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 α/β 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-Vβ 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 α/β 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 α/β 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.

It is generally accepted that T lymphocytes differentiate in the thymus and go through a process of positive or negative selection to form the repertoires of mature T cells (1–4). In earlier studies, several investigators proposed the existence of an extrathymic differentiation pathway of T cells, especially in experiments using congenitally athymic nude mice and in vitro culture systems (5–8). However, investigators have not reached a consensus, since there is no definite information yet as to where such T cells differentiate outside the thymus. In recent studies, we have shown that both α/β and γ/δ T cells with double-negative (DN)[1] $CD4^-8^-$ phenotype proliferate in the liver of humans and mice, especially under conditions of autoimmune diseases (9), malignancies (10, 11), and aging (12). These results have led us to consider the possibility that the liver might be a major site for extrathymic differentiation of certain T cells.

In the present study, we have applied a recently described system for identification of oligoclonal T cells expressing particular Vβ of α/β TCRs by using mAbs (13, 14) to demonstrate the hepatic pathway for cell differentiation. The present results support the possibility that the liver is a major site of extrathymic T cell differentiation and reveal that the hepatic pathway has several unique properties distinct from the intrathymic pathway for T cell differentiation.

Materials and Methods

Mice and Bacterial Stimulation. Male AKR/J (H-2^k, Mls-1^a) and C3H/HeJ (H-2^k, Mls-2^a) 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°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

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive, MMC, mitomycin; MNC, mononuclear cells.

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(15). 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 (Lyt2) 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-Vβ8 (F23.1) mAb was also used (20). The fluorescence-positive cells were analyzed by a FACScan® (Becton Dickinson & Co.).

Syngeneic MLR. Syngeneic MLR was performed as previously described (21). As the spleen cells consist of a large proportion of B cells and adherent macrophages, whole spleen cells were used as stimulators. Briefly, 5×10^5 responder cells and an equal number of mitomycin (MMC)-treated (50 μg/ml, 45 min) stimulator cells were mixed together in 0.2 ml of RPMI 1640 medium supplemented with 10% FCS and 5×10^{-5} M 2-ME, and were cultured in a 96-well round-bottomed microculture plate (Falcon Labware, Oxnard, CA) for 4 d. To test the cell proliferation, [^{3}H]TdR incorporation was analyzed as described (17). The data express the mean cpm ± 1 SD of triplicate cultures.

Results

Generation of Forbidden T Cell Clones in the Liver. In this study, AKR/J (H-2^k, Mls-1^a) and C3H/HeJ (H-2^k, Mls-2^a) mice, aged 6 wk, were used (Table 1 and Fig. 1). The T cell (Vβ6$^+$) oligoclones reactive to Mls-1^a were identified by the anti-Vβ6 mAb, whereas the other T cell (Vβ3$^+$) oligoclones reactive to Mls-2^a were identified by the anti-Vβ3 mAb in conjunction with the immunofluorescence test. As shown in Table 1, normal AKR/J mice had very few, if any, Vβ6$^+$ cells among MNC of various organs such as the liver, spleen, and thymus. This was due to intrathymic clonal deletion of the Vβ6$^+$ cells being self-reactive clones to Mls-1^a in these AKR/J mice. Although a significant proportion of Vβ6$^+$ cells (2.3%) was detectable in the thymus, almost all of these Vβ6$^+$ cells showed a low density of Vβ6 antigens (Fig. 1 *a, right*). It is well established that such TCR dull positive cells are premature T cells before the process of positive or negative selection (14, 22). In this figure, TCR bright positive cells of Vβ6 were not detected (<0.1%) because of complete negative selection in the thymus. On the other hand, considerable proportions of both Vβ3$^+$ and Vβ8$^+$ (except

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

Mice	Bacterial stimulation	Organ	Cell number yielded	Percent fluorescence-positive cells		
				Vβ6 (to Mls-1^a)	Vβ3 (to Mls-2^a)	Vβ8
			× 10^6/mouse			
AKR/J	–	Liver	0.8 ± 0.2	0.3	4.3	12.7
(Mls-1^a)		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	–	Liver	0.8 ± 0.2	5.9	<0.1	15.8
(Mls-2^a)		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. acnes* 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.

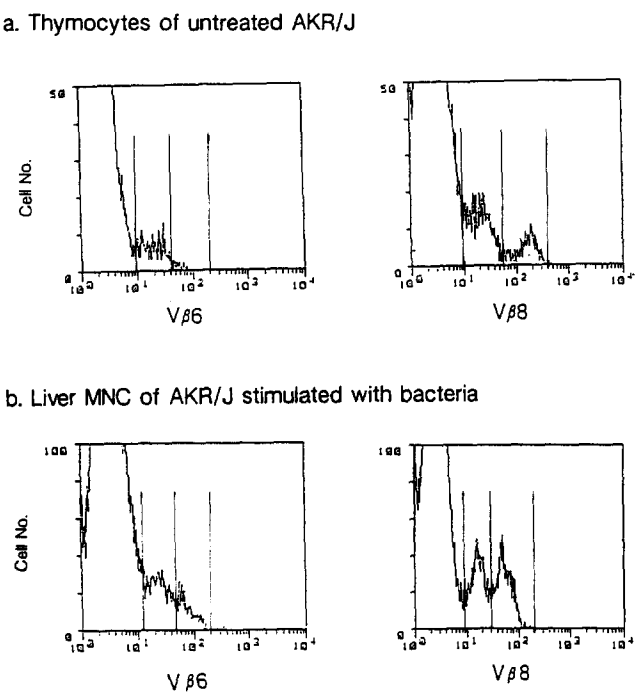


Figure 1. Surface antigen expression analyzed by immunofluorescence tests. (*a*) Vβ6 and Vβ8 expression on thymocytes of untreated AKR/J mice; (*b*) Vβ6 and Vβ8 expression on liver MNC of AKR/J mice 4 d after *P. acnes* stimulation. In this experiment, scales of relative cell number of the ordinate, 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 $V\beta6^+$ cells in the corresponding tissues are at a basal level (see Table 1).

for Vβ8.1) cells were detected in the MNC of all organs tested (see Table 1), since these T cell oligoclones are not forbidden clones in AKR/J mice. In this regard, the $V\beta8^+$ cells constitute two peaks of TCR dull positive (7.3%) and bright positive cells (2.1%) in the thymus (Fig. 1 *a*, *left*).

We then examined the levels of Vβ6, Vβ3, and Vβ8 in AKR/J mice injected intraperitoneally with heat-killed *P. acnes* (10^8/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 $V\beta6^+$ cells (6.5%) appeared in the liver of mice stimulated with the bacteria (Table 1). It is noteworthy that these $V\beta6^+$ forbidden clones generated in the liver also constituted two peaks of TCR dull positive and bright positive cells (Fig. 1 *b*, *right*) similar to the case of positive selection of T cells in the thymus. With stimula-

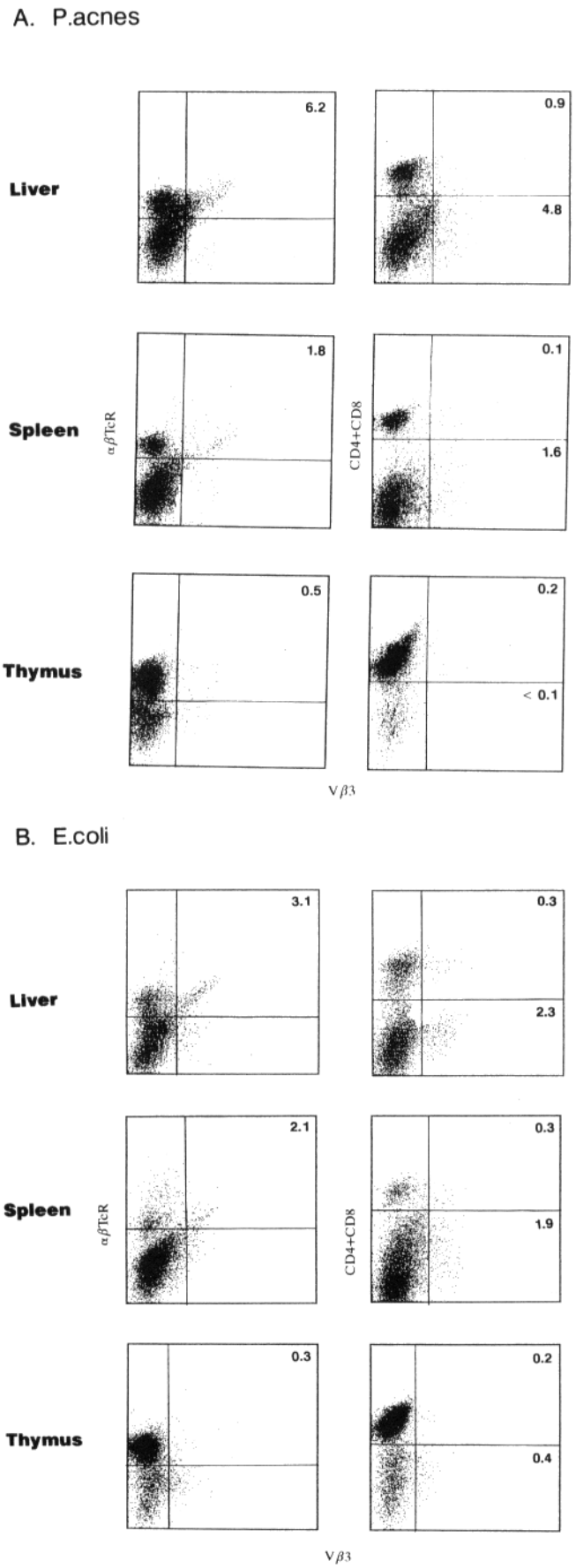


Figure 2. Phenotypic characterization of $V\beta3^+$ forbidden T cell clones induced in C3H/HeJ mice injected with *P. acnes* (*A*) and *E. coli* (*B*). C3H/HeJ mice were intraperitoneally injected with 10^8 heat-killed *P. acnes* or *E. coli* and were killed 4 d after the injection. MNC were isolated 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.

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

Responder Cells	Stimulator cells	[^{3}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

tion, it appears that oligoclones such as Vβ3 and Vβ8, i.e., oligoclones other than forbidden clones, also expand in the liver (see Table 1). These clones (e.g., Vβ8) also constitute two peaks of TCR in terms of their intensity (Fig. 1 *b*, *left*).

Almost the same results were obtained in C3H/HeJ mice (Table 1). In these mice, Vβ3$^+$ cells were self-reactive, forbidden clones that reacted to Mls-2^a, and actually were a very minor population (~0.5%) in the periphery of untreated mice. However, the forbidden clones, Vβ3$^+$, were generated in the liver (12.5%) and spleen (6.3%) by bacterial stimulation. It was noticed that a significant proportion of forbidden clones, to a lesser extent than in the liver, appeared in the spleen of both AKR/J and C3H/HeJ mice stimulated with the bacteria. Such forbidden clones, especially with the high intensity of Vβ, never appeared in the thymus, irrespective of stimulation. In another study, we also stimulated mice with various species of bacteria other than *P. acnes*, including *E. coli*, *Streptococcus pyogenes*, and *Mycobacterium bovis*. All of these heat-killed or living bacteria could induce the forbidden T cell oligoclones in the liver of these mice, despite individual differences in the magnitude of the induction.

Phenotypic Characterization of Forbidden T Cell Clones. The phenotypes of forbidden T cell clones appeared in C3H/HeJ mice stimulated with heat-killed *P. acnes* and *E. coli* were then characterized (Fig. 2). The two-color immunofluorescence tests using biotin-conjugated anti-α/β TCR mAb (red) and FITC-conjugated anti-Vβ3 mAb (green), and using a mixture of PE-conjugated anti-CD4 and anti-CD8 mAbs (red) and FITC-conjugated anti-Vβ3 mAbs (green), were performed. As shown in Fig. 2, Vβ3$^+$ forbidden clones in these mice were confirmed to be induced in the liver and, to some 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 was not seen 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%)

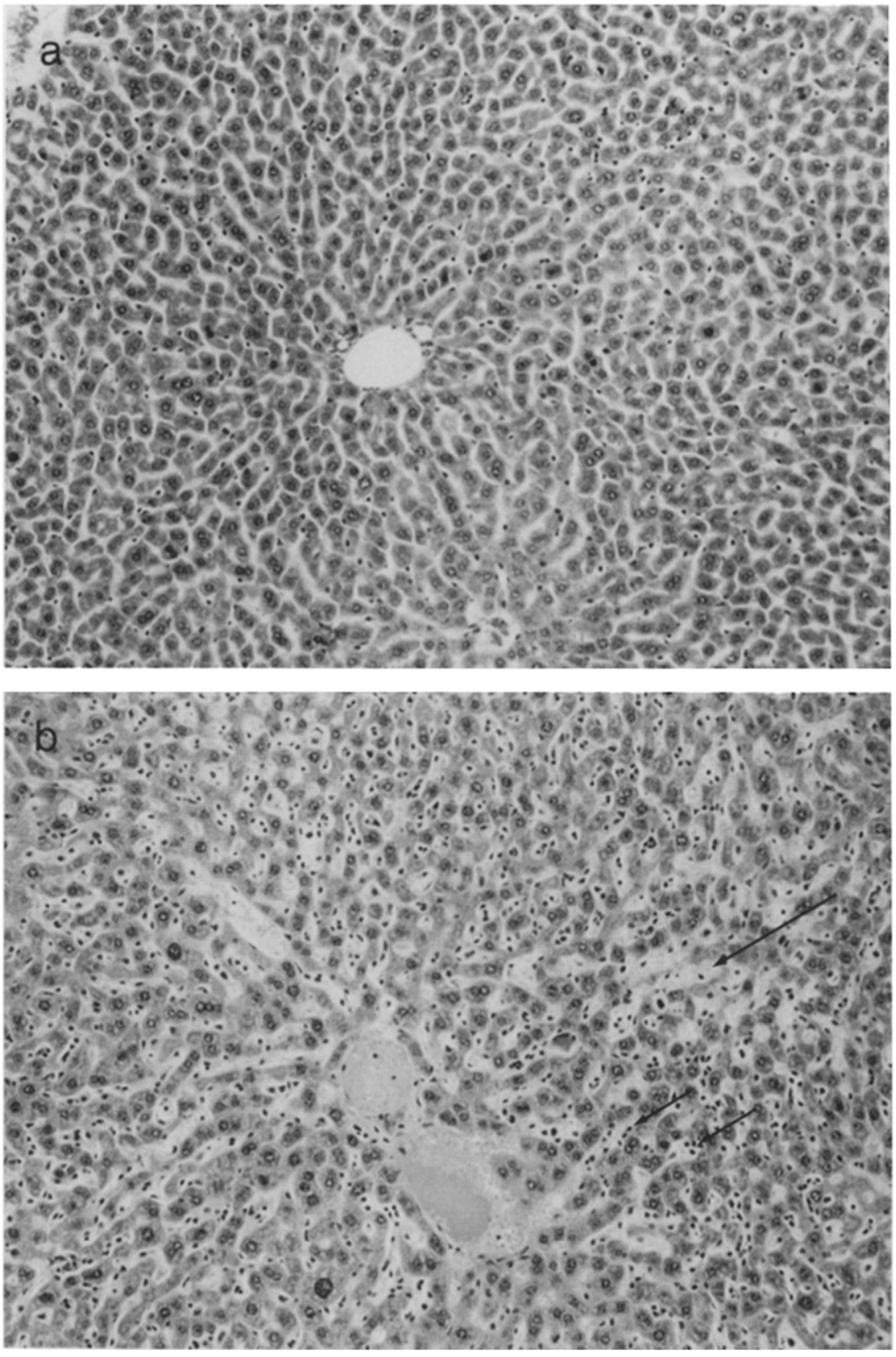
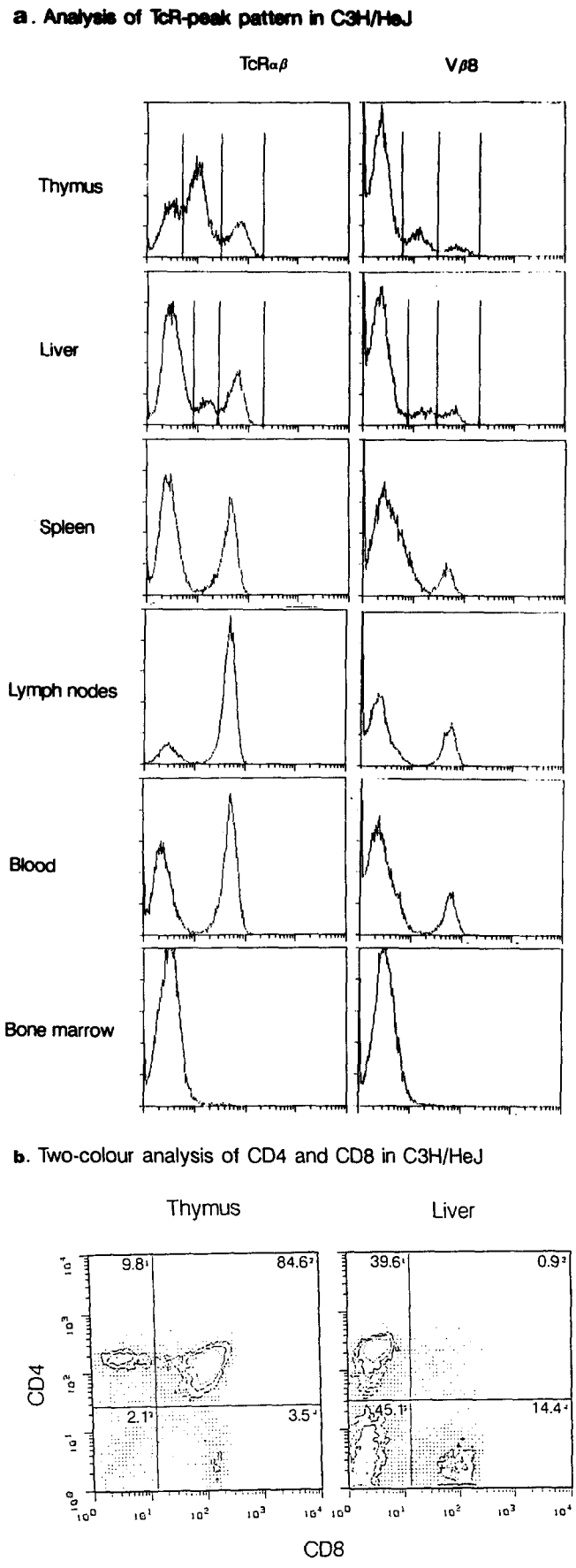


Figure 3. Histology of the liver of normal C3H/HeJ mice (*a*) and C3H/HeJ mice 4 d after *P. acnes* stimulation (*b*) (hematoxylin-eosin staining, ×400). A short arrow indicates MNC in the hepatic sinusoids, whereas a long arrow indicates sinusoidal dilatation.

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



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 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 oligoclones) 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 appear-

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.

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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