Age-Associated Increase in the Expression of T-Cell Antigen Receptor γ-Chain Gene in Conventional and Germfree Mice

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To determine whether environmental antigens such as intestinal microflora contribute to expansion of the T-cell repertoire, age-related changes in the expression level of T-cell antigen receptor genes encoding γ, β, and α chains were compared in the lymphoid tissues of conventional versus germfree mice. Irrespective of the conditions of maintenance, an age-associated increase in the expression of the γ-chain gene was evident in the thymus and spleen. Both conventional and germfree old mice (age, 40 weeks) had a relatively high proportion of Thy1+L3T4+Lyt2− cells but a reduced level of Thy1−L3T4−Lyt2+ cells in the thymus compared with their counterparts (age, 8 weeks). The thymic dysfunction but not the stimulation by intestinal microflora may contribute to this age-associated increase in γ-gene transcripts in these tissues. On the other hand, an age-associated increase in the expression of γ RNA was not evident in the mesenteric lymph nodes of germfree mice, although a remarkable increase in the γ-chain gene messages was detected in the lymph nodes of the aged conventional mice. These results suggest that the expression of γ RNA in cells of gut-associated lymphoid tissue is partly influenced by intestinal microflora.

The T-cell antigen receptor (TcR), by which T cells recognize antigens and major histocompatibility complex products, is composed of a heterodimer of an α and β chain (2, 17, 28). Analysis of the genes encoding the α and β chains revealed that these genes are formed by a somatic recombination of variable, joining, and constant gene segments (11, 12, 19, 21, 32, 38–40). Another type of TcR described as a γ-δ heterodimer has been identified (4, 8–10, 29). The γ-chain gene was also assembled from gene segments homologous to those made up of variable, joining, and constant regions of the TcR genes (20). The gene encoding the δ chain has most recently been located just 5′ to the joining,-constant,-coding region (3, 5, 6, 13, 18, 25).

T-cell precursors arise from pluripotent hematopoietic cells in the fetal liver or adult bone marrow and migrate to the thymus. These precursors proliferate and differentiate in the thymus, giving rise to functional T cells that can recognize antigens and self-major histocompatibility complex gene products. Immune-competent T cells generated in the thymus eventually emigrate to peripheral lymphoid organs such as the spleen and lymph nodes (34, 37). The thymus not only generates the full range of TcRs but also selects T cells with self-major histocompatibility complex specificities by inducing TcR gene rearrangements (1). Although the peripheral T-cell repertoire is generated primarily in the thymus, environmental antigens, including microbial flora, are responsible for the expansion of the T-cell repertoire.

In this study we investigated age-related changes in the expression of γ-, β-, and α-chain genes in conventional and germfree mice, with the objective being to determine whether microflora contribute to the expansion of the T-cell repertoire. An age-associated increase in γ-gene transcripts was evident in the thymus and spleen, irrespective of the maintenance of the mice, while germfree mice showed no age-associated increase in the messages in the gut-associated lymphoid tissue. The implication of these findings for the role of intestinal microflora in aged mice is discussed.

MATERIALS AND METHODS

Mice. BALB/c mice were raised and maintained under conventional or germfree conditions in the animal facility of Yakult Central Institute. Conventional mice were maintained in a clean, air-conditioned animal room and provided with laboratory mouse chow and water ad libitum. Germfree mice were maintained under germfree conditions in flexible plastic isolators. Cages, bedding, water, and chow for germfree mice were sterilized in an autoclave. These mice were taken out of the plastic isolator just before use for an experiment. Age-matched conventional and germfree mice were used at 8 and 40 weeks after birth. Congenitally athymic nude (nu/nu) mice and their littermate (nu/+ ) controls were obtained from Shizuuko Laboratory Animal Center (Shizuoka, Japan) and bred under conditions of protection from pathogens. The females were used for experiments at either 8 or 20 weeks of age.

Fluorescence-activated cell sorter analysis. Thymus, spleen, and mesenteric lymph nodes (MLNs) were obtained from conventional and germfree mice and teased into Hanks solution. A single-cell suspension was incubated with fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAB) and phycoerythrin-conjugated MAB. FITC-conjugated anti-Thy1.2 MAB, FITC-conjugated anti-Lyt2 MAB, FITC-conjugated anti-immunoglobulin M (IgM), and phycoerythrin-conjugated anti-L3T4 MAB were purchased from Becton Dickinson Labware (Oxnard, Calif.; Div. Becton Dickinson and Co.). Samples were analyzed on a fluorescence-activated cell sorter (440; Becton Dickinson) by flow cytometry. All data were displayed on a log scale of increasing green and red fluorescence intensities. To obtain percentages of the T-cell subpopulations, total counts were integrated into selected areas of the contour plots.

Northern blot analysis. RNA was extracted from thymocytes, spleen cells, nylon wool-passed spleen cells, and...
lymph node cells by the guanidine thiocyanate and CsCl gradient centrifugation procedure (14). A total of 10 μg each of total RNA was denatured by reacting the RNA with glyoxal in dimethyl sulfoxide and electrophoresed through a 1% agarose gel in 10 mM sodium phosphate buffer (pH 7.0).

The RNA was transferred to Gene Screen Plus (New England Nuclear Corp., Boston, Mass.). Ethidium bromide staining of the gel before and after transfer confirmed that nearly equal amounts of RNA were blotted onto the screen. Hybridization was made to the 32P-labeled, nick-translated γ (constant γ), β (constant β), and α-chain constant probes (24, 41). Following hybridization in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–10% dextran sulfate–0.1% sodium dodecyl sulfate at 65°C, the filters were washed 3 times in 2× SSC–0.1% sodium dodecyl sulfate at 65°C and once in 0.2× SSC–0.1% sodium dodecyl sulfate at 65°C. Rehybridization in sequence to γ, β, and α probes was carried out after the probe was removed by washing the filter with boiling water. Approximate sizes of the hybridizing RNA was estimated by using mouse rRNA (1.9 and 4.8 kilobases [kb]) as molecular size markers.

**RESULTS**

T-cell subpopulation analysis in thymuses, spleens, and MLNs from conventional and germfree mice. Two-color flow cytometric analysis for expression of Thy1 and L3T4-Lyt2 and for L3T4 and Lyt2 on the thymocytes of conventional young mice (age, 8 weeks) revealed the following four subpopulations: (i) Thy1+ L3T4− Lyt2− cells, which may represent the earliest lineage in the adult thymus; (ii) Thy1+ L3T4− Lyt2+ cells, which were found predominantly in the cortex; (iii) Thy1+ L3T4+ Lyt2− cells; and (iv) Thy1+ L3T4+ Lyt2+ cells, which were immunocompetent, mature thymocytes (Fig. 1). The selection of contour areas that maximized separation of the four subpopulations gave a subpopulation distribution of 2.7, 83.2, 4.3, and 9.7% for the four subpopulations listed above, respectively. Irrespective of maintenance conditions, the proportion of Thy1+ L3T4− Lyt2− cells was increased from less than 3% to 10% in the thymuses of aged mice, but the percentage of Thy1+ L3T4− Lyt2− mature cells was reduced from 4% to less than 2% in the thymuses (Fig. 1 and Table 1).

The distribution of the T-cell subpopulations was also investigated in the spleens and MLNs of conventional and germfree mice by using two-color flow cytometric analysis (Table 2). Age-related increases in the number of spleen and MLN cells was observed both in conventional and germfree mice, and these increases were mainly due to increases in the number of surface IgM-positive cells (data not shown). Although the proportion of T lymphocytes in these tissues of old mice was decreased, the ratio of L3T4+ Lyt2− cells and L3T4+ Lyt2+ cells was much the same as that in their young counterparts. There was no age-related increase in the number of Thy1+ L3T4− Lyt2− cells in these peripheral lymphoid tissues.

Expression of TCR genes in thymuses, spleens, and MLNs from conventional and germfree mice. Age-related changes in the expression of RNAs encoding TCR γ, β, and α chains was assayed in lymphoid cells of conventional and germfree mice. Conventional mice had an age-related increase in TCR γ-chain gene expression in the thymuses, spleens, and MLNs, whereas an age-related change in the expression of the α-gene transcript was not observed in these lymphoid tissues of mice. On the other hand, an age-related increase in TCR γ-chain gene expression was observed in the thymuses and spleens of germfree mice, although the increase was not evident in the MLNs of germfree mice (Fig. 2A). A two-to threefold enrichment in T cells was obtained by passing spleen cells over a nylon wool column (23). An age-related
These results suggest that the TcR γ-chain gene expression occurs with aging, in the absence of the thymus.

**DISCUSSION**

For the purpose of establishing a role for the microbial intestinal flora in the development of the TcR repertoire, which is expressed by spontaneously occurring T cells in unprimed mice, we investigated age-related changes in the expression level of the TcR genes encoding γ, β, and α chains using conventional and germfree mice. We found that age-related increases in the level of expression of γ RNA were observed in the thymuses, spleens, and MLNs of conventional mice. Interestingly, this age-related increase in γ-chain gene messages was also detected in the lymphoid tissues of germfree mice, although increases were not conspicuous in the MLNs of these same mice. These results suggest that while the expression of γ-chain gene messages is not controlled by the presence of bacterial flora, the microbial flora are, to some extent, responsible for the age-associated increase in the γ-chain gene messages in gut-associated lymph nodes.

During T-cell development of the fetal thymus, the variable γ gene is rearranged and transcribed first; and around this time or soon after, rearrangement and transcription of the joined diversity γ joining γ gene segment occurs, followed by rearrangement and transcription of the variable γ gene segments, and then finally by rearrangement and transcription of the variable δ gene segments (7, 30, 31, 35). Similar progression of γ-, β-, and α-gene expression is observed in adult thymocytes (27). The L3T4+ Lyt2− or double-negative subpopulation that may represent the earliest lineage in the adult thymus has a large amount of γ-gene transcripts and very little α RNA (33). Our results reveal that the thymus in aged mice, irrespective of the maintenance condition of the mice, contains a relatively high proportion of double-negative T cells, as compared with those of their young counterparts. It is possible that the decline in the

**TABLE 2.** Proportion of T-cell subpopulations in lymphoid organs of young (age, 8 weeks) or old (age, 40 weeks) mice bred under conventional or germfree conditions

| Organ | Mice (age [wk]) | Cell no. (10⁶/organ) | Proportion (%) of the following T-cell subpopulations:
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L3T4− Lyt2−</td>
</tr>
<tr>
<td>Spleen</td>
<td>Conventional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.6 ± 0.3</td>
<td>56.5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>40</td>
<td>3.9 ± 0.6</td>
<td>70.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Germfree</td>
<td>2.2 ± 0.2</td>
<td>54.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>3.6 ± 0.5</td>
<td>61.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>MLN</td>
<td>Conventional</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.9 ± 0.2</td>
<td>21.6</td>
<td>&lt;1</td>
</tr>
<tr>
<td>40</td>
<td>2.1 ± 0.4</td>
<td>42.9</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Germfree</td>
<td>0.9 ± 0.1</td>
<td>31.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>1.8 ± 0.3</td>
<td>38.0</td>
<td>&lt;1</td>
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*Values are means ± standard errors.

The percentage of each subpopulation was determined from a selected area of the contour plots (data not shown).
thymic function concerning maturation of T-cell precursors causes an increase in the number of double-negative cells, resulting in high levels of expression of γ-gene transcripts in the thymuses of aged mice. Although rearrangement and expression of the TcR genes are thought to occur primarily or exclusively in the thymus, some γ-gene rearrangements have been reported to occur along extrathymic pathways (16). We also found a remarkable age-related increase in the γ-gene transcripts in nude mice. Therefore, the extrathymic rearrangement and expression of TcR γ-chain genes may be responsible for the age-related increase in γ-chain gene transcripts in peripheral lymphoid tissues such as the spleen and MLN. The decline in thymic function during the elimination of the T cells which express the TcR γ protein along the extrathymic pathway may also be responsible for the increase in γ-gene transcripts in aged mice.

Differences in the locations of MLNs and spleens were also observed, as they related to the microflora-related dichotomy in TcR gene expression. Our results reveal that the difference in the amount of γ-gene expression between conventional and germfree mice is more conspicuous in MLNs than spleens. Hooijkaas et al. (22) found a similar dichotomy in background immunoglobulin synthesis between the MLN and spleen. They noted that the background immunoglobulin synthesis in the MLN, especially IgG and IgA synthesis, mainly depends on exogenous antigenic stimulation, whereas the background immunoglobulin synthesis in the spleen is mainly due to endogenous stimulation. Ernst et al. (15) have reported that although the cell-mediated immune responses of aged mice are significantly reduced compared with those of their young counterparts, the responses of aged Peyer's patch cells retained greater vigor than did the aged spleen cells. The higher level of antigenic stimulation and exposure to immunopotentiating substances produced by the microbial flora of the gut may help to explain why the age-related increase in gene transcripts is remarkable in the MLNs of conventional mice. Environmental antigens may also affect the T-cell repertoire composed of α and β chains. No difference in the expression of the TcR gene messages was detected between aged conventional mice and aged germfree mice.

Results of this study on the age-related decline of the immune system indicated that the T-cell-dependent functions are altered significantly with age, whereas the B-cell functions remain relatively intact. The T-cell functions that decline with age include helper-T-cell activity for antibody production, generation of cytotoxic T cells, and delayed-type hypersensitivity reactions (26). The exact role of cells that express the TcR γ-δ heterodimer is unknown. Recently, Bank et al. (4) have reported a natural killer cell-like cytotoxic activity in an anti-T3-stimulated CD4+ CD8+ thymocyte clone that expressed a T3 γ-δ complex but not the α-β heterodimer. It is possible that the increase in the number of T cells bearing the γ chain on their surface may contribute to the increase in the cytotoxic activity of lymphoid cells in aged mice. These cells may participate in host immune surveillance against malignancy or infection in aged mice. The gut-associated lymphoid tissue may well present the first line of defense against various antigens that are encountered in the environment. A remarkable increase in
the number of the γ-bearing T cells would protect the aged mice and substitute for the systemic immune responses that declined considerably.

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LITERATURE CITED


