The Appearance of T Cells Bearing Self-reactive T Cell Receptor in the Livers of Mice Injected with Bacteria

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Summary

We demonstrated in the present study that with bacterial stimulation, an increased number of \( \alpha/\beta \) T cells proliferated in the liver of mice and that even T cells bearing self-reactive T cell receptor (TCR) (or forbidden T cell clones), as estimated by anti-V\( \beta \) monoclonal antibodies in conjunction with immunofluorescence tests, appeared in the liver and, to some extent, in the periphery. The majority (>80%) of forbidden clones induced had double-negative CD4\(^{-}\)8\(^{-}\) phenotype. In a syngeneic mixed lymphocyte reaction, these T cells appear to be self-reactive. Such forbidden clones and normal T cells in the liver showed a two-peak pattern of TCR expression, which consisted of \( \alpha/\beta \) TCR dull and bright positive cells, as seen in the thymus. A systematic analysis of TCR staining patterns in the various organs was then carried out. T cells from not only the thymus but also the liver had the two-peak pattern of \( \alpha/\beta \) TCR, whereas all of the other peripheral lymphoid organs had a single-peak pattern of TCR. However, T cells in the liver were not comprised of double-positive CD4\(^{+}\)8\(^{+}\) cells, which predominantly reside in the thymus. The present results therefore suggest that T cell proliferation in the liver might reflect a major extrathymic pathway for T cell differentiation and that this hepatic pathway has the ability to produce T cells bearing self-reactive TCR under bacterial stimulation, probably due to the lack of a double-positive stage for negative selection.

Materials and Methods

**Mice and Bacterial Stimulation.** Male AKR/J (H-2\( ^{k} \), Mls-1\( ^{a} \)) and C3H/HeJ (H-2\( ^{k} \), Mls-2\( ^{b} \)) mice, aged 5–6 wk, were obtained from Charles River Japan Inc. (Tokyo, Japan) (15). They were fed under pathogen-free conditions. Bacterial stimulation applied here was the intraperitoneal injection of 10\(^{8} \) heat-killed Propionibacterium acnes or Escherichia coli. All bacteria used were prepared in this laboratory and heat-killed for 30 min at 100\(^{\circ}\)C (16). They were fed under pathogen-free conditions. Bacterial stimulation applied here was the intraperitoneal injection of 10\(^{8} \) heat-killed Propionibacterium acnes or Escherichia coli. All bacteria used were prepared in this laboratory and heat-killed for 30 min at 100\(^{\circ}\)C (16). After washing three times with sterile PBS (0.01 M, pH 7.2), the bacteria were suspended in PBS until use.

**Cell Preparation.** The treated mice were killed 4 d after the bacterial injection. Untreated control mice were used in parallel. Liver mononuclear cells (MNC) were prepared as previously described.
Briefly, perfusion was performed to eliminate blood contained in the liver by injection of 10 ml PBS into the portal vein before removal of the liver. To obtain MNC, the liver was cut into small pieces with scissors, pressed through 100-gauge stainless steel mesh, and suspended in RPMI 1640 medium. After washing once with the medium, the cell pellet was 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 method applied here, the proportion of contaminated phagocytes (i.e., Kupffer cells) was negligible (<4%) (15). MNC in other lymphoid organs, including the spleen, blood (heparinized), and bone marrow, were also collected by the Ficoll-Isopaque method. Thymocytes and lymph node cells were obtained by forcing the thymus and inguinal lymph nodes through 100-gauge steel mesh.

Immunofluorescence Tests. Surface phenotypes of cells were identified by using mAbs in conjunction with the single- or two-color immunofluorescence test (10). The mAbs used here included FITC-conjugated anti-CD4 (L3T4) mAb and FITC-conjugated and biotin-conjugated anti-CD8 (Lyt-2) mAbs (Becton Dickinson & Co., Mountain View, CA). Biotin-conjugated and unconjugated hamster IgG anti-α/β TCR (H57-597) mAbs were kindly provided by Dr. R. Kubo (National Jewish Center, Denver, CO) (17). A biotin-conjugated reagent was developed with PE-conjugated avidin. FITC-conjugated or unconjugated rat IgG2a anti-Vβ6 (44-22-1) mAb (18) and hamster IgG anti-Vβ3 (KJ25-606-4) mAb (19) were used. Unconjugated reagents were developed with PE-conjugated anti-rat or anti-hamster Ig (Caltag Laboratories, San Francisco, CA). FITC-conjugated anti-Vδ3 (F23.1) mAb was also used (20). The fluorescence-positive cells were analyzed by a FACScan® (Becton Dickinson & Co.).

Table 1. Generation of Forbidden T Cell Clones in the Liver

| Mice | Bacterial stimulation | Organ | Cell number yielded \( \times 10^6 \) | Percent fluorescence-positive cells |
|------|-----------------------|-------|---------------------------------|-----------------------------------|
|      |                       |       |                                 | Vβ6 to Mls-1<sup>*</sup> | Vβ3 to Mls-2<sup>+</sup> | Vβ8 |
| AKR/J (Mls-1<sup>*</sup>) | - | Liver | 0.8 ± 0.2 | 0.3 | 4.3 | 12.7 |
|      |                       | Spleen | 70.3 ± 8.4 | 0.5 | 2.8 | 9.6 |
|      |                       | Thymus | 122.3 ± 20.4 | 2.3 | 1.5 | 9.4 |
|      | + | Liver | 3.9 ± 1.2 | 6.5 | 16.5 | 15.0 |
|      | | Spleen | 68.4 ± 5.6 | 3.0 | 2.1 | 5.1 |
|      | | Thymus | 48.6 ± 7.6 | 0.4 | 0.1 | <0.1 |
| C3H/HeJ (Mls-2<sup>+</sup>) | - | Liver | 0.8 ± 0.2 | 5.9 | <0.1 | 15.8 |
|      | | Spleen | 68.7 ± 9.0 | 5.2 | 0.5 | 7.2 |
|      | | Thymus | 113.8 ± 19.4 | 8.2 | <0.1 | 17.9 |
|      | + | Liver | 4.6 ± 1.2 | 6.7 | 12.5 | 15.0 |
|      | | Spleen | 68.8 ± 8.2 | 4.0 | 6.3 | 8.3 |
|      | | Thymus | 42.3 ± 7.9 | 2.5 | 1.6 | 2.0 |

The treated mice were killed 4 d after P. a入ez injection. Here, the cell numbers yielded were represented as the mean ± 1 SD from four mice, whereas the percentages of fluorescence-positive cells were enumerated by using the pooled MNC of four mice.
for Vβ8.1) cells were detected in the MNC of all organs tested (see Table 1), since these T cell oligoclonal clones are not forbidden clones in AKR/J mice. In this regard, the Vβ8+ cells constitute two peaks of TCR dull positive (7.3%) and bright positive cells (2.1%) in the thymus (Fig. 1a, left).

We then examined the levels of Vβ6, Vβ3, and Vβ8 in AKR/J mice injected intraperitoneally with heat-killed P. acnes (10⁸/mouse). Such stimulation is known to induce the growth of γ/δ and α/β T cells in the liver of mice (10). Indeed, the number of liver MNC yielded in the treated mice was several times greater than that of the control mice. A significant proportion of Vβ6+ cells (6.5%) appeared in the liver of mice stimulated with the bacteria (Table 1). It is noteworthy that these Vβ6+ forbidden clones generated in the liver also constituted two peaks of TCR dull positive and bright positive cells (Fig. 1b, right) similar to the case of positive selection of T cells in the thymus. With stimula-

**Figure 1.** Surface antigen expression analyzed by immunofluorescence tests. (a) Vβ6 and Vβ8 expression on thymocytes of untreated AKR/J mice; (b) Vβ6 and Vβ8 expression on liver MNC of AKR/J mice 4 d after P. acnes stimulation. In this experiment, scales of relative cell number of the ordinate have been magnified eightfold and fourfold, respectively, as compared with Fig. 3. Here, the Vβ6 expression on thymocytes of AKR/J mice stimulated with bacteria and Vβ6 expression on liver MNC of untreated AKR/J mice are not represented as figures, because such Vβ6+ cells in the corresponding tissues are at a basal level (see Table 1).

**Figure 2.** Phenotypic characterization of Vβ3+ forbidden T cell clones induced in C3H/HeJ mice injected with P. acnes (A) and E. coli (B). C3H/HeJ mice were intraperitoneally injected with 10⁸ heat-killed P. acnes or E. coli and were killed 4 d after the injection. MNC were isolated from the liver, spleen, and thymus, and analyzed by two-color immunofluorescence test. The numbers in the figure indicate the percentages of fluorescence positive cells.
ordered clones that reacted to Mls-2a, and actually were a liver (see Table 1). These clones (e.g., V38) also constitute Periphery of Mice Stimulated with Bacteria

Table 2. Appearance of Self-reactive T Cells in the Liver and Periphery of Mice Stimulated with Bacteria

| Responder Cells | Stimulator cells | [H]TdR incorporation (cpm ± SD) |
|-----------------|-----------------|-------------------------------|
| Untreated C3H/HeJ mice | - + | 370 ± 85 |
| Liver MNC | - + | 10,217 ± 2,678 |
| Liver MNC | + + | 12,244 ± 1,704 |
| Spleen cells | - + | 2,296 ± 851 |
| Spleen cells | + + | 3,086 ± 983 |
| Lymph node cells | - + | 3,655 ± 931 |
| Lymph node cells | + + | 5,649 ± 828 |
| C3H/HeJ mice treated with bacteria | - + | 370 ± 85 |
| Liver MNC | - + | 16,224 ± 1,008 |
| Liver MNC | + + | 32,511 ± 1,207 |
| Spleen cells | - + | 1,966 ± 284 |
| Spleen cells | + + | 7,210 ± 985 |
| Lymph node cells | - + | 7,675 ± 89 |
| Lymph node cells | + + | 18,430 ± 877 |

extent, in the spleen by both stimulations. Such Vβ3+ cells were confined in the α/β TCR+ cell fraction (left column). Interestingly, the majority of these Vβ3+ cells (>80%) were DN CD4-8- (right column). Even after bacterial stimulations, Vβ3+ forbidden T cell clones were a small population in the thymus.

After bacterial stimulation, NK cells and monocytes with Fc receptor were increased in both the liver and spleen. However, the forbidden T cell clones predominantly appeared only in the liver, and still confined to α/β T+ cells and to mainly DN (CD4-8-) cell fractions, as shown in Fig. 2. Moreover, it was confirmed that Fc+ monocytes isolated from the liver were α/β TCR- in our experimental procedure. Monocytes were identified by the phagocytosis of yeast particles (data not shown). Therefore, the peak of dull TCR in the liver was not false positive.

Acquisition of Self-reactivity in Syngeneic MLR. The appearance of forbidden T cell clones in the liver and spleen of mice treated with bacteria led us to examine whether such T cells actually proliferate in response to self-antigens (Table 2). Here, responder cells were liver MNC, spleen cells, and lymph node cells of both untreated C3H/HeJ mice and C3H/HeJ mice treated with P. acnes, whereas stimulator cells were MMC-treated spleen cells of untreated C3H/HeJ mice in a syngeneic MLR. A significant proliferative response was induced when the responder cells from the liver, spleen, and lymph nodes of C3H/HeJ mice stimulated with bacteria were used.

Accumulation of MNC in the Liver of Mice Injected with Bacteria. We then examined the histology of the liver of C3H/HeJ mice treated with P. acnes in parallel with that of untreated mice (Fig. 3). It was obvious that many MNC (indicated by a short arrow) were present in the sinusoidal area of the liver of stimulated mice. In this case, moderate sinusoidal dilatation appeared (indicated by a long arrow) around hepatic central veins.

Formation of Two-peak Pattern of TCR in the Liver MNC. The experiments thus far described indicate that the liver is a unique organ where not only TCR bright positive cells but also TCR dull positive cells exist. A possible appearance of this two-peak pattern of TCR was systematically examined in the various organs of untreated C3H/HeJ mice (Fig. 4). The TCR expression was identified by both anti-α/β TCR and anti-Vβ8 mAbs. The appearance of the two peaks was confined to the MNC from the thymus and liver, but not in the spleen, lymph nodes, blood, and bone marrow (Fig. 4 a). Almost all of the α/β TCR+ or Vβ8+ cells in the latter organs were comprised of only TCR bright positive cells. This was true even after bacterial stimulation (data not shown). The bone marrow cells did not contain a significant proportion of T cells.

In the next experiment, we performed the two-color immunofluorescence test using FITC-conjugated anti-CD4 (green) and PE-conjugated anti-CD8 (red) mAbs to compare the CD4 and CD8 antigen expression patterns of T cells between the thymus and liver of untreated C3H/HeJ mice (Fig. 4 b). In contrast to the T cells of the thymus, those of the liver were comprised of a large proportion of DN cells (45.1%), and virtually no double-positive (DP) cells (0.9%)
were detectable in the liver. Even after bacterial stimulation, DP cells did not appear in the liver MNC. Here, TCR dull positive cells in the liver were comprised predominantly of DN cells, whereas the majority of TCR bright positive cells were single-positive cells of either CD4⁺ or 8⁺ (data not shown).

Discussion

We previously demonstrated that the liver is a possible site for the proliferation of abnormal α/β T cells with DN phenotype in autoimmune MRL-lpr/lpr mice (9). Such liver α/β T cells are generated in the hepatic sinusoids and thereafter migrate to the periphery. In subsequent studies, we revealed...
a. Analysis of TCR peak pattern in C3H/HeJ

![Diagram showing TCR peak pattern in various organs](image)

b. Two-colour analysis of CD4 and CD8 in C3H/HeJ

![Two-colour analysis of CD4 and CD8 in thymus and liver](image)

that γ/δ T cells also proliferate in the liver of humans and mice with malignancies (10, 11). These γ/δ T cells had a lymphoblastic morphology, and the freshly isolated cells could spontaneously proliferate in vitro culture. It was also demonstrated that γ/δ T cells preferentially appeared in the liver of old mice with thymic atrophy (12). We therefore proposed the possibility that some populations of α/β and γ/δ T cells undergo extrathymic differentiation in the liver after birth.

In the present study, data obtained using a different approach further support this possibility. At first, even T cells bearing self-reactive TCR (or forbidden α/β T cell oligoclonals) identified by anti-Vβ mAbs were generated in the liver of mice stimulated with bacteria. Although the most predominant site of appearance of the forbidden T cell clones was the liver, a significant proportion was also detectable in the periphery. The majority of these forbidden T cell clones had DN CD4−8− phenotype. Under bacterial stimulation, forbidden T cell clones in the liver were TCR dull and bright positive cells. More importantly, the staining pattern of α/β TCR and Vβ8 expression also constituted the two-peak pattern in the liver of normal mice. The two-peak pattern of TCR is somewhat similar to that of premature and mature T cells seen in the case of positive selection in the thymus (14, 22). The difference is that such TCR dull positive cells in the thymus are DP (CD4+8+) and go through a process of either negative or positive selection (1-4). However, the majority of those cells in the liver are DN CD4−8− and probably undergo positive selection or just random expansion. As clonal deletion of self-reactive T cells occurs in the CD4−8− DP stage in the thymus (13, 14), the lack of a DP stage in the liver may reflect one of the reasons why the hepatic differentiation pathway of T cells does not perform such clonal deletion.

In syngeneic MLR, a significant proliferative response of MNC in the liver and periphery of mice injected with bacteria was induced. Although the induction magnitude of syngeneic MLR is known to be very low in comparison with that of allogeneic MLR (21), it should be noted that the magnitude of syngeneic MLR represented here is considerably high when MNC obtained from mice under bacterial stimulation are used. The precise determination of responding cells in such syngeneic MLR remains to be investigated.

In the present study, the actual accumulation of MNC in the liver of mice injected with bacteria was also demonstrated. A lymphoblastic appearance of these MNC attached to the endothelial cells of hepatic sinusoids has already been reported (9, 10, 12). Even after the perfusion of 10 ml of PBS from the portal vein, these MNC were not released from the sinusoidal lumen. These morphological changes and the appearance of lymphoblastic morphology of MNC strongly suggest a possible proliferative response of γ/δ T cells in the liver after bacterial stimulation.

Figure 4. A comparison of the staining patterns of TCR, CD4, and CD8 antigens in various lymphoid organs. (a) α/β TCR and Vβ8 expression on MNC from various tissues of untreated C3H/HeJ mice; (b) two-color staining of CD4 and CD8 antigens on thymocytes and liver MNC of C3H/HeJ mice. The numbers in the figure indicate percentages of fluorescence positive cells.

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ance of forbidden T cell clones continued for several days after bacterial injection, showing a peak on day 4. In other words, the phenomenon described here is reversible.

As the TCR dull positive cells are rarely seen in peripheral lymphoid organs other than the liver, except for thymus, we have postulated that the liver may be the most predominant site for extrathymic differentiation pathway of T cells. Even if this is the case, it remains unclear whether progenitor cells of such an extrathymic T cell differentiation pathway are common to those of the thymic pathway, and what proportion of TCR bright positive cells in the liver are actually generated in the hepatic pathway. In recent studies, the clonal deletion of forbidden T cell clones was reported to be unsuccessful in athymic nude mice (23, 24). We have recently demonstrated that such forbidden clones also proliferate in the liver of these nude mice and thymectomized mice with or without bacterial stimulation (Ohteki, T., and T. Abo, unpublished observation). Although it is well established that the liver is the major hematopoietic organ in the fetal stage (25), the present results show that the liver is still an important organ for the production of a unique T cell population. It is conceivable that the microenvironments (e.g., Kupffer cells and endothelial cells) of hepatic sinusoids can accept homing of T cell precursors from the bone marrow and support the differentiation of unique T cell population. The unique properties of hepatic endothelial cells, such as stromal cells in the immune organs and the interaction of these cells with lymphocytes, were reported by Nagura et al. (26). Although it is conceivable that there are alternatives to extrathymic development of T cells in the liver, we have not yet reached such organs, especially those being able to supply extrathymic T cells to the periphery.

Finally, our recent study shows that the levels of T cells bearing self-reactive TCR definitely increase in the liver of mice, if we move them from specific pathogen-free conditions to conventional feeding conditions, despite the elevated level being lower than that in the bacterial injection system. It is conceivable that the living body is generally, to some extent, activated with respect to the hepatic, extrathymic differentiation pathway of T cells. An appropriate generation of self-reactive T cell clones might be beneficial for the surveillance of autologous bacterially infected cells or atypical cells generated in vivo. However, overstimulation of such T cells might be responsible for the onset of certain autoimmune diseases, since episodes of anamnestic bacterial infection are known to precede the onset of autoimmune diseases.

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