones obtained 4 to 6 days after activation fail to provide the costimulatory signals required for pre-B-cell differentiation. In contrast, sp+ fetal BM B cells and normal, adult B cells could efficiently be induced to undergo Ig isotype switching and differentiation into Ig-secreting cells in the presence of IL-4 and CD4+ T cells obtained 4 to 6 days after they had been activated. Thus, the activation state of the T cells required for pre-B-cell differentiation differs from that required for immature CD10+, CD19+, sp+ B cells, suggesting that the signal requirement for pre-B-cell differentiation also differs from that for differentiation of sp+ immature B cells.

The membrane-bound molecules on the CD4+ T cells mediating pre-B-cell differentiation remain to be determined. It has been recently shown that CD40L, transiently expressed on CD4+ T cells after activation, induces proliferation and Ig synthesis by normal, adult B cells in the presence of IL-4. Patients with X-linked hyper IgM-syndrome, who have no or minimal levels of IgG, IgA, and IgE in their sera, were subsequently shown to have mutations in their CD40L gene, showing the essential role of CD40L-CD40 interactions in induction of Ig isotype switching in vivo. We recently showed that the 26-kD membrane form of tumor necrosis factor-α, expressed on activated CD4+ T cells after activation, also is involved in T-B-cell interactions resulting in B-cell activation, isotype switching, and Ig production. Moreover, major histocompatibility complex class II molecules and CD4 have been shown to be associated with productive T-B-cell interactions resulting in Ig synthesis by normal, adult B cells. Although these molecules are likely to play a role also in T-cell—pre-B-cell interactions, anti-CD40 MoAbs, either soluble or cross-linked to L-cells transfected with FcγRII/CD32, failed to induce pre-B-cell differentiation in the presence of IL-4, which is consistent with a previous report indicating a failure of normal and leukemic human B-cell precursors to respond to an anti-CD40-mediated stimulus. These data are in line with the observation that CD40 expression on fetal BM B cells is low, and that CD34+ pro-B cells lack CD40 expression. Moreover, no expression of CD40L in fetal BM was found by PCR analysis, supporting the notion that CD40 antigen does not play a major role in early events of B-cell development. In contrast, sp+, CD10+, CD19+ fetal BM B cells ex-
press CD40 and also produce Ig when activated by anti-
CD40 MoAbs and IL-4 under the present culture condi-
tions.6 These results further support the notion that the
signals required for pre-B cells to differentiate into Ig-secre-
ting cells differ from those required for normal, adult B cells
or for immature $\mu^+$, CD10$, CD19$ fetal B cells, and that the
Ig production observed in pre-B-cell cultures was not
due to nondetectable levels of contaminating $\mu^+$ B cells.

The present data indicate that human pre-B cells differen-
tiate into Ig-secreting plasma cells in a short-term culture
system in the presence of activated, cloned CD4$^+$ T cells and
IL-4. In contrast to long-term cultures of murine B-cell pre-
cursors,22-24 this culture system did not require the presence
of BM stromal cells. Because T cells are abundantly present
in vivo, the Ig production observed in pre-B-cell cultures was not
in vivo. The present culture system allows for further studies
or for immature

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ity to switch to IgE-producing cells in response to interleukin-4
and T-helper cells may play a role in B-cell development
in vivo. The present culture system allows for further studies
on the signals regulating human pre-B-cell differentiation
and provides grounds to investigate the defects in this pro-
cess in pathologic situations.

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