*Biotherapy* 5: 167–176, 1992.


# Selective activation of extrathymic T cells in the liver by glycyrrhizin

Motohiko Kimura, Hisami Watanabe & Toru Abo
*Department of Immunology, Niigata University School of Medicine, Niigata 951, Japan*

Received 13 February 1992; accepted 20 February 1992

*Key words*: extrathymic T cells, glycyrrhizin, liver

**Abstract**

Extrathymic pathways for T cell differentiation were recently demonstrated in the liver, intestine and omentum. In this study, glycyrrhizin (GL), a plant extract was investigated as to its effect on extrathymic T cells in the liver of mice. A new method using anti-LFA-1 mAb in conjunction with anti-TCR or -CD3 mAbs to sensitively identify such extrathymic T cells is included. Single injection and repeated injections of GL increased not only the number of total hepatic MNC but also the proportion of intermediate TCR cells, which are extrathymic T cells uniquely seen in the liver. In contrast to other tested reagents (e.g., lymphotoxin and estrogen) that activated the extrathymic T cells and simultaneously induced profound thymic atrophy, GL did not affect regular T cells in the thymus. The present results suggest that the selective activation of extrathymic T cells in the liver might be intimately related to the clinical effects of GL.

*Abbreviations*: GL: glycyrrhizin; TCR: T cell receptor; mAb: monoclonal antibody; DP: double-positive; DN: double-negative; MHC: major histocompatibility complex; MNC: mononuclear cells.

## Introduction

It has been well established that T lymphocytes differentiate in the thymus [1, 2]. Since the majority of precursor T cells which enter the thymus comprise self-reactive forbidden clones, they are eliminated by negative selection at the double-positive (DP) $CD4^+8^+$ stage and only a minor population migrates to the periphery after maturation [1–5]. In this regard, T cells in the periphery primarily recognize foreign antigens in the context of self-major histocompatibility complex (MHC) antigens. On the other hand, several extrathymic pathways of T cell differentiation have been reported, e.g., in the liver [6, 7], intestine [8, 10] and omentum [10]. We demonstrated that the liver was a major site of extrathymic T cell differentiation [6, 7]. Although the hepatic pathway is extremely minor at young ages, it becomes predominant at older ages [12] and under conditions of bacterial stimulation [13], malignancies [7, 14] and autoimmune diseases [5, 15, 16]. T cells generated in the liver mainly consist of self-reactive forbidden clones (estimated with anti-V$\beta$ mAbs) and self-reactive V$\beta 8^+$ clones, possibly due to the lack of the DP stage for negative selection [13]. Activated extrathymic T cells might be beneficial for the surveillance of atypical cells generated *in vivo*, for example, under conditions of aging, bacterial infections and malignancies. However, an overstimulation of extrathymic T cells might be responsible for the onset of certain autoimmune diseases by virtue of their autoreactivity. Extrathymic T cells in the liver have been shown to have other unique properties: they had TCR of intermediate intensity (i.e., intermediate TCR cells) and comprised a significant proportion of double-negative (DN) $CD4^-8^-$ cells as well as single-positive $CD4^+$ or $CD8^+$ cells [15, 17].

Glycyrrhizin (GL), is an aqueous extract from licorice root (*Glycyrrhiza radix*) [18–21]. This

compound consists of glycyrrhetinic acid and two molecules of glucuronic acid (Fig. 1). GL is known for its anti-inflammatory property in Chinese medicine and is widely used as a therapeutic agent for viral hepatitis, chronic active hepatitis, and various allergic diseases, especially in Japan. More recently, high doses of Stronger Neo-Minophagen C, comprised mainly of GL, was found to be effective in the treatment of HIV-1 infected hemophilia patients as shown by improvements observed in their clinical symptoms, and in their immunological and liver functions [22]. The major functions so far described include inhibition of viral replication [18-21], reduction of protein kinase C activity [23], improvement of hepatocyte dysfunction [24, 25], induction of interferon [26] and immunopotentiation of macrophages and NK cells [27]. However, these functions do not seem to thoroughly explain the unique effects of GL.

In the present study, we examined whether GL modulates the extrathymic pathway of T cell differentiation in the liver of mice, since the liver seems to be a key organ for GL effects. We found that many conditions (e.g., bacterial stimulation and aging) [12, 13] and reagents (e.g., estrogen and lymphotoxin) [28], which augment extrathymic T cell differentiation, simultaneously induce a suppression of intrathymic T cell differentiation. In this regard, the effect of GL on thymocytes was examined in parallel.

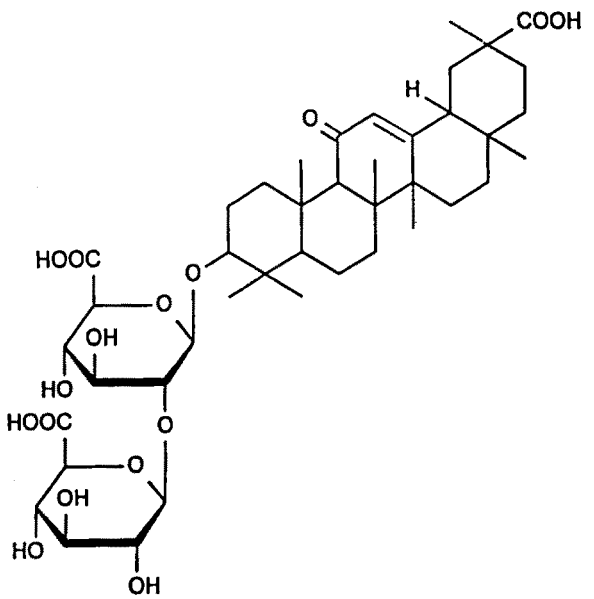


*Fig. 1.* The structure of glycyrrhizin.

## Materials and methods

*Mice.* C3H/He mice at 6 to 8 weeks of age were purchased from Charles River Japan, Inc., Atsugi, Japan [13]. They were fed under specific pathogen-free conditions in our laboratory until use.

*Cell preparations.* Hepatic mononuclear cells (MNC) were prepared as previously described [17]. Briefly, mice anesthetized with ether were killed by total bleeding by incision of the axillary artery and vein. As MNC resident in the liver are estimated to be much more predominant (> 80%) compared to the MNC contaminated by circulation in this bleeding method, PBS perfusion was not applied here. The removed liver was cut into small pieces with scissors, pressed through 200-gauge stainless steel mesh, and suspended in Eagles MEM with 5 mM HEPES (Sigma Chemical Co., St. Louis, U.S.A.) and 2% heat-inactivated newborn calf serum. After being washed once with the medium, the cell pellet was resuspended in 30 ml of the medium. MNC were isolated from parenchymal hepatocytes, nuclei of hepatocytes and Kupffer cells by Ficoll-Isopaque density (1.090) gradient centrifugation. Blood MNC and splenic MNC were also isolated by the Ficoll-Isopaque method [17]. Thymocytes and lymph node cells were obtained by forcing the thymus and the inguinal lymph nodes through 200-gauge steel mesh.

*Immunofluorescence test.* Surface phenotypes of cells were identified by using mAbs in conjunction with a single- or two-color immunofluorescence test [17]. The mAbs used here included FITC-, PE- or biotin-conjugated reagents of hamster anti-$\alpha\beta$ TCR (H57-597), anti-CD3 (145-2C11), and anti-LFA-1 (FD441.8) mAbs [17]. Biotin-conjugated reagents were developed with PE-conjugated avidin (Caltag Laboratories, San Francisco, U.S.A.). The fluorescence-positive

cells were analyzed by a FACScan (Becton-Dickinson Co., Mountain View, CA, U.S.A.).

*GL and other reagents.* GL was subcutaneously injected into mice at the indicated amounts (e.g., 2 mg/mouse). The effect of GL on immunoparameters was examined after a single injection or repeated injections. In order to show the unique effect of GL, various cytokines and hormones were used. The cytokines used here were TNF$\alpha$ (Dainippon Seiyaku, Inc., Tokyo, Japan), lymphotoxin (TNF$\beta$) (Kanegafuchi Chemical Co., Takasago, Japan), IFN$\alpha$, IFN$\gamma$, and IL-1$\alpha$ (Genentics Institute, Cambridge, U.S.A.) [28]. Estrogen (Ovahormone depo; Teikoku Zoki, Inc., Tokyo, Japan) and hydrocortisone (Sigma Chemical Co.) were also used [28].

## Results

### *Identification of intermediate TCR cells in the liver*

When hepatic MNC were stained with single-color immunofluorescence tests using anti-$\alpha\beta$TCR or anti-CD3 mAb (Fig. 2), they showed a two-peak pattern of $\alpha\beta$TCR or CD3, constituting dull and bright TCR cells (Fig. 2A and B). Two-color immunofluorescence tests using anti-$\alpha\beta$TCR (FITC) and anti-CD3 (PE) mAbs, revealed that dull TCR cells had dull CD3, and bright TCR cells had bright CD3 (Fig. 2C). Here, there was a significant proportion of $CD3^+$ (dull) $\alpha\beta TCR^-$ cells. This population corresponded to $\gamma\delta$ T cells.

In a previous study [15] and in this experiment (Fig. 3), the two-peak pattern of $\alpha\beta$TCR was observed in hepatic MNC and thymocytes, whereas a single-peak pattern, which consists of only bright TCR, was observed in the MNC of the periphery, including the spleen and lymph nodes (Fig. 3A). In this study, CD3 expression was also demonstrated to be the same as the above $\alpha\beta$ TCR expression (Fig. 3A, right column). The bone marrow cells did not have any $\alpha\beta$TCR (nor CD3)-positive cells (data not shown). It was confirmed that the bright peak of $\alpha\beta$TCR (and CD3) in hepatic MNC was present at the same position as the bright peak of

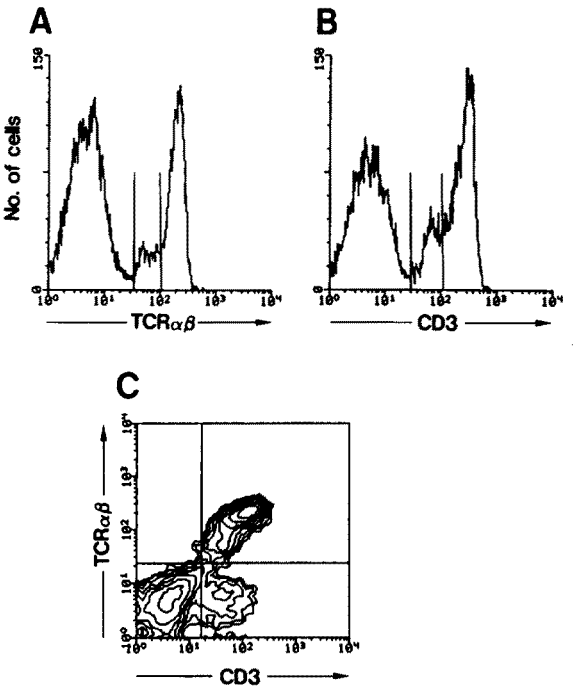


*Fig. 2.* Fluorescence profiles of $\alpha\beta$TCR and CD3 stainings. (A) Single-color staining of $\alpha\beta$TCR; (B) Single-color staining of CD3; (C) Two-color staining of $\alpha\beta$TCR (FITC) and CD3 (PE). Two-peak pattern was produced by both stainings of $\alpha\beta$TCR and CD3. Intermediate TCR cells expressed CD3 of intermediate intensity, and bright TCR cells expressed CD3 of bright intensity.

$\alpha\beta$TCR (and CD3) in thymocytes (Fig. 3B). On the other hand, the dull peak of $\alpha\beta$TCR (and CD3) in hepatic MNC was at the intermediate position between dull and bright peaks of $\alpha\beta$TCR (and CD3) in thymocytes. In this regard, we named hepatic T cells with such intermediate TCR intensity, 'intermediate TCR cells' [14]. Since congenitally athymic mice and thymectomized mice (two months after thymectomy) had only intermediate TCR cells in the liver (and the periphery), intermediate TCR cells are truly of extrathymic origin [29].

To further emphasize the unique properties of hepatic MNC, the morphology of hepatic MNC and splenic cells was compared using light microscopy (Fig. 4). Interestingly, hepatic MNC contained a considerable proportion of lymphoblasts (up to 30%). In contrast, splenic cells consisted of small and medium-sized lymphocytes. The morphology of hepatic MNC might reflect the

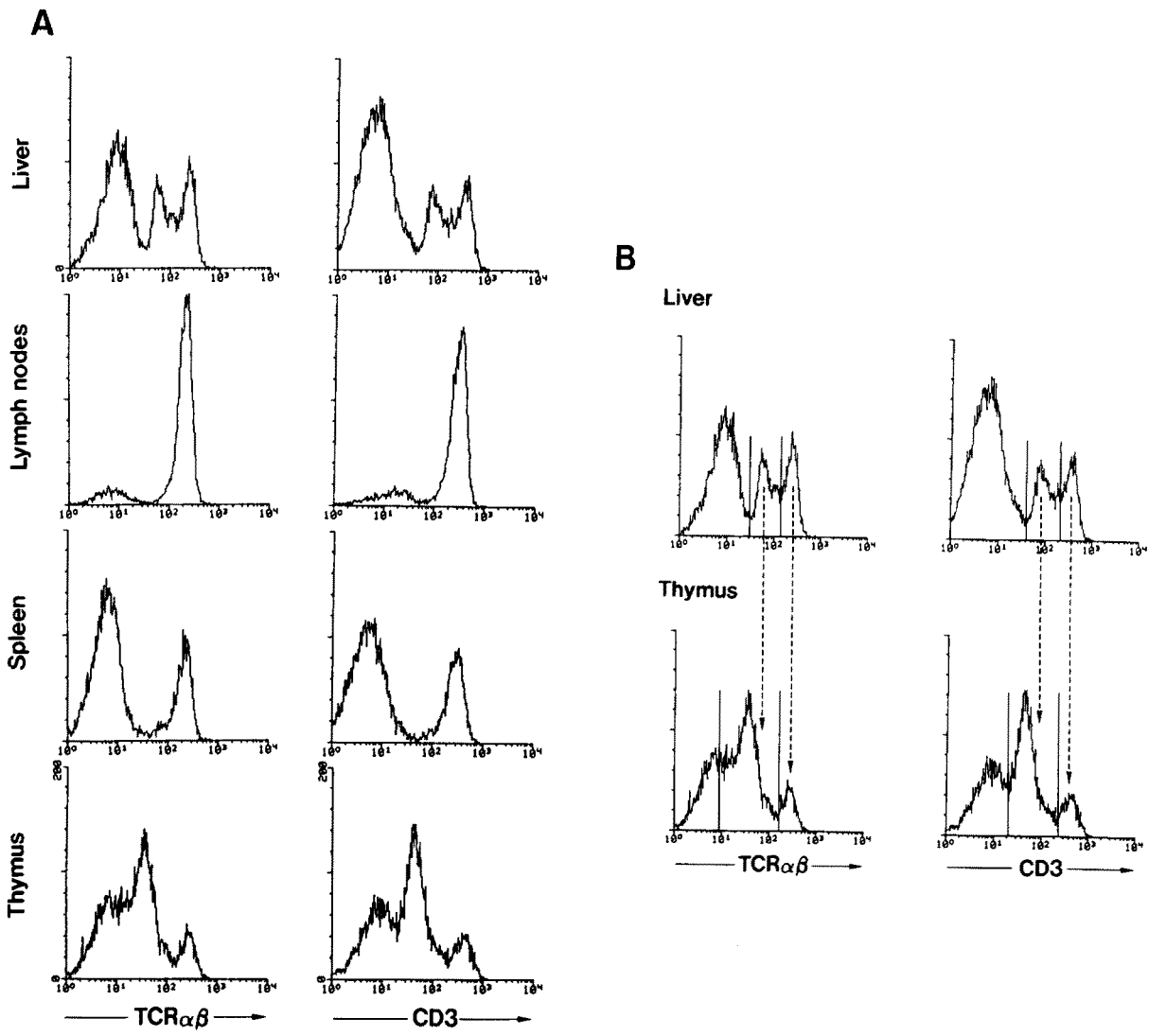


*Fig. 3.* Fluorescence profiles of αβTCR and CD3 stainings in MNC obtained from various organs. (A) Single-color stainings of MNC in the liver, thymus, spleen and lymph nodes. (B) A direct comparison of αβTCR and CD3 stainings between hepatic MNC and thymocytes. Hepatic MNC and thymocytes showed a two-peak pattern, consisting of dull and bright TCR cells, whereas MNC in the spleen and lymph nodes had a single-peak pattern, consisting of only bright TCR cells. Direct comparisons revealed that the relatively dull peak of hepatic TCR (and CD3) was at the intermediate position between the dull and bright peaks of thymic TCR.

proliferation of extrathymic T cells in the liver (i.e., hepatic sinusoids).

*Dose-dependency of GL effect on the number of MNC in the liver*

In the light of the results so far obtained, we examined whether the administration of GL affects the hepatic pathway of extrathymic T cell differentiation. In this experiment, the effect of GL on the number of MNC in liver, thymus and spleen was examined (Fig. 5). Since a preliminary experiment revealed that a single injection of GL (2 or 10 mg/mouse) increased the number of hepatic MNC 2 to 4 days after administration, the dose-kinetics of GL was examined on day 3 after injection. It was demonstrated that a single injection of 2 mg GL significantly increased the

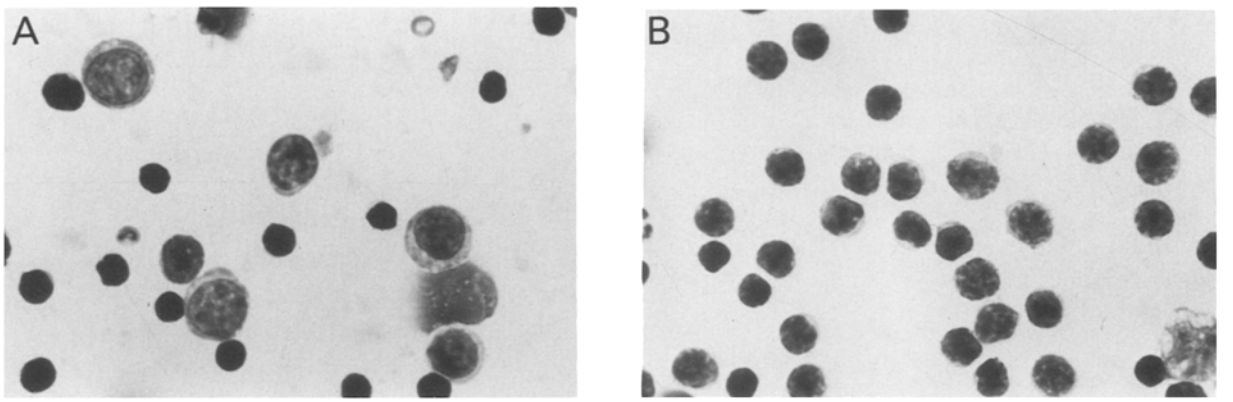


*Fig. 4.* Morphology of hepatic MNC and splenic cells as shown by light microscopy (× 1,000). (A) Hepatic MNC; (B) Splenic cells. Cell smears were stained by May-Gruenwald and Giemsa method. Only hepatic MNC contained a significant proportion of lymphoblasts.

number of hepatic MNC (up to a 70% increase) (Fig. 5A). The increase of hepatic MNC was minimal at 10 mg GL, whereas the highest dose (20 mg GL/mouse) decreased the number of hepatic MNC. On the other hand, GL did not significantly increase the numbers of thymocytes and splenic cells, irrespective of the doses (Fig. 5B and C).

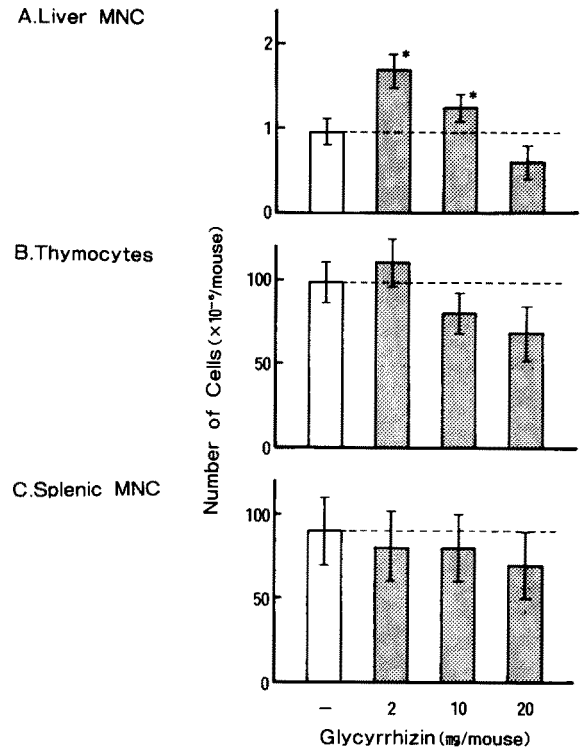


*Fig. 5.* Effect of dose-kinetics of GL on the numbers of hepatic MNC, thymocytes and splenic cells. C3H/He mice at 8 weeks of age were injected with GL at the indicated concentrations. The cell numbers yielded were enumerated 3 days after injection. Data represent the mean and one standard deviation of 6 tested mice. * indicates the statistical significance ($P < 0.05$) analyzed by Student's $t$ test.

Since GL is usually repeatedly administered in patients, the effect of repeated injections was also examined (Table 1). Almost similar to the effect of a single injection, repeated injection of GL resulted in maintenance of a high level of the number of hepatic MNC. On the other hand, the number of thymocytes and splenic cells remained relatively constant.

### *Increase in the proportion of intermediate TCR cells by GL administration*

We then investigated whether the increase in the number of hepatic MNC, which was induced by GL, was accompanied by an increase in the

*Table 1.* Effect of GL on the number of MNC yielded in terms of repeated injections or a single injection

| GL | Number of MNC ($\times 10^{-6}$) | | |
|---|---|---|---|
| | Liver | Thymus | Spleen |
| none | 0.92 ± 0.13 | 132 ± 28 | 109 ± 8 |
| Repeated injection[a] | 1.53 ± 0.20[b] | 141 ± 31 | 118 ± 11 |
| A single injection | 1.80 ± 0.21[b] | 169 ± 41 | 120 ± 13 |

[a] Mice were injected with 2 mg GL/mouse twice a week and such injections were continued for 3 weeks. Measurement was carried out 3 days after the final injection. Data represent the mean and one standard deviation of 4 tested mice.
[b] The difference is statistically significant ($P < 0.05$) by Student's $t$ test.

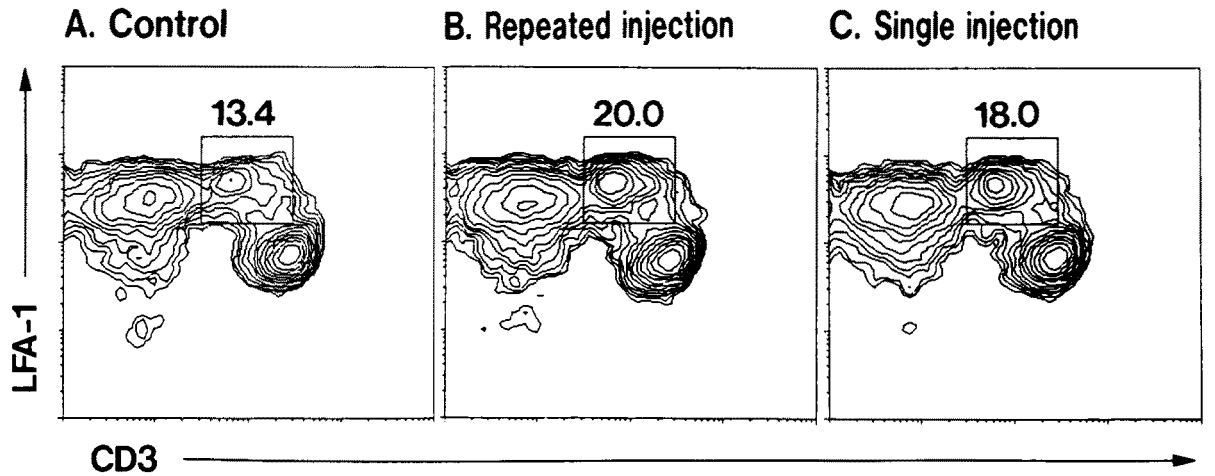


*Fig. 6.* Two-color immunofluorescence profiles of CD3 and LFA-1 expression in hepatic MNC. (A) Control; (B) Repeated injections of GL; (C) A single injection of GL. Intermediate TCR cells expressing the highest level of LFA-1 are indicated by squares. Numbers in the figures are the percentages of intermediate TCR cells.

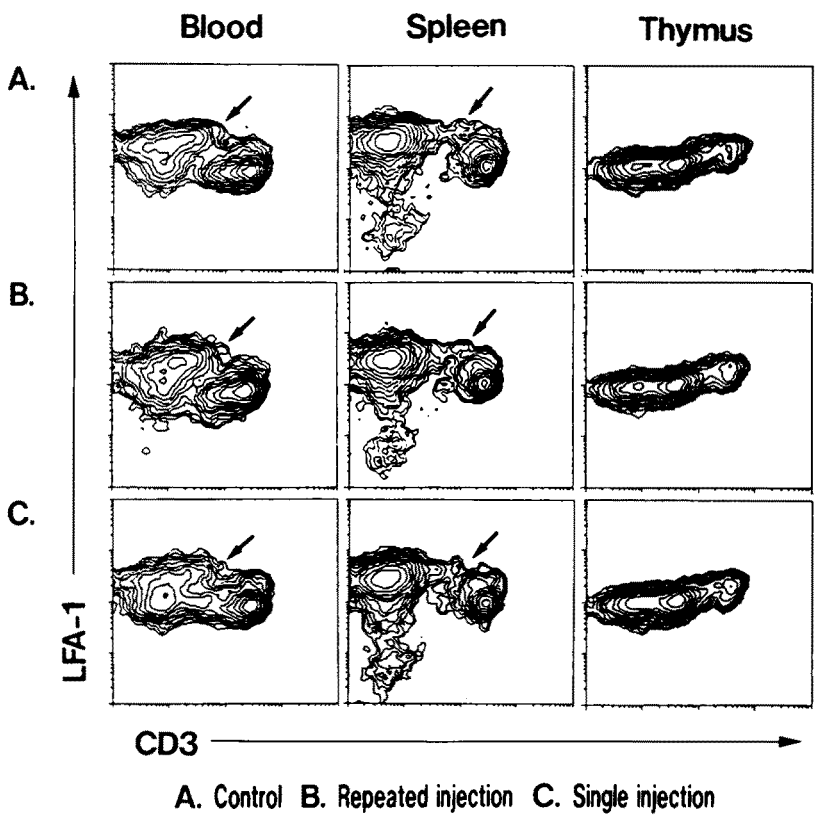


*Fig. 7.* Two-color profiles of CD3 and LFA-1 stainings in MNC of the blood, spleen and thymus. (A) Control; (B) Repeated injections of GL; (C) A single injection of GL. A small but significant proportion of intermediate TCR cells were identified in the blood and spleen, but not in the thymus. The staining patterns were not changed by GL administration.

proportion of intermediate TCR cells. Although intermediate TCR cells were able to be identified by single-color immunofluorescence test, the two-color test using anti-CD3 (FITC) and anti-LFA-1 (PE) mAbs was applied here (Fig. 6). Since intermediate TCR cells expressed a higher level of LFA-1 than did bright TCR cells, each population was clearly identified by this method. It was demonstrated that GL increased the proportion of intermediate TCR cells in the liver, irrespective of the single or repeated injection.

The two-color staining of CD3 and LFA-1 antigens also enabled us to detect a very small proportion of intermediate TCR cells in MNC of the blood (2.5%) and the spleen (3.4%) (Fig. 7). Such intermediate TCR cells were not present in the thymus. Even after the single or repeated injection of GL, the proportions of intermediate TCR cells in the blood and spleen were not changed.

*A comparison of the effects of GL and other reagents on the numbers of hepatic MNC and thymocytes*

In recent studies, we demonstrated that cytokines (e.g., lymphotoxin) and hormones (e.g., estrogen) could uniquely modulate the hepatic pathway of extrathymic T cell differentiation [27]. Lymphotoxin and estrogen induced an increase in not only the proportion but also the absolute number of intermediate TCR cells. Therefore, we systematically compared the effects of GL and other reagents with respect to the numbers of hepatic MNC and thymocytes (Fig. 8). In these experiments, the maximal effects of GL and cytokines appeared at about 3 days after administration, whereas the maximal effects of estrogen and hydrocortisone were observed at about 6 days after injection. This discrepancy is due to the fact that the maximal effects of these hormones showed delayed onsets in comparison with the other reagents used here. Interestingly, augmented effects on the number of hepatic MNC were produced by three reagents, namely, GL, lymphotoxin (TNF$\beta$) and estrogen. However, effects on the number of thymocytes greatly differed. GL did not induce thymic atrophy, whereas lymphotoxin and estrogen induced a profound thymic atrophy.

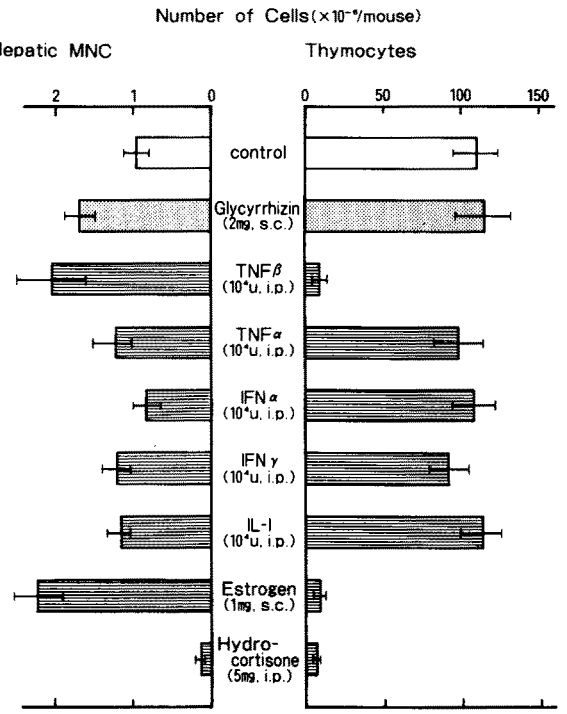


*Fig. 8.* Changes in the numbers of hepatic MNC and thymocytes after treatments with GL and other reagents. The effects of GL and cytokines were examined on day 3 after injections, whereas the effects of hormones, estrogen and hydrocortisone, were examined day 6 after injections. All reagents, except GL and estrogen, were injected intraperitoneally (i.p.) at the indicated concentrations. Estrogen, dissolved in sesame oil due to its water-insolubility, was administered by subcutaneous injection (s.c.). Date represent the mean and one standard deviation of the results from 3 to 4 isolated experiments.

TNF$\alpha$, IFN$\alpha$, IFN$\gamma$, and IL-1 did not induce a significant change in the numbers of hepatic MNC and thymocytes, at least at a concentration of $10^4$ U/mouse. On the other hand, hydrocortisone simultaneously decreased both the numbers of hepatic MNC and thymocytes.

## Discussion

In the present study, GL was demonstrated to selectively augment the absolute and relative number of intermediate TCR cells in the liver. In previous studies, we also observed that extrathymic T cells with $\alpha\beta$TCR of intermediate

intensity were activated by bacterial stimulation [13, 29], lymphotoxin and estrogen [28]. However, all of these reagents simultaneously induced a profound thymic atrophy. Since GL did not affect the number and cell distribution pattern of thymocytes, its effect is quite unique. It is therefore conceivable that among therapeutic drugs GL might have a unique position as an immunomodulator.

In a series of recent studies, we have revealed that intermediate TCR cells in the liver are of extrathymic origin and generated in the liver *per se* [16, 28]. They have several unique properties distinct from regular T cells of thymic origin. Thus, they consist of DN $CD4^-8^-$ cells as well as a single-positive $CD4^+$ or $CD8^+$ cells, and comprise a large proportion of self-reactive forbidden clones (estimated by anti-V$\beta$ mAbs) and autoreactive V$\beta 8^+$ cells [13, 15]. T cells with the same properties were also recently demonstrated in the liver by Goosens *et al.* [30] and Murosaki *et al.* [31]. In addition to the intermediate TCR cells in the liver, intraepithelial lymphocytes (IEL) in the intestine [8–10] and T cells in the omentum of thymectomized mice [11] were subsequently demonstrated to differentiate extrathymically. It seems that extrathymic T cells, which are possibly more primitive than regular T cells [29], are generated in several places in the body. However, it should be noted that intermediate TCR cells probably migrate to the periphery when activated during bacterial stimulation [13], malignancies [14], aging [12] and autoimmune diseases [6, 15, 16]. All of these conditions accompany thymic atrophy or involution. On the other hand, other extrathymic T cells, i.e., IEL in the intestine and T cells in the omentum, work mainly at the site where they are generated. Especially, IEL displays bright TCR and contains a significant proportion of DP $CD4^+CD8^+$ cells [8–10, 30]. Such cells are rarely seen in the periphery.

In the present study, we demonstrated that intermediate TCR cells expressed the highest level of LFA-1 antigens. Since LFA-1 antigens are important adhesion molecules for lymphocytes [32], the highest expression of LFA-1 on intermediate TCR cells might be responsible for their adhesion to the sinusoidal endothelial cells in the liver. Indeed, we have observed that the sinusoidal endothelial cells in the liver express a higher level of ICAM-1, which are one of the molecules that bind LFA-1 [32], than do the endothelial cells in other organs (our unpublished observation). It is postulated that the early precursors, originating in the bone marrow, migrate to the hepatic sinusoids due to their unique expression of adhesion molecules and then differentiate *in situ*. The null cell population depleted of Thy $1.2^+$ cells (i.e., $TCR^-$ cell fraction) are able to acquire $\alpha\beta$TCR in *in vitro* cultures [16]. It is therefore conceivable that early precursors of extrathymic T cells are present in the liver. On the other hand, a significant number of stem cells did not appear to be present in the liver after birth, especially in adult mice. Thus, we could not reconstitute lymphocytes in lethally irradiated mice (12 Gy) by the injection of $2 \times 10^7$ hepatic MNC, although such injection kept lymphocytes in the sinusoids for at least 3 weeks (K. Sugiura and S. Seki, manuscript in preparation). In sharp contrast, we were able to reconstitute lymphocytes well in the entire body of lethally irradiated mice by injection of the same number of bone marrow cells.

Many researchers have reported that GL inhibits that replication of certain viruses (e.g., varicella-zoster virus and HTLV-III) in *in vitro* studies [18–21]. However, this function of GL was not always complete and many investigators suspected certain immunomodulatory functions of GL *in vivo*. It was already reported that GL activated NK cells in *in vivo* and *in vitro* studies [27]. Taken together with the present findings, GL can be seen to have an important potential for the activation of certain immune functions. In a recent study, we demonstrated that preactivation of intermediate TCR cells in the liver (e.g., by stimulation with heat-killed bacteria) prevented death in mice injected with a lethal dose of influenza virus (our unpublished observation). It is conceivable that intermediate TCR cells have potent cytotoxic activity against virally infected cells. Therefore, the activation of intermediate TCR cells as well as of NK cells by GL might be intimately related to resistance against viral infections.

It was suspected that viral infected hepatocytes are destroyed by specific cytotoxic T cells (CTL) in chronically ill patients and that such process

might be associated with a deterioration of chronic active viral hepatitis [33, 34]. Since intermediate TCR cells appeared to have the ability to mediate a specific tolerance to certain antigens, e.g., allogeneic lymphocytes ([29] and our unpublished observation), such function might be related to the tolerance of CTL which was specific to the corresponding viruses. If this is the case, activated intermediate TCR cells may possibly suppress the progression of chronic active hepatitis.

It is well known that an increased IgE production, which induces allergic reaction, absolutely requires the helper function of T cells differentiated in the thymus [35]. On the other hand, extrathymic $\alpha\beta$ and $\gamma\delta$ T cells might regulate such helper T cells by virtue of their autoreactivity [29, 36–39]. It is postulated that intermediate TCR cells preactivated by GL might be responsible for the suppression of allergic reactions. In any case, an understanding of the hepatic pathway for T cell differentiation appears to clarify the mechanisms underlying many unique immunopotentiators, including GL, cytokines, and hormones.

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*Address for correspondence*: Toru Abo, Department of Immunology, Niigata University School of Medicine, Niigata 951, Japan.