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Research Article

T cell receptor (TcR)gamma delta cells are known to be a minor population of T lymphocytes in the blood (less than 10%) and other peripheral lymphoid organs in healthy donors. We demonstrated here that a large proportion of TcR gamma delta cells, i.e., up to 30% of mononuclear cells (MNC) were detectable in the liver, but not other lymphoid organs of cancer patients. More importantly, the majority of such TcR gamma delta cells (greater than 70%) were shown to be lymphoblastic by electron microscopy. An activation marker of T lymphocytes, Leu-19 (CD56) was also highly expressed on the hepatic TcR gamma delta cells. The possibility of hepatic TcR gamma delta cells being activated was further examined in mice. C3H/He mice injected with syngeneic tumor cells were demonstrated to have an increased number of liver MNC; such MNC showed an ability to proliferate in vitro. These mice eventually had a considerable proportion of TcR gamma delta cells in the liver, showing activation markers, the Ia and LFA-1 antigens. These results suggest that the liver may be an important organ for activation and probably expansion of TcR gamma delta cells especially in tumor bearing hosts.

Identification of Activated T Cell Receptor γδ Lymphocytes in the Liver of Tumor-bearing Hosts

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Abstract

T cell receptor (TcR)γδ cells are known to be a minor population of T lymphocytes in the blood (< 10%) and other peripheral lymphoid organs in healthy donors. We demonstrated here that a large proportion of TcRγδ cells, i.e., up to 30% of mononuclear cells (MNC) were detectable in the liver, but not other lymphoid organs of cancer patients. More importantly, the majority of such TcRγδ cells (> 70%) were shown to be lymphoblastic by electron microscopy. An activation marker of T lymphocytes, Leu-19 (CD56) was also highly expressed on the hepatic TcRγδ cells. The possibility of hepatic TcRγδ cells being activated was further examined in mice. C3H/He mice injected with syngeneic tumor cells were demonstrated to have an increased number of liver MNC; such MNC showed an ability to proliferate in vitro. These mice eventually had a considerable proportion of TcRγδ cells in the liver, showing activation markers, the Ia and LFA-1 antigens. These results suggest that the liver may be an important organ for activation and probably expansion of TcRγδ cells especially in tumor bearing hosts. (*J. Clin. Invest.* 1990. 86:409–415.) Key words: TcRγδ cells • liver • cancer

Introduction

A large population of T lymphocytes in the immune organs of humans and mice are T cell receptor (TcR)$\alpha\beta$[1] cells, whereas a relatively small population are TcRγδ cells (1–6). A considerable proportion of these TcRγδ cells have a double negative (DN) phenotype of $CD2^+3^+4^-8^-$ and appear early in thymic ontogeny (7, 8). It is established that TcRγδ cells are a separate lineage from TcR$\alpha\beta$ cells (9, 10). Although TcRγδ cells become a minority in the thymus at birth (11–13), a significant number of TcRγδ cells still exist after birth in the peripheral blood, intestines, skin, and lungs (14–19). In terms of the uniqueness of tissue distribution, structure of TcR gene products and cytotoxic function, they are considered to recognize a different set of antigens and have distinct functions from those of TcR$\alpha\beta$ cells.

In studying tissue localization of human TcRγδ cells, we observed that nonparenchymal mononuclear cells (MNC) of the liver obtained from cancer patients consisted of a considerably larger proportion of TcRγδ cells compared with those from the other lymphoid organs (i.e., the blood, spleen, lymph nodes, tonsils, bone marrow, and thymus). Moreover, such hepatic TcRγδ cells, localized in the hepatic sinusoids, were lymphoblastic in morphological appearance in these cancer patients. It is, therefore, conceivable that TcRγδ cells may be in an activated state in the liver of cancer patients. In the present study, we investigated the possibility of TcRγδ cells being activated in the hepatic sinusoids of tumor-bearing hosts in both human and murine systems. Although it is well established that the liver is a major hematopoietic organ in the fetal period (20), the present results have evidenced that the liver may still be an important organ for the activation of TcRγδ cells after birth.

Methods

Cell preparations. MNC were isolated from small pieces of the liver and spleen in autopsy cases. The cases included patients (age 50–75 d) with gastric, intestinal, or pulmonary cancers who had no apparent hepatic metastasis. Also they had not been exposed to any intensive chemotherapies for at least 1 mo before death (n = 8). The other age-matched control patients included those who had died of cardiac attack or apoplexy (n = 8). To purify MNC, the pieces of liver were cut into tiny pieces with scissors, pressed on a 100-gauge steel mesh, and overlaid on a Ficoll-Isopaque gradient (1.077) column, as shown previously (21). This gradient centrifugation method yielded hepatic MNC without significant contamination of Kupffer cells (< 4%). The cell yields of hepatic MNC by the method applied here were ~ 20% (21). Splenic MNC were purified by the Ficoll-Isopaque method (22). Blood MNC for control studies were also isolated from heparinized blood of healthy adult donors (23).

Immunofluorescence tests. The phenotype of lymphocytes was analyzed by the immunofluorescence tests using MAbs (23). The MAbs used were FITC- or phycoerythrin (PE)-conjugated aliquots of anti-TcR-1 (specific for a α/β chain framework determinant) (24) and anti-TcRδ1 (specific for a δ chain framework determinant and representing a pan-TcRγδ marker) (25), and anti-CD16 (Leu-11) and anti-CD56 (Leu-19) antibodies (Becton Dickinson & Co., Mountain View, CA and T cell Science Inc., Cambridge, MA). The fluorescence positive cells were enumerated by a FACScan analyzer (Becton Dickinson & Co.) (23).

Electron microscopy after immunostaining. MNC were fixed in 4% paraformaldehyde in a cacodylate buffer (pH 7.2) by microwave irradiation (26). They were embedded in optimal cutting temperature compound. Frozen sections were cut and incubated with anti-TcRδ1 antibody (T cell Science Inc.) and left overnight. After washing in PBS, the sections were stained with streptavidin-biotin-complex-peroxidase method and were reacted with 3,3'-diaminobenzidine. After postfixation with osmium tetroxide, these specimens were embedded in Epon

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1. *Abbreviations used in this paper:* DN, double negative; MNC, mononuclear cells; NK, natural killer; PE, phycoerythrin; TcR, T cell receptor.

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812-filled gelatin capsules. Then the ultrathin sections were stained with lead citrate.

Mice and tumor. Male C3H/He mice were obtained from the Institute of Experimental Animals, Tohoku University School of Medicine, and used at 6–8 wk of age. A syngeneic MH134 hepatoma cell line and a FM3A/R mammary carcinoma cell line were cultured in this laboratory (27). 10 million MH134 or FM3A/R cells were subcutaneously injected and the mice were killed to examine immunoparameters 3 d after the injection. MNC were isolated from the liver and spleen by the same method as used with humans, using Ficoll-Isopaque gradient (1.090 for mouse) centrifugation. The liver was obtained after perfusion with 10 ml of PBS (21).

DNA synthesis assay. To test the proliferation of MNC, [^{3}H]thymidine incorporation was analyzed as described previously (21). The cell culture was performed in a medium supplemented with 1% heat-inactivated mouse sera and 5×10^{-5}M mercaptoethanol. MNC (2.5×10^6/ml) were cultured for 18 h with 0.5 μCi of [^{3}H]thymidine in a 96-well flat-bottomed plastic microculture plate (0.2 ml/well) (Falcon Labware, Oxnard, CA). Data expressed the mean cpm and 1 SD of triplicate cultures.

Immunofluorescence tests for murine study. The phenotype of murine MNC was analyzed by immunofluorescence tests using MAbs (21). The MAbs used here included biotin-conjugated aliquots of anti-Thy1 and anti-CD3 antibodies, and FITC- or PE-conjugated aliquots of anti-CD4 (L3T4) and anti-CD8 (Lyt2) antibodies (Becton Dickinson & Co.). The biotin-conjugated reagents were developed with PE-conjugated avidin. FITC-conjugated aliquots of anti-Ia and anti-LFA-1 MAbs were kindly provided by Dr. K. Nishimura at Tokai University (Isehara, Japan) (28). Unconjugated hamster anti-TcR$\gamma\delta$ (3A10) MAb was kindly provided by Dr. S. Tonegawa at the Massachusetts Institute of Technology (Cambridge, MA) (29) and developed with PE-conjugated anti-hamster Ig. Polyclonal rabbit anti-asialo GM_1 antibody was developed with FITC-conjugated anti-rabbit Ig.

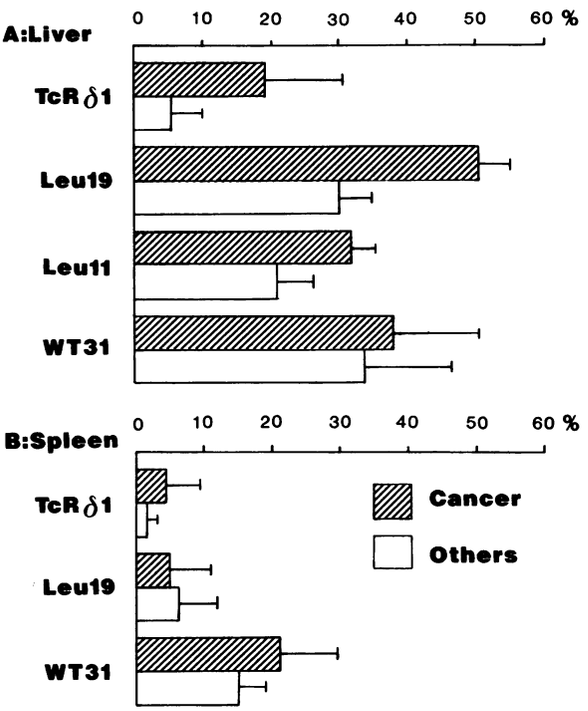

Figure 1. Phenotype of MNC in the liver and spleen of cancer patients and noncancer patients. The surface phenotype of MNC isolated from the liver (*A*) and spleen (*B*) in eight cancer patients (▨) and eight noncancer patients (□) were analyzed by the immunofluorescence test using MAbs, TcRδ1 (TcR$\gamma\delta$ cells), Leu-19 (NK and activated T cells), Leu-11 (NK cells), and WT31 (TcR$\alpha\beta$ cells).

Results

Increased proportion of TcR$\gamma\delta$ cells in the liver of cancer patients. The surface phenotype of MNC isolated from the liver and spleen in eight cancer and eight noncancer patients was analyzed by the immunofluorescence tests (Fig. 1). TcR$\gamma\delta$ cells were identified by the MAb, TcRδ1, whereas TcR$\alpha\beta$ cells were identified by the MAb, WT31. Natural killer (NK) cells were detected by the MAbs, Leu-19 (anti-CD56) and Leu-11 (anti-CD16). It was shown that liver MNC consisted of larger proportions of TcR$\gamma\delta$, TcR$\alpha\beta$, and NK cells than did spleen MNC. This was due to the enrichment of surface Ig$^+$B cells in the spleen (30). Interestingly, further increased proportions of TcR$\gamma\delta$ and NK cells were demonstrated in the liver of cancer patients, especially, where the percentages of TcR$\gamma\delta$ cells were 18.5±12.0. Such high levels of TcR$\gamma\delta$ cells have never been seen in any of the lymphoid organs, including peripheral blood, lymph nodes, and tonsils in either cancer or noncancer patients (unpublished observation). As suggested from the great variation in proportions of TcR$\gamma\delta$ cells in cancer patients (Fig. 1), three out of eight cancer patients did not show such increased proportions of TcR$\gamma\delta$ cells (< 5%).

NK cells identified by the MAbs, both Leu-19 and Leu-11, were increased in the cancer patients. However, there was a considerable discrepancy between the percentages of Leu-19$^+$ and Leu-11$^+$ cells. The Leu-19 antigens were known to be expressed on the activated T lymphocytes (31). We therefore examined whether the TcR$\gamma\delta$ cells coexpressed the Leu-19 antigens on the cell surface by two-color immunofluorescence analysis (Fig. 2). The MNC of the liver, spleen, and lymph nodes were obtained from a cancer patient with intestinal malignancy, whereas the MNC of blood were from a healthy donor. It is interesting that more than 70% of hepatic TcR$\gamma\delta$

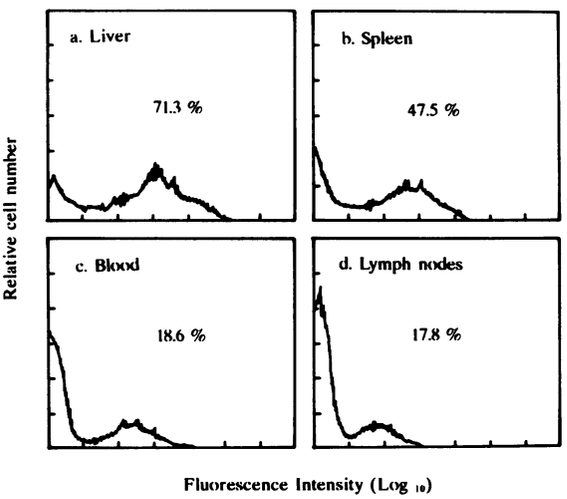

Figure 2. A comparison of the Leu-19 antigen coexpression on TcR$\gamma\delta$ cells of various lymphoid organs. The MNC of liver, spleen, and lymph nodes were obtained from a cancer patient with intestinal malignancy, whereas the blood MNC were obtained from a healthy donor. All MNC were stained by the FITC-conjugated Leu-19 and the PE-conjugated TcRδ1 MAbs and analyzed by the FACScan analyzer. A gate was set to TcR$\gamma\delta$ cells in two-color flow cytometry and the Leu-19 antigen expression within this gate was obtained as the figures represent.

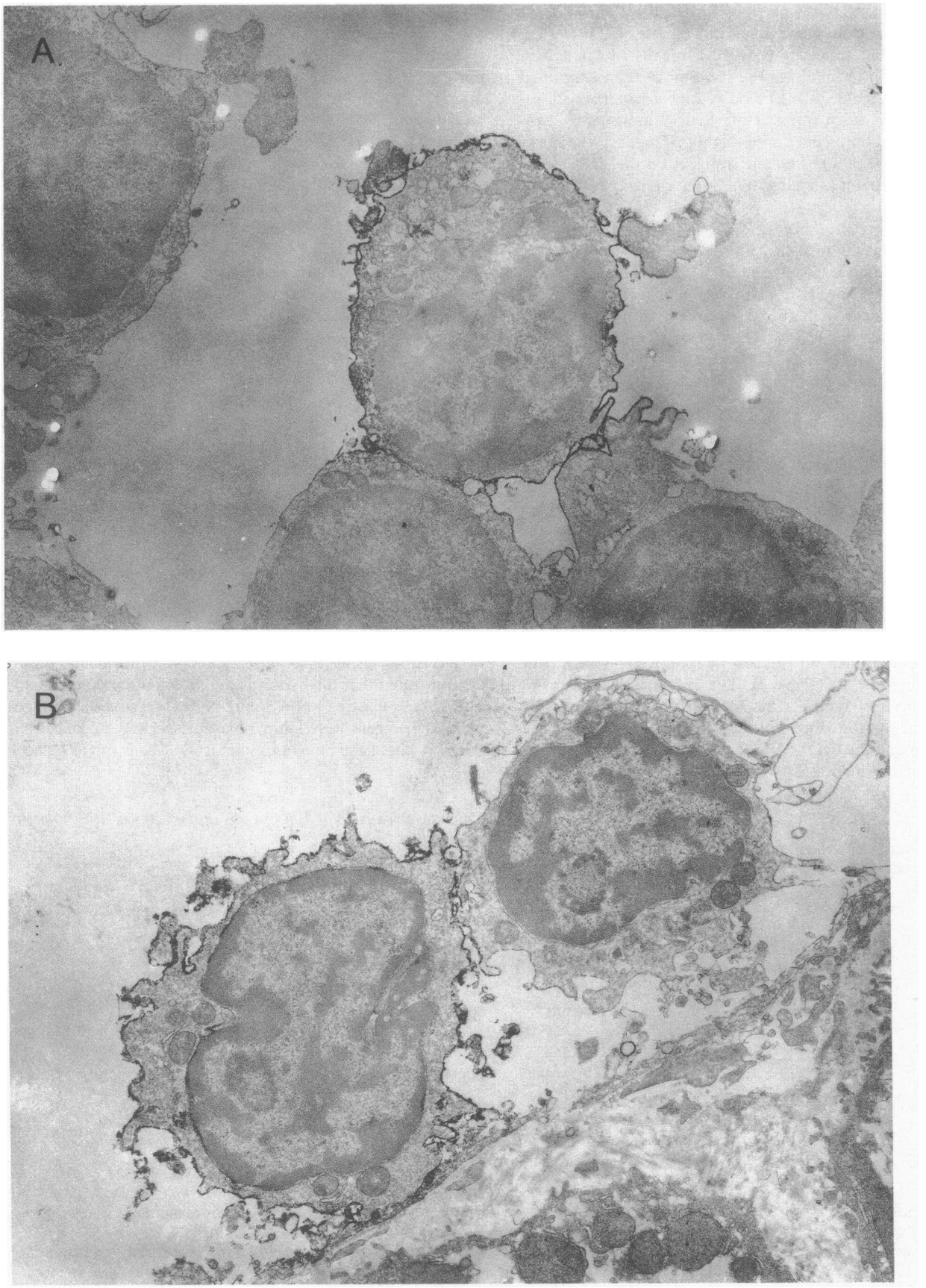

Figure 3. Morphology of TcR$\gamma\delta$ cells (×8,500). The morphology of TcR$\gamma\delta$ cells in the peripheral blood and liver of cancer and noncancer patients was analyzed by electron microscopy after immunostaining with TcRδ1 MAb. Representative features of the positive cells (showing a dense staining on the cell surface) in the blood of a normal donor (*A*) and the liver of a cancer patient (*B*), are represented.

cells expressed the Leu-19 antigens with TcR$\gamma\delta$ cells of blood and lymph nodes expressed only at low levels (< 20%) of the Leu-19 antigens. The value of TcR$\gamma\delta$ cells in the spleen seemed to be intermediate. We have since then examined whether TcR$\gamma\delta$ cells, in the blood of cancer patients with intestinal malignancy, expressed the Leu-19 antigens by two-color immunofluorescence tests highly. However, the coexpression level (< 30%) of Leu-19 antigens on the TcR$\gamma\delta$ cells in the blood was not as high as that on the hepatic TcR$\gamma\delta$ cells (data not shown).

Morphological study of TcR$\gamma\delta$ cells. The morphological appearance was then investigated in TcR$\gamma\delta$ cells derived from the peripheral blood, spleen, and liver (Fig. 3). In each organ, small resting TcR$\gamma\delta$ cells and large lymphoblastic TcR$\gamma\delta$ cells were demonstrated in both cancer and noncancer patients. However, almost all TcR$\gamma\delta$ cells (> 90%) in the peripheral blood and spleen of both cancer and noncancer patients were small resting lymphocytes (Fig. 3 *A*), whereas a high number of TcR$\gamma\delta$ cells in the liver of cancer (> 70%) and noncancer patients (~ 30%) were large lymphoblasts (Fig. 3 *B*). The hepatic TcR$\gamma\delta$ cells adhered to the endothelial cells in the sinusoids. It seems that TcR$\gamma\delta$ cells, in the liver, are in an activated state, especially in cancer patients.

Increased number and DNA synthesis of liver MNC in tumor bearing mice. We extended the experiment to murine study in order to further clarify the unique existence of activated TcR$\gamma\delta$ cells in the liver. Here, C3H/He mice injected subcutaneously with 1×10^7 syngeneic tumor cells, MH134, or FM3A/R, were used as a murine model of tumor-bearing hosts. MNC derived from the liver, spleen, blood, and lymph nodes were examined on the cell yield and the spontaneous proliferation in vitro (Table I). The increased number and DNA synthesis of lymphocytes in the liver and spleen were shown in the mice bearing tumor cells (3 d after the injection). It is of note that the liver MNC derived from the mice bearing tumors had the highest DNA synthesis in an 18-h in vitro culture.

Table I. Increased Number and DNA Synthesis of Lymphocytes in the Liver of Mice Injected with Tumor Cells

Organs	Stimulation with tumors	Number of cells*	DNA synthesis/ 5×10^5 cells‡
		$\times 10^6$	*cpm±SD*
Liver	—	0.6±0.1	913±137
	MH134	2.2±0.3	8,399±810
	FM3A/R	2.1±0.4	7,962±670
Spleen	—	19.2±4.9	1,199±84
	MH134	31.8±6.3	4,129±209
	FM3A/R	30.6±5.8	5,042±462
Blood	—	1.0±0.1	288±61
	FM3A/R	1.1±0.2	1,051±78
Lymph nodes	—	3.0±0.6	246±62
	FM3A/R	3.5±0.7	725±27

* Number of cells in the liver and spleen shows the mean±1 SD from four mice. The values of blood are the number of MNC obtained from 1 ml of heparinized peripheral blood, whereas those of lymph nodes are the number of lymphocytes of a lymph node obtained from the lateral abdominal wall.
‡ DNA synthesis represents the mean cpm±1 SD of triplicate cultures of 5×10^5 pooled cells derived from four mice.

Expansion of TcR$\gamma\delta$ cells in the liver. Phenotypic analysis was then performed using the immunofluorescence tests on these mice (Table II). Of particular interest, a significant proportion of TcR$\gamma\delta$ cells were induced in only the liver of mice injected with tumors. Such cells were at an undetectable level in the spleen, blood, and lymph nodes. A considerable proportion of TcR$\gamma\delta$ cells (> 70%) in the liver were $CD4^-8^-$ (data not shown). TcR$\gamma\delta$ cells were a minority in the liver of normal mice, and were at undetectable levels in the spleen, irrespective of the injection of tumors. Here, the vast majority of asialo-GM_1^+ (bright) NK cells were shown to be $Thy1^-$ or $Thy1^+$ (dull), whereas almost all TcR$\gamma\delta$ cells were asialoGM_1^+ (dull) as shown by the two-color immunofluorescence test ([21] and our unpublished observation).

We then investigated whether murine TcR$\gamma\delta$ cells in the liver also coexpressed the lymphocyte-activation markers, as in the case of humans. Here, the activation markers, Ia and LFA-1, on the hepatic TcR$\gamma\delta$ cells in mice injected with or without tumor cells were examined by two-color immunofluorescence tests (Fig. 4). Although the Ia expression on TcR$\gamma\delta$ cells were originally 9% in the control mice, 25% of the hepatic TcR$\gamma\delta$ cells in mice injected with FM3A/R acquired the Ia antigens (Fig. 4 *A*). Similarly, the increased expression was observed on the LFA-1 antigens (Fig. 4 *B*). The hepatic TcR$\gamma\delta$ cells in the mice injected with FM3A/R expressed the LFA-1 antigens at a greater level of intensity than those in the control mice.

Discussion

In this study using both human and murine materials, we demonstrated that the liver was the most predominant site of TcR$\gamma\delta$ cells existing in the living body. Moreover, as the hepatic TcR$\gamma\delta$ cells were lymphoblastic especially in cancer patients, it raised the possibility that TcR$\gamma\delta$ cells might be acti-

Table II. Expansion of TcR$\gamma\delta$ Cells in the Liver of Mice Injected with Tumor Cells

Organs	Stimulation with tumors	% Positive cells				
		Thy1	CD3	CD4 + 8*	asialoGM_1	TcR$\gamma\delta$
Liver	—	31.1	29.0	24.5	25.4	4.2
	MH134	60.2	45.2	29.6	38.3	13.4
	FM3A/R	59.6	44.8	30.2	38.4	12.8
Spleen	—	32.1	39.4	39.8	11.1	0
	MH134	7.9	10.9	10.7	16.6	0
	FM3A/R	13.2	14.6	14.5	15.9	ND
Blood	—	74.2	ND	74.8	20.4	0.6
	FM3A/R	44.1	ND	43.9	18.2	0.4
Lymph nodes	—	90.6	ND	92.6	6.7	0
	FM3A/R	93.4	ND	93.1	9.7	0

* The MNC or lymphocytes of each organ were obtained as described in Table I and the pooled cells from four mice were analyzed by immunofluorescence tests.
Data represent the mean percent fluorescence positive cells from four mice. Here, the mean proportion and 1 SD of TcR$\gamma\delta$ cells in the liver of control mice and mice injected with MH134 and FM3A/R were 4.2±1.3, 13.4±1.0, and 12.8±1.3%, respectively. The mean differences were significant ($P < 0.01$) between control mice and mice injected with both tumor cells, when analyzed with a *t* test.

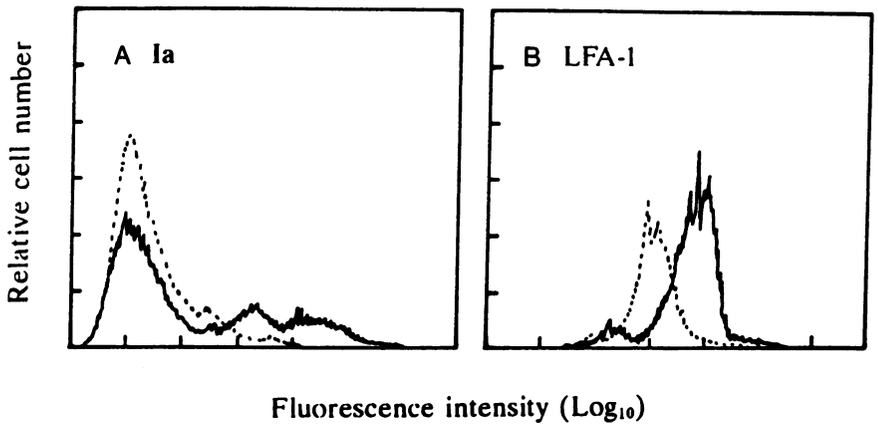

Figure 4. The increased expression of the Ia (*A*) and LFA-1 (*B*) antigens on hepatic TcR$\gamma\delta$ cells in mice injected with tumor cells. The MNC of the liver in mice injected with (—) or without (---) 1×10^7 FM3A/R tumor cells were stained with FITC conjugated aliquots of anti-Ia and anti-LFA-1 murine MAbs, and a combination of PE-conjugated anti-hamster Ig and 3A10 MAb (hamster Ig). A gate was set to TcR$\gamma\delta$ cells in two-color flow cytometry and the Ia and LFA-1 antigen expressions within this gate were obtained as the figures represent.

vated, and probably increased in number (from a murine study) in the liver. The increased proportion of Leu-19$^+$ (CD56) cells and the coexpression of Leu-19 antigens on TcR$\gamma\delta$ cells also supported this possibility. Thus, it can be said that the activated T lymphocytes acquired the Leu-19 antigens on their surface (31). Similarly, the activation markers of lymphocytes, Ia and LFA-1 antigens (28, 32), were also highly expressed on the hepatic TcR$\gamma\delta$ cells in mice injected with tumor cells. The present results have evidenced, for the first time, that the liver is an important immune organ not only as the hematopoietic site at the fetal periods (20) but also as the site for the activation of TcR$\gamma\delta$ cells after birth.

In the morphological study of humans, it was also demonstrated that TcR$\gamma\delta$ cells existed in the hepatic sinusoids where they adhered to the sinusoidal endothelial cells. The adhesion of TcR$\gamma\delta$ cells to the endothelial cells might be firm, since the hepatic TcR$\gamma\delta$ cells were not eliminated by the perfusion of liver with PBS as shown in murine experiments. Such unique localization of TcR$\gamma\delta$ cells (i.e., the adhesion to endothelial cells) was reported in the splenic sinusoids of humans and chickens (33–35). In this regard, we have recently observed that TcR$\gamma\delta$ cells, of blood MNC, proliferated preferentially in vitro in a medium supplemented with high amounts of autologous serum (e.g., 20–50%). Here, the stimuli for TcR$\gamma\delta$ cells were either heat-killed bacteria or mitomycin-treated tumor cell lines. On the other hand, the proliferation of TcR$\alpha\beta$ cells induced by lectins was completely inhibited in the medium with such a high amount of serum (Seki, S. and T. Abo, manuscript in preparation). It is presumed that the activation and proliferation of TcR$\gamma\delta$ cells occurred in vivo in serum-rich areas such as the sinusoids in the liver and spleen. Morphological study revealed that TcR$\gamma\delta$ cells in the liver had few electron-dense granules, despite their lymphoblastic appearance (see Fig. 3 *B*). In contrast, it is well established that NK cells in the liver (i.e., pit cells) have a number of electron-dense granules in the cytoplasm (36, 37).

It is also well established that TcR$\gamma\delta$ cells are widely distributed in various tissues, blood, intestines, skin, and lungs (14–19). It is still a matter of debate whether such TcR$\gamma\delta$ cells originate from a small population of TcR$\gamma\delta$ cells in the thymus, or if they generate by an absolutely extrathymic differentiation. In a recent study, we demonstrated that the liver is also a possible site for the proliferation of abnormal TcR$\alpha\beta$ CD3$^+$4$^-$8$^-$ DN lymphocytes in autoimmune MRL-1pr/1pr mice (38). These abnormal DN cells constitute the prominent lymphadenopathy and are considered to be responsible for the autoimmunity in these mice, probably due to their autoreactive cytotoxicity. As the hepatic TcR$\gamma\delta$ cells of humans and mice in the present study were DN lymphocytes, it is conceivable that in certain circumstances both TcR$\alpha\beta$ and TcR$\gamma\delta$ DN lymphocytes can expand in the liver. Indeed, it is known that some TcR$\gamma\delta$ DN cells are characterized by autoreactive cytotoxicity (39, 40). If we postulate that hepatic TcR$\gamma\delta$ DN cells as well as TcR$\alpha\beta$ DN cells expand by extrathymic differentiation, it is easy to consider that such DN cells may have such autoreactivity because of their escape from thymic education. The possibility of extrathymic maturation of TcR$\alpha\beta$ and TcR$\gamma\delta$ cells has been intensively proposed in recent studies (41–44). If either hypothesis (i.e., the intrathymic vs. extrathymic generation of TcR$\gamma\delta$ cells) is true, there is the possibility that a predominant site for the proliferation of TcR$\gamma\delta$ cells in vivo may be the liver.

In this regard, it is of note that human TcR$\gamma\delta$ cells consist of nonoverlapping populations with either Cγ1-encoded or Cγ2-encoded $\gamma\delta$ heterodimer (45). The usage of MAbs revealed that such populations were identified by the MAbs BB3 (or anti-TiγA) and δTCS-1, respectively (46). Thus, the BB3 (or anti-TiγA) MAb reacted with the gene product of Vδ2 (or Vγ9), which was preferentially clustered with Cγ1, whereas the δTCS-1 reacted with that of Vδ1 which was clustered with Cγ2. It is known that TcR$\gamma\delta$ cells in the thymus expressed Cγ2 and the predominant population of TcR$\gamma\delta$ cells in the peripheral blood expressed Cγ1 (45). In the most recent study, we demonstrated that more than 70% of TcR$\gamma\delta$ cells in the liver of cancer patients ($n = 3$) are BB3$^+$ (peripheral type), but not δTCS-1$^+$ (Seki, S., and T. Abo, unpublished observation). On the other hand, the usage of gene segments in $\gamma\delta$ heterodimer of hepatic TcR$\gamma\delta$ cells were under investigation in the mice injected with syngeneic tumor cells, by using the polymerase chain reaction method and the Southern blot analysis.

We previously established that MNC could be isolated from the liver of mice after perfusion with PBS from the portal vein, in conjunction with the Ficoll-Isopaque gradient centrifugation (21). The isolated MNC mainly consists of T lymphocytes and NK cells but only a small proportion of Kupffer cells (< 4%). Interestingly, the decreased cell yield was only 10% by the perfusion, and the contents of lymphocyte constitution was not changed greatly (21). We could also observe a unique distribution pattern of lymphocytes in the human liver without perfusion. On the other hand, the results in mice represented were produced after perfusion.

In previous studies of humans and mice, TcR$\gamma\delta$ cells were

induced in vitro by the stimulation of allogeneic or syngeneic tumor cells (47, 48). Such cells, and the cell lines cultured in the presence of IL-2, had nonspecific NK-like cytotoxicity. The present results revealed that TcR$\gamma\delta$ cells were also inducible in vivo by the stimulation of tumor cells, especially in the liver. Although it is well established that NK cells are the first line of defense of tumor surveillance (30, 31), it raises the possibility that TcR$\gamma\delta$ cells may act as the second line of defense for the suppression of tumor growth, since TcR$\gamma\delta$ cells in the liver appear in cancer patients or in mice injected with tumor cells. Whether such hepatic TcR$\gamma\delta$ cells migrate to the tumor-filtrating sites remains to be investigated. However, TcR$\gamma\delta$ cells can eventually be identified in the tumor-infiltrating lymphocytes of cancer patients with malignant melanoma and renal cell carcinoma (Seki, S., and T. Abo, unpublished observation). It is, therefore, conceivable that the present results may include important evidence not only as to the liver function as an immune system but also for cancer immunology.

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References

1. Meuer, S. C., K. A. Fitzgerald, R. E. Hussy, J. C. Hodgdon, S. F. Schlossman, and E. L. Reinherz. 1983. Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. *J. Exp. Med.* 157:705–719.

2. Brenner, M. B., I. S. Trowbridge, and J. L. Strominger. 1985. Cross-linking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. *Cell.* 40:183–190.

3. Saito, H., D. M. Kranz, Y. Takagaki, A. C. Hayday, H. N. Eisen, and S. Tonegawa. 1984. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. *Nature (Lond.).* 309:757–762.

4. Hayday, A. C., H. Saito, S. D. Gillies, D. M. Kranz, G. Tanigawa, H. N. Eisen, and S. Tonegawa. 1985. Structure, organization, and somatic rearrangement of T cell gamma genes. *Cell.* 40:259–269.

5. Yachie, A., Y. Ueno, N. Takano, T. Miyawaki, and N. Taniguchi. 1989. Developmental change of double-negative ($CD3^+4^-8^-$) T cells in human peripheral blood. *Clin. Exp. Immunol.* 76:258–261.

6. Groh, V., S. Porcelli, M. Fabbi, L. L. Lanier, L. J. Picker, T. Anderson, R. A. Warnke, A. K. Bhan, J. L. Strominger, and M. B. Brenner. 1989. Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. *J. Exp. Med.* 169:1277–1294.

7. Crispe, I. N., M. W. Moore, L. A. Husman, L. Smith, M. J. Bevan, and R. P. Shimonkevitz. 1987. Differentiation potential of subsets of $CD4^-8^-$ thymocytes. *Nature (Lond.).* 329:336–339.

8. Lanier, L. L., and A. Weiss. 1986. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. *Nature (Lond.).* 324:268–270.

9. Pardoll, D. M., B. J. Fowldes, J. A. Blueston, A. Kruisbeek, W. L. Maloy, J. E. Coligan, and R. H. Schwartz. 1983. Differential expression of two distinct T-cell receptors during thymocyte development. *Nature (Lond.).* 326:79–81.

10. Winoto, A., and D. Baltimore. 1989. Separate lineages of T cells expressing the $\alpha\beta$ and $\gamma\delta$ receptors. *Nature (Lond.).* 338:430–433.

11. Raulet, D. H., R. D. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T-cell receptor gene expression. *Nature (Lond.).* 314:103–107.

12. Pardoll, D. M., B. J. Fowlkes, R. I. Lechler, R. N. German, and R. H. Schmartz. 1987. Early genetic events in T cell development analyzed by in situ hybridization. *J. Exp. Med.* 165:1624–1638.

13. Haynes, B. Y., K. H. Singer, S. M. Denning, and M. E. Martin. 1988. Analysis of expression of CD2, CD3, and T cell antigen receptor molecules during early human fetal thymic development. *J. Immunol.* 141:3776–3784.

14. Borst, J., R. J. van de Griend, J. W. van Oostveen, S.-L. Ang, C. J. Melief, J. G. Seidman, and R. L. H. Bolhuis. 1987. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. *Nature (Lond.).* 325:683–688.

15. Goodman, T., and L. Lefrancois. 1988. Expression of the γ-δ T-cell receptor on intestinal $CD8^+$ intraepithelial lymphocytes. *Nature (Lond.).* 333:855–858.

16. Bonneville, M., C. A. Janeway Jr., K. Itoh, W. Haser, I. Ishida, N. Nakanishi, and S. Tonegawa. 1988. Intestinal intraepithelial lymphocytes are a distinct set of $\gamma\delta$ T cells. *Nature (Lond.).* 336:479–481.

17. Koning, F., G. Stingl, W. M. Yokoyama, H. Yamada, W. L. Maloy, E. Tschachler, E. M. Shevach, and J. E. Coligan. 1987. Identification of a T3-associated $\gamma\delta$ T cell receptor on Thy-1^+ dendritic epidermal cell lines. *Science (Wash. DC).* 236:834–837.

18. Kuziel, W. A., A. Takashima, M. Bonyhadi, P. R. Bergstresser, J. P. Allison, R. E. Tigelwar, and P. W. Tucker. 1987. Regulation of T-cell receptor γ-chain RNA expression in murine Thy-1^+ dendritic epidermal cells. *Nature (Lond.).* 328:263–266.

19. Augustin, A., R. T. Kubo, and G.-K. Sim. 1989. Resident pulmonary lymphocytes expressing the γ/δ T-cell receptor. *Nature (Lond.).* 340:239–241.

20. Kamps, W. A., W. Timens, G. J. DeBoer, H. H. Spanjer, and S. Poppema. 1989. *In situ* study of haematopoiesis in human fetal liver. *Scand. J. Immunol.* 30:399–408.

21. Itoh, H., T. Abo, S. Sugawara, A. Kanno, and K. Kumagai. 1988. Age-related variation in the proportion and activity of murine liver natural killer cells and their cytotoxicity against regenerating hepatocytes. *J. Immunol.* 141:315–233.

22. Abo, T., C. A. Miller, and C. M. Balch. 1984. Characterization of human granular lymphocyte subpopulations expressing HNK-1 (Leu-7) and Leu-11 antigens in the blood and lymphoid tissues from fetuses, neonates and adults. *Eur. J. Immunol.* 14:616–623.

23. Abo, T., S. Sugawara, A. Amenomori, H. Itoh, H. Rikiishi, I. Moro, and K. Kumagai. 1986. Selective phagocytosis of gram-positive bacteria and interleukin 1-like factor production by a subpopulation of large granular lymphocytes. *J. Immunol.* 136:3189–3197.

24. Brenner, M. B., I. S. Trowbridge, J. McLean, and J. L. Strominger. 1984. Identification of shared antigenic determinants of the putative human T lymphocyte antigen receptor. *J. Exp. Med.* 160:541–551.

25. Faure, F., S. Jitsukawa, F. Triebel, and T. Hercend. 1988. Characterization of human peripheral lymphocytes expressing the CD3-γ/δ complex with anti-receptor monoclonal antibodies. *J. Immunol.* 141:3357–3360.

26. Ohtani, H., H. Naganuma, and H. Nagura. 1990. Microwave-stimulated fixation for histocytochemistry: application to surgical pathology and preembedding immunoelectron microscopy. *Acta Histochem. Cytochem.* In press.

27. Tsuchiya, Y., M. Igarashi, R. Suzuki, and K. Kumagai. 1988. Production of colony-stimulating factor by tumor cells and the factor-mediated induction of suppressor cells. *J. Immunol.* 141:699–708.

28. Nishimura, T., and T. Itoh. 1988. Higher level expression of lymphocyte function-associated antigen-1 (LFA-1) on in vivo natural killer cells. *Eur. J. Immunol.* 18:2077–2088.

29. Bonneville, M., K. Itoh, E. G. Krecko, S. Itohara, D. Kappes, I. Ishida, O. Kanagawa, C. A. Janeway, Jr., D. B. Murphy, and S. Tonegawa. 1989. Recognition of a self major histocompatibility complex TL region product by $\gamma\delta$ T-cell receptors. *Proc. Natl. Acad. Sci. USA.* 86:5928–5932.

30. Abo, T., C. A. Miller, G. L. Gartland, and C. M. Balch. 1983. Differentiation stages of human natural killer cells in lymphoid tissues from fetal to adult life. *J. Exp. Med.* 157:273–284.

31. Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK and cytotoxic T lymphocytes. *J. Immunol.* 136:4480–4485.

32. Sawada, H., T. Abo, S. Sugawara, and K. Kumagai. 1988. Prerequisite for the induction of lymphokine-activated killer cells from T lymphocytes. *J. Immunol.* 140:3668–3673.

33. Falini, B., L. Flenghi, S. Pileri, P. Pelicci, M. Fagiori, M. F. Martelli, L. Moretta, and E. Ciccone. 1989. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. *J. Immunol.* 143:2480–2488.

34. Bucy, R. P., C. H. Cheu, J. Cihak, U. Losch, and M. D. Cooper. 1988. Avian T cells expressing $\gamma\delta$ receptors localize in the splenic sinusoids and the intestinal epithelium. *J. Immunol.* 141:2200–2205.

35. Bucy, R. P., C. H. Chen, and M. D. Cooper. 1989. Tissue localization and CD8 accessory molecule expression of T$\gamma\delta$ cells in human. *J. Immunol.* 142:3045–3049.

36. Kaneda, K., C. Dan, and K. Wake. 1983. Pit cells as natural killer cells. *Biomed. Res.* 4:567–576.

37. Kaneda, K. 1989. Liver-associated large granular lymphocytes: morphological and functional aspectes. *Arch. Histol. Cytol.* 52:447–459.

38. Ohteki, T., S. Seki, T. Abo, and K. Kumagai. 1990. Liver is a possible site for the proliferation of abnormal $CD3^+4^-8^-$ double negative lymphocytes in autoimmune MRL-1pr/1pr mice. *J. Exp. Med.* In press.

39. Brenner, M. B., J. McLean, H. Scheft, J. Riberdy, S.-L. Ang, J. G. Seidman, P. D. Devlin, and M. S. Kragel. 1987. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. *Nature (Lond.).* 324:689–694.

40. Bluestone, J. A., R. Q. Cron, M. Cotterman, B. A. Houlden, and L. A. Matis. 1988. Structure and specificity of T cell receptor γ/δ on major histocompatibility complex antigen-specific $CD3^+$, $CD4^-$, $CD8^-$ T lymphocytes. *J. Exp. Med.* 168:1899–1916.

41. Kishihara, K., Y. Yoshikai, G. Matsuzaki, T. W. Mak, and K. Nomoto. 1987. Functional alpha and beta T cell chain receptor messages can be detected in old but not young athymic mice. *Eur. J. Immunol.* 17:477–482.

42. Yoshikai, Y., M. D. Reis, and T. W. Mak. 1986. Athymic mice express a high level of functional gamma-chain but greatly reduced levels of alpha- and beta-chain T-cell receptor messages. *Nature (Lond.).* 324:482–485.

43. Pardoll, D. M., B. J. Fowlkes, A. M. Lew, W. L. Maloy, M. A. Weston, J. A. Bluestone, R. H. Schwartz, J. E. Coligan, and A. M. Kruisbeek. 1988. Thymus-dependent and thymus-independent development pathways for peripheral T cell receptor-$\gamma\delta$-bearing lymphocytes. *J. Immunol.* 140:4091–4096.

44. Preffer, F. I., C. W. Kim, K. H. Fisher, E. M. Sabga, R. L. Kradin, and R. B. Colvin. 1989. Identification of pre-T cells in human peripheral blood. Extrathymic differentiation of $CD7^+3^-$ cells into $CD3^+$ γ/δ^+ or α/β^+ T cells. *J. Exp. Med.* 170:177–190.

45. Ferrini, S., I. Prigione, C. Bottino, E. Ciccone, G. Tambussi, S. Mammoliti, L. Moretta, and A. Moretta. 1989. Monoclonal antibodies which react with the T cell receptor γ/δ recognize different subsets of $CD3^+WT31^-$ T lymphocytes. *Eur. J. Immunol.* 19:57–61.

46. Borst, J., A. Wicherink, J. J. M. Van Dongen, E. De Vries, W. M. Comans-Bitter, F. Wassenaar, and P. Van Den Elsen. 1989. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. *Eur. J. Immunol.* 19:1559–1568.

47. Koide, J., A. Rivas, and E. G. Engleman. 1989. Natural killer (NK)-like cytotoxic activity of allospecific T cell receptor-γ,δ^+ T cell clones. Distinct receptor-ligand interactions mediate NK-like and allospecific cytotoxicity. *J. Immunol.* 142:4161–4168.

48. Sperling, A. I., and H. H. Wortis. 1989. $CD4^-$, $CD8^-$ γ/δ cells from normal mice respond to a syngeneic B cell lymphoma and can induce its differentiation. *Inter. Immunol.* 1:434–442.