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Immunologic States Induced by Starvation: Suppression of Intrathymic T-cell Differentiation Contrasting with the Relative Resistance of Extrathymic T-cell Differentiation

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Summary. **Immunologic states induced by starvation were investigated in mice. Attention was focused on not only T cells of thymic origin but also extrathymically differentiated T cells. It was demonstrated that the numbers of lymphocytes yielded by the various immune organs, including the liver, spleen and thymus, prominently decreased during starvation (4-day period). At this time, intrathymic T-cell differentiation was profoundly suppressed and the number of thymus-derived T cells in the peripheral organs decreased. On the other hand, extrathymic T cells did not significantly decrease in number, especially in the liver. During starvation, a stress-associated glucocorticoid, corticosterone, was elevated in the serum of these mice. Since the *in vivo* injection of hydrocortisone induced a response similar to that seen during starvation, it is likely that glucocorticoids as well as a cessation of energy supply participates in immunologic states induced by starvation.**

INTRODUCTION

Awareness of the immunologic states in subjects during starvation, including that due to medical causes (e.g., surgery) is important. It is presumed that starvation not only causes a decrease in the supply of energy, but also subjects the body to critical stress. In this regard, such starvation may induce considerable immunosuppression and result in the possibility of infection and other problems.

A recent advance in immunology has revealed that there are two pathways of T-cell differentiation, including the intrathymic pathway and the extrathymic pathways.[1-4] We recently demonstrated that not only thymus-derived T cells but also extrathymically differentiated T cells mediate important immunological functions[5-8]; for example, extrathymic T cells play important roles in the host defense mechanisms involved in infections of the intracellular pathogens and in malignancies. Since we heve recently established a simple method to detect these thymus-derived T cells and extrathymic T cells simultaneously in mice,[9] we applied it to elucidate the immunologic states induced by starvation. It was demonstrated that the immunomodulation pattern of each pathway was quite different from that of the other pathway during starvation. Namely, intrathymic T-cell differentiation was very sensitive to starvation, and starvation resulted in a decrease in the number of thymus-derived T cells in the peripheral organs, whereas the extrathymic pathways appeared to be relatively resistant to such starvation and showed no apparent decrease in the number of extrathymic T cells. Additional experiments further revealed that some stress-associated glucocorticoids might be involved in the induction of these unique changes.

MATERIALS AND METHODS

Mice

Male C3H/He mice aged 8 weeks were used, the animals being maintained under specific pathogen-free conditions in the animal facilities of Niigata University.

Starvation

Mice were starved by withholding food, but were given water *ad libitum*. Under this condition, mice

lived for 4 to 5 days.

Administration of drugs

Hydrocortisone (Sigma Chemical Co., St. Louis, MO, USA) was subcutaneously injected at the specified concentrations. All effects of hydrocortisone were examined on Day 3 after administration by using 4 mice for one group. The day of the maximal effects had been determined by our previous study.[7,10]

Cell preparation

Thymocytes were obtained by forcing the thymus through 200-gauge stainless steel mesh. Splenocytes were also obtained by forcing the spleen through steel mesh with subsequent Ficoll-Isopaque gradient (1.090 g/cm^3) centrifugation. To accurately determine the number of cells, cell counts were performed before the gradient centrifugation.

Hepatic MNC were prepared as previously described.[9] Briefly, mice anaesthetized with ether were sacrificed by total bleeding from the incised axillary artery and vein. The liver was removed, cut into small pieces with scissors, pressed through steel mesh, and suspended in Eagles minimal essential medium containing 5 mM HEPES (Nissui Pharmaceutical Co., Tokyo, Japan) and 2% heat-inactivated newborn calf serum. MNC were separated from parenchymal hepatocytes, the nuclei of hepatocytes, and Kupffer cells by Ficoll-Isopaque centrifugation. This MNC preparation contained 96% lymphocytes and less than 4% Kupffer cells. The number of hepatic lymphocytes was confirmed at this stage, cell yields ranging from 5 to 10% as previously estimated.[9]

Immunofluorescence tests

The surface phenotypes of the cells were identified by using monclonal antibodies (mAb) in conjunction with a two-color immunofluorescence test.[9] The mAb used were fluorescein isothiocyanate (FITC)- or biotin [or phycoerythrin (PE)]-conjugated anti-CD3(145-2C11), anti-CD4(L3T4) and anti-CD8(Lyt-2) mAbs. FITC- or biotin-conjugated anti-IL-2 receptor β-chain (anti-IL-2Rβ)(TM-β1) mAb was kindly provided by Dr. T. Tanaka (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Biotin-conjugated reagents were developed with PE-conjugated avidin (Caltag Laboratories, San Francisco, CA, USA). The fluorescence-positive cells were analysed by a FACScan (Becton Dickinson Co., Mountain View, CA, USA).

Plasma corticosterone

Plasma corticosterone of the mice was detected by radioimmunoassay by the method of Murphy, with minor modifications.[11]

RESULTS

Decrease in the numbers of hepatic MNC, splenic MNC and thymocytes during starvation

Time-kinetics of the numbers of hepatic MNC, splenic MNC and thymocytes during starvation were investigated (Fig. 1). All numbers of hepatic MNC,

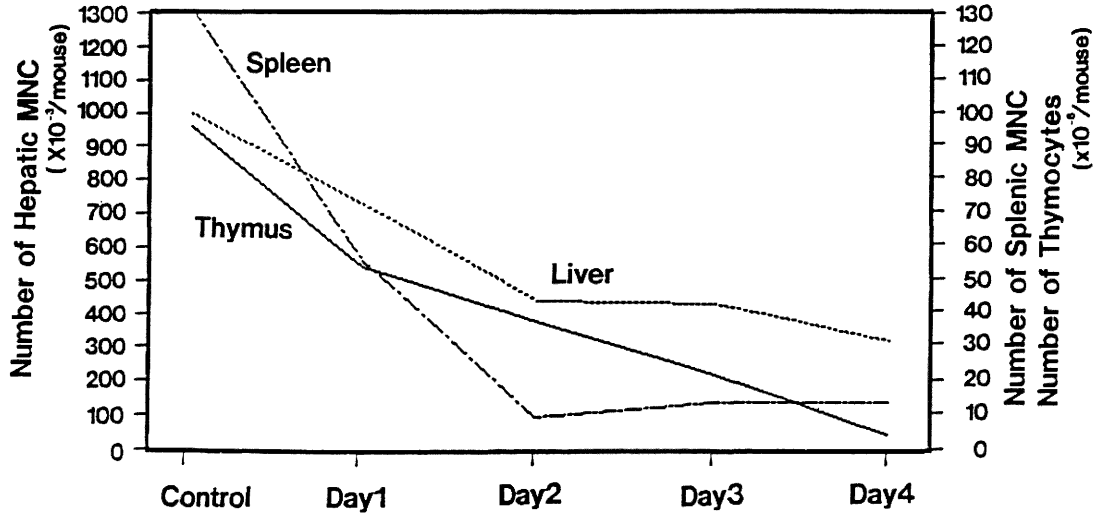

Fig. 1. Decrease in the numbers of MNC yielded by the liver, spleen and thymus during starvation. Male C3H/He mice aged 8 weeks were used. To standardize the number, the number of MNC pooled from 4 mice was counted at each point and the mean value was calculated.

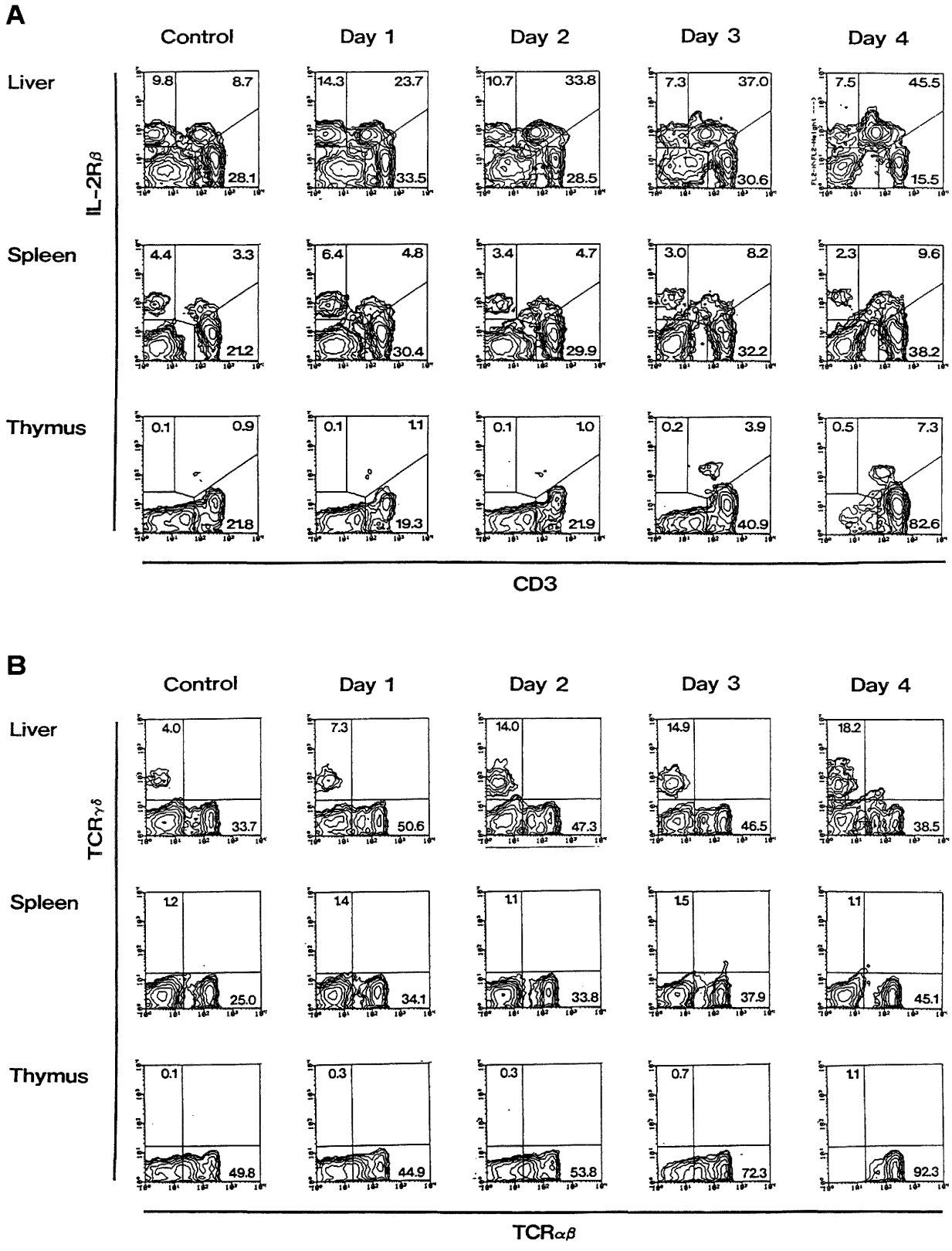
A
Control
Day 1
Day 2
Day 3
Day 4
Liver
Spleen
Thymus
IL-2Rβ
9.8
8.7
28.1
14.3
23.7
33.5
10.7
33.8
28.5
7.3
37.0
30.6
7.5
45.5
15.5
4.4
3.3
21.2
6.4
4.8
30.4
3.4
4.7
29.9
3.0
8.2
32.2
2.3
9.6
38.2
0.1
0.9
21.8
0.1
1.1
19.3
0.1
1.0
21.9
0.2
3.9
40.9
0.5
7.3
82.6
CD3
B
Control
Day 1
Day 2
Day 3
Day 4
Liver
Spleen
Thymus
TCRγδ
4.0
33.7
7.3
50.6
14.0
47.3
14.9
46.5
18.2
38.5
1.2
25.0
1.4
34.1
1.1
33.8
1.5
37.9
1.1
45.1
0.1
49.8
0.3
44.9
0.3
53.8
0.7
72.3
1.1
92.3
TCRαβ

Fig. 2.

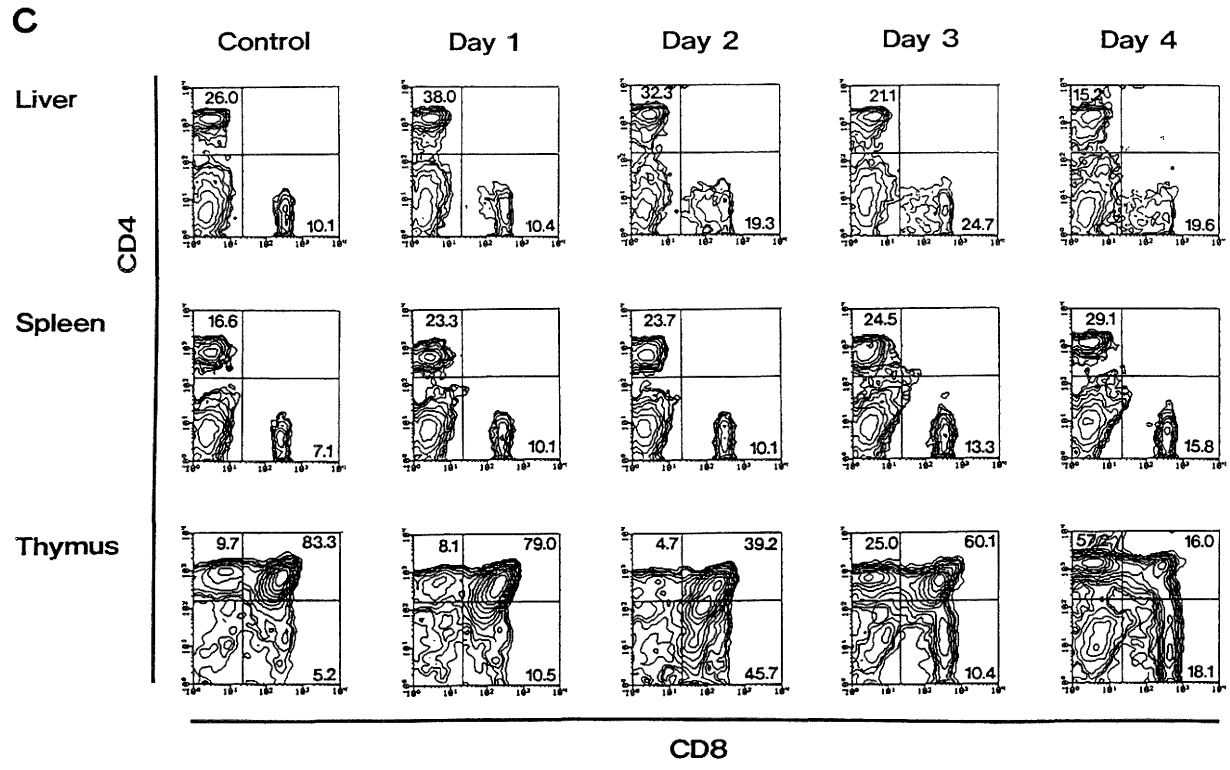

Fig. 2. Increase in the proportion of intermediate TCR cells in the liver and thymus during starvation. **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$, and **C.** Two-color staining for CD4 and CD8. Two-color staining for CD3 and IL-2Rβ was performed to identify NK cells (CD3$^-$IL-2Rβ^+), extrathymic T cells (CD3-intermediate IL-2Rβ^+), thymus-derived T cells (CD3-bright $^+$IL-2Rβ^-) and B cells (CD3$^-$IL-2Rβ^-).

splenic MNC and thymocytes were demonstrated to prominently decrease after starvation. Almost all of the starved mice died by Day 5.

Phenotypic characterization of MNC obtained from the liver, spleen and thymus

To determine how the distribution of lymphocyte subsets in various organs were modulated during starvation, two-color staining for CD3 and IL-2Rβ was performed (Fig. 2). As shown in our previous study,[12] this staining stimultaneously identified B cells (CD3$^-$IL-2Rβ^-), NK cells (CD3$^-$IL-2Rβ^+), extrathymic T cells (CD3-intermediate $^+$IL-2Rβ^+) and thymus-derived T cells (CD3-bright$^+$IL-2Rβ^-). In this regard, we call extrathymic T cells intermediate CD3(or TCR) cells and thymus-derived T cells bright TCR cells, respectively. In this experiment, it was clearly demonstrated that the proportion of intermediate TCR cells gradually increased throughout the organs tested as a function of time after starvation (Fig. 2A). Here, intermediate CD3 cells were most abundant in the liver, the proportion of such cells reaching 45.5% in this organ on Day 4. Interestingly, the proportion of NK cells (CD3$^-$IL-2Rβ^+) seen in the liver and spleen was relatively constant during starvation. When the thymus became atrophic on Day 4, CD3$^-$ and CD3^{dull} immature T cells disappeared, with a relative increase in the proportion of bright TCR cells observed instead.

Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$ was then performed (Fig. 2B). $\gamma\delta$ T cells were seen only in the liver among the organs tested here, and the proportion of such hepatic $\gamma\delta$ T cells gradually increased after starvation. Two-color staining for CD4 and CD8 antigens was carried out (Fig. 2C). There was a tendency for the proportion of CD4$^+$ cells to decrease but that of CD8$^+$ cells to reciprocally increase, especially in the liver and spleen after starvation. In the case of the thymus, a large proportion of double-positive (DP) CD4$^+$8$^+$ was initially observed. Thymocytes underwent a process of negative selection at this stage. During starvation, the proportion of DP cells decreased significantly.

Relative resistance of extrathymic T cells to starvation

From our data compiled thus far, the absolute numbers of lymphocyte subsets in the liver were calculated (Fig. 3). For purposes of simplification, only the mean value of each subset is represented (n=4). It is noteworthy that extrathymic, intermediate TCR cells were shown to be quite resistant to starvation. They seemed even to increase in number during starvation. On the other hand, thymus-derived, bright

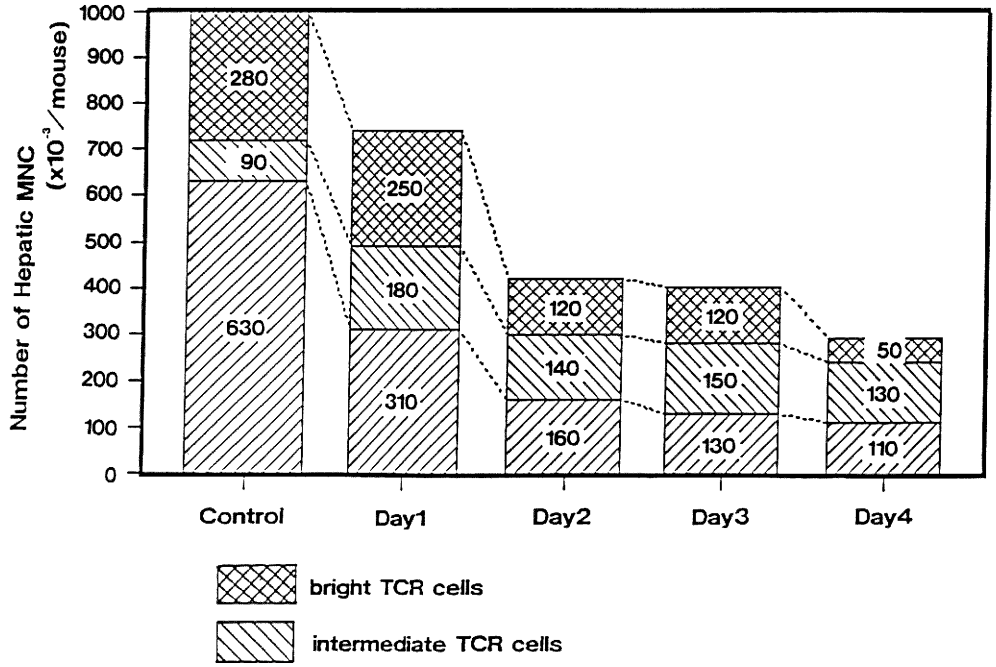

Fig. 3. Resistance of intermediate TCR cells in the liver by starvation. The absolute number of each lymphocyte subset was calculated by using the data of Fig. 1 and 2.

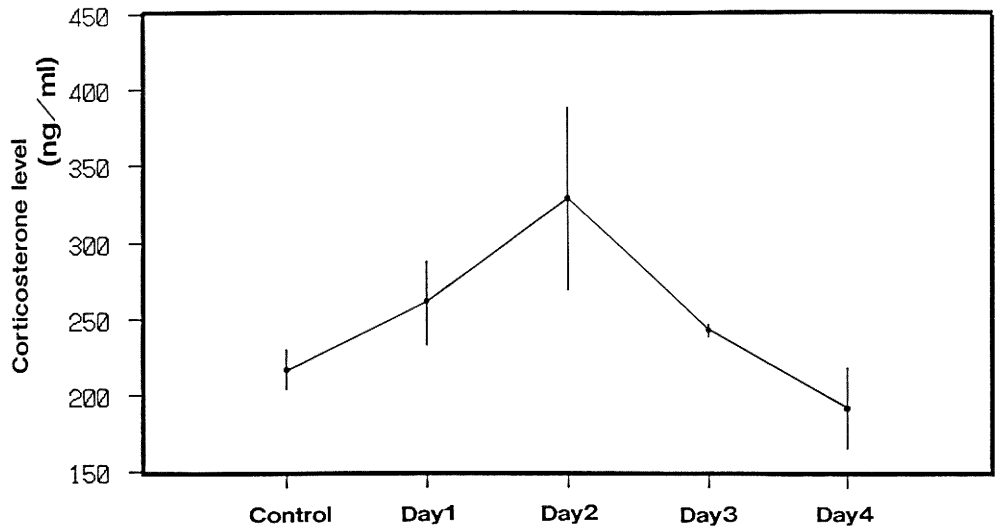

Fig. 4. Variation of serum corticosterone levels during starvation. The elevation of serum corticosterone was induced by starvation, showing a peak at Day 2.

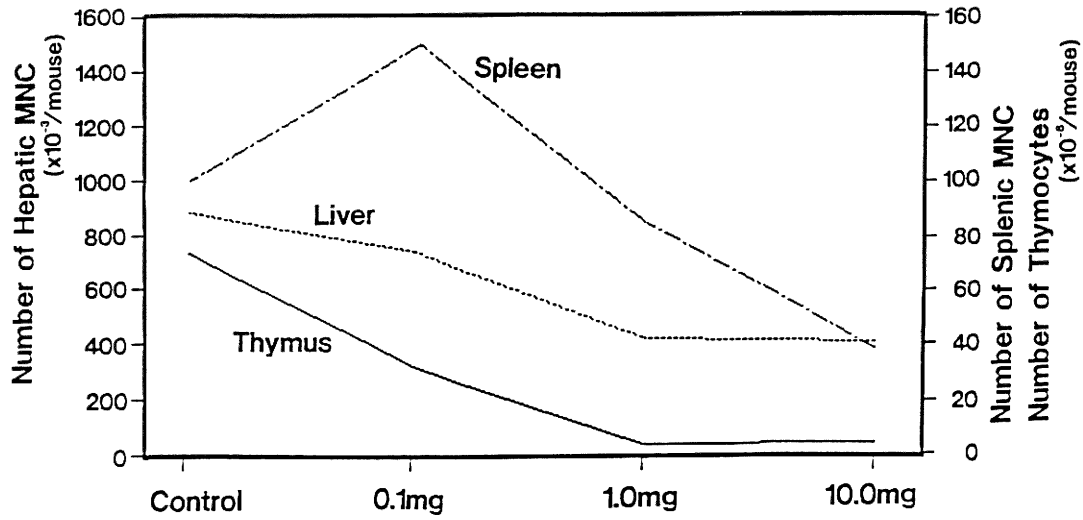

Fig. 5. Decrease in the numbers of MNC yielded by the liver, spleen and thymus after a hydrocortisone administration. Male C3H/He mice aged 8 weeks were injected with hydrocortisone at the indicated concentrations. The number of MNC pooled from 4 mice was counted at each point and the mean values were calculated.

TCR cells were quite sensitive to starvation.

Corticosterone levels in the serum during starvation

Obviously, starvation placed great stress on the mice. Therefore, a stress-associated glucocorticoid, corticosterone, in the serum was measured in these mice during starvation (Fig. 4). As expected, the level of corticosterone was elevated, showing a peak on Day 2.

Effect of glucocorticoid on the mouse immune system

To compare the immunomodulation pattern resulting from both starvation and glucocorticoid, the *in vivo* effect of glucocorticoid was then investigated (Fig. 5). Since the maximal change was observed on Day 3 after administration in the preliminary experiments,[10] only the data on Day 3 are represented. Mice were subcutaneously administered with the specified amounts of hydrocortisone and examined as to the numbers of hepatic MNC, splenic MNC and thymocytes yeilded. At a low dose of hydrocortisone (0.1 mg/mouse), a profound thymic atrophy was still induced, while the number of splenic MNC was rather elevated. On the other hand, the higher doses (1.0 and 10 mg/mouse) stimultaneously induced a decrease in the numbers of hepatic MNC, splenic MNC and thymocytes.

An increase in the proportion of intermediate CD3 cells after hydrocortisone administration

Two-color staining for CD3 and IL-2Rβ was performed to determine which lymphocyte subsets were sensitive or resistant to hydrocortisone administration (Fig. 6A). Similar to conditions of starvation, a relative increase in the proportion of intermediate CD3 cells was seen in the liver and thymus. The proportion of CD3$^-$IL-2Rβ^+ NK cells was constant. At the highest dose of hydrocortisone (10 mg/mouse), almost all CD3$^-$ and CD3^{dull} immature thymocytes disappeared.

Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$ showed that the proportion of $\gamma\delta$ T cells did not significantly change in all tested organs (Fig. 6B). The distribution of CD4$^+$ and CD8$^+$ cells was then examined (Fig. 6C). Such distribution was stationary in the liver and spleen, while the proportion of DP CD4$^+$ 8$^+$ cells prominently decreased, and was accompanied by thymic atrophy. This change was highly dependent on the doses.

Relative resistance of intermediate CD3 cells to hydrocortisone treatment

The absolute numbers of lymphocyte subsets in the liver were then calculated (Fig. 7). It was clearly demonstrated that intermediate TCR cells were quite resistant to hydrocortisone treatment. The cell fraction which contained B and NK cells decreased most prominently, depending on the increased doses.

Relative increase in the proportion of intermediate CD3 cells in the liver of mice due to stress resulting from restriction

In a final phase of these experiments, mice were

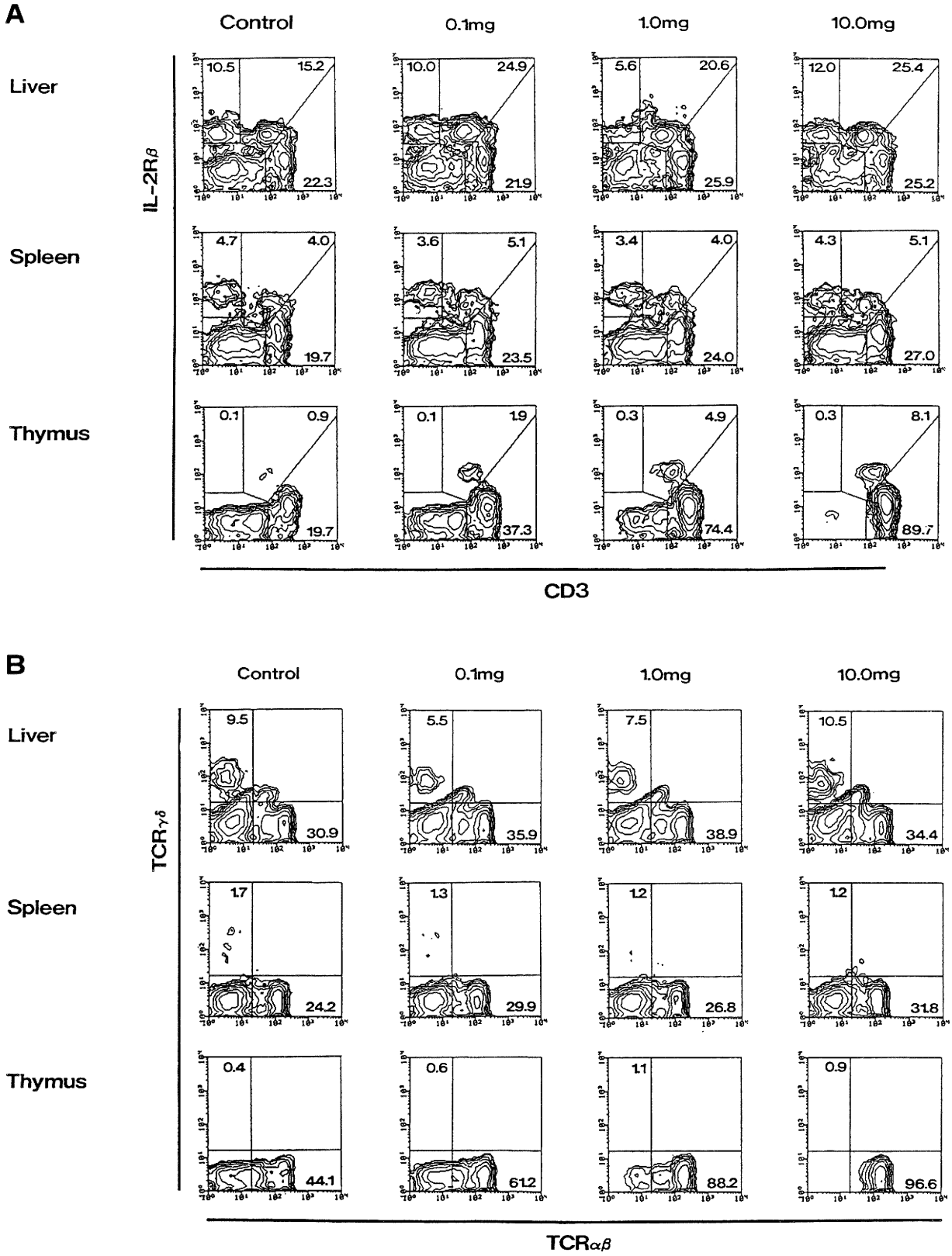
A
Control
0.1mg
1.0mg
10.0mg
Liver
Spleen
Thymus
IL-2Rβ
10.5
15.2
22.3
10.0
24.9
21.9
5.6
20.6
25.9
12.0
25.4
25.2
4.7
4.0
19.7
3.6
5.1
23.5
3.4
4.0
24.0
4.3
5.1
27.0
0.1
0.9
19.7
0.1
1.9
37.3
0.3
4.9
74.4
0.3
8.1
89.7
CD3
B
Control
0.1mg
1.0mg
10.0mg
Liver
Spleen
Thymus
TCRγδ
9.5
30.9
5.5
35.9
7.5
38.9
10.5
34.4
1.7
24.2
1.3
29.9
1.2
26.8
1.2
31.8
0.4
44.1
0.6
61.2
1.1
88.2
0.9
96.6
TCRαβ

Fig. 6.

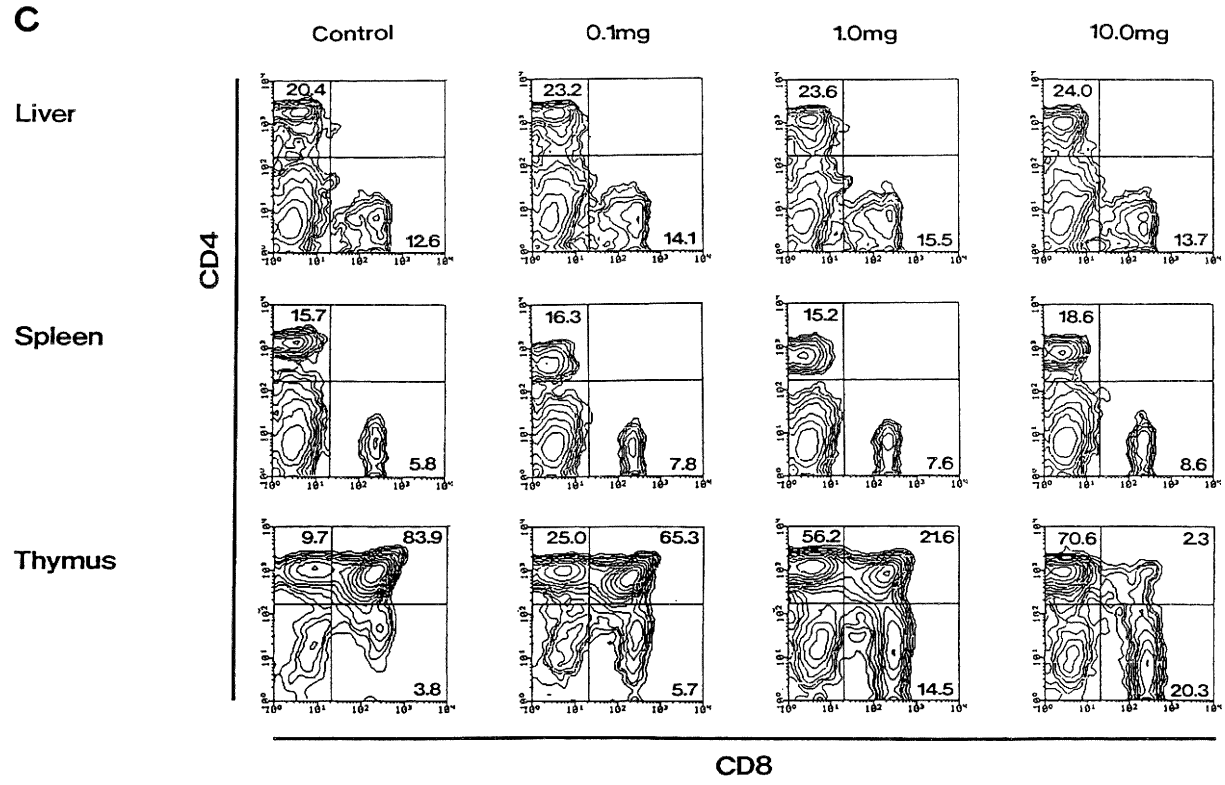

Fig. 6. Increase in the proportion of intermediate TCR cells in the liver and thymus after a hydrocortisone administration. **A.** Two-color staining for CD3 and IL-2Rβ, **B.** Two-color staining for TCR-$\alpha\beta$ and TCR-$\gamma\delta$, and **C.** Two-color staining for CD4 and CD8.

restricted to small cages for 10 h and the immunoparameters examined several hours after restriction. Mice could neither move nor feed at all during that time. As a result of this treatment, the numbers of hepatic MNC and splenocytes decreased up to 30%, while that of thymocytes decreased up to 70%. Two-color staining for CD3 and IL-2Rβ showed taht the proportion of intermediate TCR cells, especially in the liver, relatively increased (Fig. 8A). The proportion of $\gamma\delta$ T cells, which was included in the intermediate TCR cell fraction, was also elevated (Fig. 8B). All the other markers remained generally unchanged.

DISCUSSION

It was demonstrated that the numbers of MNC yielded by the liver, spleen and thymus in mice prominently decreased during starvation. When attention was focused on the thymus, intrathymic T-cell differentiation was found to be greatly suppressed, showing a selective decrease in the proportion of DP $CD4^+8^+$ cells. The proportion of thymus-derived T cells (i.e., bright TCR cells) was constant in a peripheral immune organ, the spleen. Since the total number of MNC in this organ fell profoundly, the absolute number of thymus-derived T cells decreased. However, the proportion of extrathymic T cells (i.e., intermediate TCR cells) in the liver, the thymus and, to some extent, in the spleen, was inversely elevated. Even the absolute number of intermediate TCR cells, especially in the liver, was stationary or elevated during starvation. It can be concluded that the intrathymic pathway of T-cell differentiation is very sensitive to starvation, while the extrathymic pathways of T-cell differentiation are resistant.

It is easily conceivable that starvation not only cuts off supply of energy but also places great stress on the body. Toward this assumption, the level of a stress-associated glucocorticoid, i.e., corticosterone, was measured in these mice. As expected, the level of cortisosterone was elevated, showing a peak on Day 2. Since hydrocortisone administration also induced a pattern of immunomodulation similar to that seen during starvation, glucocorticoids might be one of the

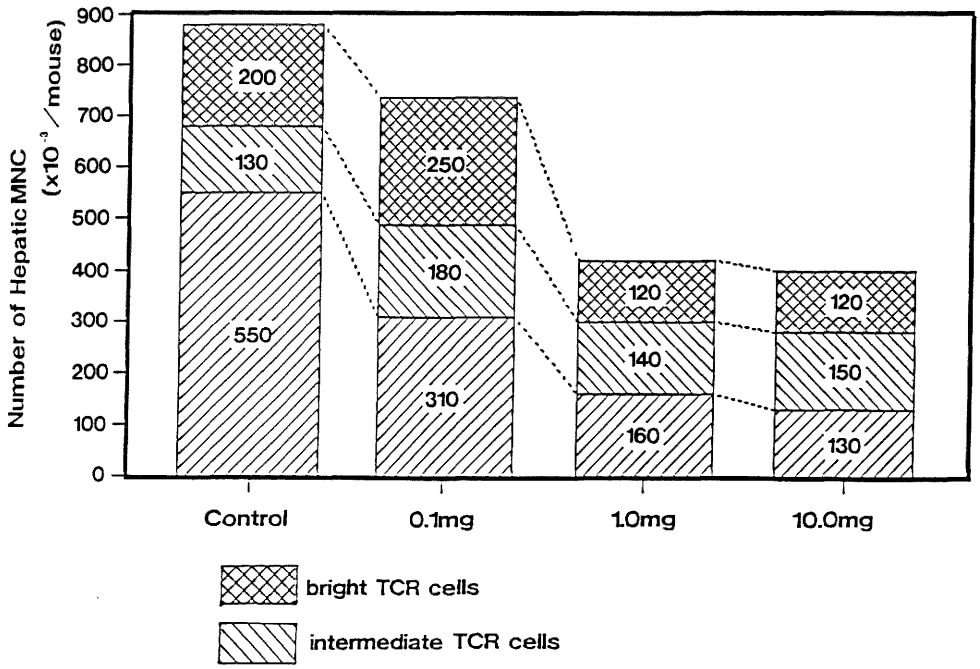

Fig. 7. Resistance of intermediate TCR cells in the liver to hydrocortisone administration. The absolute number of each lymphocyte subset was calculated by using the data from Fig. 5 and 6.

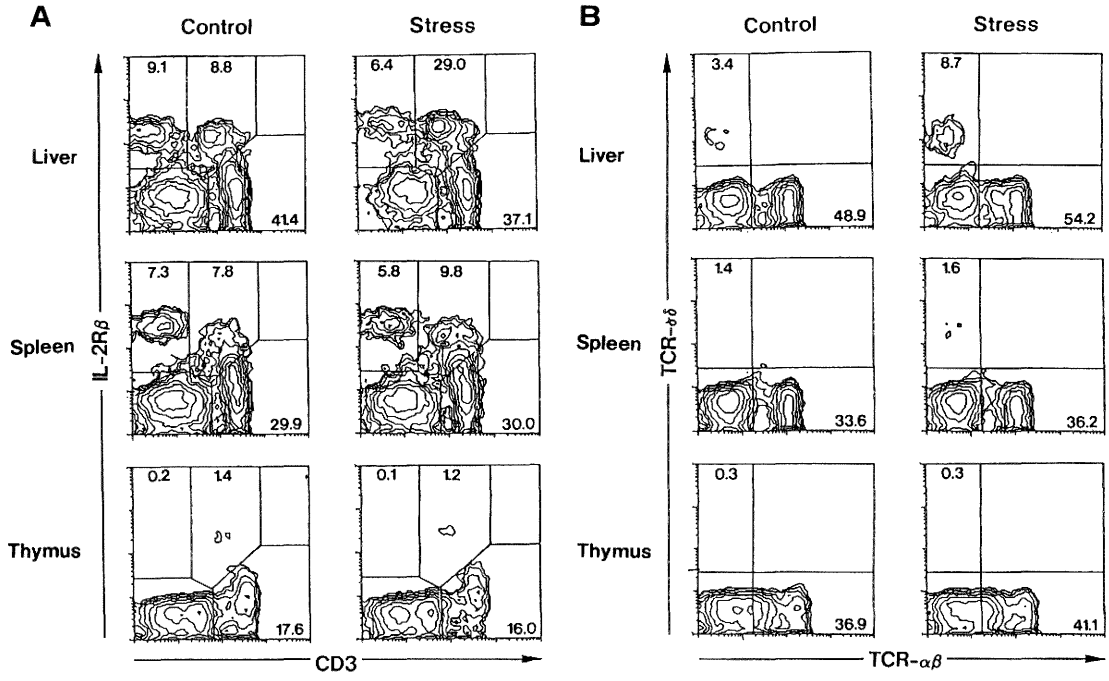

Fig. 8. Variation pattern of the proportions of lymphocyte subsets in mice subjected to restriction stress. The proportion of intermediate TCR cells and that of $\gamma\delta$ T cells have been elevated by such stress.

factors associated with immunologic states induced by starvation.

In the present study, we applied two-color staining for CD3 and IL-2Rβ to identify the lymphocyte subsets in mice. As shown previously,[12] this staining could simultaneously identify four lymphocyte subsets, namely, NK cells, extrathymic T cells, thymus-derived T cells and B cells. Major reasons for using this method are that: 1) NK cells and extrathymic T cells constitutively express IL-2Rβ, and 2) extrathymic T cells have TCR of intermediate intensity while thymus-derived T cells have TCR of bright intensity. Indeed, the *in vivo* injection of anti-asialo GM_1 antibody completely eliminated CD3$^-$IL-2Rβ^+ NK cells but did not at all eliminate CD3-intermediate$^+$IL-2Rβ^+ extrathymic T cells.[13] A more detailed characterization of each subset has been reported elsewhere.[13] Here, all $\gamma\delta$ T cells were intermediate TCR cells[13] and, reflecting this situation, the proportion of $\gamma\delta$ T cells and that of intermediate TCR cells varied almost in parallel.

The immunomodulation induced by starvation was not significantly accompanied by a modulation of CD4 and CD8 expression pattern in the peripheral immune organs. When profound thymic atrophy was induced at the final stage of starvation, the proportion of DP CD4$^+$8$^+$ cells decreased. As shown previously,[14] this pattern was produced when intrathymic T-cell differentiation was suppressed. This was true for both starvation and hydrocortisone administration.

When immunosuppression was induced by starvation or glucocorticoid administration, intermediate TCR cells became prominent even in the thymus. In our recent studies[14,15] we demonstrated that small proportion of intermediate TCR cells exist in the thymic medulla. Usually, they are obscured by a large number of immature T cells. T cells with properties similar to those of the intermediate TCR cells in our studies have been demonstrated by other investigators.[16-20] They considered population as T cells which are generated by an alternative pathway of T-cell differentiation rather than by the conventional pathway in the thymus. However, no one has yet proposed that this alternative pathway in the thymus and the extrathymic pathways seen in the liver should both be categorized as primitive pathways of T-cell diferentiation. We have proposed the possibility that such primitive pathways exist at multiple sites in the body.[21,22]

In the final experiment, we demonstrated that stress due to physical restriction also induced suppression of intrathymic T-cell differentiation. However, the extrathymic pathways were quite resistant to such suppression, and even seemed to be rather activated. As shown in a series of our studies,[21,22] extrathymic pathways of T-cell differentiation and T cells derived from such pathways appear to be primitive in phylogenetic development. These primitive T cells as well as NK cells were quite resistant to whole body irradiation (e.g., 6 to 9 Gy).[10] Taken together with the present data, it is concluded that primitive lymphocytes and their differentiation pathways are also resistant to starvation and to glucocorticoid-induced immunosuppression.

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