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Lymphotoxin activates hepatic T cells and simultaneously induces profound thymic atrophy

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SUMMARY

We have recently demonstrated that the liver may be a major site of extrathymic T-cell differentiation. This hepatic pathway was shown to be activated in mice injected with heat-killed bacteria. It is conceivable that the resulting activation of macrophages or lymphocytes, and the production of cytokines may be responsible for a subsequent activation of hepatic T cells. In this context, we investigated the possibility of whether certain cytokines may activate hepatic T cells. It was demonstrated that the administration of lymphotoxin [tumour necrosis factor-β (TNF-β)] more than doubled the number of hepatic mononuclear cells (MNC) yielded 3–5 days after the treatment. More strikingly, such treatment induced profound thymic atrophy and resulted in a decrease of more than 95% in the number of thymocytes. Spontaneous proliferation in an *in vitro* culture of hepatic MNC from treated mice increased, and inversely such activity of thymocytes decreased. The increased number of hepatic MNC was mainly due to an increase in intermediate $\alpha\beta$ T-cell receptor (TcR) cells, which are extrathymic T cells uniquely seen in the liver. On the other hand, the thymic atrophy was caused by the prompt apoptotic death of dull $\alpha\beta$ TcR cells with double-positive (DP) $CD4^+$ $CD8^+$ phenotype. These results indicate that lymphotoxin may be one of the factors that activates extrathymic T cells in the liver and at the same time inhibits intrathymic T-cell differentiation.

INTRODUCTION

It is well established that T cells differentiate in the thymus.[1,2] However, to account for the presence of unique T cells in the peripheral lymphoid organs of athymic nude mice[3,4] and aged mice bearing the involuted thymus,[5,6] the existence of extrathymic pathways has been postulated. We recently proposed that the liver might be a major site for extrathymic T-cell differentiation.[7–9] Although the hepatic pathway is relatively minor in the young, this pathway becomes prominent under conditions of autoimmune diseases,[7,10,11] malignancies,[8,9] ageing[6] and bacterial stimulation.[12,13]

Precise characterization of $\alpha\beta$ T cells in the liver was therefore performed in our recent studies. A most striking evidence was that not only thymocytes but also liver mononuclear cells (MNC) showed a two-peak pattern of $\alpha\beta$ T-cell receptor (TcR) in terms of their immunofluorescence staining intensity.[10] Dull TcR cells in the thymus are immature T cells occurring before a process of negative or positive selection and predominantly expressing the double-positive (DP) $CD4^+$ $CD8^+$ phenotype.[1,2] On the other hand, bright TcR cells in the thymus are mature T cells occurring after the selection and expressing the single-positive $CD4^+$ or $CD8^+$ phenotype. In contrast, dull TcR cells in the liver should be called 'intermediate' TcR cells, since hepatic dull TcR is somewhat brighter than thymic dull TcR.[10,14] Intermediate TcR cells in the liver consist of double-negative (DN) cells and single-positive $CD4^+$ or $CD8^+$ cells. These cells might be extrathymic T cells, since athymic nude mice[14] and thymectomized mice have only intermediate TcR cells in the liver.[15] Bright TcR cells seen in the liver, i.e. the intensity of such TcR being as bright as that of bright TcR in the thymus, are of thymic origin. An abnormally expanding population of DN $CD4^-$ $CD8^-$ $\alpha\beta$ T cells in autoimmune MRL-*lpr*/*lpr* mice was also demonstrated to be extrathymic T cells generated in the liver.[7,10,11]

We have recently demonstrated that the hepatic pathway in mice was activated by the injection of heat-killed bacteria.[12,13] Such stimulation coincidentally inactivated the intrathymic pathway of T-cell differentiation. Macrophages or lymphocytes activated by bacterial stimulation or the production of cytokines might be responsible for a subsequent event of each pathway. We have investigated the possibility of whether certain

Abbreviations: DN, double-negative; DP, double-positive; MNC, mononuclear cells.

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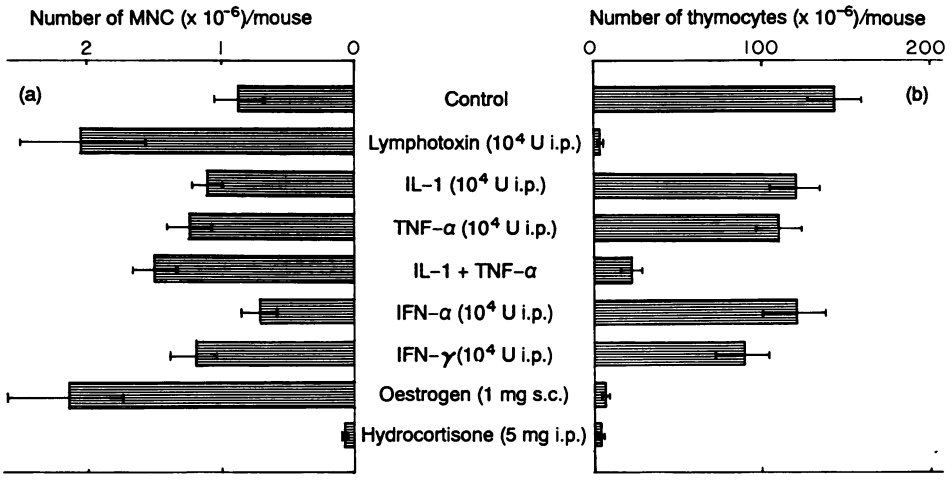

Figure 1. A comparison of total number of MNC in the liver (a) and thymus (b) in mice treated with cytokines and others. Three days after the injection of various agents, mice were killed and the numbers of MNC in the liver and thymus were enumerated. Data express the mean ± 1 SD of four tested mice.

cytokines mediate such functions. Among the many cytokines tested including interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, interferon-α (IFN-α), IFN-γ, tumour necrosis factor-α (TNF-α) and lymphotoxin (TNF-β), lymphotoxin induced a prominent increase in hepatic MNC. Such treatment induced a decrease of more than 95% in the number of thymocytes. It was suggested that lymphotoxin might play an important role in modifying the two pathways of T-cell differentiation.

MATERIALS AND METHODS

Mice

Male C3H/He mice, aged 6–8 weeks, were obtained from Charles River Japan Inc. (Tokyo, Japan). They were fed under specific pathogen-free conditions.[8]

Lymphotoxin administration

Human recombinant lymphotoxin (Kanegafuchi Chemical Industry Co., Takasago, Japan) was used.[16] Lymphotoxin units were determined by cytotoxicity assay against TNF-α and lymphotoxin-sensitive mouse fibroblasts in the presence of 1 μg/ml actinomycin D.[16,17] Units of lymphotoxin used here corresponded to those of human recombinant TNF-α (Dainippon Seiyaku, Inc., Tokyo, Japan), which was an internal standard of TNF-α and lymphotoxin bioassays. Lymphotoxin was subcutaneously injected and mice were killed at the indicated days. To investigate the unique effects of lymphotoxin, murine recombinant IL-1α, TNF-α, IFN-α and IFN-γ, which were kindly provided by Genetics Institute (Cambridge, MA) were used. Oestrogen (Ovahormone depo; Teikoku Zoki, Inc., Tokyo, Japan) and hydrocortisone (Sigma Chemical Co., St Louis, MO) were also used.[18]

Cell preparations

Mononuclear cells were prepared from the liver, thymus and spleen. Liver MNC were obtained as previously described.[7,8] To obtain MNC, the liver was pressed through 100-gauge stainless steel mesh and suspended in RPMI-1640 medium supplemented with 5% foetal calf serum. After being washed once with the medium, the cells were resuspended in 20 ml of the medium and MNC were isolated from parenchymal hepatocytes by Ficoll–Isopaque density (1·090) gradient centrifugation. In the MNC preparation, the proportion of Kupffer cells was negligible (<4%). Mononuclear cells of the spleen were also collected by the Ficoll–Isopaque method, whereas thymocytes were obtained by forcing the thymus through 100-gauge mesh.

Cell proliferation assay

Spontaneous cell proliferation was analysed by [^{3}H]thymidine uptake into DNA.[7,8] Briefly, freshly isolated MNC (5×10^5 cells/0·2 ml medium) were cultured in a 96-well round-bottomed microplate (Falcon, Oxnard, CA) for 18 hr at 37° in a CO_2 incubator. One-half μCi of [^{3}H]thymidine (Amersham International, Amersham, U.K.) was added at the initiation of culture. The medium used was RPMI-1640 supplemented with 1% fresh mouse serum and 5×10^{-5} M 2-mercaptoethanol. The data are expressed as the mean c.p.m. ± 1 SD of triplicate cultures.

Immunofluorescence test

Surface phenotypes of cells were identified by using monoclonal antibodies (mAb) in conjunction with a single- or two-colour immunofluorescence test. The mAb used included biotin-conjugated hamster anti-αβ TcR (H57-597),[19] unconjugated anti-γδ TcR (3A10)[20] and fluorescein isothiocyanate (FITC)-conjugated hamster anti-CD3 (145-2C11) mAb.[7] Biotin-conjugated reagent was developed with PE-conjugated avidin, whereas unconjugated hamster reagent was developed with PE-conjugated goat anti-hamster Ig. FITC-conjugated rat anti-CD4 (L3T4) and PE-conjugated anti-CD8 (Lyt-2) mAb were also used (Becton Dickinson, Mountain View, CA). The fluorescence-positive cells were analysed by a FACScan (Becton Dickinson). Ten thousand cells were analysed to make the profiles.

Electron microscopy

Thymus specimens were quickly fixed in 3% glutaraldehyde, followed by post-fixation in 1% OsO_4. They were embedded in Epon 812. The ultrathin sections were stained with lead citrate

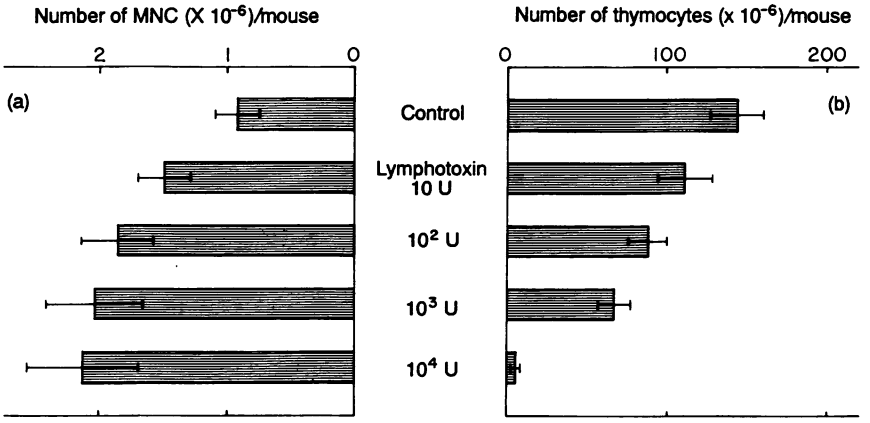

Figure 2. Dose kinetics of lymphotoxin based on the effects of the numbers of hepatic MNC (a) and thymocytes (b). Three days after lymphotoxin administration, the numbers of hepatic MNC and thymocytes were enumerated. Data express the mean ± 1 SD of four tested mice.

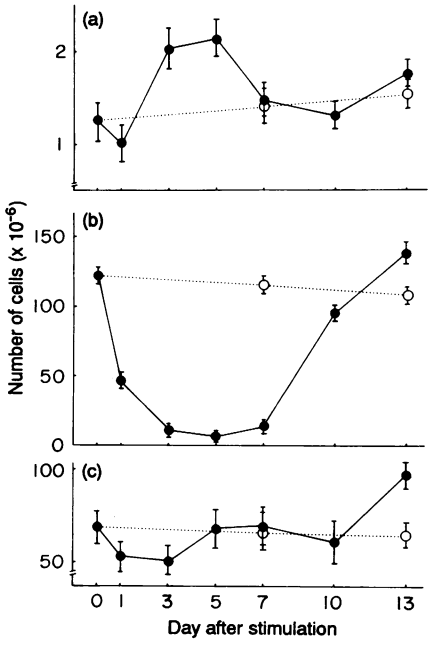

Figure 3. Variation in the number of MNC in the liver (a), thymus (b) and spleen (c) after lymphotoxin administration. After a single intraperitoneal injection of lymphotoxin (10^4 U/mouse), four mice (●) were examined at each of the indicated days. Untreated mice were examined in parallel (○). Data express the mean ± 1 SD of four tested mice.

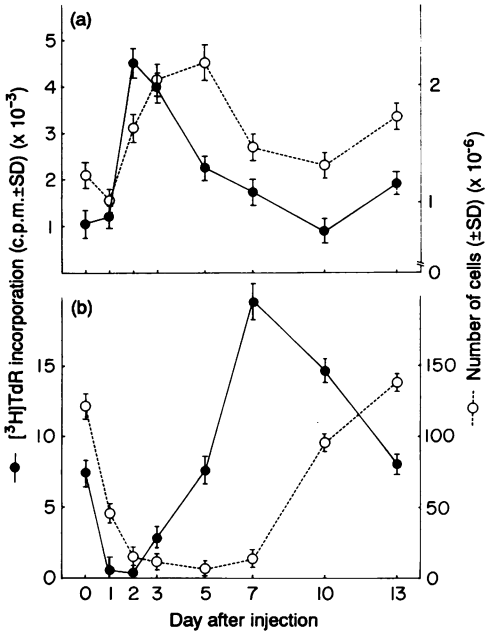

Figure 4. Time kinetics of the spontaneous proliferating activity and the number of hepatic MNC (a) and thymocytes (b) after lymphotoxin administration. Spontaneous proliferating activity (●) was examined for an 18-hr incubation of freshly isolated MNC. The numbers (○) of hepatic MNC and thymocytes are represented in parallel. The value at each of the indicated days is the average of four treated mice or four untreated mice (Day 0). Data express the mean ± 1 SD.

and were examined with a JEOL 100B electron microscope (JEOL, Peabody, MA).[11]

RESULTS

Lymphotoxin increased the number of hepatic MNC and decreased the number of thymocytes

Several cytokines, including lymphotoxin, IL-1α, TNF-α, IFN-α and IFN-γ (10^4 U of each per mouse) were subsequently injected and the effects on the number of liver MNC and the number of thymocytes were examined 3 days after the injection (Fig. 1). Since a preliminary study suggested that administrations of lymphotoxin and other reagents (e.g. hydrocortisone) induced the thymic atrophy on Days 2–7, the effects of these injections on Day 3 were represented. The effects of oestrogen (1 mg s.c./mouse) and hydrocortisone (5 mg i.p./mouse) were examined in parallel. As shown in the figure, approximately $0{\cdot}9 \times 10^6$ cells were yielded by the liver, whereas 140×10^6 cells were yielded by the thymus. Interestingly lymphotoxin induced a drastic increase in the number of hepatic MNC and inversely decreased the number of thymocytes less than 5×10^6 cells were yielded by the atrophic thymus of mice injected with lymphotoxin. On the other hand, a single injection of IL-1α or TNF-α induced only a

Table 1. Phenotypic analysis of MNC in the liver, spleen and thymus of mice with or without lymphotoxin administration

Organ	Lymphotoxin	% fluorescence positive cells of MNC					
		αβ TcR	γδ TcR	CD3	CD4	CD8	CD4$^+$ CD8$^+$
Liver	(−)	46·0±17·0	11·6±3·0	52·3±10·0	35·9±9·6	10·2±2·9	<1·0
	(+)	51·3±11·3	5·7±3·2*	53·8±11·8	36·3±9·6	8·3±2·4	<1·0
Spleen	(−)	22·5±3·0	0·8±0·4	20·5±1·1	17·6±2·0	6·7±2·2	<1·0
	(+)	34·0±2·1*	3·6±3·0*	29·2±3·2*	28·9±4·1*	10·5±2·3*	<1·0
Thymus	(−)	56·5±5·1	<0·1	54·0±8·0	13·6±3·2	3·5±1·5	79·9±5·8
	(+)	86·7±3·3*	<0·1	91·0±1·7*	50·2±5·2*	16·1±4·5*	27·9±9·3*

* Significant effects of lymphotoxin on the phenotype ($P<0·01$).

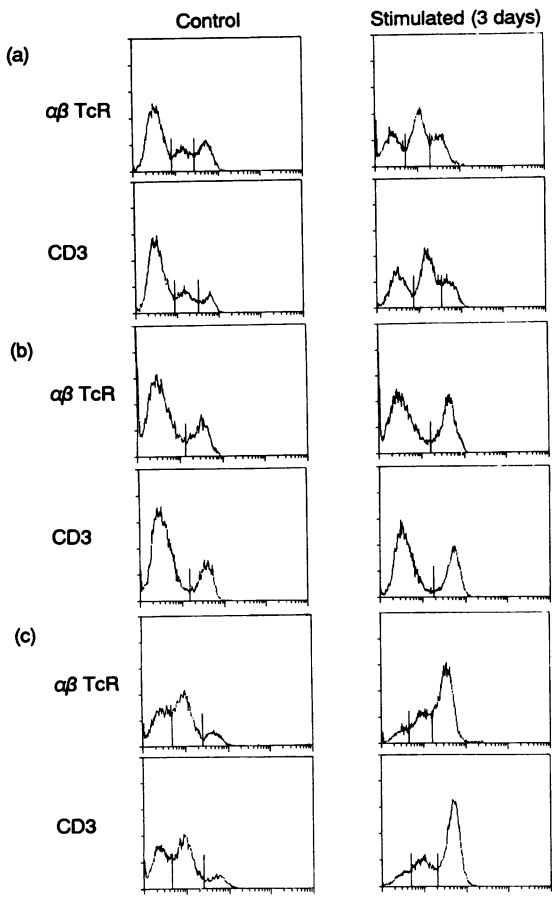

Figure 5. A comparison of the phenotypic pattern of αβ TcR and CD3 stainings of the liver, spleen and thymus before and after lymphotoxin administration. MNC isolated from the liver (a), spleen (b) and thymus (c) were stained by a single-colour immunofluorescence test using anti-αβ TcR or anti-CD3 mAb.

mild increase in the number of MNC and a mild decrease in the number of thymocytes. However, a simultaneous injection of both IL-1α or TNF-α induced a considerable increase in the number of liver MNC and considerable decrease in the number of thymocytes. Interferon-α and IFN-γ did not significantly change the number of hepatic MNC, whereas they reduced slightly the number of thymocytes. Oestrogen also increased the number of hepatic MNC and decreased the number of thymocytes.[18] Hydrocortisone injections decreased the number of cells yielded from both the liver and thymus.

Dose kinetics of lymphotoxin was then investigated, based on the numbers of hepatic MNC and thymocytes on Day 3 after treatment (Fig. 2). Although the maximum effect of lymphotoxin was induced at a dose of 10^4 U/mouse, the smaller doses also induced the increase in the number of hepatic MNC and the decrease in the number of thymocytes up to 10^2 U/mouse ($P<0·01$ by Student's t-test).

Time kinetics of the cell number and the activity of spontaneous proliferation of MNC from the liver, thymus and spleen after lymphotoxin administration

We investigated precise time kinetics of variation in the numbers of MNC in the liver, thymus and spleen of mice injected with lymphotoxin (Fig. 3). An increased number of heptaic MNC was seen on Days 3 and 5 after the injection. In contrast, the number of thymocytes rapidly decreased on Day 1, reached the baseline on Day 5 and then returned to the normal level until Day 10 after the injection. The variation of splenic MNC was minimal.

The amount of spontaneous cell proliferation was then investigated (Fig. 4), since we previously reported that freshly isolated hepatic MNC and thymocytes, but not MNC of the spleen and lymph nodes, could spontaneously proliferate in *in vitro* cultures. Such proliferation continued for more than 12 hr after isolation. Interestingly, the peak in the increased amount of spontaneous proliferation of hepatic MNC (Day 2) appeared to precede the increase in the number of heptaic MNC (Day 5). Similarly, the period of decreased spontaneously proliferation of thymocytes (Days 2–7). Moreover, the restoration in the amount of spontaneous cell proliferation of thymocytes (Days 5–7) also preceded the restoration in the number of thymocytes (Days 10–13).

Phenotypic analysis of lymphocytes in the liver, spleen and thymus before and after lymphotoxin administration

Phenotypic analysis of MNC in the liver, spleen and thymus were then performed by a single-colour immunofluorescence test (Table 1). It was confirmed that hepatic MNC in untreated mice consisted of large proportions of both αβ and γδ T cells. After lymphotoxin administration, i.e. 3 days after the injection,

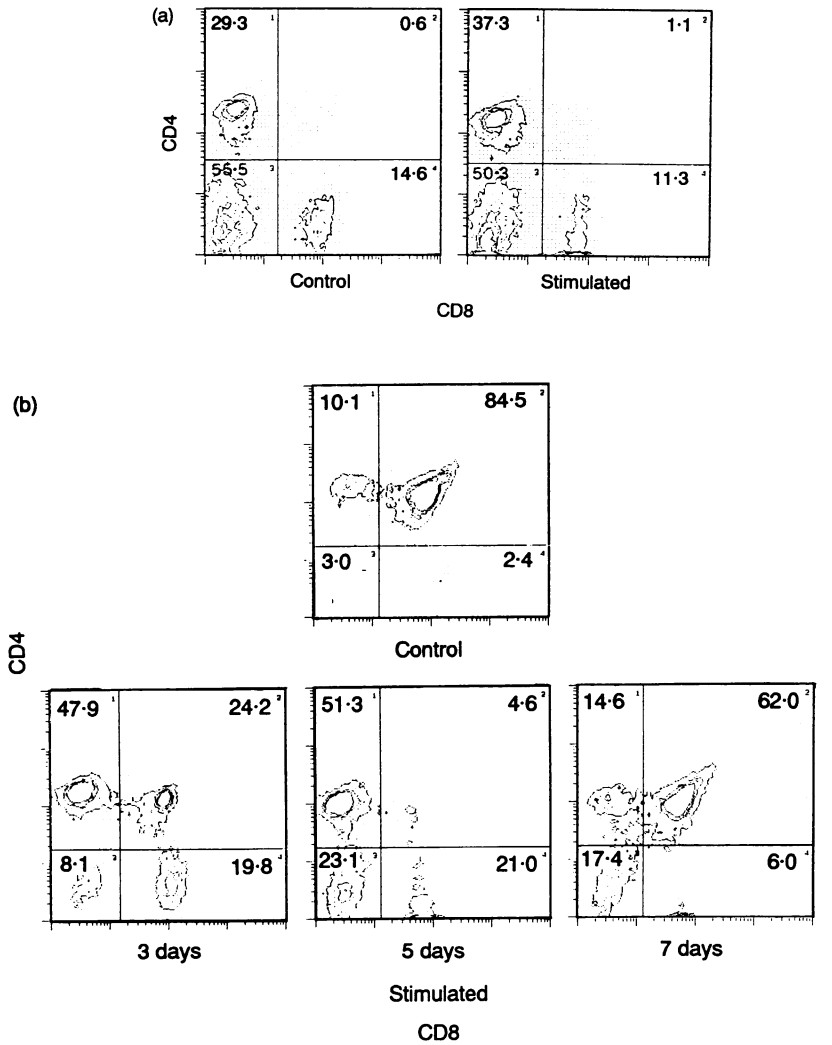

Figure 6. Profiles of flow cytometry in the staining of a two-colour immunofluorescence test using FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 mAb. Liver MNC (a) and thymocytes (b) in mice with or without the treatment were examined. The numbers in the figure express the percentages of fluorescence positive cells in each square.

none of the T-cell markers tested significantly changed, except that the percentage of $\gamma\delta$ TcR$^+$ cells slightly decreased (11·6–5·6%). It was estimated that the absolute number of both CD4$^+$ and CD8$^+$ $\alpha\beta$ T cells increased after the treatment, because the total number of hepatic MNC more than doubled. Two-colour immunofluorescence test using PE-conjugated anti-$\alpha\beta$ TcR mAb and a mixture of FITC-conjugated anti-CD4 and anti-CD8 mAb also demonstrated that a significant proportion of DN $\alpha\beta$ T cells (up to 12%) appeared in the liver after the injection (data not shown). Although the number of splenic MNC was not changed, the proportions of both $\alpha\beta$ and $\gamma\delta$ T cells in the spleen were elevated. Most striking changes were observed in the phenotype of thymocytes. The major population of DP CD4$^+$ CD8$^+$ cells in the thymus of untreated mice decreased drastically after the treatment (79·9–27·9%). Inversely, relative proportions of single-positive CD4$^+$ and CD8$^+$ cells were elevated, although the absolute numbers of CD4$^+$ and CD8$^+$ cells decreased due to a drastic decrease in the total number of thymocytes after the treatment.

Changes in the staining pattern of $\alpha\beta$ TcR and CD3 in liver MNC and thymocytes

In previous studies,[10,14] we demonstrated that MNC obtained from not only the thymus but also the liver showed a two positive-peak pattern of $\alpha\beta$ TcR and CD3 in terms of their immunofluorescence staining intensity. We then examined whether the two positive-peak pattern varied with lymphotoxin administration (Fig. 5). Three days after the injection, the relative proportion of intermediate TcR cells in the liver significantly increased as estimated by both $\alpha\beta$ TcR and CD3 staining (Fig. 5a). Concerning the increased number of hepatic MNC after treatment, the absolute number of intermediate TcR cells increased drastically. A single-peak pattern of TcR in the spleen did not vary irrespective of the treatment (Fig. 5b). On the other hand, the two-peak pattern of TcR in thymocytes showed a unique change (Fig. 5c). A major population of dull TcR cells in the thymus decreased and bright TcR cells inversely increased as a result. Again, due to the drastic decrease in the

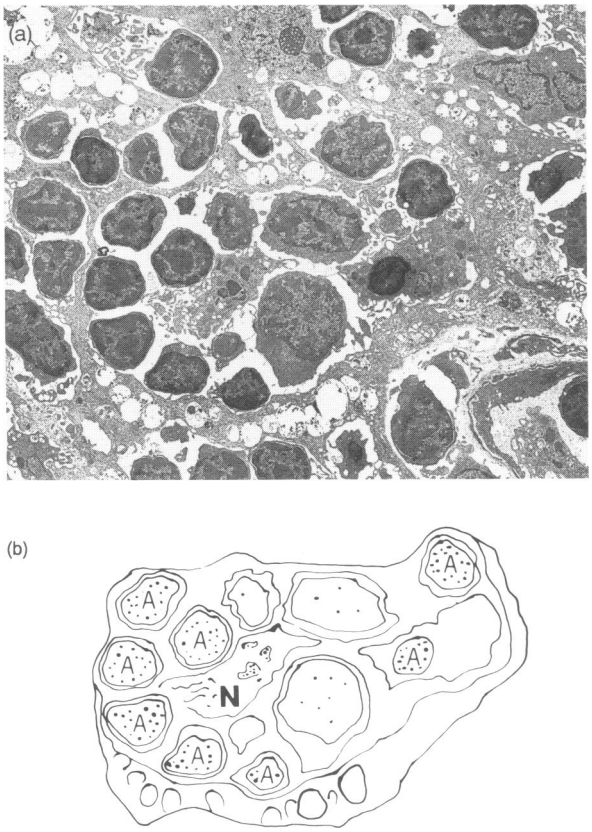

Figure 7. (a) Electron microscopy of the thymus in mice after lymphotoxin administration (×3500). Morphological change in the thymus was examined in mice 1 day after administration. (b) Schematic representation: thymic nurse cells contained many apoptotic lymphocytes (A) and normal lymphoblasts in their cytoplasms.

number of thymocytes caused by the treatment, the absolute number of bright TcR cells decreased.

The actual profiles of the two-colour immunofluorescence test for CD4 and CD8 staining were also represented (Fig. 6). This figure shows a representative result from the experiments outlined in Table 1. In hepatic MNC, DP cells were a minor population irrespective of lymphotoxin administration (Fig. 6a). Significant populations of single-positive $CD4^+$ and $CD8^+$ cells were present. As shown previously,[10] $\alpha\beta$ T cells constituted 10–15% of the DN $CD4^-$ $CD8^-$ cell population seen in this figure. The rest of the cells lacking CD4 and CD8 antigens were sIg^+ B cells and asialo $GM1^+$ natural killer (NK) cells. In sharp contrast, thymocytes constituted a large population of the DP cells (Fig. 6b). However, this population decreased promptly after lymphotoxin administration on Days 3 and 5. This effect was reversible. Almost normal patterns of CD4 and CD8 staining, accompanying the restoration of thymocyte number yielded, were observed 7 days after treatment.

Appearance of many apoptotic cells in the thymus after lymphotoxin administration

The morphology of the thymus was investigated by electron microscopy before and after treatmemt (Fig. 7). Although negative selection of autoreactive T-cell clones was seen to be intense in the thymus, apoptotic cells in the thymus were not so prominent in normal mice. On the other hand, apoptotic cells became prominent Day 1 after treatment (Fig. 7a). As shown in the scheme of this figure (Fig. 7b), many apoptotic cells (indicated as A) resided in the cytoplasm of thymic nurse cells (N, nucleus of thymic nurse cell). Two days after the treatment only a few cells, including both normal and apoptotic thymocytes, were seen in the atrophic thymus.

DISCUSSION

We demonstrated that lymphotoxin activates hepatic T cells and simultaneously induces profound thymic atrophy, that is a single injection of lymphotoxin increased the number of hepatic MNC and decreased the number of thymocytes. More importantly, the increased number of hepatic MNC was accompanied by an increase in the spontaneous proliferation in *in vitro* culture and the enrichment of intermediate TcR cells which are extrathymic $\alpha\beta$ T cells only seen in the liver.[10,13] The decreased number in thymocytes was accompanied by a decrease in spontaneous proliferation and in immature T cells with DP phenotype and dull TcR. All these changes were reversible. As shown by our previous data, when the hepatic pathway was in an activated state, the intrathymic pathway was reciprocally inactivated, especially under conditions of ageing,[6] bacterial stimulation[12,13] and malignancies.[8,9] Therefore, both pathways might be modified or regulated by common, undetermined factors, and lymphotoxin might be one of the potent physiological mediators regulating the above responses.

Lymphotoxin is one of the first lymphokines to be discovered and it is produced by activated T lymphocytes.[21] Such lymphotoxin is thought to mediate the cytotoxic functions of certain tumours[22,23] and also to activate macrophages,[24] endothelial cells and other types of cells.[25,26] Due to the homology of molecules and the similarity in biological activities between lymphotoxin and TNF, the latter of which is produced by macrophages, they are termed TNF-β and TNF-α, respectively.[21] However, the ability of lymphotoxin to induce a modulation of the pathways was stronger than that of TNF-α. TNF-α showed considerable activity as described above in combination with IL-1. As hepatic $\alpha\beta$ T cells resided on the sinusoidal endothelial cells or Kupffer cells, the activating effect of lymphotoxin on these cells might be responsible for the subsequent activation process. On the other hand, the well-known ability of lymphotoxin to fragment DNA[27] might be related to the acceleration of apoptosis in thymocytes. It is known that an apoptotic process of thymocytes is associated with DNA fragmentation.[28]

Cumulative evidence has revealed that thymic atrophy is always present when the hepatic pathway is activated,[8,9,12,13] Most recently, we have demonstrated that higher invertebrates without thymic development (e.g. *Crustacea*) and the most primitive vertebrates having only the protothymus (e.g. lamprey) have abundant resting and blastic lymphoid cells in the sinusoids of the liver. Therefore, the hepatic pathway seems to be the earliest phylogenetic site for lymphocyte differentiation.[15] The hepatic pathway lacks a DP stage for negative selection and is even able to produce self-reactive T-cell clones.[12] In this respect, this pathway may be beneficial for the surveillance of

atypical cells generated in higher and lower vertebrates. However, an overstimulation of the hepatic pathway might be responsible for the onset of certain autoimmune diseases.[7,10,11]

Intermediate TcR cells seen in the liver may be unique extrathymic T cells. Thus, athymic nude mice[14] and thymectomized mice (i.e. those 2 months after thymectomy)[15] had only intermediate TcR cells. A considerable proportion of intermediate TcR cells showed lymphoblastic morphology and the ability to proliferate in *in vitro* culture.[7,8] Such proliferation continued for approximately 12 hr after cell isolation. Taken together, these findings indicate that early precursors, which probably come from the bone marrow, differentiate in the hepatic sinusoids. Although extrathymic and intrathymic pathways were independent processes, their earliest progenitors may share common stem cells,[14] because scid mice, which lack lymphocytes of both T-cell and B-cell lineage, have neither intrathymic T cells in the thymus nor extrathymic T cells in the liver. A small proportion of intermediate TcR cells seemed to migrate to the periphery in athymic nude mice[14] and in mice subjected to bacterial stimulation,[12] and a large proportion of intermediate TcR cells could migrate to the periphery in autoimmune MRL-*lpr/lpr* mice.[7,10,11] However, a single injection of lymphotoxin did not induce such cells in the periphery. We consider that lymphotoxin treatment, especially the single injection, was not strong enough to induce the migration of intermediate TcR cells to the periphery.

It is well established that steroid hormones, glucocorticoids, induce profound thymic atrophy.[29] However, lymphotoxin effects on thymocytes may not be mediated by the secondary effect of glucocorticoids, because the induced levels of glucocorticoids were not high enough (A. Kusmi, T. Abo, T. Masuda, K. Sugiura, S. Seki, T. Ohteki, R. Okuyama and K. Kumagai, unpublished data). To induce profound thymic atrophy, we found that 2–5 mg hydrocortisone should be injected into mice and this dose corresponds to at least more than 50 μg/ml plasma corticosterone.[13] Moreover, the high doses of glucocorticoids simultaneously induced the decrease in the numbers of hepatic MNC and thymocytes (see Fig. 1). In other words, glucocorticoid is a potent immunosuppressant of both extrathymic and intrathymic T cells. Fu *et al.* have recently demonstrated that even thymic atrophy induced by tumour burden in mice was mediated by cytokines (e.g. IL-1) rather than stress-associated hormones, glucocorticoids.[30] We revealed that such tumour burden also induced the activation of extrathymic in the liver.[9] Taken together, it is conceivable that cytokines such as lymphotoxin, TNF-α and IL-1 may be physiological factors which modulate the extra- and intrathymic pathways of T-cell differentiation.

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