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Maturation of B Cell Precursors Is Impaired in Thymic-Deprived Nude and Old Mice

Paul Szabo,* Kesheng Zhao,* Irena Kirman,* Joel Le Maoult,* Rubendra Dyall,† William Cruikshank,‡ and Marc E. Weksler*‡*

We have previously reported that bone marrow B cell precursors from thymic-deprived nude and old mice express less recombination-activating gene-1 (RAG-1) mRNA than they do in young euthymic mice. We now report that both nude and old mice have decreased bone marrow pre-B cells and that fewer pre-B cells express RAG protein. This combination of events appears to be the basis for the lower level of bone marrow RAG mRNA in thymic-deprived mice. A link between thymic function and B cell development was suggested by the similar kinetics of thymic involution and of declining bone marrow RAG-1 gene expression during aging. Support for this hypothesis was obtained by demonstrating that injection of supernatant medium from activated CD8⁺ but not CD4⁺ young T cells from mice increases RAG mRNA, RAG protein, and the number of bone marrow pre-B cells in nude and old mice. Furthermore, in vivo CD8⁺ T cells also regulate bone marrow RAG gene expression. Thus, mice deficient in CD8⁺ T cells expressed levels of RAG-1 mRNA in their bone marrow that were only 10% of those observed in normal or CD4⁺ T cell-deficient mice. IL-16 was detected in the supernatant medium from activated T cell cultures, and injection of nanogram quantities of recombinant IL-16 (rIL-16) into nude or old mice increased the levels of RAG mRNA in bone marrow B cell precursors and the number of bone marrow pre-B cells. We conclude that the impaired development of B cells within the bone marrow of thymic-deprived nude and old mice can be reversed, at least in part, by the administration of rIL-16. The Journal of Immunology, 1998, 161: 2248–2253.

Bone marrow B cell precursors pass through a series of developmental stages during their maturation into B cells (1). During the pro-B and pre-B cell stages, the germline Ig heavy and light chain gene segments begin to rearrange, respectively. The expression of the two recombination-activating genes, RAG-1 and RAG-2, is an absolute requirement for the expression of surface Ig (slg) molecules and the appearance of mature B cells (2). Normal B cell development had been thought to be independent of the thymus gland, T cells, or their products because the numbers of splenic B cells in nude, adult thymectomized, or αβ-TCR-deficient mice are normal (3, 4).

We previously reported that the expression of the RAG-1 gene by bone marrow B cell precursors declines with age in mice (5). Thus, the level of RAG-1 mRNA is maximal in mice between the ages of 2 and 5 mo and thereafter diminishes. Bone marrow B cell precursors from mice >10 mo of age have levels of RAG-1 mRNA that are only 10% of their peak levels. The rate of decline in the levels of RAG-1 mRNA with age occurs later but is similar to that of thymic involution, which in mice begins after 6 wk when the total thymic cell number and migration of T cells from the thymus declines (6). By 6 mo, the total number of thymic cells has dropped to ~30% but the number of T cells released is only 5% of peak production (6). This observation, together with the low levels of RAG-1 mRNA in nude mice and the increased levels of RAG-1 mRNA in nude mice following the injection of supernatant medium from activated T cells, suggested that the thymus gland, functional T cells, or T cell products are required for the normal expression of the RAG-1 gene and the normal development of bone marrow B cell precursors (7).

We now offer evidence that a decreased number of bone marrow pre-B cells in old and nude mice and a lower percentage of pre-B cells expressing RAG is the cellular basis for the low levels of RAG mRNA (and RAG protein) in bone marrow B cell precursors. We also show that supernatant medium from activated CD8⁺ T cells induces an increase not only in RAG mRNA but also in the number of bone marrow pre-B cells. Finally, IL-16, present in the supernatant medium from activated T cells, when injected in nanogram quantities into nude or old mice increases the levels of RAG mRNA and the numbers of pre-B cells in nude and old mice.

Materials and Methods

**Mice**

Female C57BL/6 and BALB/c mice between 3 and 80 wk of age were obtained from the National Institutes of Health aging colony (Charles River Laboratories, Wilmington, MA). Nude mice, 6 to 8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). MHC class II knockout mice, C57BL/6.FDR-KO-Aβ*, obtained from Taconic Farms (Germantown, NY), are CD4⁺ T cell deficient. TAP1 deficient mice, provided by A. Berns, are CD8⁺ T cell deficient (8). Mice of both transgenic strains were 6 to 8 wk old. C57BL/6 mice were used in the initial experiments on RAG and terminal deoxynucleotidyl transferase (TdT) mRNA in old mice; BALB/c mice were used for all subsequent experiments. Mice were maintained at the Cornell University Medical College under laminar flow and killed by cervical dislocation. Routine serologic assays for viral, bacterial, and parasitic pathogens were negative. All experiments were performed in...
Preparation of supernatant from activated T cells

Single spleen cell suspensions were prepared, washed three times with HBSS, resuspended in RPMI 1640–5% FCS at a concentration of 2 × 10^9 cells/ml, and incubated on nylon wool columns incubated at 37°C After 1 h, T cells were eluted with RPMI 1640–5% FCS. For analysis of T cell subsets, nylon wool-purified T cells were incubated with rat anti-murine CD4 or CD8 Ab (Boeringer-Mannheim, Indianapolis, IN) and separated into CD4- or CD8-enriched preparations using magnetic beads (Dynal, Lake Success, NY) coated with goat anti-rat IgG Ab (9). The T cell preparations were >90% pure and were adjusted to 10^7 cells/ml per ml of RPMI 1640–5% FCS and incubated for 72 h at 37°C with 10 µg/ml PHA (Sigma, St. Louis, MO) added either at the beginning or end (control T cell supernatant [ATCS]) of the culture period. The activated T cell supernatant (ATCS) was collected by centrifugation and was stable for at least 3 mo when stored frozen at −80°C.

Identification of bone marrow B cell precursors

Bone marrow cells, flushed from the marrow cavity of leg bones, were collected by centrifugation and washed three times with HBSS. Where indicated, bone marrow cells were depleted of slg^+ cells by panning with anti-mouse Ig (Sigma) and stained with anti-mouse CD43-FITC and anti-mouse B220-phcoerythrin for 30 min at 4°C, washed three times, and analyzed using a FACScan cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). Selection for lymphoid cells was on the basis of F5 vs SS. The gates were chosen to exclude cell debris and granulocytes; these gates included 75% of the total bone marrow cells. Absolute numbers of bone marrow cells were determined by direct counting of the cells flushed from the bone and percentages of cells at different stages of development obtained by flow cytometry analysis. Fluorescence was measured on a log scale.

RT-PCR of bone marrow cell RNA

Bone marrow cells were pelleted, and RNA was isolated using the Ultra-spec RNA isolation kit as specified by the manufacturer (Life Technologies, Gaithersburg, MD). cDNAs were prepared from 5 µg of total cellular RNA by mixing with 200 ng of oligo(dT), for TdT cDNA reactions, or specific primers for the RAG mRNAs as previously described (10). The PCR reactions were conducted in a final volume of 50 µl containing 1 to 5 µl of DNA reaction, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 20 µg/ml gelatin, 0.5 mM concentrations each deoxynucleotide triphosphate, 10 µCi of [α-32P]dCTP, 125 ng of oligonucleotide primers, and 0.5 units of Taq polymerase. The primer pairs used have been published: TdT (11); RAG-1 (10); RAG-2 (10); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (12). The PCR cycle was begun at 94°C for 2 min, followed by 25 to 30 cycles of 1 min at 62°C, 1 min at 72°C, and 45 s at 94°C. Preliminary studies established that these reaction conditions yielded PCR reactions within the linear range. Aliquots of the PCR reactions were fractionated on 2.5% agarose gels, fixed in 7% TCA, dried under vacuum, visualized by exposure to x-ray film, and quantitated by densitometry.

Quantitative PCR

The method of Pannetier et al. (13) was used for quantitative PCR determinations. A competitive standard plasmid was prepared by cloning RAG-1 PCR product into pCRII (Invitrogen, San Diego, CA), linearizing the resultant plasmid with AccI, and removing terminal nucleotides by partial exonuclease digestion using T4 DNA polymerase (14). The resultant digested DNA was religated, and a clone containing a plasmid with a 18-base pair deletion was selected as the standard. For the quantitative PCR reactions, a constant amount of cDNA was added to tubes containing 0.01 to 100 pg of plasmid DNA, and standard RAG-1 PCR reactions were run to 40 cycles. Run off reactions (10 cycles) were done using a 32P-end-labeled, nested primer, 5'-GGAAAATTTGATTTGTGGGTGTTG-3', and the products were displayed on a DNA-sequencing gel. The amount of each product was determined by densitometry, and the ratio of standard to unknown was plotted against the amount of standard. The point at which the ratio of products equaled 1 was determined, and the number of cDNA molecules calculated from the known amount of indicator plasmid.

RAG protein detection

Bone marrow cells (2 × 10^7) were first depleted of Ig^+ and CD43^+ cells by panning using culture dishes coated with anti-mouse Ig (Sigma) and anti-mouse CD43 (PharMingen, San Diego, CA). B220^+ cells in the non-adherent cell fraction were selected using magnetic beads coated with anti-
would lead to the changes in the levels of RAG and TdT mRNA observed. This prediction was confirmed when the numbers of pre-B and pro-B cells in the bone marrow of young, old, and nude mice were compared (Table I). There was a significant (p < 0.01) decrease in the number of pre-B cells from old and nude mice compared with young mice, while there was no significant difference in the number of pre-B cells from young, old, or nude mice.

**ATCS increases RAG mRNA and RAG protein expression by bone marrow B cell precursors in nude and old mice**

A causal relationship between the thymic-deprived state and the decreased levels of RAG mRNA in nude and old mice was suggested by the capacity of activated T cells or ATCS to increase the expression of the RAG-1 gene by B cell precursors in nude mice (5). Figure 3 shows that injection of ATCS also increases RAG gene expression in bone marrow B cell precursors from old and nude mice.

ATCS increased the percentage of pre-B cells with detectable intracellular RAG protein (Fig. 4). Ten and 13.6% of bone marrow pre-B cells from two CTCS-treated nude mice had detectable intracellular RAG-2 protein. In contrast, 66.3 and 68.8% of pre-B cells from two ATCS-treated nude mice had detectable intracellular RAG-2 protein; this same percentage of RAG-2-expressing cells from two ATCS-treated nude mice had detectable intracellular RAG protein. In contrast, 66.3 and 68.8% of pre-B cells from two CTCS-treated nude mice had detectable intracellular RAG protein.

10 and 13.6% of bone marrow pre-B cells in the bone marrow of young, old, and nude mice were compared (Table I). There was a significant (p < 0.01) decrease in the number of pre-B cells from old and nude mice compared with young mice, while there was no significant difference in the number of pre-B cells from young, old, or nude mice.

**Table I. Fewer bone marrow pre-B cells in old and nude compared with normal young mice**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>No. of Cells × 10⁶</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Pre-B cells (B220⁺CD43⁺Ig⁺)</td>
</tr>
<tr>
<td>2–3 mo BALB/c (11)</td>
<td>1.3 ± 0.6</td>
</tr>
<tr>
<td>16–18 mo BALB/c (11)</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>2–3 mo nude (11)</td>
<td>0.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Single-cell suspensions of bone marrow cells in HBSS were prepared from both femurs and tibias of young, nude, and old mice. The bone marrow cells were depleted of Ig⁺ cells and stained with anti-mouse B220-phycocerythrin and anti-mouse CD43- FITC. The total number of B220⁺drg CD43⁺ or B220⁺drg CD43⁻ cells were calculated, and the mean ± SD of cells identified is shown. The number of mice studied is given in parentheses. The number of pre-B cells from the bone marrow of young mice is statistically greater than those of nude or old mice (p < 0.01; Student t test).*
nude and old mice. In Figure 7, representative gels show that one to three injections of 100 ng of rIL-16 into four nude (one shown) or three old mice, respectively, increased their RAG-1 gene expression by bone marrow B cell precursors compared with untreated nude or old control mice. Furthermore, injection of IL-16 increased the number of pre-B cells an average of 2.6-fold in the four nude mice and by 1.6-fold in three old mice compared with untreated controls.

Discussion

The key findings in this study are that the levels of bone marrow RAG mRNA and protein are decreased in thymic-deprived nude and old mice compared with control mice and that the injection of ATCS or rIL-16 into these thymic-deprived mice reverses these defects. Thus, it appears that T cells influence B cell development, a conclusion that differs from the more conventional view that B cell development is independent of T cell influence. Peripheral B cells appear to be normal in thymic-deprived nude (18) and old mice (7) because these animals have the same or greater number of peripheral B cells as do young control mice and also because the number of splenic B cells 2 mo after irradiation and bone marrow transplantation is comparable in nude and normal mice (3). However, these and other studies do not distinguish between the steady state number of peripheral B cells and the rate of their generation by the bone marrow. In contrast, nude/sid mice exhibit decreased levels of pre-B cells which could be corrected by thymus transplants from normal, young mice, suggesting a role for T cells in B cell development (19). Additionally, some studies have provided evidence of altered B cell development in old mice, primarily at the pro-B stage of development and involving the interactions of pro-B cells with stroma and IL-7 (20–22). However, this defect(s) was not related to altered T cell function. We have reported a defect in the rate of splenic B cell reconstitution from the bone marrow of thymic-deprived mice (7). Specifically, the number of donor splenic B cells in irradiated nude mice given Ly-5 disparate bone marrow cells during the first month after bone marrow reconstitution was significantly less than in similarly treated control young mice. In contrast, as previously reported, the number of splenic B cells 2 mo after reconstitution was comparable in nude

FIGURE 4. ATCS increases the number of pre-B cells that express RAG-2 gene product. Bone marrow pre-B cells (B220<sup>+</sup>, CD43<sup>−</sup>, SIg<sup>−</sup>) were isolated from two nude mice injected with ATCS 5 days before harvest as described and from two control nude mice. The pre-B cells were immunostained using standard procedures with affinity-purified rabbit anti-mouse RAG-2 Ab and a monoclonal peroxidase-labeled anti-rabbit Ig. The signal was amplified using a tyramine-based signal amplification system. Two representative microscopic fields of fluorescein-stained pre-B cells are shown from control nude mouse bone marrow (A) and from ATCS-treated nude mouse bone marrow (B). Negative controls, using nonimmune rabbit serum, yielded a lower percentage of immunostained pre-B cells (<5%) than was seen for specific staining of pre-B cells from nude mice (11.7%).

FIGURE 5. Bone marrow RAG-1 mRNA expression is induced in nude mice by supernatant from activated CD8<sup>+</sup> cells. Supernatants from activated purified T cells, CD8<sup>+</sup> T cells, and CD4<sup>+</sup> T cells prepared as described were injected into nude mice. After 5 days, bone marrow cells were isolated and assayed by RT-PCR for the expression of RAG-1 and GAPDH mRNA, as above.

FIGURE 6. RAG-1 gene expression is impaired in CD8-deficient mice. Bone marrow RNA was prepared from two C57BL/6, two CD8<sup>−/−</sup>-deficient (TAP<sup>−/−</sup>), and two CD4<sup>−/−</sup> T cell-deficient (MHC class II<sup>−/−</sup>) mice and subjected to quantitative RT-PCR assay. The amount of competing standard plasmid is plotted vs the ratio of band intensities of the standard vs that of the 18-bp larger RAG-1 cDNA product. Where the ratio is 1, the amount of cDNA equals the known amount of competing plasmid standard. Thus, C57BL/6 and CD4<sup>−/−</sup> T cell-deficient mice have equal amounts of RAG-1 mRNA, while CD8<sup>−/−</sup> deficient mice have −10% of the control levels.
It has not been determined whether the decrease in pre-B cells totally explains the decreased expression of the RAG genes in thymic-deprived mice. A low number of pre-B cells in thymic-deprived might explain the lower level of RAG mRNA in bone marrow of thymic-deprived mice. In this view ATCS or rIL-16 acts as a growth or differentiation factor to increase the steady state number of pre-B cells in thymic-deprived mice. It is known that IL-16 acts on cells that express CD4 (23). In preliminary studies, we have confirmed previous results that demonstrated the presence of CD4 on early bone marrow B cell precursors (24). This raises the possibility that IL-16 may act directly on B cell precursors to favor their differentiation. Compatible with this view is our recent preliminary observation that the level of IL-16 produced by PHA-activated T cells from old mice was 10% or less of the level seen in young mice, as determined by bioattractant activity and ELISA (W. Cruikshank et al., unpublished studies).

Alternatively, the decreased expression of the RAG genes by bone marrow pro-B cells in thymic-deprived mice might impair the rearrangement of Ig heavy chain gene segments and thereby inhibit the transition of pro-B to pre-B cells or their survival. The generation of pre-B cells requires the expression of both RAG genes and the rearrangement of the Ig heavy chain gene segments. Mice deficient in one of the two RAG gene products do not rearrange their Ig genes and do not generate pre-B cells in the bone marrow. However, the introduction of a rearranged Ig transgene into RAG knockout mice was associated with the appearance of pre-B cells (2). Thus, it is also possible that the primary defect of B cell development in thymic-deprived mice is the impaired expression of RAG genes and the consequent impairment in Ig gene rearrangement. This process might, in turn, impair the generation or survival of bone marrow pre-B cells. According to this view, injection of ATCS or rIL-16 into thymic-deprived mice increases their expression of RAG genes, favors their rearrangement of Ig gene segments, and results in their having more bone marrow pre-B cells. Studies are under way to distinguish these alternatives.

The recognition that thymic-derived factors might increase lymphocyte generation may have clinical applications. Inadequate lymphocyte reconstitution is an important source of morbidity and mortality in patients undergoing intensive chemotherapy or bone marrow transplantation. Recently, it has been reported that age is an important determinant of the rate of lymphocyte regeneration following intensive chemotherapy in patients with neoplastic disease (25). In these studies, the rate of regeneration of peripheral CD4+ T cells was inversely proportional to the age of the patient but directly related to the size of the thymus gland determined by radiographic imaging. Furthermore, with increasing age there was an increase in the fraction of regenerating T lymphocytes that were derived from thymic-independent pathways including peripheral renewal. It is possible that the administration of T cell factors, including IL-16, may increase the rate of T as well as B lymphocyte regeneration in older, thymic-deprived patients and thereby speed their recovery following chemotherapy or bone marrow transplantation.

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FIGURE 7. IL-16 increases the level of RAG-1 mRNA in bone marrow B cell precursors of nude and old mice. A. Nude mice were injected i.v. with a single dose (100 ng) of human rIL-16. Five days after injection, RNA was isolated from total bone marrow cells, and the level of RAG-1 and GAPDH mRNA was determined by RT-PCR. The labeled product of this reaction was separated on a 2.5% agarose gel and visualized by radiography. B. Old mice were injected with three doses of 100 ng of human rIL-16, every other day. The RT-PCR reactions using bone marrow RNA were run on 2.5% agarose gels and visualized by ethidium bromide staining.
References


