Acta Medica et Biologica
Vol. 45, No. 3, 107-114, 1997

Detection of Intermediate TcR Cells, NK3.2.3-positive T Cells, and CD5$^+$B Cells in Older Rats

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Received June 19 1997; accepted August 19 1997

Summary. **We investigated the presence of autoreactive T and B cells, extrathymic T cells (intermediate TcR cells), natural killer (NK) marker-positive T cells, and CD5$^+$B cells in the liver of older rats. Results showed that lymphocyte function-associated antigen-1 (LFA-1)$^{+++}$CD3$^+$ cells, which have a phenotype similar to that of intermediate TcR cells of mice, increased in the liver, spleen, and peripheral blood of older rats (55 weeks and 64 weeks of age) compared with young, normal rats. Conversely, LFA-1^{++}CD3$^+$ cells decreased in older rats. The LFA-1^{+++}CD3$^+$ cells displayed an intermediate staining intensity for CD3 compared with that of LFA-1^{++}CD3$^+$ cells and they seemed similar to extrathymic T cells in mice. However, the LFA-1^{+++} CD3$^+$ cells were almost all CD8$^+$, in contrast to those of mice. Double negative (DN) CD4$^-$CD8$^-$T cells comprised less than 1% of the T-cell population in rats. NK3.2.3^{++} T cells also increased in the liver, but not in the peripheral blood of older rats; conversely, NK cells decreased in older rats. CD5$^+$ B cells increased in the liver but not in the spleen and peripheral blood of older rats. Furthermore, class II positive T cells and interleukin-2 receptor (IL-2R) positive T cells increased in the liver of older rats. These results suggest that T cells with a phenotype similar to the extrathymic T cells of mice are present in the rat liver.**

Key words—rat, liver, ageing, extrathymic T cell, NK-marker positive T cell, CD5-positive B cell.

INTRODUCTION

T-cell dysfunction and thymic atrophy, including decreased T-cell response to mitogens or antigens and altered cytokine expression and phenotype, are major immunologic abnormalities associated with ageing.[1-3] Recently, it has been demonstrated that extrathymic T cells and CD5$^+$B cells or NK-marker positive T cells increase in autoimmune diseases and aging.[4-6] Moreover, it has been demonstrated that the activation of extrathymic T cells induces thymic involution. Abo et al. have demonstrated that extrathymic T cells (intermediate TcR cells) in the mouse liver have a CD44$^+$ L-selectin lymphocyte function-associated antigen-1 (LFA-1)$^{++}$ICAM-1$^+$ phenotype.[7] Almost all these studies, however, have been performed in mice or humans. Therefore, we examined whether similar phenomena are present in the liver of older rats. We demonstrated the presence of LFA-1^{+++}CD3$^+$ cells in rat liver, which are phenotypically similar to extrathymic T cells in mice. Moreover, NK3.2.3^{++}T cells and CD5$^+$B cells were found to increase in the liver of older rats.

MATERIALS AND METHODS

Experimental animals

Lewis rats, aged 9 (n=6), 55 (n=3), and 64 (n=3) weeks, were obtained from the Centre d'Elevage R. Janvier (Le Genest-St-Isle, France). They were fed under specific pathogen-free conditions. Animals were cared for observing the standard procedures described in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (N.I.H 80-23).

Cell preparation

Under ether anesthesia, the infrahepatic vena cava

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was severed and 5 mL of saline was gently injected into the portal vein to eliminate the blood contained in the liver. The liver was then removed, chopped into small pieces, and digested with collagenase (Type 1, C5138, Sigma) for 30 min in RPMI supplemented with 2% fetal calf serum (FCS) at 37℃ as previously described for other organs. The digest was then squeezed through a wire mesh and filtered. The cell suspension was washed three times in RPMI with 2% FCS and purified on a single-step density gradient (Ficoll-Hypaque, d=1.083, Sigma). All procedures were performed in siliconized tubes at 4℃ to reduce the loss of macrophages by adherence as well as their potential activation by uptake of cell debris or other substances. This cell population comprised greater than 95% viable leukocytes, as estimated by FDA staining and flow cytometry analysis. Contamination by hepatocytes was less than $1/10^4$.

Flow cytometry

The phenotype of the leukocytes was assessed by flow cytometry as previously described. In brief, freshly isolated single-cell suspensions were incubated at 4℃ for 60 min with a panel of murine antibodies, in the form of tissue culture supernatants, specific for rat leukocyte-common antigen (MRC OX1 and OX30), kappa chain (MRC OX12), polymorphic class II antigens (MRC OX6), CD5 (MRC OX19), CD3 (G4.18), CD4 (W3/25 and MRC OX35), CD8 (MRC OX8), NKR-P1 (3.2.3(NK3.2.3)), Pta.A2 antigen, which is present on the majority of peripheral T cells (P4/16), and CD11a (WT.1), as well as human factor I (MRC OX21, negative control). After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (FO257, Sigma Chemical Co., Poole, England) and analysed with an EPICS PROFILE flow cytometer (Coultronics, Hialeah, FL, USA). In addition, two-color analysis was performed in a separate series of rats on a Coulter Profile by first incubating cells with an unlabeled antibody and a phycoerythrine (PE)-conjugated GAM(HA 16, Murex Diagnostics) and further incubating the cells with an anti-CD3- OR CD5-FITC labeled antibody (G4.18 and OX19 respectively, Pharmingen, San Diego, CA, USA). Triple-staining experiments were performed with two fluorochromes (FITC- conjugated OX19 Versus W3/25-OX35 and OX8 indirectly labeled with PE- GAM) to assess the percentage of $OX19^+CD^4$-CD^8-cells, as previously described.

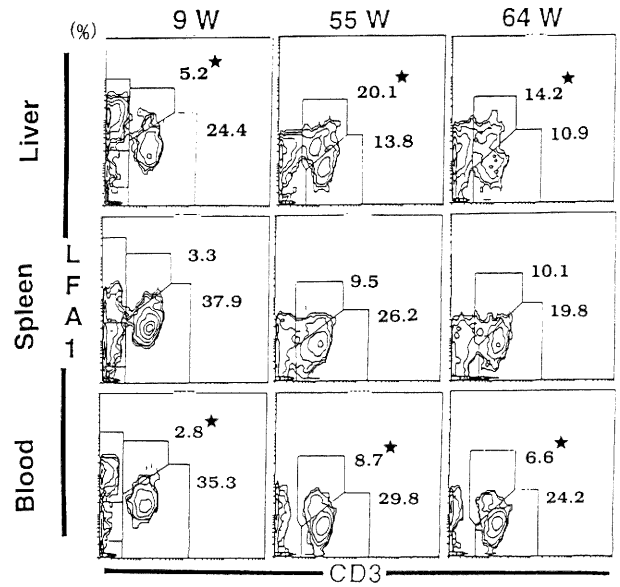

Fig. 1. Changes in extrathymic T cells in older rats. Extrathymic T cells ($LFA\text{-}1^{+++}CD3^+$ cells) increased markedly with age in each organ. Moreover extrathymic T cells displayed a staining of intermediate intensity for CD3, similar to mice. Conversely, thymic T cells ($LFA\text{-}1^{++}$ $CD3^+$ cells) decreased with age. Data are the mean of measurements performed on 3-6 animals. (Liver and Blood, ★; 9w vs 55w and 64w, $p<0.05$).

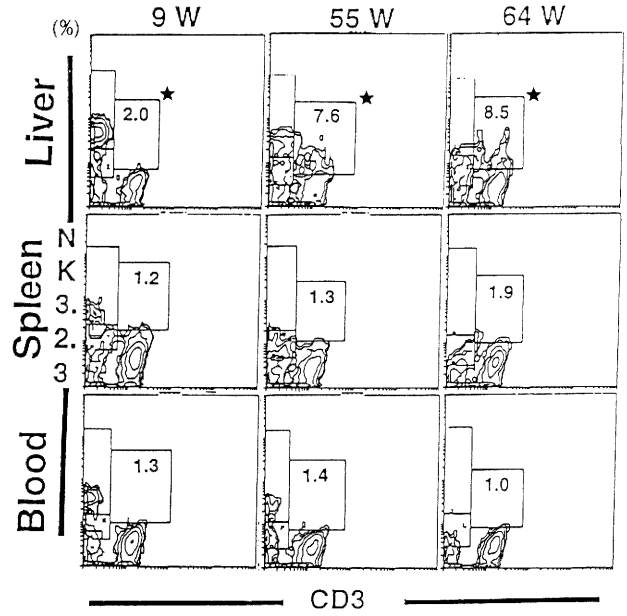

Fig. 2. Changes in $NK3.2.3^{++}$T cells in older rats. $NK3.2.3^{++}$T cells increased markedly with age in the liver, but not in the spleen or blood. Data are the mean of measurements performed on 3-6 animals. (Liver, ★; 9w vs 55w and 64w, $p<0.05$).

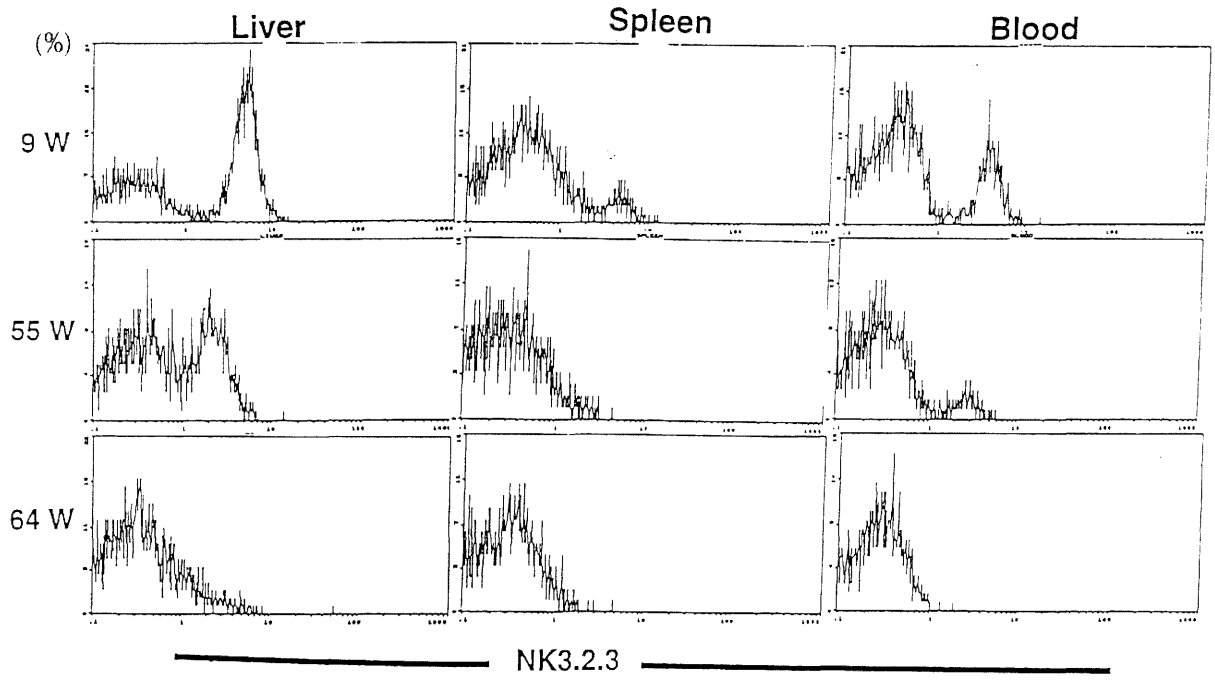

Fig. 3. Changes in NK cells in each organ analyzed by single-color staining with a mAb directed against NK3.2.3 in older rats. In contrast to the NK3.2.3^{++}T cells, NK cells decreased with age.

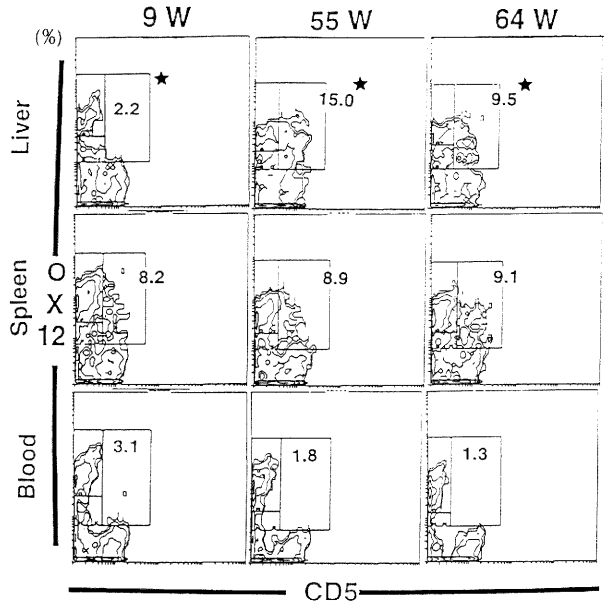

Fig. 4. Changes in CD5^{+}B cells in older rats. CD5^{+}B cells were preferentially found in the spleen in younger rats. CD5^{+}B cells markedly increased in the liver with age, but not in the spleen or blood. Data are the mean of measurements performed on 3-6 animals. (Liver, ★; 9w vs 55w and 64w, $p<0.05$).

Statistical analysis

Student's *t*-test was used to analyze the data.

RESULTS

1. Changes with age in organ weight and mononuclear cell number

The liver weight/body weight ratio (LW/BW×100) increased in parallel with the number of hepatic mononuclear cells (MNC) in older rats as follows: 9 w; $2.88/3\times10^{6}$, 55 w; $3.1/7\times10^{6}$, 64 w; $4.1/12\times10^{6}$. Conversely, thymus weight and thymocyte number decreased with age, as follows: 9w; 0.57 g/1.5×10^{9}, 55 w; 0.48 g/8×10^{7}, 64 w; 0.33 g/5×10^{7}. Spleen weight and splenocyte number increased slightly with age: 0.53 g/6×10^{7}, 0.63 g/7.5×10^{7}, 0.72 g/8.4×10^{7}. Data are expressed as a mean of measures performed on 3-6 animals.

2. Changes in LFA-1^{+++}CD3^{+} cells in older rats

In this study, LFA-1^{+++}CD3^{+} cells increased in the liver (5% to 20%), spleen (3.3% to 10%), and peripheral blood (2.8% to 8.7%) of older rats. In contrast,

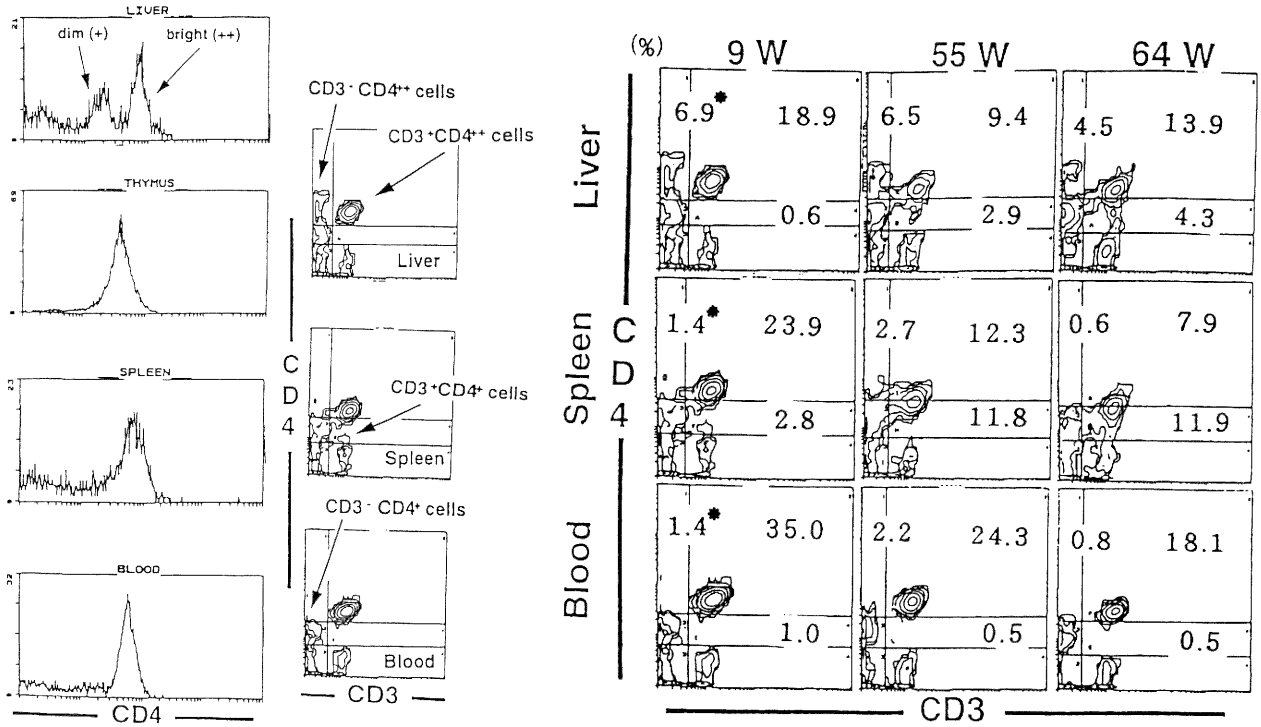

Fig. 5. Analysis of MNC by mAbs against CD4 and CD3 in older rats. MNC were divided into two subpopulations by single-color staining with a mAb directed against CD4. Almost all of the $CD4^+$T cells expressed CD4 strongly, while macrophages expressed CD4 weakly. Interestingly, $CD4^{high}CD3^-$ cells were preferentially found in the liver in younger rats. $CD4^{low}$T cells were preferentially found in the spleen in younger rats. $CD4^{low}$T cells increased in the liver in older rats. $CD4^{high}CD3^-$ cells did not change in the liver in older rats. $CD4^+$T cells markedly decreased in the thymus in 64-week-old rats. Data are the mean of measurements performed on 3–6 animals. (●; 9w$CD3^-CD4^{++}$cells, Liver vs Spleen and Blood, $p<0.05$).

$LFA\text{-}1^{++}CD3^+$ cells decreased from 24% to 13.8% in the liver of older rats. Moreover, $LFA\text{-}1^{+++}CD3^+$ cells displayed an intermediate staining intensity for CD3 similar to intermediate TcR cells of mice (Fig. 1).

3. Changes in $NK3.2.3^{++}$ T cells in older rats

We have reported that NK-marker positive T cells displayed a staining intensity for NKR-P1 molecules between normal T cells and NK cells in hepatectomized rats.[8] In this study, $NK3.2.3^{++}$T cells increased from 2% to 8% in the liver of older rat, but were not changed in the spleen and peripheral blood (Fig. 2). In contrast to $NK3.2.3^{++}$T cells, NK cells decreased with age (Fig. 3).

4. Changes in $CD5^+$B cells in older rats

$CD5^+$B cells were preferentially located in the spleen in younger rats. $CD5^+$B cells increased from 2% to 15% in the liver of older rats, but were not changed in the spleen and blood (Fig. 4).

5. Analysis of CD4 and CD8 expression in MNC

Single staining for CD4 divided MNC into two subpopulations, $CD4^{high}$ and $CD4^{low}$. Almost all the CD4-positive T cells expressed CD4 strongly while macrophages expressed CD4 weakly. Interestingly, $CD4^{high}$ $CD3^-$ cells were primarily found in the normal young liver, while $CD4^{low}CD3^+$ cells were found in the normal young spleen. $CD4^{high}CD3^-$ cells did not change in the liver of older rats, while $CD4^{low}CD3^+$ cells increased in the spleen and liver of older rats (Fig. 5).

$CD8^+$T cells clearly displayed an intermediate intensity staining for CD3 compared to $CD4^+$T cells. $CD8^+CD3^-$ cells, which correspond to NK cells, decreased with age in all organs as did $NK3.2.3^{+++}CD3^-$ cells (Fig. 6).

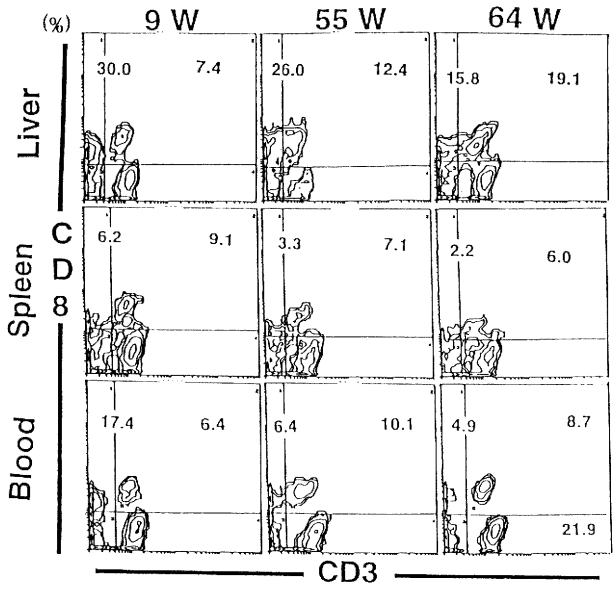

Fig. 6. Analysis of MNC by staining with a mAb directed against CD8 in older rats. $CD8^+$T cells clearly displayed a staining of intermediate intensity for CD 3 compared to $CD4^+$T cells. $CD8^+CD3^-$ cells (NK cells) decreased with age in rats. Data are the mean of measurements performed of 3-6 animals.

6. Single-color analysis of thymocytes in older rats

In 64-week-old rats, $DPCD4^+CD8^+$ cells decreased markedly in combination with simultaneous thymic atrophy. Conversely, $DNCD4^-CD8^-$ and single positive CD4 and CD8 cells increased. Almost all thymocytes showed highly intense staining for OX1-30; hepatic MNC fell into two subpopulations, OX1-30^{high} and OX1-30^{low}, in the younger rats. In contrast, almost all thymocytes were OX1-30^{low} in 64-week-old rats. Moreover, almost all thymocytes showed low staining for LFA-1 in older rats; LFA-1 negative thymocytes were few in number but were clearly increased in 64-week-old rats (Fig. 7).

DISCUSSION

Along with thymus-derived T cells, extrathymically differentiated T cells have recently been demonstrated to localize mainly in the liver[9] and intestine.[10] Their extrathymic origin is supported by the observation that they are present in congenitally athymic nude mice and express the mRNAs for recombination activating gene-1 (RAG-1) and RAG-2 in the liver

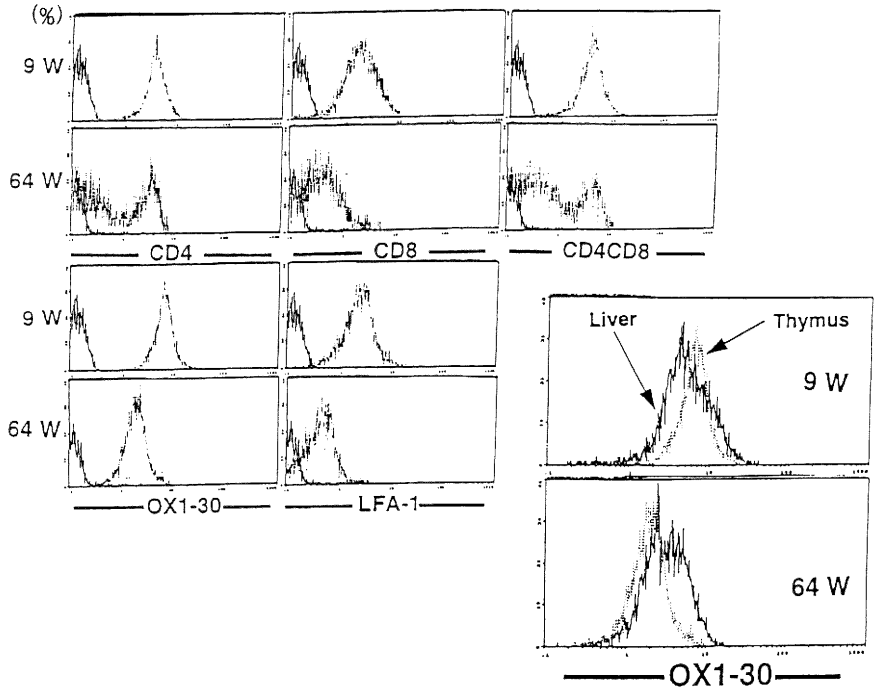

Fig. 7. Single-color analysis of thymocytes in older rats. DPCD4CD8 cells decreased in older rats, while DNCD4CD8 cells and single CD4 or CD8 cells increased in the thymus in older rats. Almost all of the thymocytes stained intensely for OX1-30 in younger rats. However, two subpopulations, OX1-30^{high} and OX1-30^{low}, were observed in the liver. Interestingly, almost all thymocytes were OX1-30^{low} in older rats.

and intestine.[11,12] Ohteki and MacDonald[13] reported that the liver is a major source of NK1.1$^+$TCR-α/β^+ cells, which have a phenotype clearly corresponding to "intermediate" T cells (extrathymic T cells) in the mouse liver. Besides extrathymic T cells, NK-marker positive T cells and CD5$^+$B cells were reported to be activated in autoimmune diseases and ageing.[14,15] Therefore, we investigated whether similar phenomena were present in the liver of older rats. In this study, LFA-1^{+++}CD3$^+$ cells, NK3.2.3^{++}T cells, and CD5$^+$B cells were all activated in the liver of older rats. LFA-1^{+++}CD3$^+$ cells of rats clearly displayed an intermediate staining intensity for CD3, similar to those of mice. Although many of the extrathymic T cells in mice are DNCD4$^-$CD8$^-$ cells, the prevalence of DNCD4$^-$CD8$^-$ cells among LFA-1^{+++}CD3$^+$ cells was less than 1%. Many of the LFA-1^{+++}CD3$^+$ cells were CD8$^+$T cells. Compared with normal young mice and rats, LFA-1^{+++}CD3$^+$ cells in rats were about half as prevalent as intermediate TcR cells of mice.[16] In mice, intermediate CD3$^+$ cells were identified as cells with higher levels of IL-2β and LFA-1; however, IL-2R^{high}CD3$^+$ cells did not display the intermediate intensity of CD3. Thus there were some differences between the rat and mouse. Although it would be difficult to say that LFA-1^{+++} CD3$^+$ cells are definitely extrathymic T cells in rats, they have a phenotype similar to extrathymic cells, and the changes between LFA-1^{+++}CD3$^+$ cells and LFA-1^{++} CD3$^+$ cells seem to correspond to those seen in extrathymic T cells and thymic T cells in mice. Alan et al.[16a] reported that the expression of LFA-1 was demonstrated on various cell types: CTL and PHA-activated T cells, PBL, thymocytes, B lymphoblastoid cells. And that LFA-1 MAb significantly blocked cytolysis, not only by HLA-DR-specific CTL, but also by HLA-A, B-specific CTL and cell lines displaying NK activity. NK cells have a CD8 surface marker; CD8$^+$T cells displayed an intermediate staining for CD3 in the rats. NK3.2.3^{++}T cells amounted to almost half the CD8$^+$ intermediate CD3$^+$ cells and almost one third the LFA-1^{+++}CD3$^+$T cells. NK3.2.3^{++}T cells displayed a staining intensity for NKR-P1 between NK cells and normal T cells.[8] Abo et al.[17] postulated that extrathymic T cells may be intermediate between T cells and NK cells. Our results suggest that extrathymic T cells are intermediate between T cells and NK cells phenotypically. Although NK cells markedly decreased in the liver, spleen, and blood of older rats, LFA-1^{+++}CD3$^+$ cells and NK3.2.3^{++}T cells increased in the liver of older rats. There are two possible origins for the extrathymic T cells and NK3.2.3^{++}T cells. Extrathymic T cells may proliferate in response to foreign antigens in the liver, which is a major site of extrathymic T-cell generation. It has been shown that all extrathymic T cells in the liver, intestine, and other organs are CD44$^+$L-selectin$^-$LFA-1^{++}ICAM-1$^+$[7]. These cells are presumably generated as primitive T cells early in phylogeny, but thereafter develop independently according to microenvironmental changes at different sites occurring with time.[18] Alternatively, common progenitors of NK cells and T cells might differentiate into extrathymic T cells to protect the host against suppression of the immunosurveillance system by a NK cell crisis.

Hayakawa et al.[19] proposed that CD16$^-$CD56^{bright} cells are an immature common progenitor of NK cells; the expression of RAG-1 and 2 mRNAs by these cells is a marker for their immaturity. These cells possibly differentiate into CD3$^+$ extrathymic T cells in the decidua under the influence of trophoblastic cells such as thymic nurse cells or intestinal mucosal epithelial cells. Philips et al.[20] reported that common progenitors of NK cells and T cells express CD3 δ and CD3 ε molecules in the cytoplasm even if they lack surface CD3. These findings support the second possibility for extrathymic T-cell generation discussed above.

Interestingly, cells with phenotypes similar to extrathymic T cells increased in older animals and in autoimmune diseases.

CD5$^+$B cells participate in autoimmune diseases and lymphoid malignancies in fashion similar to extrathymic T cells.[6,21] Sekigawa et al.[22] reported that the production of IgM and IgG anti-DNA antibodies is controlled by different T-cell subsets. The pathogenic production of anti-DNA autoantibodies in both spontaneous and experimentally induced systemic lupus erythematosus is Th cell dependent; these Th cells are predominantly CD4$^+$.[23] Since the pathogenic Th clones found in lupus preferentially help the select population of B cells that produce the pathogenic anti-DNA autoantibodies, it is likely that those autoimmune B cells in turn present autoantigens with cationic residues to these Th cells.[24] In this study, MHC class II antigen-positive T cells increased from 2.2% to 7.7% and IL-2R-positive T cells increased from 1.8% to 4.5%, but only in the liver. Therefore we postulate that extrathymic T cells or NK3.2.3^{++} T cells may play an important role in the development of CD5$^+$ B cells.

Extrathymic T cells may be involved in thymic involution or T-cell apoptosis. It has been demonstrated that NK1.1$^+$T thymocytes directly kill CD4$^+$ CD8$^+$thymocytes expressing Fas antigens.[25] The Fas antigen, a cell-surface protein that mediates apoptosis, is expressed in various tissues including

the thymus. Fukunaga et al.[26] reported that the Fas antigen has an important role in the negative selection of autoreactive T cells in the thymus. In this study, DNCD4CD8 cells or single positive CD4 or CD8 (CD5 negative) cells were increased in the thymus in 64-week-old rats with simultaneous thymic involution. Moreover, almost all of the thymocytes in older rats stained both LFA-1 and OX1-30 at low intensity. These cells may be associated with thymic atrophy. Both $CD5^+$B cells and $NK3.2.3^{++}$T cells also increased in association with thymic involution along with a marked decrease in $CD5^+$T cells (data not shown). These results raise the possibility that $CD5^+$B cells and $NK3.2.3^{++}$T cells may function together to induce thymic atrophy or the suppression of thymic T cells in the liver and thymus via the MHC class II pathway.

In conclusion, $LFA\text{-}1^{+++}CD3^+$ cells, which have a phenotype similar to the intermediate TcR cells of mice, are present in the liver of rats as well as mice. However, at present, it is difficult to say definitively that these cells are extrathymic T cells in the rat. In older rats, $LFA\text{-}1^{+++}CD3^+$ cells, $CD5^+$B cells, and $NK3.2.3^{++}$ T cells may be activated, especially in the liver, as seen in autoimmune diseases.

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