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# The Presence of $CD4^{bright}CD3^-$ Cells in the Liver after Partial Hepatectomy in Rats

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***Summary.*** **Background/Aims: We investigated intrahepatic leukocytes in the regenerating rat liver following 70% partial hepatectomy, using anti-CD4 and CD8 antibodies on a FACScan.**

**Materials and Methods: Rat leukocytes were divided into two groups by CD4 single staining, $CD4^{bright}$ and $CD4^{dull}$. $CD4^{bright}$ cells were mainly $CD3^+$T lymphocytes, while $CD4^{dim}$ cells were mainly macrophages in the blood or normal spleen as previously reported.[1,2]**

**Results: We found $CD4^{bright}CD3^-$ cells specifically in the liver in normal rats by two-color flow cytometry. This cell type was not present in the blood or spleen in normal rats. However, their numbers decreased in the liver and increased in the spleen following 70% partial hepatectomy. $CD8^+$T cells displayed a lower expression of CD3 molecules than did $CD4^+$T cells.**

**Conclusions: $CD4^{bright}CD3^-$ cells were demonstrated mainly in the liver. Their numbers were affected by the 70% partial hepatectomy. They therefore may play a role in liver regeneration after partial hepatectomy.**

**Key words**—rat, partial hepatectomy, liver regeneration, CD4, macrophage, intrahepatic leukocyte.

## INTRODUCTION

Recent studies have reported on the activation of extrathymic T cells in ageing and autoimmune diseases of mice.[1,2] We have also demonstrated the activation of extrathymic T cells in regenerating liver after partial hepatectomy in mice.[3] However, these data are not necessarily applicable to rats. For instance, double negative $CD4^-CD8^-$ cells were hardly detected in the liver of rats (<1%) as well as mice, and intermediate CD3 cells were not so clear as those of mice.[4] Moreover, the rat macrophages have CD4 molecules, and natural killer (NK) cells of rats have CD8 molecules, but neither holds true for mice.[5,6] The CD4 or CD8 molecules play an important role in the recognition of antigens by major histocompatibility complex (MHC) class I and class II molecules. We have investigated the relationship between MHC class II or class I antigen expression and liver regeneration in rats by immunohistochemical staining focused on Kupffer cells.[7-10] Nevertheless, there are few reports on intrahepatic leukocytes in rats. Hence, the functions of CD4 and CD8 molecules on intrahepatic leukocytes in rats are not clear. Therefore, in this study we investigated intrahepatic leukocytes in rats, using the basic cell surface molecular markers CD4 and CD8.

## MATERIALS AND METHODS

Nine-week-old Lewis rats were obtained from the Centre d'Elevage R. Janvier (Le Genest-St-Isle, France). They were cared for under specifically pathogen-free conditions. An approximately 70% partial hepatectomy was performed under ether anesthesia according to Higgins and Anderson.[11]

Animals were cared for according to the standard procedures indicated 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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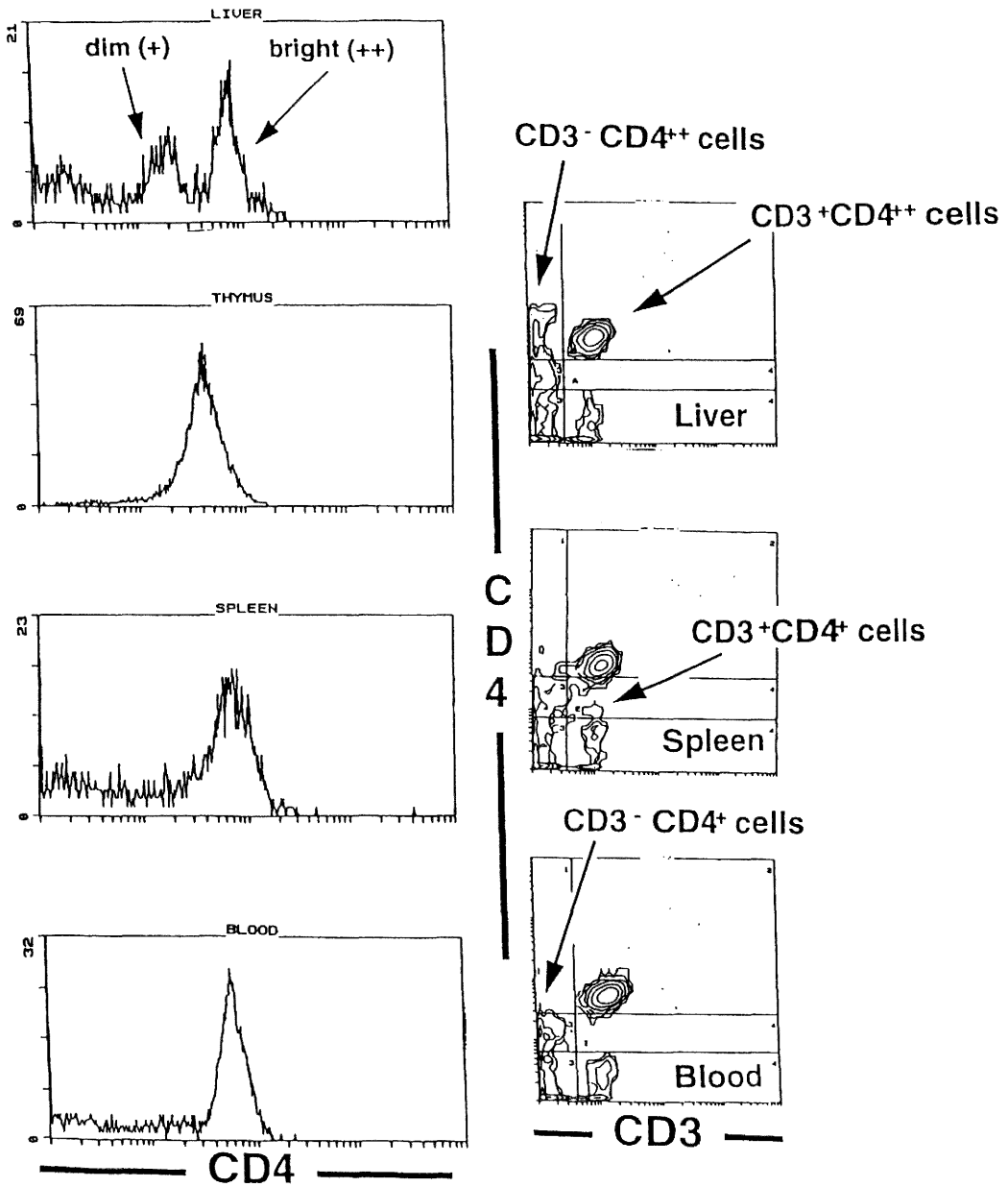


**Fig. 1.** Classification of rats' MNC by anti-rat anti-CD4 and CD3 antibody in normal young rats. MNC were divided into two subpopulations, $CD4^{bright}$ and $CD4^{dim}$, using single staining by CD4. Using double staining by CD4 and CD3, they were divided into four subpopulations: $CD4^{bright}CD3^-$, $CD4^{dim}CD3^-$, $CD4^{bright}CD3^+$, and $CD4^{dim}CD3^+$ cells. $CD4^{bright}CD3^-$ cells were present almost exclusively in the liver and $CD4^{dim}CD3^+$ cells almost exclusively in the spleen.

was severed and 5ml of saline was gently injected into the portal vein to eliminate blood contained in the liver. The liver was thereafter removed, chopped into small pieces and digested with collagenas (Type 1, C5138, Sigma) for 30 min in RPMI 2% FCS at 37℃ as previously described for other organs. The digest was further squeezed through a wire mesh and filtered. The cell suspension was washed three times in RPMI 2% FCS and purified on a single step density gradient (Ficoll-hypaque, d=1.083, Sigma). All procedures were performed in siliconized tubes and at 4℃ to reduce any loss by the adherence of macrophages as well as their potential activation by the uptake of cell debris or other, substances. This cell population

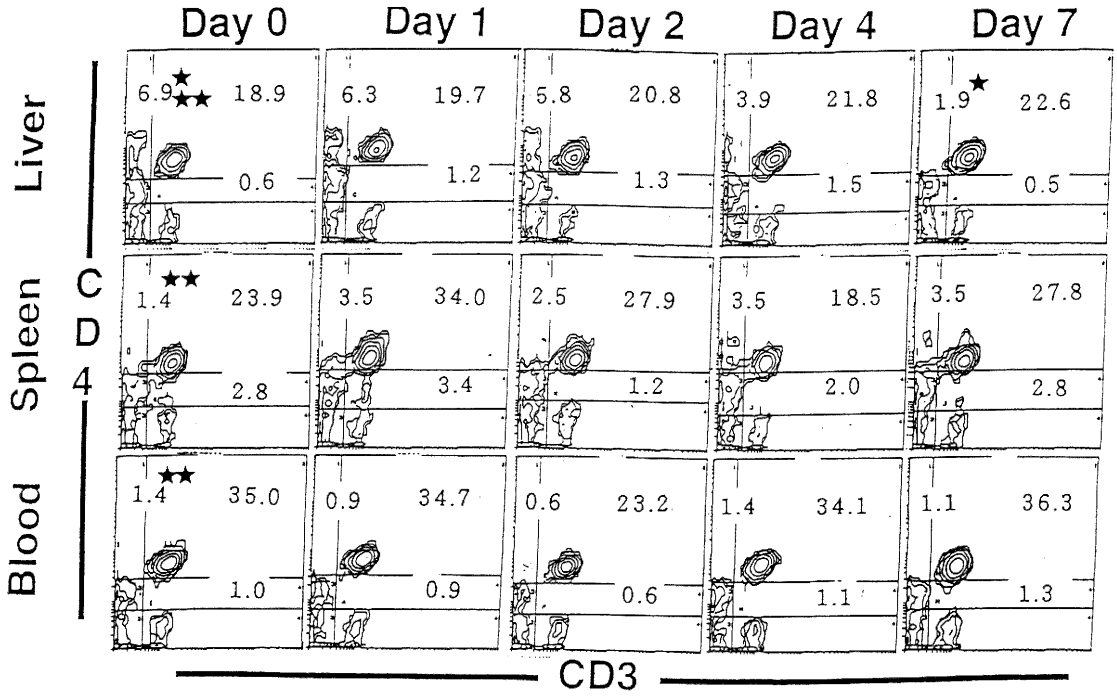


**Fig. 2.** Changes in $CD4^+$ cells after partial hepatectomy in rats. $CD4^{bright}CD3^-$ cells decreased in the liver at a statistically significance and increased in the spleen. They had no changes in the blood after partial hepatectomy. $CD4^{dull}CD3^+$ cells increased in the spleen on day 1; however there was no significance. The data are the mean of measurements performed on 3-5 animals. ★, Liver $CD4^{bright}CD3^-$ cells Day0 vs Day7 $p<0.05$, ★★, Liver $CD4^{bright}CD3^-$ cells Day0 vs Spleen or Blood $CD4^{bright}CD3^-$ cells Day0 $p<0.05$.

was composed of greater than 95% viable cell leukocytes as estimated by FDA staining and flow analysis, respectively. Contamination by hepatocytes was less than $1/10^4$.

### Flow cytometry

The phenotype of the leukocytes was assessed by flow cytometry as previously described.[12] In brief, freshly isolated single cell suspensions were incubated at 4℃ for 60 min with a panel of murine antibodies, in the form of a tissue culture supernatant specific for rat leukocyte-common antigen (MRC OX1 and OX30), kapper chain (MRC OX12), polymorphic class II antigens (MRC OX6), the CD5 (MRC OX19), CD3 (G4.18), CD4 (W3/25 and MRC OX35), CD8 (MRC OX8) and Pta. A2 (RT6) antigen which is present on the majority of peripheral T cells (P4/16) as well as human factor I (MRC OX21, negative control). After washing, cells were incubated with FITC-conjugated goat anti-mouse Ig (FO257, Sigma Chemical Co., Poole, England) and analysed by 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 the unlabeled antibody and a 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). 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 $OX19+CD4^-CD8^-$ cells as previously described.

### Statistical analysis

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

## RESULTS

### Changes in $CD4^+$ or $CD8^+$ T cells after partial hepatectomy

Using single staining by CD4, mononuclear cells (MNC) were divided into two subpopulations, $CD4^{bright}$ and $CD4^{dim}$. It has been reported that $CD4^{bright}$cells correspond to T cells, and that $CD4^{dim}$

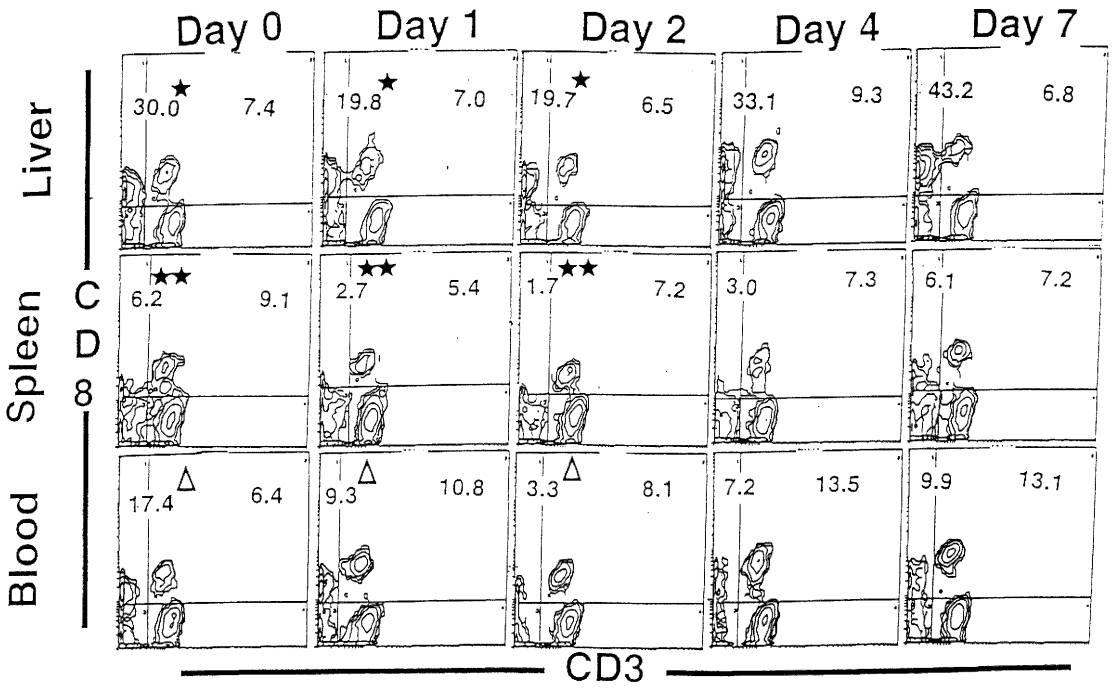


**Fig. 3.** Changes in $CD8^+$ cells after partial hepatectomy in rat. $CD8^+$ T cells displayed less intensity for CD3 compared with $CD8^-$ T cells ($CD4^+$ T cells) in the liver, Spleen and blood. $CD8^+CD3^-$ cells (NK cells) showed a statistically significant decrease in the liver, spleen and blood after partial hepatectomy. The data are mean of measures performed on 3-5 animals. ★, Liver $CD8^+CD3^-$ cells Day0 vs Day1 or Day2, $p<0.01$. ★★, Spleen $CD8^+CD3^-$ cells Day0 vs Day1 or Day2, $p<0.05$. △, Blood $CD8^+CD3^-$ cells Day0 vs Day1 or Day2, $p<0.01$.

cells correspond to macrophages.[1,2] Using double staining by CD4 and CD3, we divided MNC into four subpopulations: $CD4^{bright}CD3^-$ cells, $CD4^{dull}CD3^-$, $CD4^{bright}CD3^+$, and $CD4^{dim}CD3^+$ cells (Fig. 1). Interestingly, in normal young rats, $CD4^{bright}CD3^-$ cells were present almost exclusively in the liver, and $CD4^{dim}$ $CD3^+$ cells almost exclusively in the spleen. After partial hepatectomy, $CD4^{bright}CD3^-$ cells decreased in the liver and increased in the spleen. $CD4^{dim}CD3^+$ cells increased in the liver and spleen following partial hepatectomy (Fig. 2). $CD8^+$ T cells displayed an intermediate intensity for CD3 compared with $CD4^+$ T cells. $CD8^+CD3^-$ cells have been reported to be natural killer (NK) cells.[2] NK cells displayed a low staining intensity for CD8 when compared with $CD8^+$ T cells. $CD8^+CD3^-$ cells decreased in the early regenerating phase after partial hepatectomy in the liver, spleen, and blood, as did $NK3.2.3^{+++}$ $CD^{3-}$ cells (Fig. 3).

### Changes in MHC class II positive or IL-2R positive T cells after partial hepatectomy

MHC class II positive T cells were preferentially located in the spleen in normal young rats. These cells increased in the liver in the early regenerative phase; however, there was no significance, and they conversely decreased in the spleen at a statistically significant rate (Fig. 4).

IL-2R positive T cells were also preferentially located in the spleen in normal rats. After hepatectomy, these cells showed statistically significant increases in the liver in the early regenerative phase (Day 2), and increased in the blood in the late regenerative phase (Days 4 through 7) (Fig. 5). Interestingly, these cells decreased at a statistically significant rate in the spleen after partial hepatectomy.

### Changes in $RT6^+$ T cells after partial hepatectomy

$RT6^+$ T cells were preferentially present in the spleen in normal young rats. In contrast with extrathymic T cells or $NK3.2.3^{++}$ T cells, $RT6^+$ T cells decreased in the liver and thymus in the early regenerative phase, and subsequently increased. In contrast to the situation in the liver, $RT6^+$ T cells increased in the blood in the early phase after partial hepatectomy (Fig. 6).

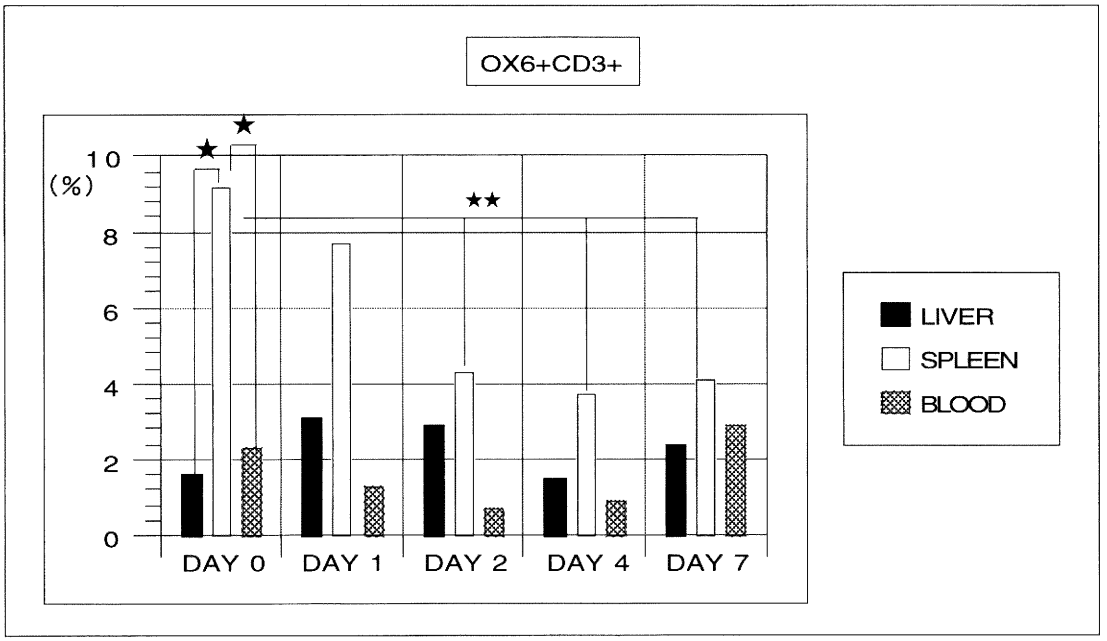


**Fig. 4.** Changes in MHC class II positive T cells after partial hepatectomy MHC class II positive T cells were preferentially located in the spleen in normal young rats (DAY0). These cells increased in the liver and decreased in the spleen and blood in the early regenerative phase. ★, $p<0.01$; Spleen ($9.18\pm0.47$) vs Liver ($1.79\pm0.28$) or Blood ($2.32\pm0.32$) (DAY0). ★★, $p<0.05$; Spleen Day0 ($9.18\pm0.47$) vs DAY2, 4 and 7 ($4.28\pm1.24$, $3.02\pm1.31$, $4.11\pm1.98$) respectively. The data are mean of measures performed on 3-5 animals. ★: $p<0.01$, ★★: $p<0.05$

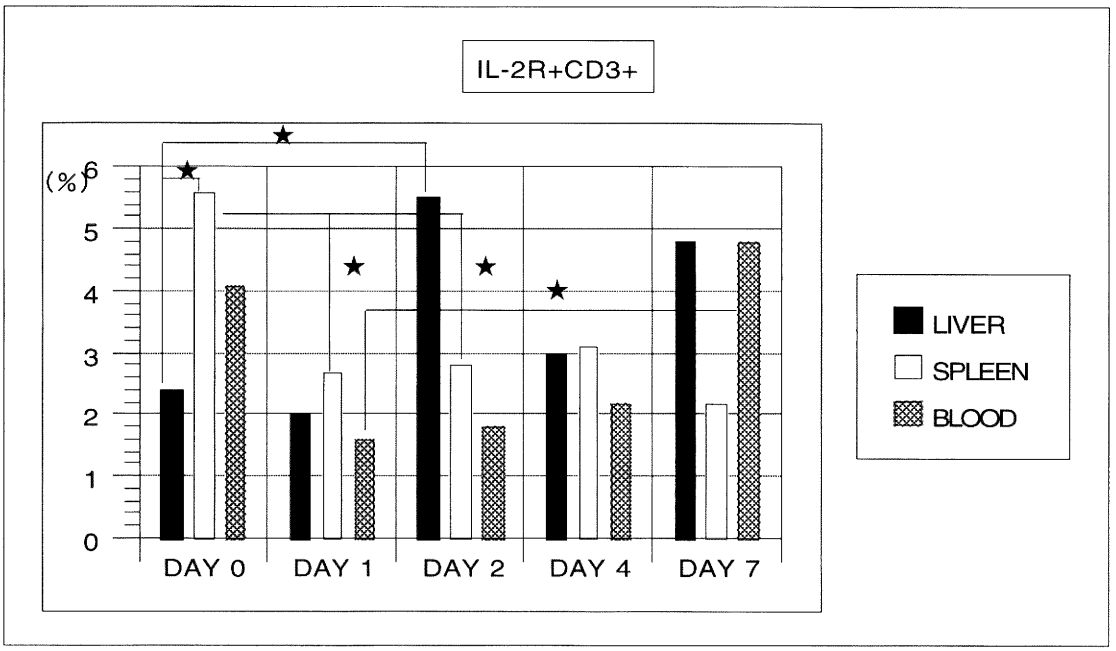


**Fig. 5.** Changes in IL- 2R+ T cells after partial hepatectomy IL- 2R+ T cells were also preferentially located in the spleen in the normal rats (DAY0 Liver: $2.38\pm1.01$, Spleen: $5.56\pm1.25$). These cells increased in the liver on day2 ($5.48\pm0.89$) postoperatively after partial hepatectomy. Conversely, they decreased in the spleen (DAY1: $2.71\pm1.04$, DAY2: $2.82\pm0.57$) and blood (DAY0: $4.08\pm1.52$, DAY1: $1.62\pm0.30$, DAY7: $4.72\pm1.02$) in the early regenerative phase. The data are mean of measures performed on 3-5 animals. ★: $p<0.05$

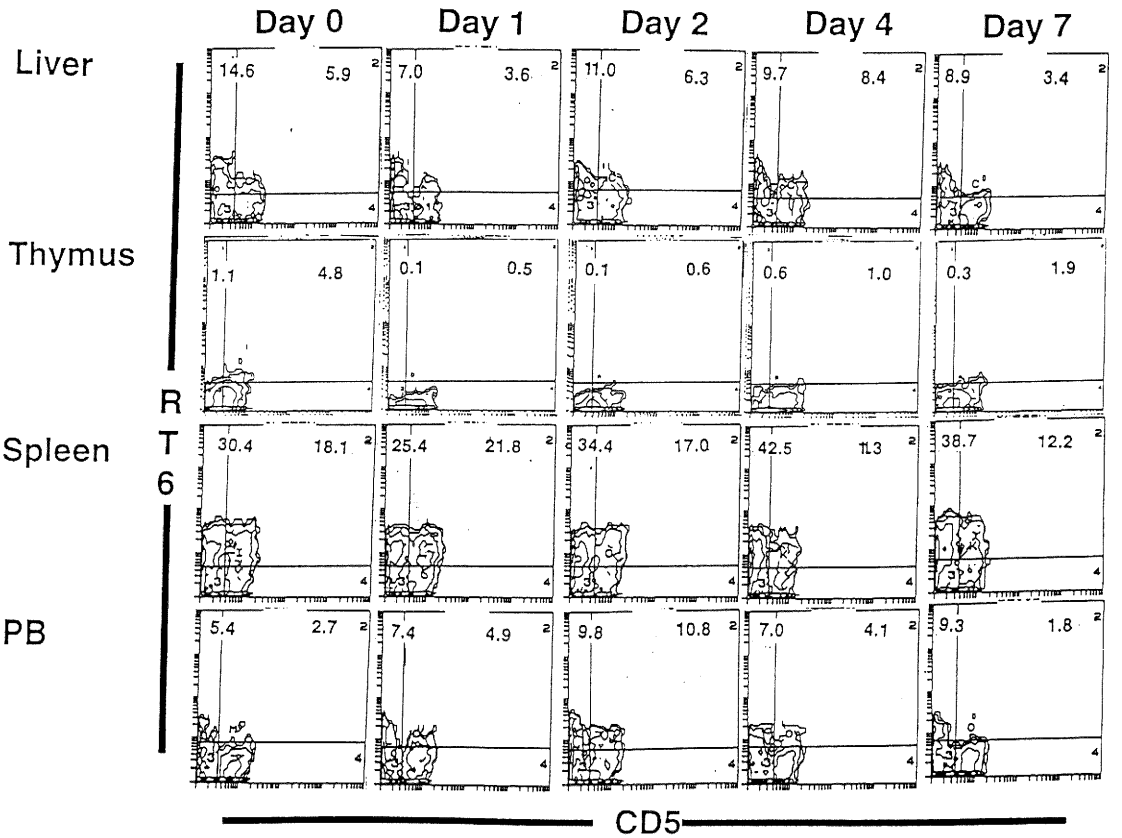


**Fig. 6.** Changes in RT6+ T cells after partial hepatectomy RT6+ T cells were preferentially present in the spleen in the normal young rats (Day0). These cells decreased in the liver and thymus and increased in the spleen and blood, but there was no statistical significance. The data are the mean of measurements performed on 3-5 animals.

## DISCUSSION

It is well known that the $CD4^+$ and $CD8^+$ T lymphocytes play an important role in cellular immunity by MHC class II and I restricted responses. It is of interest that CD4 in rats is expressed on the macrophages in addition to T lymphocytes, and CD8 is also expressed on NK cells,[5,6] although the roles of T and B lymphocytes present in the liver are unknown in rats as well as mice.

Therefore, in this study, we demonstrated that $CD4^-$ positive $CD3^-$ cells (macrophages) were heterogeneous only in the liver in normal rats. Interestingly, $CD4^{bright}CD3^-$ cells decreased in the liver and appeared in the spleen after partial hepatectomy. There are two possible explanations for this finding. The first is that the $CD4^{bright}CD3^-$ cells were liver-specific macrophages, and shifted from the liver to the spleen after partial hepatectomy. If this is true, the heterogenicity of $CD4^+$ cells might indicate a difference between dendritic cells and Kupffer cells in the rat. A second possibility is that these cells proliferated locally because of changes in the microenvironment. Abo et al.[13] postulated that extrathymic T cells may proliferate in several organs besides the liver or small intestine. Naito et al.[14] demonstrated that Kupffer cells could proliferate in the liver. Therefore $CD4^{bright}CD3^-$ cells may also proliferate in the liver and spleen. We are currently studying these macrophages. Otherwise, although there was the possibility of the influence of operative stress, we recognized that there was no difference between normal and sham operations with only an abdominal incision (data not shown).

We also found that in rats, $CD8^+$ T cells expressed CD3 at a slightly lower level than $CD4^+$ T cells. We demonstrated that extrathymic T cells which stain for CD3 at intermediate intensity (i.e., intermediate T-cell receptor (TcR) cells) are composed of $DNCD4^-$ $CD8^-$ cells as well as single-positive $CD4^+$ or $CD8^+$

cells, which are enriched in the mouse liver. In rats, the size of the $DNCD4^-$ $CD8^-$ cell compartment differed by less than 1% (data not shown) from that in mice. However, $CD8^+$ T cells stained for CD3 less intensely compared with $CD4^+$ T cells. This result was not as clear-cut in young rats as in mice. However, it was unambiguous in older rats. We also demonstrated that lymphocyte function-associated antigen-1 $(LFA\text{-}1)^{+++}$ T cells stain for CD3 at an intermediate intensity in older rats as well as mice.[4,15] This suggests that extrathymic T cells exist in rats; however, their phenotypes may differ from those in mice. Alternatively, extrathymic T cells may not exist in rats. If present, their relative number would be less than half those in mice.

We observed here that MHC class II antigen-positive Kupffer cells increase in the regenerating liver after partial hepatectomy. FK506 suppresses[8] MHC class II antigen expression in these cells and accelerates hepatocyte regeneration. Conversely IFN-$\gamma$ augments class II antigen expression in Kupffer cells and inhibits liver regeneration remarkably.[9] In this study, MHC class II antigen- positive T cells or IL-2R-positive T cells tended to increase in the regenerating liver after partial hepatectomy. These findings suggest that liver regeneration is mainly regulated by a MHC class II dependent pathway. Moreover, it is interesting to note that MHC class II antigen- positive T cells decrease in the spleen in the early regenerative phase contrary to the liver. This result suggests that a part of these cells might be circulated from the spleen.

A striking observation was the marked thymic involution: mean thymus weight decreased by approximately two- thirds by day 4 after partial hepatectomy. Surprisingly, over the same time period, the mean spleen weight increased by approximately 50%. These weight changes were reflected in the total leukocyte count in that leukocytes in the liver decreased and recovered in parallel with changes in the liver weight, while thymocyte numbers declined almost 30-fold and splenocyte numbers increased about 2.5-fold. The greatest increase in leukocyte number occurred in the blood, where a 16-fold increase was observed (data not shown). The changes in $RT6^+$ T cells after partial hepatectomy were paticularly interesting. RT6 is a T cell-specific membrane alloantigen in rats that has been detected on peripheral T lymphocytes (mature T cells).[16] $RT6^+$ T cells decreased in the liver and increased in the blood in the early regenerative phase on day 1 after partial hepatectomy in the rats. These findings suggest that there are two types of intrahepatic leukocytes; one type would tend to stay associated with sinusoidal endothelial cells (SEC), while the other would not. For instance, $RT6^+$ T cells and $CD4^+$ T cells easily dissociate from SEC and macrophages, while $CD8^+$ T cells and $LFA\text{-}1^{+++}$ T cells tend to stay with or adhere on to SEC in the early period after partial hepatectomy. To explain these phenomena after partial hepatectomy, we have advanced a hypothesis[17] that acute portal hypertension reflecting wall shear stress triggers liver regeneration following hepatectomy. It has been demonstrated that shear stress inhibits the adhesion of cultured mouse endothelial cells to lymphocytes by downregulating VCAM-1 expression.[18] Therefore it may influence the adhesion mechanism between SEC and leukocytes immediately after partial hepatectomy.

In conclusion, we have characterized some of the phenotypic and functional properties of liver leukocytes in rats using a hepatectomized model. Currently we are investigating morphologic examinations for $CD4^{bright}CD3^-$ cells.

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