Immature Surface Ig+ B Cells Can Continue to Rearrange \( \kappa \) and \( \lambda \) L Chain Gene Loci

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Summary

Pro and pre B cells possess the long-term capacity to proliferate in vitro on stromal cells and interleukin 7 (IL-7) and can differentiate to surface immunoglobulin (slg+) cells upon removal of IL-7 from the cultures. A key event in this differentiation is the extensive cell loss due to apoptosis. Because the proto-oncogene \( bcl-2 \) can promote cell survival, we established pre-B cell lines from \( E\mu-bcl-2 \) transgenic mice. These pre-B cells have the same properties as those derived from non-\( bcl-2 \) transgenic mice except that they do not die by apoptosis. This allowed us to study the fate of newly formed B cells in vitro for a longer period of time. Here we show that early during the differentiation of pre-B cells, upregulation of RAG-1 and RAG-2 expression go hand in hand with rearrangements of the Ig gene loci. Moreover, the newly formed slg+ B cells continue to express RAG-1 and RAG-2 and continue to rearrange L chain gene loci, even in the absence of proliferation, in an orderly fashion, so that \( \kappa L^+ \) slg+ cells can become \( \lambda L^+ \) slg+ or slg cells, whereas \( \lambda L^+ \) slg+ cells can become slg-, but not \( \kappa L^+ \) slg+ cells. Thus, deposition of a complete Ig molecule on the surface of a B cell does not automatically stop the Ig-rearrangement machinery.

The \( bcl-2 \) gene encodes a 26-kD \( \alpha \) protein and a \( \beta \) form of 21 kD varying at the COOH-terminal end (1). The \( bcl-2 \) protein is mainly expressed in B- and T-lymphocyte-lineage cells at defined stages of their differentiation (2-4). It was originally identified as the locus translocated to the Ig H chain locus in the (t 14:18) (q32;q21) translocations found in most cases of human follicular lymphomas (5-9). Translocations leave the coding regions intact but put the (onco)gene under the control of expression of the IgH locus, leading to constitutive high expression along the B-lineage pathway from early precursors to plasma cells. Although the exact functions of the \( bcl-2 \) gene are still unknown, it plays the role of rescuing cells from programmed cell death (apoptosis, characterized by chromatin condensation and by endonucleolytic cleavage of DNA into characteristic nucleosome ladders) (10, 11), thereby prolonging the lifespan of the cell in which it is expressed. This has been shown for B-lineage, T-lineage, and other hematopoietic cells by transfection of the \( bcl-2 \) gene (12, 13) and in \( bcl-2 \)-transgenic (tg) mice (14-20). The \( bcl-2 \) expression pattern in normal T- and B-lymphoid lineage cells suggests that it may play a role in changing the life span of antigen-selected lymphocytes to longevity (21, 22). From all these studies it appeared reasonable to expect that constitutive \( bcl-2 \) expression in precursor (pre-) B cells of \( E\mu-bcl-2 \)-tg mice (16-18) would inhibit the apoptosis of B cells differentiating from long-term proliferating pre-B cells. This differentiation can be induced by removal of IL-7 from cultures of these pre-B cells which need stromal cells and IL-7 for continued proliferation (23).

This paper compares the differentiation of normal pre-B with \( bcl-2 \)-tg pre-B cells in vitro to surface Ig+ (slg+) and slg- cells. The results show that proliferation on stromal cells and IL-7, and differentiation of \( bcl-2 \)-tg pre-B cells upon removal of IL-7, is normal except that the differentiated B cells do not die by apoptosis. This allows an investigation of the state of the slg+ and slg- cells, generated by productive and nonproductive, respectively, rearrangements of the H and L chain gene loci, their expression of pre-B cell-specific and -related genes such as \( V_{\text{preB}} \) and \( \lambda_5 \) (24), and RAG-1 and RAG-2 (25, 26), and their use of \( \kappa L \) and \( \lambda L \) chain gene loci during differentiation to slg+ B cells.

Materials and Methods

Animals and Cell Lines. \( E\mu-bcl-2 \) transgenic mice originally obtained from Drs. A. Harris and S. Cory, (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and previously described (16-18) were bred in the breeding facilities of the

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Abbreviations used in this paper: HU, hydroxyurea; slg, surface Ig; tg, transgenic.
Blasts transfected with the murine I1:7 cDNA were grown as described (23).

Interleukins, Monoclonal Antibodies, Immunofluorescence, and Flow Cytometric Analysis. rIL-7, produced by the fibroblast cell line 3T3 transfected with the mouse I1-7 gene, was used at a concentration of 50-200 U/ml. The FITC-, PE- or biotin-conjugated mAb RAB-6B2 (anti-B220), R26-46 (M + X2 L chains), and 6C3 (anti-BP-1) were obtained from Pharmingen (San Diego, CA). The mAb S7 (anti-CD43), Ack4 (anti-c-kit), M41 (anti-μH chain), 187.1 (anti-κL chain), and 4/1-101 (anti-λL chain) were purified from culture supernatant and conjugated as previously described (23). Cell surface immunofluorescence and flow cytometric analyses were performed as described (27). Cells expressing κ or λ L chains were sorted using the FACStar Plus® (Becton and Dickinson, Mountain View, CA).

Preparation of Pre-B Cell Lines. Pre-B cell lines that possess the long-term capacity to proliferate on stromal cells in the presence of IL-7 were prepared from day 17-18 fetal liver and from 2-wk-old bone marrow cells as previously described (23, 27). Throughout these experiments PA-6 cells were used as stromal cells. The influence of hydroxyurea on the proliferation and differentiation of our pre-B cells was studied using different concentrations. For proliferation, pre-B cell cultures were initiated at 5 × 10⁴ cells/ml on PA-6 plus IL-7 in the presence of different concentrations of hydroxyurea. Cell growth was determined by [³H]thymidine uptake during the last 6 h of a 72-h culture period. For differentiation, pre-B cell cultures were initiated at 1-2 × 10⁴ cells/ml on PA-6 stromal cells without IL-7 and in the presence of different concentrations of hydroxyurea. Differentiation was determined by measuring sIg* cells after a 72-h culture period.

Northern Blot and Dot Blot Analysis. Total cellular RNA was extracted from cells with acid guanidium-thiocyanate-phenol-chloroform (28). For Northern blot analysis 15 μg denatured total RNA per lane was fractionated on a 1.2% agarose 0.4 M formaldehyde gel and then blotted onto Gene Screen Plus® nylon membranes (Du Pont-Biotechnology Systems NEN, Boston, MA) by downward alkaline blotting. For dot blot analysis, 15 μg total RNA was denatured in 55% formamide, 7% formaldehyde, 50 mM NaH₂PO₄, pH 7.2, for 10 min at 65°C, serially diluted in steps of three in the same buffer, and applied to a Gene Screen Plus® nylon membrane with a Unifold® 96-well blotting apparatus (Schleicher & Schuell, Inc., Keene, NH) by gentle suction. The membrane was washed in 0.2 M NaH₂PO₄, pH 7.2, for 10 min, in H₂O (tridist.) for 2 min and air-dried.

Filters were prehybridized in 7% SDS, 1 mM EDTA, 0.5 M NaH₂PO₄, pH 7.2, for 4 h at 65°C and hybridized in the same buffer overnight at 65°C with cloned fragments of Cκ, VpreB1, RAG-2, and β-actin genes in M13mp18, labeled by second strand synthesis in a reaction primed by a 17-mer sequencing primer (New England Biolabs, Beverly, MA) and α-[³²P]dCTP. The probe for Cα1 was a kind gift of Dr. Shunichi Takeda in our Institute and was labeled using a "Quick Prime®" kit (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol.

Hybridized membranes were washed twice in 5% SDS, twice in 1% SDS, both in 40 mM NaH₂PO₄, pH 7.2, 1 mM EDTA at 65°C for 20 min each and were autoradiographed with a Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY). Radioactivity on the blots was also visualized and quantified by means of a PhosphorImager® with ImageQuan® software 3.0 (Molecular Dynamics, Inc., Sunnyvale, CA). Before rehybridization, blots were stripped of the hybridized radioactivity by washing the membranes four to five times in 0.01 × SSC, 0.01% SDS at 95°C for 2-3 min.

RNA Isolation, cDNA Synthesis, and PCR. BCL2-5 cells were FACs® sorted after 5 d of differentiation and total RNA was isolated from 20,000 κ*, λ*, or κ*/λ* cells.

The cells were lysed in 100 μl guanidinium-isothiocyanate solution (28), 10 μl 2 M Na-acetate, pH 4, and 100 μl phenol/chloroform/isoamylalcohol 25:24:1 (water saturated) were added, mixed (15 min, 4°C). The upper phase was added to 120 μl isopropanol and the RNA precipitated by centrifugation. The pellet was washed with 70% ethanol, air dried, and resuspended in 20 μl water.

First strand synthesis was carried out using random hexamer primers (Boehringer-Mannheim, Mannheim, FRG) and Superscript Reverse Transcriptase (GIBCO BRL, Basel, Switzerland) in a total volume of 20 μl.

1 μl of the cDNA preparation was subsequently used for PCR amplification. The primers for RAG-1 and RAG-2 were kindly provided by Dr. G.-K. Sim, Basel Institute for Immunology, and had the following sequences: RAG-1 5' CCAAGCTGAGACATTCTAGACACTCTCAGCCTCAGTGGAC- TCC; RAG-2 5' CACATCCAAACAGGAGGACAACTCAGGCACTCTTCACCTAAG; The control primers for the housekeeping gene HPRT were kindly provided by Dr. M. V. Wiles, Basel Institute for Immunology. The sequences are published (29).

All primers were designed to be intron-spanning to distinguish DNA contamination.

PCR reactions were performed on 1 μl of the cDNA preparation (i.e., RNA from 1,000 cells) in a total volume of 20 μl according to the enzyme manufacturer's conditions (Boehringer Mannheim). The temperature settings for the Perkin Elmer Cetus PCR Cycler (Norwalk, CT) were 94°C 20 s, 58°C 20 s, 72°C 30 s for 35 cycles.

Semiquantitative PCR Titration for κ Rearrangements. BCL2-5 cells on days 0, 1, 2, 3, and 4 of differentiation were counted by Trypan blue exclusion, lysed by boiling, and DNA prepared as described elsewhere (27). DNA was diluted in threefold steps in 10 mM Tris-Cl, pH 8.3, leading to DNA equivalents of 300, 100, 30, 10, and 3 cells, respectively, per 5 μl of the dilution. The PCR reaction was performed according to the enzyme manufacturer's conditions (Boehringer Mannheim) in a final volume of 20 μl. The PCR primers (universal V, and JL primers detecting Vκ-Jκ1 and Vκ-Jκ2 rearrangements) and reaction conditions are described elsewhere (30).

Half of the resulting PCR product was separated on a 1.5% agarose gel by electrophoresis. Bands of 536 (Vκ-Jκ1) and 130 bp (Vκ-Jκ2) were visualized under UV light.

Results

Establishments of Pre-B Cell Lines from bcl-2 tg Mice. Pre-B-I cells of normal mice can proliferate for long periods of time on stromal cells in the presence of IL-7 (23, for the nomenclature of pre-B cells used by us see reference 24). To establish pre-B-I cell lines from bcl-2 tg mice, cell suspensions of fetal liver at day 17–18 of gestation and of bone marrow 2 wk after birth were cultured under conditions of limiting dilution on PA6 stromal cells in the presence of exogenously added IL-7. Growth of cells was scored at day 6 of culture, and lines and clones were established from positive cultures.
by continued growth on stromal cells in the presence of IL-7. Fetal liver cells were obtained from individual embryos of crosses of mice heterozygous for the bcl-2 tg and from bone marrow of bcl-2 tg mice. The presence of the bcl-2 tg in pre-B cell lines was ascertained by the presence of SV-40 DNA sequences flanking the bcl-2 gene construct, and was furthermore evident from the unusual survival capacity of cells in tissue culture upon differentiation as described below. Three bcl-2 transgenic cell lines (bcl-2-1, bcl-2-5, and bcl-2-6) were derived from fetal liver of three individual embryos which were typed as tg positive.

Proliferation of bcl-2 tg pre-B cells on stromal cells in the presence of IL-7 was indistinguishable from pre-B cells of normal mice in several parameters. Both needed the simultaneous presence of stromal cells and IL-7 for proliferation, both proliferated at the same rates and for the same long periods of time (data not shown). The frequencies of pre-B cells (1 in 50 at day 17–18 of gestation in fetal liver; 1 in 50–100 in 2-wk-old bone marrow) were within the ranges found for normal cells.

All bcl-2 tg pre-B-I cell lines, just as those from normal mice, expressed mRNA for the pre-B cell-specific genes VpreB, RAG-1, and RAG-2, but not κL and λL chain genes, as detected in Northern blot analyses (Fig. 1). They expressed CD43, c-kit, B220, and BP-1, but not slg (μ, κ), as detected by immunofluorescence with specific mAbs. Pre-B-I cells from bcl-2 tg mice were found to be DαH+ rearranged in their H chain loci (data not shown).

We conclude that bcl-2 tg pro and pre-B-I cells have many of the properties of normal pre-B-I cells.

**Differentiation of bcl-2 tg Pre-B-I Cell Lines In Vitro**

Differentiation of normal pre-B-I cells occurs in vitro when IL-7 is removed from tissue culture (23). Differentiating cells lose the capacity to grow on stromal cells in the presence of IL-7 (23), and a portion of them become slg+ and mitogen-reactive cells within 2–3 d (23, 31, 32). Normal cells die rapidly by apoptosis, as evidenced by the endonucleolytic degradation of DNA and a loss of live cells (23).

When IL-7 was removed from cultures of bcl-2 tg pre-B-I cell lines, the cells lost the capacity to grow on stromal cells in the presence of IL-7, determined as frequencies of clonable cells (Fig. 2, bottom). In contrast to normal pre-B-I cells, bcl-2 tg pre-B cells did not die by apoptosis (Fig. 2, top). Cells remained viable during differentiation. However, the vast majority of the cells changed their phenotype. Within 3 d

![Figure 1](image-url)
they lost surface expression of c-kit, CD43, BP-1, and the surrogate L chain V<sub>preb</sub>/X5. Fetal liver–derived cell lines were indistinguishable in these differentiation capacities from bone marrow–derived cells (data not shown).

On the level of specific RNA expression, the surrogate L chain disappeared within 2–3 d, whereas expression of κL chain mRNA became detectable after 2 d, and of λL chain mRNA after 3 d of differentiation (Fig. 1). Surprisingly, the expression of RAG-1 and RAG-2 mRNA actually increased for the first 2 d of in vitro differentiation, before it began to decrease. This increase was seen in Eμ-bcl-2-tg as well as in normal cells. Using a limiting dilution PCR, IgL chain rearrangements were analyzed. About 1 in 300 cells at day 0 had a rearrangement, this was 1 in 100 at day 1, and more than 1 in 10 from day 2 of differentiation onwards.

**Differentiation to slg<sup>+</sup> B Cells Expressing Either κ or λL Chains.** Within 3 d of in vitro differentiation of normal as well as bcl-2 pre-B cells, ~10% of all cells became slg<sup>+</sup>. At the same time, >85% of the slg<sup>+</sup> cells expressed κL chains (Fig. 3). Extensive apoptosis of the normal cells prevents an analysis of cells which have differentiated in vitro for periods exceeding 3 d. Such an analysis could, however, be done with bcl-2-tg cells. It is evident from the data shown in Fig. 3 that the total number of slg<sup>+</sup> B cells increased with time of differentiation in vitro, reaching levels of 40% slg<sup>+</sup> cells. Furthermore, the ratio of κL chains versus λL chains expressed in these slg<sup>+</sup> cells changed. At 3 d of in vitro differentiation it was near 5, and at one week or later around 0.7.

These results suggested that the differentiated cells might be capable of continuous, successive rearrangements in their κ and λL chain gene loci. This was substantiated by the fact that sorted κL<sup>+</sup> slg<sup>+</sup> and λL<sup>+</sup> slg<sup>+</sup> were still positive for RAG-1 and RAG-2 expression as determined by RT-PCR. Therefore, bcl-2-tg cells differentiated for 5 d in vitro were sorted for κL<sup>+</sup> slg<sup>+</sup> and λL<sup>+</sup> slg<sup>+</sup> cells. Thereafter, they were recultured in the absence of IL-7 for a further 3 d and then assayed again for κL<sup>+</sup> slg<sup>+</sup>, λL<sup>+</sup> slg<sup>+</sup>, and slg<sup>-</sup> cells. Of the κL<sup>+</sup> slg<sup>+</sup> cells, about half remained κL<sup>+</sup> slg<sup>+</sup> cells, ~10% became λL<sup>+</sup> slg<sup>-</sup> cells, and ~40% changed to slg<sup>-</sup> cells. Of the λL<sup>+</sup> slg<sup>+</sup> cells, 75–80% remained λL<sup>+</sup> slg<sup>+</sup> cells, ~20–25% became slg<sup>-</sup> cells, whereas 1% or less became κL<sup>+</sup> slg<sup>+</sup> cells. Several experiments with different fetal liver– and bone marrow–derived Eμ-bcl-2-tg pre-B cell lines yielded similar results. Taken together, these results indicate that the slg<sup>+</sup> B cells differentiated in vitro from bcl-2-tg, and probably also from normal pre-B cells, can continue to rearrange L chain gene loci.

**Differentiation in the Presence of Hydroxyurea.** Hydroxyurea (HU) inhibits the replication of DNA and, thereby, the proliferation of cells. It was added to Eμ-bcl-2-tg pre-B cells cultured on stromal cells in the presence or absence of IL-7 to see whether it would inhibit pre-B cell proliferation, and
whether that would still allow differentiation to slg+ B cells. Results in Fig. 4 show that HU at 10^{-4} M or higher completely inhibits proliferation as measured by uptake of radioactive thymidine. On the other hand, the proportion of slg+ B cells differentiating at these high concentrations of HU remained constant. The development of κL+ slg- and λL+ slg- B cells within 3 d in the presence of 2 \times 10^{-4} M HU yielded ratios of λL to κL chain-expressing slg- B cells indistinguishable from those obtained in the absence of HU (Fig. 3). The results collectively suggest that differentiation to slg+ B cells and maybe even the continued rearrangement of L chain gene loci can occur in the absence of DNA synthesis and cell proliferation.

**Discussion**

Pre-B cells that possess the long-term capacity to proliferate on stromal cells and IL-7 can differentiate to slg+ B cells by removal of IL-7 from the culture system (23, 31, 32). So far, extensive apoptosis (23) has hampered a more detailed analysis at the cellular and molecular level during this differentiation. Here we show that constitutive expression of the bcl-2 proto-oncogene in these pre-B cells can prevent this apoptosis and therefore allows us to follow the fate of newly formed slg+ B cells in vitro. One important question concerning the relevance of these studies is whether constitutive expression of the bcl-2 gene interferes with the normal pre-B cell differentiation pathway. The findings that the pre-B cells derived from Eμ-bcl-2 transgenic mice show the properties as those derived from non-bcl-2 transgenic mice like: (a) loss of clonability on stromal cells and IL-7 after removal of IL-7 from the culture system; (b) loss of markers like CD43, c-kit, BF-1, VpreB, and λ5 during differentiation; (c) upregulation of RAG-1 and RAG-2 expression during differentiation; and (d) identical kinetics for the appearance of slg+ B cells strongly argue that constitutive expression of bcl-2 does not interfere with the normal pre-B cell differentiation pathway.

High κ/λ ratios can also be explained by stochastic models that postulate an intrinsic rearrangement activity which is higher for κ than for λ loci (36–39). The stochastic model has recently gained support from studies with mice in which the Ig κ locus was inactivated (40–42). These studies demonstrate that rearrangements at the Ig κ locus and the production of XL chain-expressing B cells can occur without rearrangements at the Ig λ locus.

A third model argues that high κ/λ ratios in the periphery reflect antigen selection and stimulation and that newly generated B cells have κ/λ ratios near unity (42). Our earlier published findings (31) as well as the data shown above have strongly argued against this model. We have shown that immature slg+ B cells obtained from normal non-bcl-2 tg pre-B cells that differentiate in vitro in the absence of exogenous antigen have already κ/λ ratios of >10.

Our findings described here support the scenario in which κ gene rearrangements are initially preferred over λ genes. However, when the immature B cells can survive longer, the chances for λ chain gene rearrangements increase and consequently the ratio of κ over λ light chain–expressing B cells decreases. These and other findings (35–41) reaffirm a stochastic model in which a higher intrinsic rearrangement activity of the κ over the λ gene loci appears to be responsible for the high κ/λ ratio found in normal mice.

Secondary rearrangements can in principle occur at several Ig loci. Thus far, two examples of Vr-gene replacement in which an already rearranged V(D)Jκ-rearranged H chain locus could be altered with a second Vκ gene have been described (43, 44). The κL chain locus has a series of Vk- and Jκ- segments that allow subsequent rearrangements in an already rearranged locus by inversion or deletion of intervening sequences (45–48). In normal B cells, circular DNAs of rearranged VJs have been demonstrated of which a large part is in-frame and thus potentially functional (49). Moreover in the μH, κL chain–expressing NFS-5 tumor cells, κL chain rearrangement may occur and thus κL chains can be exchanged.

**Figure 4.** Proliferation and differentiation of bcl-2-5 pre B cells in the presence of hydroxyurea. Proliferation of bcl-2-5 pre B cells on PA6 stromal cells in the presence (●) or absence of IL-7 (△) and in the presence of different concentrations of hydroxyurea was determined by [3H]thymidine uptake as described (23). The expression of slgM was determined at day 3 of culture on PA6 stromal cells without IL-7 and in the presence of different concentrations of hydroxyurea as described (23). Similar results were obtained with other (fetal liver- or bone marrow–derived) Eμ-bcl-2 pre-B cell lines.
by λL chains (50). Hence, the rearrangement machinery of a B cell does not automatically stop after deposition of a complete Ig in the cell membrane.

Secondary rearrangements, also termed "receptor editing" (51, 52) could either silence unwanted specificities and/or salvage B cells with new desirable specificities. Recent studies (51, 52) using mice expressing an autoantibody as a transgene have shown that immature B cells in the bone marrow of these mice can be rescued after encounter with their autoantigen by exchanging the transgenic L chain in the Ig molecule with an endogenously encoded L chain. This L chain replacement is possible because these immature B cells still express RAG-1 and RAG-2 (51). On the other hand, cross-linking of slg on immature B cell lines from N-myctg mice which express slg as well as RAG-1 and RAG-2 simultaneously has been found to lead to a rapid and reversible down-regulation of RAG-1 and RAG-2 expression (53). Therefore, occupancy of slg on (immature?) B cells can either down-regulate (at least in N-myctg mice), or preserve (at least in autoantibody-tg mice) the expression of RAG-1 and RAG-2 and, consequently, the capacity to express another L chain. This indicates that the signals given to an immature B cell via slg, as in mature B cells, can result in different reactions of these cells. It remains to be seen how the mode of Ig-receptor occupancy, additional cell contacts with the antigen-presenting environment, and secondary signals by cytokines influence these reactions.

Ongoing Ig gene rearrangements in slg+ B cells are likely to generate B cells with more than one antibody specificity. In practice, this may be avoided by the rapid apoptosis of the immature B cells which leaves little time for secondary rearrangements, before the cells die, or before they are selected into the peripheral pool of mature B cells. This might also be the reason for the fact that the rescued peripheral B cell pool in mice with transgenic H and L chain genes encoding an autoantibody is relatively small (51). These scenarios of early B cell development are reminiscent of similar stages of T cell development, where immature α/β TcR+ thymocytes keep expressing RAG-1 and RAG-2 and continue to rearrange α chain gene loci until they are positively selected into the pool of MHC-restricted CD4+ or CD8+ T cells (54, 55).

Immature lymphocytes from Eμ-bcl-2-tg mice of both T and B cell lineages appear suitable target cells to study the cellular and molecular requirements of positive selection into the peripheral, long-lived pools of cells.

We thank Drs. Alan Harris and Suzanne Cory for the gift of Eμ-bcl-2 transgenic mice and Drs. Klaus Karjalainen and Jean-Marie Buerstedde for critical reading of this manuscript. The able technical assistance of Ms. Andrea Groenewegen is gratefully acknowledged.

The Basel Institute for Immunology is founded and supported by F. Hoffmann-La Roche Ltd., Basel, Switzerland.

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Received for publication 17 May 1993.

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