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Induction of Unique Populations of Hepatic T Cells in Mice during Infection with *Toxocara canis*

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Abstract

When mice were orally administered with mature eggs of *Toxocara canis (T. canis)* (100 eggs/mouse), unique populations of hepatic T cells were induced during infection. On day 1 after administration, double-positive (DP) $CD4^+8^+$ cells were transiently demonstrated in the liver. These DP cells were rarely seen in the liver of control mice. Detailed characterization showed that they contained a unique population carrying the $CD4^+8\alpha^+\beta^-$ phenotype. This raised the possibility that they were generated extrathymically in the liver. On day 3 and day 7, the proportion and absolute number of extrathymic T cells expressing IL-2 receptor β-chain and CD3 of intermediate levels (i.e., intermediate CD3 cells) were also elevated. During this early phase of infection, the thymus tended to become atrophic while the spleen remained unchanged in terms of the number and the surface phenotype. The early phase of *T. canis* infection is known to be the time when hatched larvae go to the liver; they thereafter stay in the liver or migrate to other tissues. Therefore, these results suggest that a unique interaction between *T. canis* and host defence system occur in the liver during this initial time-period.

Key words: hepatic T cells; *T. canis* infection; double-positive cells; extrathymic T cells; liver int TCR cells.

Introduction

The interaction of ascaris larvae with host defence system is an interesting subject for investigation, because ascaris larvae are often able to escape such host defence system, irrespective of their being final hosts or not (Beaver *et al.*, 1952; Cypess *et al.*, 1977). It is speculated that ascaris may have subtle mechanisms enabling it to adapt to the host tissues. Indeed, cellular immune responses during ascariasis are quite obscure, and only eosinophilia and hyperglobulinemia have been demonstrated (Inoue *et al.*, 1989). As a result, the migration of larvae through various tissues occurs and induces a clinical syndrome of visceral larva migrans, especially in children. It is known that ascaris larvae penetrate the intestinal walls and reach the liver via the portal vein (Glickman *et al.*, 1987). In this regard, the liver is one of the important target organs in ascariasis and hepatomegaly is often observed.

In a series of recent studies (Abo *et al.*, 1991; Seki *et al.*, 1991; Watanabe *et al.*, 1993; Iiai *et al.*, 1992; Kimura *et al.*, 1993; Koyamada *et al.*, 1993), we reported the existence of extrathymic pathways of T-cell differentiation and characterized the properties of extrathymic T cells, especially in the liver. The liver is a major site of extrathymic T-cell differentiation and extrathymic T cells are most abundant in this organ. Since these hepatic T cells of extrathymic origin have many properties distinct from those of thymus-derived T cells, they can be easily distinguished from each other. Hepatic T cells express TCR (or CD3) of intermediate levels (i.e., intermediate TCR cells), constitutively express IL-2 receptor β-chain (IL-2Rβ), and contain a significant proportion of self-reactive forbidden

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clones.

In light of the above-mentioned findings, it would be of interest to know how extrathymic T cells as well as thymus-derived T cells in the liver and other organs are modulated during ascariasis. In the present study, we used mice (they are not final hosts) infected with *Toxocara canis (T. canis)*. When larvae of *T. canis* reached the liver after penetration through the intestine, unique populations of hepatic T cells were observed, i.e., double-positive (DP) $CD4^+8^+$ cells. The proportion of intermediate CD3 cells was also elevated thereafter. The present results suggest that ascaris interacts with extrathymic T cells existing in the liver in a unique manner. As already reported (Iiai *et al.*, 1992; Ohteki *et al.*, 1992), these extrathymic T cells increase in number with aging. The present results, therefore, might contain one factor responsible for the high incidence of viceral larva migrans in children.

Materials and Methods

Mice

Male C3H/He mice at 15 weeks of age were used. They were originally obtained from Charles River Japan, Kanagawa, Japan, and were maintained in the animal facility of Niigata University.

T. canis *infection*

T. canis adult worms were obtained from the intestine of a puppy, and eggs of *T. canis* were then collected from these worms (Inoue *et al.*, 1989). These eggs were maturated in an incubator until the development of second-stage larvae in the eggs. Mice were orally administered with maturated eggs just before hatching (100 eggs/mouse). Such eggs hatched in the intestine of mice and the larvae penetrated the intestinal walls and reached the liver via the portal vein within 2 days after administration. All parameters of the experiments were produced by an individual mouse.

Cell preparations

Hepatic mononuclear cells (MNC) were isolated by a previously described method (Watanabe *et al.*, 1992). Briefly, mice anesthetized with ether were sacrificed by total exsanguination by cardiac puncture. To obtain MNC, the liver was removed, pressed through 200-gauge stainless steel mesh, and suspended in PBS (pH 7.2). After one washing with PBS, MNC were isolated from hepatocytes and hepatocyte nuclei by Ficoll-Isopaque density (1.090) gradient centrifugation. To avoid selective cell loss by the gradient centrifugation method (Huang *et al.*, 1994), sufficient dilution of mashed liver samples with the medium (i.e., 30 ml for two livers) before they were overlaid on the gradient cushion was important. MNC collected from the interface were then suspended in MEM medium supplemented with 2% fetal calf serum. The preparations of hepatic MNC contained less than 4% Kupffer cells. Spleen cells were also collected by the Ficoll-Isopaque method, while thymocytes were obtained by forcing the thymus through 200-gauge steel mesh.

Immunofluorescence test

The surface phenotype of cells was analyzed using mAbs in conjunction with two- or three-color immunofluorescence tests (Kusumi *et al.*, 1992). FITC-conjugated anti-CD3 (145-2C11) mAb was obtained from PharMingen Co., San Diego, CA. Biotin-conjugated anti-IL-2Rβ (TM-β1) mAb was also used (kindly provided by Dr. T. Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) (Tanaka *et al.*, 1991). FITC- or biotin-conjugated reagents of anti-CD4 (L3T4), anti-CD8α (Lyt-2) and anti-CD8β (Lyt-3) were obtained from Becton-Dickinson Co., Mountain View, CA. Biotin-conjugated reagent was developed with phycoerythrin (PE)-conjugated avidin or Red 613-conjugated streptavidin (Becton-Dickinson Co., Mountain View, CA). The fluorescence-positive cells were analyzed with a FACScan (Becton-Dickinson Co.). Ten thousand cells were analyzed.

Statistical analysis

Statistical difference was estimated by Student's *t*-test.

Results

Variation of MNC in the liver, spleen and thymus during T. canis *infection*

Mice were orally administered with maturated eggs of *T. canis* (100 eggs/mouse), and the variation

of MNC in the liver, spleen and thymus was observed thereafter (Fig. 1). A statistically significant decrease in the number of thymocytes was induced on day 3 and day 7 after administration. The variation of MNC in the liver and spleen was minimal, although the numbers of splenic MNC tended to increase slightly at the late phase of infection.

Increase in the proportion of intermediate CD3 cells in the liver

When thymic atrophy (possibly the suppression of intrathymic T-cell differentiation) is induced under various conditions, extrathymic T cells are often activated in the liver. With regard to this, we then investigated how lymphocyte subsets in various immune organs were modulated during *T. canis* infection (Fig. 2). Two-color staining for CD3 and IL-2Rβ was performed to identify T cells (CD3-high$^+$IL-2Rβ^-), extrathymic T cells (CD3-intermediate$^+$IL-2Rβ^+) and NK cells (CD3$^-$IL-2Rβ^+) (Iiai *et al.*, 1992). It was demonstrated that the proportion of IL-2Rβ^+ intermediate CD3 cells was highly elevated in the liver on day 3 and 7 after administration. At this time, the proportion of CD3-high$^+$IL-2Rβ^- cells (i.e., thymus-derived T cells) decreased in the liver and spleen. The proportions of other subsets in the liver and other organs remained unchanged.

To confirm the induction of intermediate CD3 cells in the liver during infection, repeated experiments (n=5) to identify the variation in the absolute number of lymphocyte subsets were carried out (Fig. 3). There was a tendency for all subsets to decrease on day 1 and to increase thereafter. However, the increase in the absolute number of intermediate CD3 cells was statistically significant ($p<0.05$).

A transient appearance of a population of DP CD4$^+$8$^+$ cells in the liver on day 1 after infection

To determine the composition pattern of CD4$^+$ and CD8$^+$ among T cells, two-color staining for CD4 and CD8 was then carried out (Fig. 4). The most prominent change was seen in the liver. Namely, a unique population of DP CD4$^+$8$^+$ cells, which is rarely present in the liver under normal conditions, appeared in this organ on day 1 after infection. During the infection, especially from day 1 to day 7, an increased proportion of CD4$^+$ cells was also

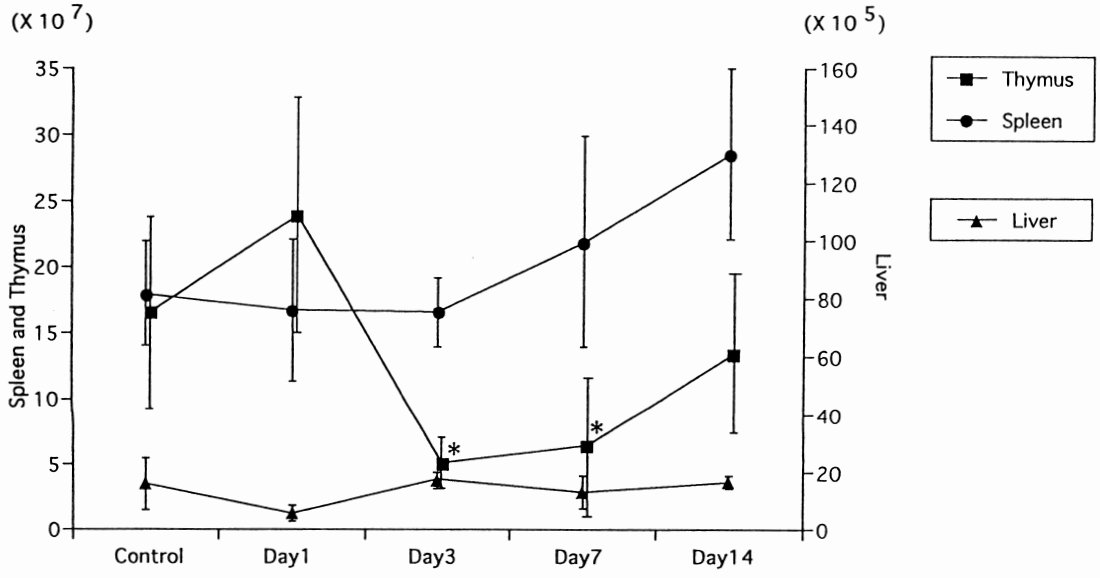

Fig. 1 Variation of the number of MNC in the thymus, spleen and liver during *T. canis* larva infection. Five mice were orally administered with eggs of *T. canis* (100 eggs/mouse) and the number of MNC were enumerated at the indicated days after administration. The number of MNC was estimated in an individual mouse. The mean and one S. D. are represented. *$p<0.05$.

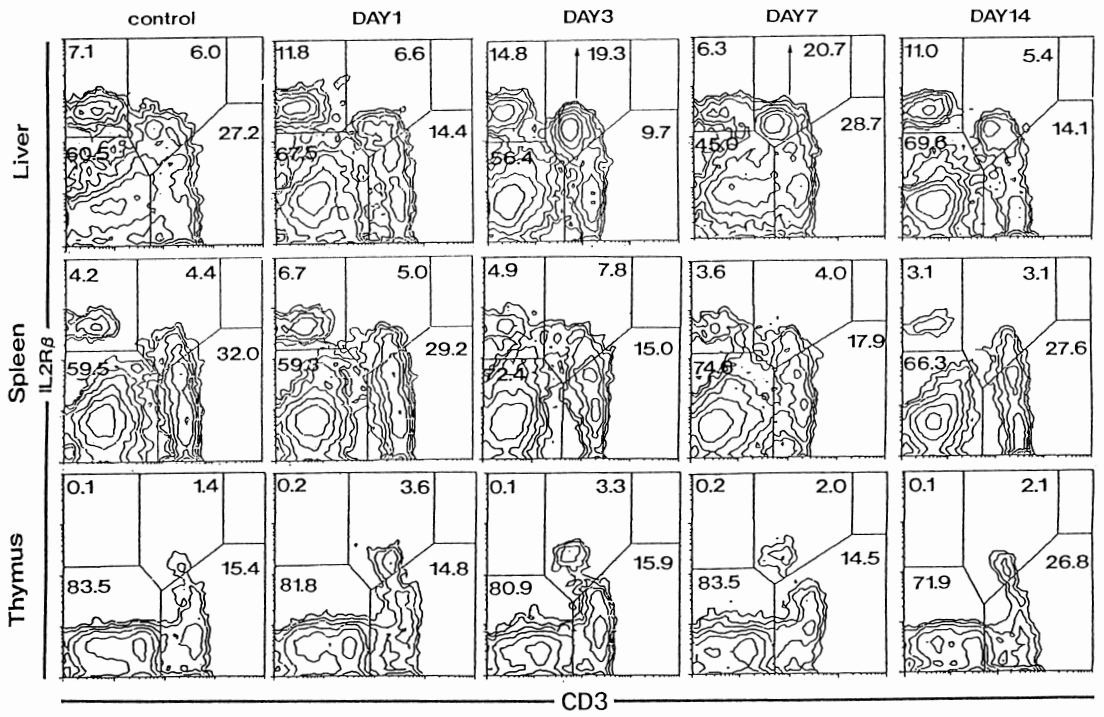

Fig. 2 Phenotypic characterization of MNC in the liver, spleen and thymus during *T. canis* infection. Two-color staining for CD3 and IL-2Rβ was carried out. Representative results from three experiments (= three mice) are depicted. The numbers in the figure represent the fluorescence-positive cells in the corresponding areas. A prominent increase in the proportion of IL-2Rβ^+ intermediate CD3 cells was seen in the liver on day 3 and day 7 after infection (indicated by arrows).

observed in the liver. The variations in the spleen and thymus were minimal.

To confirm the above results, repeated experiments (n=5) to identify $CD4^+$, $CD8^+$ and DP $CD4^+8^+$ cells were performed in the liver (Fig. 5). Although the absolute number of DP $CD4^+8^+$ cells was very low, the appearance of such population was consistent in the liver on day 1. The absolute number of $CD4^+$ cells tended to increase on day 3 and day 7.

In a final portion of this experiment, we further characterized DP $CD4^+8^+$ cells and $CD8^+$ cells, since some T cells expressing CD8 antigens have $CD8\alpha^+\beta^+$ or $CD8\alpha^+\beta^-$ phenotype. For this purpose, three-color staining for CD4, CD8α and CD8β was performed (Fig. 6). By gating analysis, the expression level of CD8β antigens on $CD4^+CD8\alpha^+$ and $CD4^-CD8\alpha^+$ cells was determined. It was clearly demonstrated that both DP $CD4^+CD8\alpha^+$ cells and single-positive $CD4^-CD8\alpha^+$ cells in the liver contained a $CD8\beta^-$ population. Although the proportion of DP $CD4^+8^+$ cells in the spleen was very low, such population also contained $CD8\beta^-$ cells. In sharp contrast, $CD8\alpha^+$ cells in the spleen and DP $CD4^+CD8\alpha^+$ cells and single-positive $CD4^-CD8\alpha^+$ cells in the thymus were all $CD8\beta^+$.

Discussion

In the present study, we demonstrated that the infection of *T. canis* induced a unique population of hepatic T cells from day 1 to day 7 after the oral administration. Such populations included DP $CD4^+8^+$ cells on day 1 and IL-2Rβ^+ intermediate CD3 cells from day 3 to day 7 in the liver. Since hatched larvae penetrated the intestine, travelled through the portal vein, and reached the liver at this early phase of infection (Beaver *et al.*, 1952; Cypess *et al.*, 1977; Inoue *et al.*, 1989), these changes might

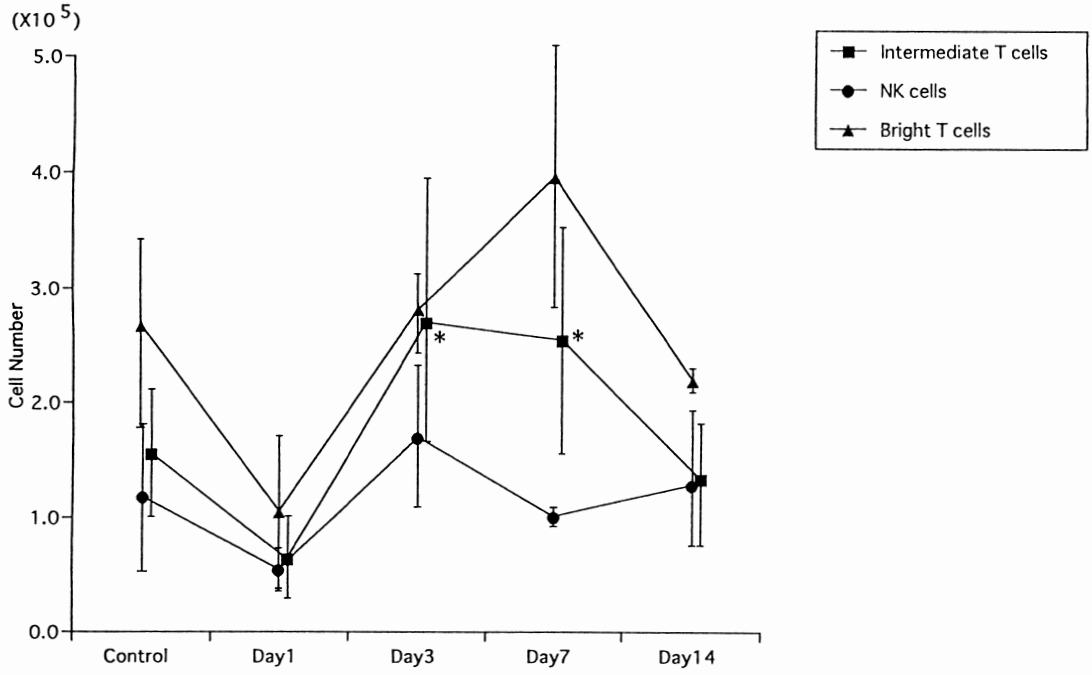

Fig. 3 Variation of the absolute number of intermediate CD3 cells, NK cells and bright CD3 cells in the liver during *T. canis* infection. Five mice were used to determine the variation of the absolute number of various lymphocyte subsets in the liver of mice infected with *T. canis*. Each parameter was produced in an individual mouse. The mean and one S. D. are represented. *$p<0.05$.

be the results of the interaction of *T. canis* with host defence system. In parallel experiments, we investigated the variation in the spleen. However, no apparent signs were observed in this organ, except the decrease in the proportion of thymus-derived T cells. To date, most experiments on such infections have focused on the spleen. The present results suggest that we should pay greater attention to the major target organ, i.e., the liver, and to the residential lymphocytes, i.e., hepatic T cells.

When the unique variation occurred in the liver after infection, the number of thymocytes also showed such a unique variation pattern. Namely, thymic atrophy was induced consistently from day 3 to day 7. When extrathymic T cells are activated under various conditions in the liver, intrathymic T-cell differentiation tends to be suppressed (Abo *et al.*, 1992; Ohmori *et al.*, 1993; Seki *et al.*, 1991; Kusumi *et al.*, 1992). With regards to this, it is possible that the thymic atrophy seen in this experiment may be also the case.

Of the two unique populations seen in the liver, IL-2Rβ^+ intermediate CD3 cells were estimated to be of extrathymic origin. Thus, all T cells in the peripheral immune organs, except in the intestine, are IL-2Rβ^+ intermediate CD3 cells in congenitally athymic nude mice (Iiai *et al.*, 1992; Ohtsuka *et al.*, 1994). Intermediate CD3 cells have IL-2R$\alpha^-\beta^+$ phenotype (i.e., intermediate affinity IL-2R), similar to NK cells (Watanabe *et al.*, 1993). In this regard, intermediate CD3 cells can immediately respond to stimuli. On the other hand, thymus-derived T cells were IL-2R$\alpha^-\beta^-$ under resting conditions and acquired IL-2R$\alpha^+\beta^+$ phenotype (i.e., high affinity IL-2R) only after activation. Therefore, their responses are very retarded, but the final magnitude of the responses is quite high.

Even in the liver, DP CD4$^+$8$^+$ cells are rarely seen (Iiai *et al.*, 1992; Ohtsuka *et al.*, 1994). This population is rather common in the intestine (Ohtsuka *et*

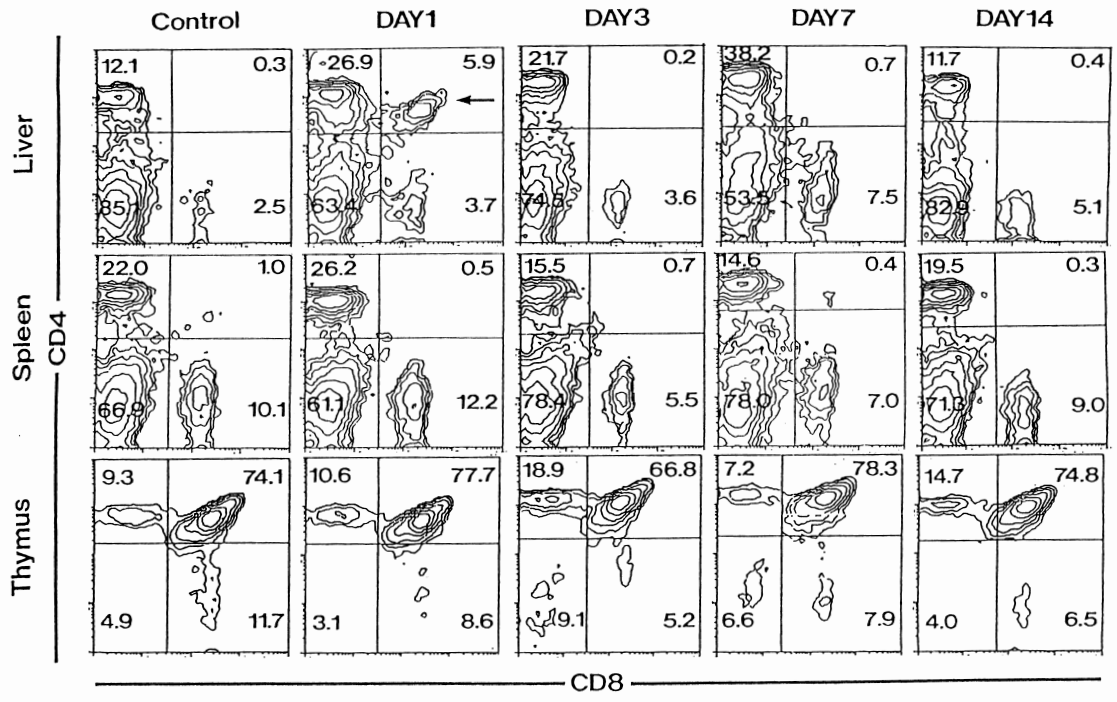

Fig. 4 The appearance of DP $CD4^+8^+$ cells in the liver during *T. canis* larva infection. Two-color staining for CD4 and CD8 was carried out at the indicated days after infection. Representative results from three experiments (= three mice) are depicted. The numbers in the figure indicate the fluorescence-positive cells in the corresponding areas. DP $CD4^+8^+$ cells appeared in the liver on day 1 (indicated by an arrow).

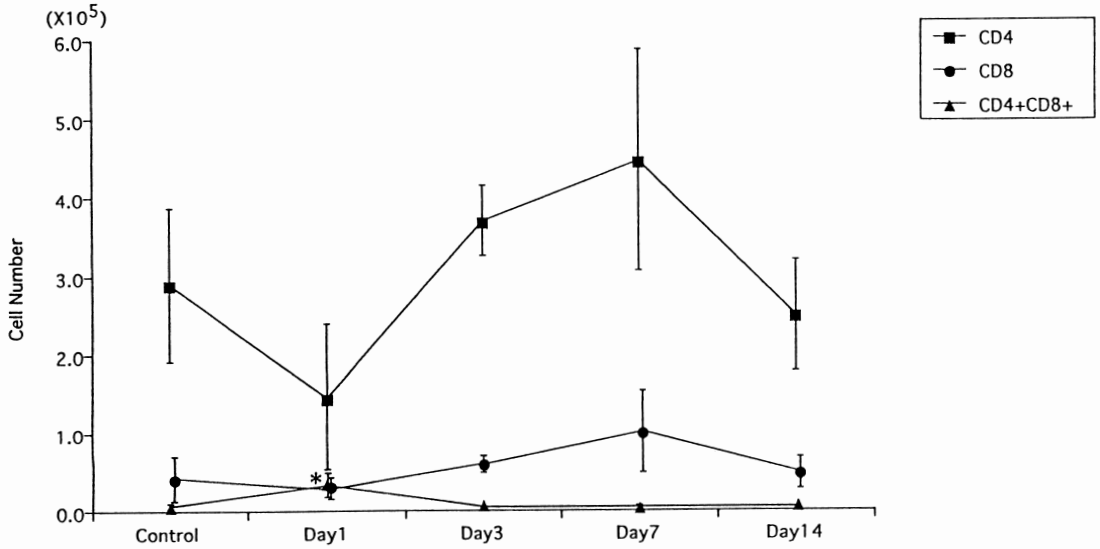

Fig. 5 Variation of lymphocyte subsets, $CD4^+$, $CD8^+$ and DP $CD4^+8^+$ cells, in the liver of mice infected with *T. canis*. Five mice were used to determine the variation of various lymphocytes subsets, and two-color staining for CD4 and CD8 was performed. Each parameter was produced in an individual mouse. The mean and one S. D. are represented. *$p<0.05$.

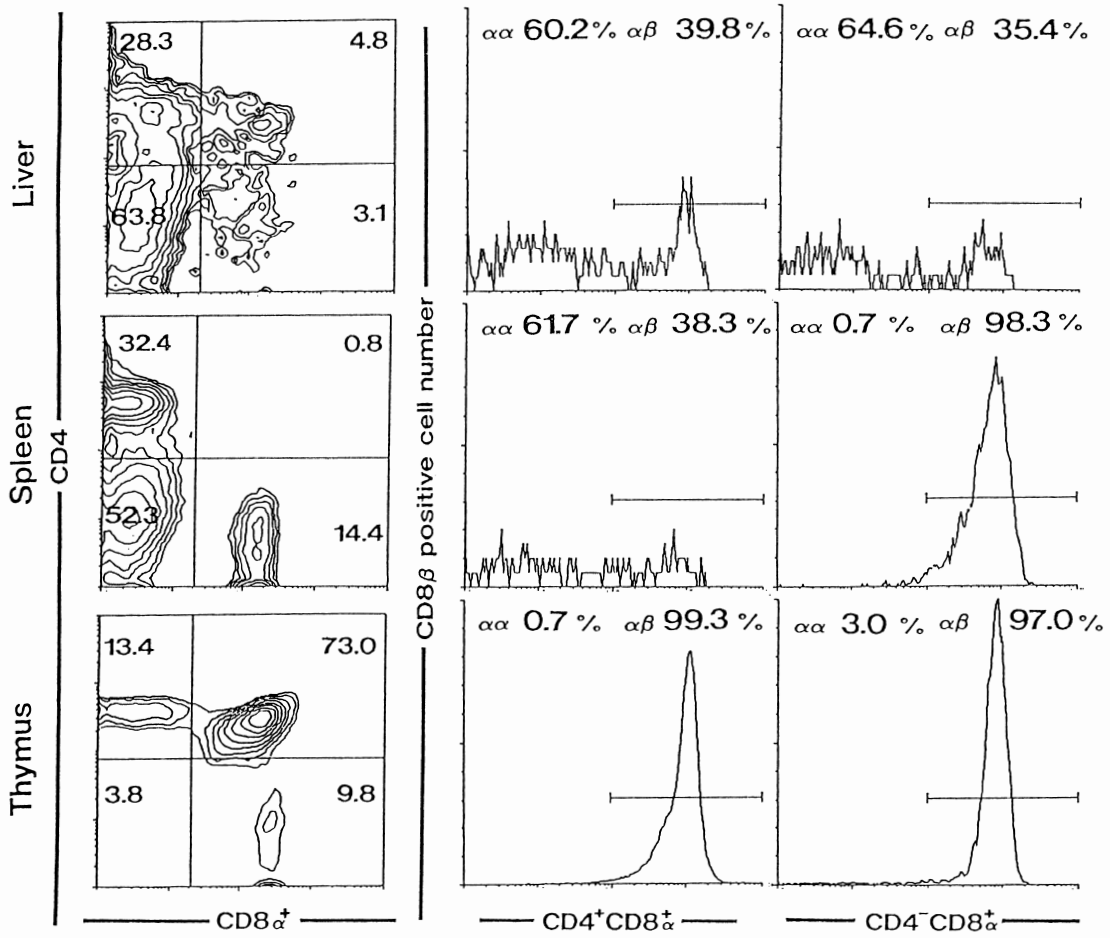

Fig. 6 DP $CD4^+8^+$ cells appearing in the liver are a mixture of $CD4^+8\alpha^+\beta^+$ and $CD4^+8\alpha^+\beta^-$ phenotype. Three-color staining for CD4, CD8α and CD8β was carried out by using liver MNC obtained from mice on day 1 after *T. canis* larva infection. Gated analysis of $CD4^+CD8\alpha^+$ and $CD4^-8\alpha^+$ cells was performed to determine their expression of CD8β antigens. The numbers in the figure indicate the fluorescence positive cells in corresponding areas. Representative results from three experiments (= three mice) are depicted.

al., 1994). One possibility is that DP $CD4^+8^+$ cells transiently seen in the liver during infection were derived from the intestine. Another possibility is that the liver has the potential to produce such a unique population under specific conditions. We consider the latter possibility for the following reasons. Almost all DP $CD4^+8^+$ cells in the intestine are known to be $CD4^+8\alpha^+\beta^-$ (Ohtsuka *et al.*, 1994, Sato *et al.*, 1993). This was true in the intestine during *T. canis* infection (our unpublished observation). On the other hand, DP $CD4^+8^+$ cells which appeared in the liver were a mixture of DP $CD4^+8\alpha^+\beta^+$ and DP $CD4^+8\alpha^+\beta^-$. Based on these data, these DP $CD4^+8^+$ cells seemed to be specific to the liver. Although the proportion of such DP $CD4^+8^+$ cells in the spleen was very low, they had properties similar to those in the liver. All DP $CD4^+8^+$ cells seen in the thymus were $CD4^+$ $CD8\alpha^+\beta^+$.

In any case, the present study showed that characterization of a target organ, the liver itself, and of residential hepatic T cells is very important if we want to determine the immunologic response during

T. canis infection. These results bring to mind the high incidence of toxocariasis as well as general ascariasis in children. Namely, extrathymic intermediate TCR cells increase in number with aging. In a recent study (Takii *et al.*, 1994), we reported that granulated $CD56^+$ T (or $CD57^+$ T) cells, that are also abundant in the liver, may be a counterpart of extrathymic T cells in humans. Since extrathymic T cells are the major lymphocyte population in the liver, it is possible that both phenomena i.e., the high incidence of toxocariasis and ascariasis in children and the age-dependent increase of extrathymic T cells in the liver, might be closely related to each other. In other words, immaturity of the immune system in the liver of children permits toxocariasis or ascariasis, possibly irrespective of their being final hosts or not.

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