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業績報告書シリーズ

マウスリンパ系組織におけるリコンビネイション活性化遺伝子（RAG-1）の局限的発現§

Restricted Expression of Recombination Activating Gene (RAG-1) in Mouse Lymphoid Tissues

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要　約

マウス胸腺，脾臓およびリンパ節におけるリコンビネース活性の分布を決定するために，in situ ハイブリダイゼイション法を用いて，リコンビネイション活性化遺伝子，RAG-1およびRAG-2の発現を調べた。大部分の皮質胸腺細胞にRAG-1の発現が見られたが，髄質内の胸腺細胞には，その発現は見られなかった。RAG-2のハイブリダイゼイション・シグナルはRAG-1ほど強くなかったが，RAG-2 transcriptはRAG-1と同様の局在を示した。脾臓においては，洞（sinus）付近の限られた細胞にのみRAG-1の発現が見られたが，濾胞内のほとんどの細胞はRAG-1 transcript陰性であった。ヌードマウスにおいても，RAG-1を発現している細胞は正常マウスと同じ領域に見いだされた。このことは，脾臓において見られるRAG-1のin situ ハイブリダイゼイション・シグナルはB細胞起源の細胞によるものであることを示唆している。リンパ節におけるRAG-1の発現は髄質部位にのみ見られた。脾臓とリンパ節におけるRAG-2 transcriptの発現は，たとえあるとしても，あまりにも弱く特異的局在を決定できなかった。これらの結果は，ほとんどの皮質胸腺細胞と脾臓のある種の細胞は，それぞれ，T細胞リセプター遺伝子および免疫グロブリン遺伝子を再構成し得ることを示唆している。しかし，脾臓とリンパ節におけるRAG-1陽性細胞のRAG-1 transcriptが，遺伝子再構成以外の機能にかかわっている可能性は排除できない。

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Restricted Expression of Recombination Activating Gene (RAG-1) in Mouse Lymphoid Tissues[§]

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Summary

In an attempt to determine the distribution of recombinase activity in the mouse thymus, spleen, and lymph nodes, we used the in situ hybridization method to examine the expression of the recombination activating genes RAG-1 and RAG-2. Expression of RAG-1 was found in most cortical thymocytes but not in the majority of medullary thymocytes. Although hybridization signals of RAG-2 were not as intense as those of RAG-1, the localization of RAG-2 transcripts was similar to that of RAG-1. In the spleen, expression of RAG-1 was found only in limited cells near the splenic sinus, and the majority of the cells within the follicle were negative for RAG-1 transcript. In nude mice, RAG-1–expressing cells were detected in the same regions, which suggests that in situ hybridization signals of RAG-1 in the spleen are due to the cells of B-cell origin. In the lymph nodes, expression of RAG-1 was found only in the medullar region. Expression of RAG-2 transcript in the spleen and the lymph nodes, if any, was too faint to allow determination of the specific localization. These results suggest that most of the cortical thymocytes and some cells in the spleen are capable of rearranging T-cell receptor genes and immunoglobulin genes, respectively, but the possible involvement of the RAG-1 transcript in RAG-1–positive cells of the spleen and the lymph nodes in functions other than the rearrangement of genes could not be ruled out.

Introduction

The primary repertoire of mammalian T-cell receptor and immunoglobulin genes is generated by a series of genomic rearrangements that occur during

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lymphoid development.[1,2] The recombination takes place among variable (V), diversity (D), and joining (J) gene segments. Recently, the recombination activating genes RAG-1 and RAG-2, which can induce V(D)J-specific recombination in fibroblast cells with cotransfection, were isolated.[3,4,5] Coexpression of RAG-1 and RAG-2 was observed in immature lymphoid cell lines, bone marrow, and thymus and to a small extent in the spleen using Northern blot hybridization methods,[4,5,6] suggesting that together RAG-1 and RAG-2 encode the lymphoid-specific component of the V(D)J recombination machinery.

Lymphoid cells within the thymus consist of three broad subpopulations, and the majority of cortical cells are composed of $CD4^+CD8^+$ immature thymocytes.[7,8] Within the thymus, high levels of expression of RAG-1 and RAG-2 were detectable in the cortex but not in the medullary region by in situ hybridization.[9,10] Using the polymerase chain reaction (PCR) method, RAG-1 mRNA was also detected in peripheral lymph nodes (LNs) but was undetectable in B-cell–depleted LNs, suggesting that B cells express RAG-1 mRNA in LNs.[11] A further study using extrachromosomal plasmids indicated that V(D)J recombinase activity is found not only in the thymus but also in the spleen and LNs.[12] For the identification of the specific location of recombinase-positive cells within their active tissues such as the spleen and LNs, we examined the expression of RAG-1 and RAG-2 using the in situ hybridization method.

Materials and Methods

Tissues

All tissues were isolated from BALB/c mice and BALB/c nude mice at age 4–5 weeks, immediately embedded in Tissue Tek OCT compound (Miles, Elkhart, Indiana, USA), and rapidly frozen on dry ice. In the cortisone-treated group, the mice were sacrificed about 1 d after intramuscular administration of 5 mg/body of cortisone-acetate, and the tissues were isolated and embedded in OCT compound.

Riboprobe synthesis

The RAG-1 cDNA (R1A, nucleotides 1322–1837; R1B, nucleotides 1838–2250), RAG-2 cDNA (R2, nucleotides 162–1013), and T-cell receptor β gene (0.7 kb; *Eco*RI fragment of β-chain constant region) were transcribed from T_3 or T_7 RNA polymerase promoter of Bluescript II KS^- (Stratagene, La Jolla, Calif, USA) to yield sense or antisense probes. The transcription reactions in 25 μL contained 1 μg of linearized template DNA; 40 mM Tris-HCl (pH 8.0); 8 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl; 30 mM dithiotheritol; 10 U of RNase inhibitor (TAKARA, Tokyo); 1 mM each of ATP, CTP, and GTP; 0.65 mM UTP; 0.4 mM digoxigenin-labeled UTP (Boehringer Mannheim, Mannheim, Germany); and 20 U of T_3 or T_7 RNA polymerase (Stratagene). The reaction was allowed to continue for 2 h at 37°C, resulting in full-length transcripts. After the template DNA was digested with DNase I (Boehringer Mannheim), the unincorporated nucleotides were removed by ethanol precipitation with 2.5 M ammonium acetate. The synthesized RNA was measured under UV light by ethidium bromide staining and compared with a standard series of known RNA concentrations. The labeling of probes with digoxigenin was checked by direct filter-spot tests. The

probes were degraded by alkaline hydrolysis to an average length of 150 nucleotides.[13] The specificity and asymmetry of transcription from these recombinant templates were checked by RNA blot analysis.

In situ hybridization

Frozen tissue sections 8 μm thick were made and mounted on glass slides treated with 50 μg/mL poly-L-lysine (Sigma, St Louis, Mo). After air-drying for about 1 h at room temperature, sections were fixed for 15 min in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) at room temperature. Sections were washed three times in PBS and acetylated[14] in 0.25% (w/v) acetic anhydride for 10 min at room temperature. The slides were washed twice in PBS and once in 2 × SSC (1 × SSC; 0.15 M sodium chloride, 0.015 M sodium citrate), followed by preincubation in 50% formamide and 2 × SSC for 20 min at 50°C. Hybridization was performed at 53–60°C for 12–16 h in a solution containing 50% formamide, 2 × SSC, 5% blocking powder (Boehringer Mannheim), 10% dextran sulfate, 0.02% sodium dodecyl sulfate, 0.1% Sarkosyl, 10 mM Tris-HCl (pH 7.5), 500 μg/mL each of salmon sperm DNA and yeast tRNA, 100 μg/mL polyadenylate, and 2.5 μg/mL of digoxigenin-labeled riboprobes. For the competitive inhibition assay, the slides were incubated at 50°C for 12 h in the presence of 125 μg/mL of nonlabeled antisense RNA in hybridization solution with labeled riboprobe. After the hybridization was complete, slides were washed six times in 2 × SSC at room temperature for 10 min each time; treated with 20 μg/mL of RNase A in 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM ethylenediaminetetraacetate (EDTA) (RNase buffer) at 37°C for 30 min; and washed in RNase buffer at room temperature for 30 min. Sections were washed for 30 min each in 2 × SSC at room temperature, 1 × SSC at room temperature, and 0.1 × SSC at 62°C. Slides were immersed in 0.5% blocking powder in 0.1 M Tris-HCl (pH 7.5) and 0.15 M NaCl (buffer 1) at room temperature for 1 h and incubated with 150 mU/mL polyclonal sheep anti-digoxigenin Fab-fragments conjugated to alkaline phosphatase at room temperature for 1 h. Sections were washed twice in buffer 1 at room temperature for 10 min each time and then twice in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, and 50 mM $MgCl_2$ (buffer 2) at room temperature for 5 min each time. The substrate solution contained 45 μL of 75 mg/mL nitroblue tetrazolin and 35 μL of 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate in 10 mL of buffer 2. For color development, slides were incubated in the dark at room temperature with substrate solution for 12–32 h. After development, the slides were immersed in PBS, counterstained with or without 0.5% methyl green, and mounted with Perma Fluor (Nippon Tanner, Tokyo).

Northern blot analysis

Total cellular RNA was extracted using acid phenol[15] with some modification. RNA samples (15 μg) were separated on 1% agarose-formaldehyde gels and transferred to Gene Screen Plus membranes (Du Pont, Boston). Northern blot hybridization with riboprobe was performed in 50% formamide, 1 M NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/mL of denatured salmon sperm DNA at 42°C for 12–16 h. Filter washes were carried out on the whole as described for in situ hybridization. The RNA probes for Northern blot hybridization were

labeled with [α-^{32}P]CTP (800 Ci/mmol; Amersham, Little Chalfont, Buckinghamshire, UK).

Results

Expression of RAG-1 and RAG-2 in the thymus

We used the locus shown in Figure 1 as a probe for RAG-1 and RAG-2. We used the following four techniques to confirm that the in situ hybridization signal is the result of specific hybridization with the target RNA: (1) use of the sense riboprobe; (2) use of the antisense riboprobe on various regions of the same gene; (3) competitive inhibition assay; and (4) Northern blot analysis. In situ hybridization to the T-cell receptor gene, expressed in T cells within the thymus, was included as an internal control. Hybridization signals with antisense riboprobe to the constant region of the T-cell receptor β chain were observed throughout the thymus (Figure 2i). In situ hybridization of RAG-1 (R1B, Figure 1) to the thymus showed that the expression was confined to the thymic cortex and was not found in the medulla (Figure 2a). No significant difference in the intensity of hybridization signals was found among cortical thymocytes (Figure 2g). Expression of RAG-1 seen in most cortical thymocytes was inhibited near the cortico-medullary junction. Experiments using two independent riboprobes (R1A and R1B, Figure 1) to the various regions of RAG-1 cDNA revealed a coincidence of hybridization patterns (Figures 2a, 2b). No signals of hybridization were detected anywhere in the thymus with any of the sense riboprobes (Figures 2d, 2e). We used cortisone to determine whether the absence of hybridization signals in the medulla is due to the consumption of riboprobes by the large amount of RAG-1 mRNA expressed in the cortical thymocytes. As a result of cortisone-treatment for 1 d, a large portion of cortical cells disappeared but most medullary cells remained intact. Even in the cortisone-treated thymus, expression of RAG-1 was found only in the remaining part of the cortex and not in the medulla (Figures 3a, 3b). Furthermore, in the competitive inhibition assay, a significant reduction of hybridization with labeled antisense R1B riboprobe was observed when nonlabeled antisense R1B RNA was used as an inhibitor (Figure 2k). When antisense R1A RNA was used as an inhibitor there was no reduction of hybridization (Figure 2j). More-

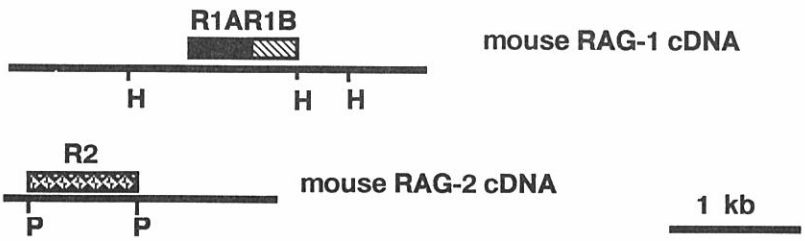

Figure 1. Schematic diagram of RAG-1 and RAG-2 probes. The approximate positions of RAG-1 and RAG-2 riboprobe are indicated as boxes, which are part of coding regions. Restriction enzyme sites are indicated: H, *Hind*III; P, *Pst*I.

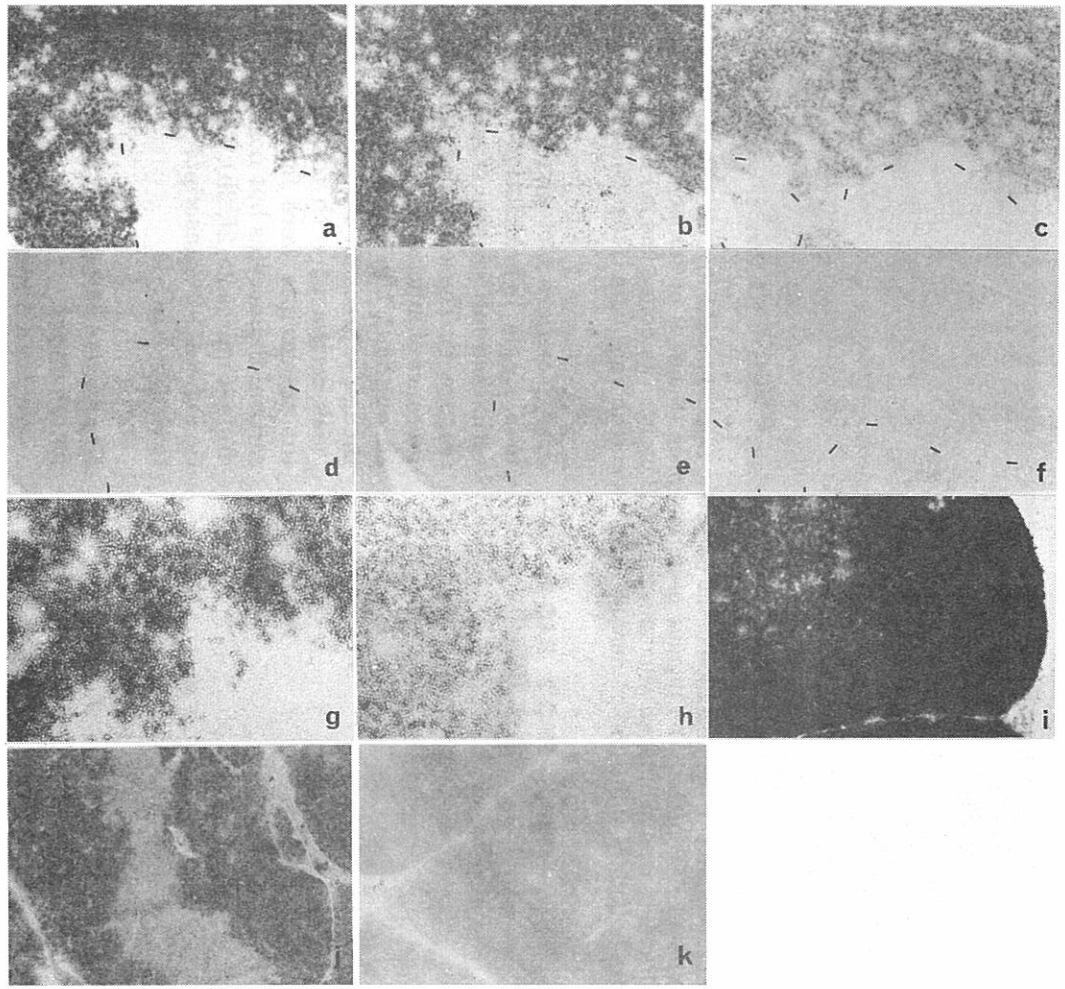

Figure 2. In situ hybridization of the thymus with digoxigenin-labeled RAG-1 and RAG-2 riboprobes: (a) antisense R1B probe; (d) sense R1B probe; (b) antisense R1A probe; (e) sense R1A probe; (c) antisense R2 probe; (f) sense R2 probe; (g) high-magnification view of the cortex in (a); (h) high-magnification view of the cortex in (c). Digoxigenine-labeled antisense T-cell receptor β was hybridized to the thymus (i). Competitive inhibition assay was carried out with antisense R1B probe and nonlabeled antisense R1A RNA (j) or nonlabeled antisense R1B RNA (k) as an inhibitor. Dotted line in (a)–(f) indicates the boundary of the cortico-medulla. Only sections in (j) and (k) were counterstained with methyl green.

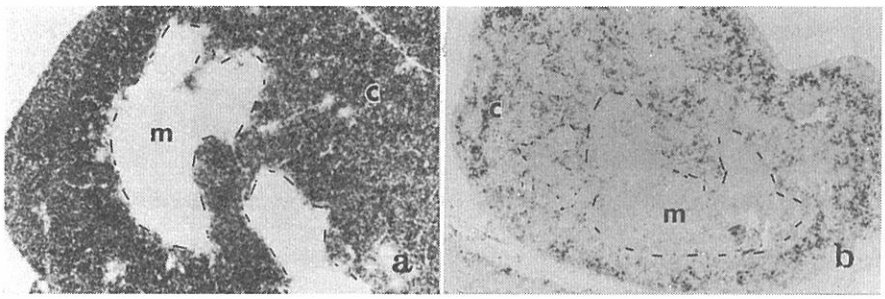

Figure 3. In situ hybridization of the cortisone-treated mouse thymus with digoxigenine-labeled R1B probe. The thymus of normal mice (Panel a) or cortisone-treated mouse (Panel b) was hybridized with antisense R1B probe. The dotted line in Panel b indicates the boundary of the cortico-medulla: c is the cortical region, m is the medullary region.

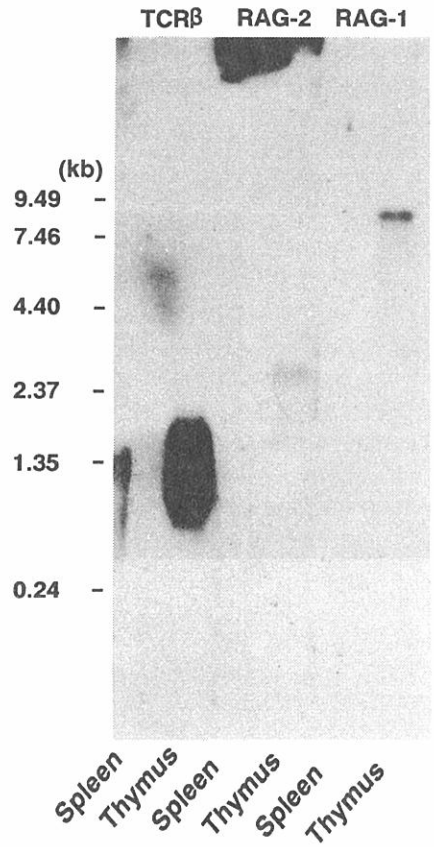

Figure 4. Northern blot analysis of thymic RNA with ^{32}P-labeled antisense RAG-1 (R1B), RAG-2 (R2), and T-cell receptor β-chain (TCRβ) probes.

over, Northern blot analysis resulted in a single hybridization band with estimated mRNA size of about 6.8 kb (Figure 4). These results indicate the presence of functional mRNA of RAG-1 throughout the thymic cortex. Although expression of RAG-2 was very weak compared with that of RAG-1, the transcripts were found in many cortical thymocytes but not in the medulla (Figures 2c, 2f, 2h). The localization of RAG-2 transcripts was similar to that of RAG-1.

Expression of RAG-1 in the spleen and lymph nodes

We then investigated the expression of RAG-1 in the spleen and LNs as a condition of in situ hybridization established in the thymus. The expression of RAG-1 was extremely weak in the spleen. Although weak, however, the expression of RAG-1 was observed in a few cells near the splenic sinus (Figures 5a, 5d). This pattern of expression was reproducible. In the nude mouse, this kind of signal was similarly observed near the splenic sinus (Figures 5b, 5e), suggesting that the cells expressing RAG-1 in the spleen are of B-cell origin.

The expression of RAG-1 in the LNs is as weak as that in the spleen; that

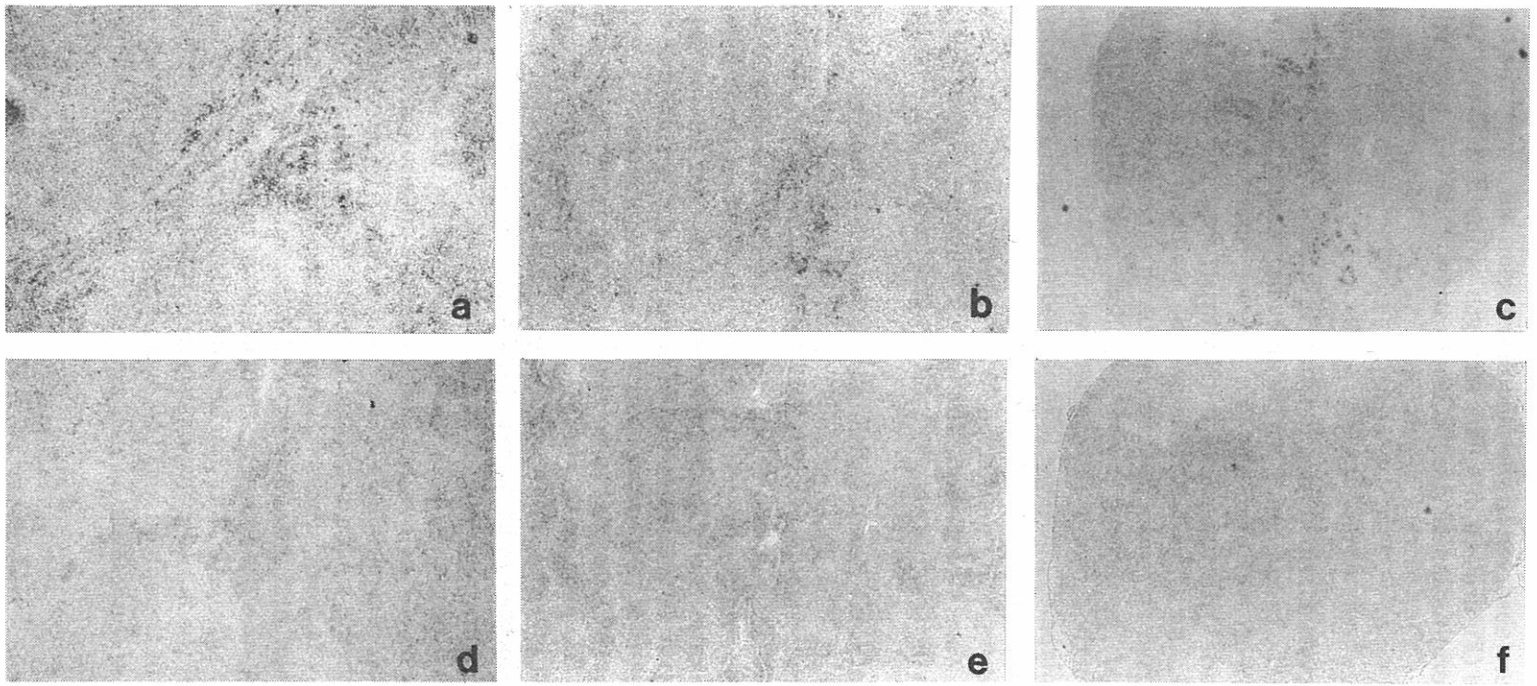

Figure 5. In situ hybridization of the spleen and lymph node with digoxigenine-labeled RAG-1 riboprobe. The spleen was hybridized with antisense R1B probe (a) or sense R1B probe (d). The spleen of the nude mouse was hybridized with antisense R1B probe (b) or sense R1B probe (e). The lymph nodes were hybridized with antisense R1B probe (c) or sense R1B probe (f).

is, faint signals were observed in the medullar region but not in the cortical or subcortical regions (Figures 5c, 5f). The strength of the RAG-2 signal was too weak to allow determination of specific localization of RAG-2–positive cells in these organs.

Discussion

To show that signals obtained from in situ hybridization result from a specific hybridization with the target genes, we began by implementing the four criteria described earlier. With Northern blot analysis, we confirmed that only bands derived from hybrids with functional mRNA exist; that is, that there are no additional bands. Using sense mRNA as a probe, we observed no hybridization signals. When we used antisense RNA as a probe on various regions of the same gene, we found that the various probes hybridize with cells within the same regions. Our final method, that is, the competitive inhibition assay, disclosed that when the same nonlabeled antisense RNA is used as an inhibitor the hybridization signal is significantly reduced but that when different antisense RNAs are used the signal is completely unaffected. With regard to RAG-1 in the thymus, these four criteria were all satisfied. This shows that the in situ hybridization signals of RAG-1 in the thymus were a result of hybridization of authentic RAG-1 mRNA.

The expression of RAG-1 and RAG-2 in the thymus is observed in the cortex but not in the medulla. This observation is consistent with the report[10] that RAG expression was found in immature $CD4^+CD8^+$ cells but not in the mouse mature $CD4^+CD8^-$ or $CD4^-CD8^+$ subpopulations. In the spleen, expression of RAG-1 in cells was weak, making it difficult to clarify its location. It is interesting, however, that the expression of RAG-1 observed in cells near the splenic sinus of normal mice was also detectable in the same region of the spleen of the nude mouse. The number of RAG-1–expressing cells might increase in the spleen of nude mice as compared with that in normal mice. The report by Guy-Grand et al[11] suggests that the expression of RAG-1 in the LNs, a type of peripheral lymphoid tissue, is of B-cell origin. The expression of RAG-1 in the spleen, therefore, is also expected to be observed in B cells. Indeed, the results of our experiments with nude mice suggested that the RAG-1–positive cells in the spleen were of B-cell origin. In both the spleen and LNs, the cells expressing RAG-1 are likely to be of B-cell origin. This raises the question of whether these cells actually display recombinase activity. In the report by Abe et al,[12] it was suggested that both the spleen and LNs have V(D)J recombinase activity, although this activity is weak. These findings indicate that the cells expressing RAG-1 in the spleen and LNs display V(D)J recombinase activity and are involved or could be involved in the rearrangement of immunoglobulin genes. It was not possible to clarify the location of RAG-2–expressing cells in the spleen and LNs because of the faint intensity. It is important now to identify the cells expressing RAG-2 in the spleen and LNs by improving the sensitivity of our in situ hybridization technique or by raising specific monoclonal antibodies for these gene products.

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