ACTIVATION OF EXTRATHYMIC T CELLS IN THE LIVER OF MICE BEARING SYNGENEIC TUMORS

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ABSTRACT

A major site of extrathymic T cell differentiation exists in the liver. We investigated herein the responses of such extrathymic T cells in mice bearing syngeneic tumor cells. To identify extrathymic T cells (i.e., intermediate TCR cells), several new methods were introduced. When C57BL/6 mice were subcutaneously injected with 1×10^6 MCA105 tumor cells of syngeneic origin, a prominent increase in the number of hepatic MNC was induced at the initial period, i.e., until 2 weeks after tumor inoculation. The number of hepatic MNC then decreased gradually. On the other hand, the number of thymocytes began to decrease 20 days after the injection, whereas the number of splenic lymphocytes increased due to the occupation of macrophages. Analysis of the surface phenotypes revealed that the increase in the number of hepatic MNC was accompanied by an increase in the proportion of intermediate TCR cells and an induction of tumor-specific cytotoxicity in the liver. Intermediate TCR cells consisted of double-negative CD4⁻8⁻ cells and single-positive CD4⁺ or CD8⁺ cells, and highly expressed IL-2 receptor β chains and LFA-1 antigens. Interestingly, the extrathymic T cell activation in the liver and profound thymic atrophy were not induced by the injection of allogeneic tumor cells or virally transformed syngeneic tumor cells. These results suggest that extrathymic T cells, rather than CTL of thymic origin, may play important roles in host immune responses against regular syngeneic tumors.

Host immune responses during the growth of tumors have been intensively investigated. In general, studies on humans and experiments using mice and syngeneic tumor systems have documented that immune down-regulation is induced during tumor burden due to a decreased function of peripheral T cells and an involution of the thymus which is the major organ for T cell differentiation (i.e., a decrease of T cell supply) (8, 16, 27). As specific anti-tumor activity is believed to be attributable to CTL, much attention has been focused

on the characterization of such T cells of thymic origin (4, 13, 21).

In a series of recent studies (3, 18, 19, 22), we demonstrated that an extrathymic pathway of T cell differentiation exists in humans and mice and that one of the major sites of support for this pathway is the hepatic sinusoids. Although extrathymic T cell differentiation in the liver is extremely minor at young age, it becomes predominant under conditions of aging (18), bacterial stimulation (3) and autoimmune diseases (14, 19, 23). Moreover, in a murine study using mitomycin (MMC)-treated syngeneic tumors, extrathymic T cells were demonstrated to be rather activated after tumor injection, despite the simultaneous induction of thymic atrophy (24).

In subsequent studies, we have demonstrated

Abbreviations: DN, double-negative; MNC, mononuclear cells; IL-2R β , interleukin-2 receptor β chain; DP, double-positive; Mls, minor lymphocyte stimulatory; TL, thymus leukemia antigen, mAb, monoclonal antibody

many unique properties of extrathymic T cells in the liver, which are distinct from those of regular T cells of thymic origin. For example, the extrathymic T cells in the liver have TCR- $\alpha\beta$ of intermediate intensity in terms of immunofluorescence staining, comprise a significant proportion of double-negative (DN) CD4⁻8⁻ T cells (14), and consist of autoreactive forbidden T cell clones estimated with a series of anti-V β mAbs (3). The object of the present study was to investigate the extrathymic T cell response in mice bearing syngeneic tumors (i.e., MMC-untreated tumors). To emphasize the unique effects of regular syngeneic tumor burden, injections of virally transformed syngeneic tumor cells and allogeneic tumor cells were used in parallel. We propose the possibility that extrathymic T cells, but not T cells of thymic origin (i.e., CTL), may play a major role in host immune response against tumors.

MATERIALS AND METHODS

Mice

Female C57BL/6 and BALB/c mice aged 6 weeks were purchased from Jackson Laboratory Japan, Tokyo. They were fed under specific pathogen-free conditions until use.

Tumors

MCA105 cells are methylcholantolene-induced fibrosarcoma of C57BL/6 origin (26) and B16 cells are melanoma of the same origin (28). Meth A cells are also methylcholantolene-induced fibrosarcoma of BALB/c origin (28). AT1 and FBL-3 cells are virally transformed fibrosarcoma (12) and lymphoma of C57BL/6 origin (6), respectively. Mice were subcutaneously injected with various numbers of syngeneic or allogeneic tumor cells and were sacrificed at the indicated days after injection to examine various immunoparameters. MMC-treatment of tumors was performed for 1 h at 37°C at a concentration of 50 μ g/ml (24).

Cell Preparations

Hepatic mononuclear cells (MNC) were prepared as previously described (31). Briefly, mice anesthetized with ether were sacrificed by total bleeding (induced by incision) of the axillary artery and vein, and their livers were removed. As resident MNC in the liver are estimated to be much more predominant (>80%) than the MNC contaminated

by circulation in this total bleeding method, PBS perfusion was not applied here. The removed liver was cut into small pieces by scissors, pressed through 200-gauge stainless steel mesh, and suspended in Eagles MEM with 20 mM HEPES (Sigma Chemical, St. Louis, MO) 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. The MNC preparation isolated by this method contained less than 4% Kupffer cells.

Immunofluorescence Tests

Surface phenotypes of cells were identified by using mAbs in conjunction with a single-, two- or three-color immunofluorescence test (31). The mAbs used here included FITC or biotin (or phycoerythrin; PE)-conjugated reagents of anti-TCR- $\alpha\beta$ (H57-597) (10), anti-CD3 (145-2C11) (24), anti-CD4 (L3T4), anti-CD8 (Lyt-2), anti-Thy 1.2, anti-IL-2 receptor β chain (IL-2R β) (30), and LFA-1 (FD441.8) mAbs (31). Biotin-conjugated reagents were developed with PE-conjugated avidin or Red 613-conjugated streptoavidin (Caltag Laboratories, San Francisco, CA). The fluorescence-positive cells were analyzed by a FACScan (Becton-Dickinson, Mountain View, CA) (31).

Cytotoxicity Assay

Cytotoxicity was examined by a ⁵¹Cr-specific release assay of 16-h incubation time, as previously described (2). Target cells were labelled with sodium chromate (Amersham International, Arlington Heights, IL), whereas effector cells were either hepatic MNC or splenic lymphocytes isolated from mice with or without tumor burden.

RESULTS

Identification of Intermediate TCR Cells in the Liver

At the beginning of this study, we confirmed that hepatic MNC comprised a unique T cell population with TCR- $\alpha\beta$ of intermediate intensity in terms of immunofluorescence staining (Fig. 1). In addition to bright TCR- $\alpha\beta$ cells which are regular T cells of thymic origin, intermediate TCR- $\alpha\beta$ cells were present at the intermediate position between TCR-

Fig. 1 Single and dual color flowcytometric analysis of hepatic MNC in normal C57BL/6 mice aged 8 weeks. A, staining of TCR- $\alpha\beta$; B, staining of CD3; C, two-color staining of TCR- $\alpha\beta$ and CD3. Intermediate TCR- $\alpha\beta$ cells express CD3 of intermediate intensity, whereas bright TCR- $\alpha\beta$ cells express CD3 of bright intensity.

$\alpha\beta^-$ cells and bright TCR- $\alpha\beta$ cells (Fig. 1A). As shown previously (23), the intermediate position of these cells was greater than that of dull TCR cells in the thymus. A similar pattern was produced by CD3 staining (Fig. 1B). The two-color immunofluorescence test clearly demonstrated that T cells with CD3 of intermediate intensity expressed TCR- $\alpha\beta$ of intermediate intensity (Fig. 1C). This was due to crosslinking of one TCR- $\alpha\beta$ molecule with one CD3 molecule. Here, 30% of hepatic MNC were CD3 $^+$, whereas 26% of hepatic MNC were TCR- $\alpha\beta$ $^+$. As shown previously (24), the remainder were TCR- $\gamma\delta$ $^+$.

To further characterize intermediate TCR cells, a three-color immunofluorescence test using FITC-anti-CD4, PE-anti-CD8, and biotin-anti-CD3 mAbs was performed (Fig. 2). Biotin-anti-CD3 mAb was developed with Red 613-streptavi-

din. It was demonstrated that total hepatic CD3 $^+$ T cells (i.e., both intermediate and bright TCR cells) consisted of not only single-positive CD4 $^+$ (69%) or CD8 $^+$ (12%) cells but also DN CD4 $^-$ CD8 $^-$ cells (18%) (Fig. 2A). This was striking when compared with the pattern of splenic lymphocytes (Fig. 2B), because splenic lymphocytes comprised only bright TCR cells which consisted of only single-positive CD4 $^+$ (69%) or CD8 $^+$ (27%) cells. DN cells in the spleen were negligible (1%). When the analysis was gated to bright TCR cells in the liver, only single-positive CD4 $^+$ (79%) or CD8 $^+$ (15%) cells were revealed (see Fig. 2A, right column), as with the spleen. On the other hand, when the analysis was gated to intermediate TCR cells in the liver, a large proportion of DN cells (33%) was demonstrated as well as single-positive CD4 $^+$ (58%) or CD8 $^+$ (8%) cells.

Fig. 2 Three-color flowcytometric analysis of hepatic MNC and splenic lymphocytes in normal C57BL/6 mice aged 8 weeks. A, hepatic MNC; B, splenic lymphocytes. Cells were stained by a three-color immunofluorescence test using FITC-conjugated anti-CD4, PE-conjugated anti-CD8 and biotin-conjugated anti-CD3 mAbs. Biotin-conjugated reagent was developed with Red 613-streptoavidin. Intermediate and bright peaks of CD3 were gated to analyze CD4 and CD8 expression. Only intermediate TCR cells in the liver comprised DN CD4⁺CD8⁻ T cells.

Fig. 3 Time-kinetics in the number of MNC in various organs of mice after syngeneic tumor injection. A, hepatic MNC; B, splenic cells; C, thymocytes. C57BL/6 mice aged 8 weeks were subcutaneously injected with 1×10^6 MCA105 cells and the cell numbers were enumerated at the indicated days.

Time-Kinetics in the Number of MNC in Various Organs of Mice after Syngeneic Tumor Injection

C57BL/6 mice were subcutaneously injected with 1×10^6 syngeneic MCA105 tumor cells, and the numbers of MNC in the liver, spleen and thymus were successively enumerated until death (up to 60 days after the injection) (Fig. 3). Here, the normal value of the cell number in each organ is indicated by the dotted area; $1.8 \pm 0.5 \times 10^6$ /mouse in the liver, $1.6 \pm 0.5 \times 10^8$ /mouse in the spleen and $11.5 \pm 2.0 \times 10^7$ /mouse in the thymus. Interestingly, just after the tumor injection the number of hepatic MNC increased up to 5×10^6 /mouse and the increased level continued for approximately 10 days (Fig. 3A). A relatively high level also continued until 40 days after the injection and then the level declined. In sharp contrast, the number of splenic cells did not change during the initial period (until 2 weeks after the injection), but subsequently increased (Fig. 3B). Almost all splenic cells in mice in the latter phase consisted of Mac-1⁺ macrophages (data not shown). In the case of the

thymus, thymic atrophy began 20 days after the injection and profound atrophy was observed at the end stage (Fig. 3C).

Phenotypic Characterization of MNC in Various Organs of Mice with Tumor Burden

As a prominent increase in the number of hepatic MNC was observed during the initial period after the injection of tumor cells, phenotypic characterization was performed (Fig. 4). To determine the relationship between CD3 expression and Thy 1 expression, two-color staining of these markers was carried out in the liver, spleen and thymus of control C57BL/6 mice and mice injected with MCA105 tumors (5 days after the injection). In normal mice, intermediate TCR cells (i.e., those expressing CD3 of intermediate intensity) in the liver (indicated by an arrowhead) were identified; these cells had a considerably high level of Thy 1 expression (a slightly lower expression of Thy 1 than bright TCR cells) (Fig. 4A, left). A small proportion of intermediate TCR cells (indicated by an

Fig. 4 Dual color flowcytometric analysis of hepatic MNC, splenic lymphocytes and thymocytes in normal mice and tumor-bearing mice. A, normal C57BL/6 mice; B, tumor-bearing mice. C57BL/6 mice injected with 1×10^6 MCA105 were examined on day 5. Two-color staining of Thy 1.2 and CD3 was performed. Intermediate TCR cells are indicated by arrowheads in the liver and spleen.

arrowhead) were present even in the spleen of normal mice by this staining method (Fig. 4A, center). As expected, thymocytes consisted of dull and bright TCR cells, and also lacked intermediate TCR cells (Fig. 4A, right). Five days after tumor-cell inoculation, intermediate TCR cells were highly prominent in the liver (Fig. 4B, left) and, to some extent, in the spleen (Fig. 4B, center). Some activated intermediate TCR cells in these organs had a population expressing a low density of Thy 1 antigen. On the other hand, the staining pattern of thymocytes did not change significantly (Fig. 4B, right).

Further Phenotypic Characterization of Intermediate TCR Cells in the Liver

In these experiments, attention was focused on the characterization of intermediate TCR cells in the liver with respect to their expression of IL-2R β and LFA-1 molecules (Fig. 5). In contrast to Thy 1

expression, IL-2R β and LFA-1 were highly expressed on intermediate TCR cells, and it was therefore easy to accurately enumerate the proportions of intermediate and bright TCR cells at the same time. As shown in untreated mice (Fig. 5A), intermediate and bright TCR cells in the liver had clear independent peaks. CD3⁻ IL-2R β ⁺ cells were subsequently estimated to be NK cells. In contrast to IL-2R β staining, LFA-1 staining with CD3 did not distinguish NK cells from CD3⁻ B cells. As a function of MCA105 cell numbers injected (Fig. 5B, 1×10^6 /mouse; C, 5×10^6 /mouse; and D, 2.5×10^7 /mouse), the proportion of intermediate TCR cells increased up to 25% of the total hepatic MNC. Here, the analysis was performed 5 days after the tumor injection.

Similarly, the two-color stainings using PE-anti-CD3 mAb and a mixture of FITC-anti-CD4 and -anti-CD8 mAbs, as well as using FITC-anti-CD4 and PE-anti-CD8 mAbs, were carried out to determine the variation in the proportions of DN T cells

Fig. 5 Dual color flowcytometric analysis of IL-2R β and LFA-1 in conjunction with CD3 for hepatic MNC. A, untreated C57BL/6 mice; B, mice injected with 1×10^6 MCA105; C, mice injected with 5×10^6 MCA105; D, mice injected with 2.5×10^7 MCA105. To clearly indicate intermediate and bright TCR cells, the corresponding areas are encompassed by squares, R₁ and R₂, respectively. The numbers in the figure express the percentages of intermediate TCR cells. Tumor effect was examined on day 5 after the injection. The increased number of tumor cells resulted in the prominent induction of intermediate TCR cells.

and single-positive T cells in the liver (Fig. 6). C57BL/6 mice injected with 1×10^6 MCA105 tumor cells were examined on days 5 and 15 after the injection. It was demonstrated that only on day 5, but not on day 15, did intermediate TCR cells

with both DN (6%→11%) and single-positive phenotype increase as shown in Fig. 6A and B. It was confirmed that in contrast to thymocytes, hepatic MNC did not comprise double-positive (DP) CD4⁺8⁺ cells (<1%) (Fig. 6B).

Fig. 6 Dual color flowcytometric analysis of hepatic MNC in mice with or without tumor injection. A, two-color staining of CD4 plus CD8 and CD3; B, two-color staining of CD4 and CD8. A considerably large proportion of DN CD4⁺8⁻ T cells were demonstrated in the liver of C57BL/6 mice injected with 1×10^6 MCA105 on day 5, but not on day 15. Hepatic MNC did not comprise DP CD4⁺8⁺ cells.

A Comparison of the Responses of Extrathymic T Cells in the Liver Between Mice Injected with Syngeneic and Allogeneic Tumor Cells

In this experiment, 1×10^6 MCA105 tumor cells of C57BL/6 origin were injected into C57BL/6 (syngeneic combination) and BALB/c mice (allogeneic combination), and the responses of intermediate TCR cells in the liver were compared (Fig. 7). In the syngeneic combination, inoculated tumor cells showed intensive tumor growth *in vivo*, especially 2 weeks after the injection, which resulted in the formation of a tumor mass in the subcutaneous injected region. On the other hand, allogeneic tumor cells could not survive in BALB/c mice. In contrast to the increase in the proportion of intermediate TCR cells and the decrease in the proportion of bright TCR cells in the liver of C57BL/6 mice (day 5) (Fig. 7A), a unique response was observed in the

liver of BALB/c mice (Fig. 7B). Namely, the decrease in the proportion of intermediate TCR cells and the increase in the proportion of bright TCR cells were induced in the liver of BALB/c mice (allogeneic combination). On day 15 after the injection, the staining patterns of both types of mice were once again almost normal (Fig. 7, bottom). It should also be emphasized that the number of hepatic MNC yielded by C57BL/6 mice injected with syngeneic tumor cells increased up to 5×10^6 /mouse on day 5, whereas the number of hepatic MNC in BALB/c mice with allogeneic tumors remained constant (1.8×10^6 /mouse) or decreased. In other words, the activation of extrathymic T cells in the liver was induced only in the syngeneic combination of mouse and tumor.

In a subsequent study, we also examined BALB/c mice injected with 1×10^6 Meth A (syngeneic

Fig. 7 Dual color flowcytometric analysis of LFA-1 and CD3 in hepatic MNC of mice injected with syngeneic or allogeneic tumors. A: Control C57BL/6 mice and C57BL/6 mice injected with 1×10^6 syngeneic MCA105 tumor cells. B: Control BALB/c mice and BALB/c mice injected with 1×10^6 allogeneic MCA105 tumor cells. Mice injected with tumor cells were examined on days 5 and 15 after the injection. The numbers of the figure express the percentages of intermediate and bright TCR cells. The increase in the proportion of intermediate TCR and the decrease in the proportion of bright TCR cells were induced only in a syngeneic combination on day 5.

combination) and C57BL/6 mice injected with 1×10^6 Meth A (allogeneic combination) on day 5 after inoculation. Again, the increase in the proportion of intermediate TCR cells and the decrease in the proportion of bright TCR cells were induced only in a syngeneic combination (data not shown).

Induction of the Cytotoxicity Against Syngeneic Tumors in Hepatic MNC

Thus far, our experiments indicated that syngeneic tumors selectively activated hepatic MNC (i.e., extrathymic T cells with intermediate TCR). It was then investigated whether such activated MNC in

Fig. 8 Cytotoxic activity of hepatic MNC and splenic lymphocytes in untreated mice, mice injected with syngeneic tumor, and mice injected with allogeneic tumor cells. (A) Target: MCA105, (B) Target: Meth A. C57BL/6 mice were previously injected with 1×10^7 MMC-treated MCA105 or Meth A. After 2 weeks, these mice were again injected with 1×10^6 corresponding live tumor cells for the induction of secondary response. Hepatic MNC (●) or splenic lymphocytes (○) were used as effector cells at the indicated effector to target (E/T) ratios. The figures of the ordinate represent the percentages of ^{51}Cr -specific release from targets after 16-h incubation analysis. Mice injected with syngeneic MCA105 tumor cells showed specific cytotoxicity against the injected MCA105 tumor cells, whereas mice injected with allogeneic Meth A tumor cells showed specific cytotoxicity against only the corresponding Meth A tumor cells. Liver MNC were always better effector cells than splenic lymphocytes in both combinations.

the liver contained cytotoxic activity against syngeneic tumor cells (Fig. 8). Effector cells used here were either hepatic MNC or splenic lymphocytes obtained from untreated C57BL/6 mice, C57BL/6

mice injected with MCA105 (syngeneic combination) and C57BL/6 mice injected with Meth A (allogeneic combination), whereas target cells were either MCA105 or Meth A tumor cells. To induce

Fig. 9 A comparison of the effects of various tumor cells on the numbers of hepatic MNC and thymocytes. BALB/c mice aged 8 weeks were injected with cells of various 1×10^6 syngeneic tumors as listed. The number of hepatic MNC were enumerated on day 10 after the tumor inoculation, whereas the number of thymocytes were enumerated on day 20. Only regular MCA105 and B16 tumor cells, but not virally transformed AT1 and FBL-3 tumor cells, induced the increase in the number of hepatic MNC and profound thymic atrophy. Figures are represented as a logarithmic scale.

significant cytotoxicity, C57BL/6 mice were previously stimulated with either 1×10^7 MMC-treated MCA105 or Meth A tumor cells, and 2 weeks after stimulation each group of mice was again stimulated with corresponding 1×10^6 live MCA105 or Meth A as a secondary stimulation. One week after the second stimulation, mice were sacrificed for cytotoxicity assay. As shown in Fig. 8, cells isolated from untreated mice did not show any cytotoxic activity against either target, i.e., MCA105 or Meth A (Fig. 8, top). However, C57BL/6 mice stimulated with syngeneic MCA105 tumor cells showed considerable cytotoxicity against syngeneic MCA105 but not against allogeneic Meth A (Fig. 8, middle). Here, hepatic MNC, which contained a large proportion of intermediate TCR cells (20%), had a stronger cytotoxicity than splenic lymphocytes. On the other hand, neither hepatic MNC nor splenic lymphocytes from mice stimulated with allogeneic Meth A tumor cells showed cytotoxicity against MCA105 (Fig. 8A, bottom).

When C57BL/6 mice were stimulated with allogeneic Meth A tumor cells, these mice showed the strongest activity against the corresponding target, Meth A, but not against MCA105 (Fig. 8B, bot-

tom). In this case, since splenic lymphocytes also had considerable cytotoxicity, bright TCR cells might be effectors in the liver as well as in the spleen (i.e., specific CTL of thymic origin).

Virally Transformed Syngeneic Tumors Did Not Activate Extrathymic T Cells in the Liver and Did Not Induce Profound Thymic Atrophy

Many cases of the syngeneic combination of mice and tumors in this and previous studies (24) have shown that such tumor burdens almost always induce the activation of intermediate TCR cells in the liver and inversely induce profound thymic atrophy. However, during the course of these studies we observed that virally transformed tumors did not induce the above phenomena despite the syngeneic combination. In this experiment, C57BL/6 mice were injected with 1×10^6 syngeneic tumor cells, including regular tumor cells of MCA105 and B16 and virally transformed tumor cells of AT1 and FBL-3 (Fig. 9). Here, the numbers of hepatic MNC were enumerated on day 10, whereas the numbers of thymocytes were enumerated on day 20 after the injection. As shown in the figure, MCA105 and B16 tumor cells were

Fig. 10 Dual color flowcytometric analysis of IL-2R β and CD3 in hepatic MNC, splenic lymphocytes and thymocytes of mice injected with tumor cells. A, untreated C57BL/6 mice; B, C57BL/6 mice injected with 1×10^6 B16 tumor cells; C, C57BL/6 mice injected with 1×10^6 syngeneic, but virally transformed FBL-3 tumor cells. B16 tumor cells induced an increase in the proportion of intermediate TCR cells in the liver, whereas virally transformed FBL-3 tumor cells increased the proportion of bright TCR cells with a high level of IL-2R β expression (indicated by an arrow) in the liver.

confirmed to induce an increased number of hepatic MNC. However, virally transformed AT1 and FBL-3 tumor cells did not significantly increase the number of hepatic MNC. More importantly, AT1 and FBL-3 tumor cells did not induce profound thymic atrophy even when inoculated tumor cells resulted in a large tumor mass *in vivo* on day 20 after the injection.

Furthermore, during the initial period (day 10 after tumor injection), B16 tumor cells actually induced an increase in the proportion of intermediate TCR cells in the liver, whereas virally transformed FBL-3 tumor cells did not induce such a response (Fig. 10). Rather, in the case of FBL-3 injection, the proportion of bright TCR cells appeared to increase in the liver. Especially, it was noted that a large proportion of such bright TCR

cells in the liver expressed the increased level of IL-2R β (see Fig. 10C, top). On the other hand, the staining pattern of splenic cells and thymocytes did not change significantly in either the case of B16 injection or FBL-3 injection.

DISCUSSION

In the present study, we systematically examined T cell responses in mice during the entire periods after syngeneic tumor cells were injected. Although many studies on host immune response against malignancies have been performed, the focus was mainly on peripheral T cells of thymic origin in those studies (8, 16, 27). Here, our focus was on extrathymic T cells, especially in the liver. In addition, we applied several newly developed

methods to identify such extrathymic T cells, i.e., intermediate TCR cells. The most striking finding was that mice bearing syngeneic tumors showed prominent increases in the number of hepatic MNC and in the proportion of intermediate TCR cells in the liver and, to some extent, in the spleen. This phenomenon was most prominent in the initial period (2 weeks) after syngeneic tumor-cell injection. There was no evidence that T cells of thymic origin (i.e., bright TCR cells) were activated in the thymus, spleen and liver during the same period. In other words, mice bearing syngeneic tumors showed only the activation of extrathymic T cells in the liver. When these extrathymic T cells increased in the liver, potent cytotoxic activity against the injected tumor cells was observed in the hepatic MNC. Although the syngeneic fibrosarcoma, MCA105, is known not to induce a strong anti-tumor immunity (see the data on splenic lymphocytes in this study), the cytotoxic activity of hepatic MNC induced here was noteworthy. It therefore raises the possibility that extrathymic T cells seen in the liver and periphery may play important roles in anti-tumor immunity.

It was also evident that allogeneic tumors and virally transformed syngeneic tumors could not activate extrathymic T cells in the liver as shown by there being no change in the number of hepatic MNC and in the proportion of intermediate TCR cells. It seemed that these tumor cells activated regular T cells of thymic origin (i.e., bright TCR cells) rather than extrathymic T cells. Virally transformed tumors may activate regular T cells via their viral antigens on the cell surface as shown by our observation that viral infection of mice preferentially activated regular T cells, but not extrathymic T cells (1). It is therefore conceivable that both allogeneic tumor cells and virally transformed syngeneic tumor cells are mainly recognized by T cells of thymic origin (i.e., CTL).

Since extrathymic T cells in the liver have the ability to produce autoreactive forbidden T cell clones (estimated by anti-V β mAbs against certain MIs antigens) and autoreactive V β 8⁺ T cell clones (3), the autoreactivity of extrathymic T cells may be beneficial for the surveillance of syngeneic tumor cells. The anti-tumor activity of V β 8⁺ T cells has already been reported (15). Extrathymic T cells in the liver also comprise DN T cells (14, 23) which are known to have autoreactivity (12, 15). In a recent study, we applied the most sensitive method, which was also introduced here, to detect intermediate TCR cells in tumor infiltrating lym-

phocytes (TIL). Interestingly, the TIL in mice with syngeneic combinations (e.g., C57BL/6 mice with B16 and BALB/c mice with Meth A) consisted mainly of NK cells, intermediate TCR cells and macrophages. Bright TCR cells were a minor population in the TIL (Iiai T. and Abo T., manuscript in preparation). It should be emphasized that extrathymic T cells may play important roles in anti-tumor immunity by virtue of their autoreactivity. However, the overstimulation of extrathymic T cells with similar properties may be responsible for the onset of certain autoimmune diseases (14, 19, 23).

There is a question as to what kinds of antigens on the syngeneic tumor cells are recognized by extrathymic T cells. In this regard, we have recently observed an interesting phenomenon in TL-transgenic mice. TL-transgenic mice were reported to have thymic atrophy even in adulthood, in which many $\gamma\delta$ T cells with DN CD4⁻8⁻ phenotype were present, as shown by Obata *et al.* (17). A collaborating study by Obata and the present authors (manuscript in preparation) have revealed that these TL-transgenic mice have abundant $\alpha\beta$ and $\gamma\delta$ T cells (approximately 2:1) with DN CD4⁻8⁻ phenotype in the liver. There was a similarity in the activated state of extrathymic T cells between TL-transgenic mice and tumor-bearing mice which showed a shared property of profound thymic atrophy. It is postulated that primitive extrathymic T cells with DN phenotype in the liver recognized monomorphic class I MHC antigens, TL. It is also conceivable that the specific tumor-immunity of extrathymic T cells may be produced by the recognition of tumor-specific antigens in the context of TL antigens. Since there were also significant proportions of single-positive CD4⁺ or CD8⁺ cells in the liver, such population might recognize specific tumor antigens in the context of regular polymorphic class II or I MHC antigens, respectively.

Two-color immunofluorescence tests directly demonstrated that intermediate TCR cells in the liver consisted of DN T cells and single-positive CD4⁺ or CD8⁺ cells. Intermediate TCR cells are a minor population in the periphery (23). However, a two-color test using anti-Thy 1.2 and anti-CD3 mAbs revealed that a small but significant proportion of intermediate TCR cells existed even in the periphery, e.g., the spleen. As shown previously (9, 25), thymocytes consist of dull and bright TCR cells, which are immature T cells with DP CD4⁺8⁺ phenotype and mature T cells with single-positive CD4⁺ or CD8⁺ phenotype, respectively. We postu-

late that intermediate TCR cells seen in the periphery come from the other organs (e.g., the liver). In autoimmune MRL-*lpr/lpr* mice, we previously demonstrated that extrathymic DN $\alpha\beta$ T cells generated in the liver, migrated to the periphery and reconstituted lymphadenopathy (19). In this case, DN $\alpha\beta$ T cells appeared to be already mature T cells *per se*, since further phenotypic switching of these cells could not be induced in an *in vitro* culture (19).

As well established (5, 7, 29), the numbers of thymocytes and splenic lymphocytes showed unique variations in tumor-bearing mice. It was confirmed that profound thymic atrophy was induced beginning 20 days after syngeneic tumor-cell injection, whereas an increase in the number of splenic lymphocytes was observed in similar periods. It was previously reported that thymic atrophy was due to the accelerated programmed death of DP CD4⁺8⁺ cells (24). On the other hand, the increase in the number of splenocytes was due to the occupation of the spleen by Mac-1⁺ macrophages. At the end stage of tumor burden, even the number of hepatic MNC was reduced due to the decrease in the number of bright TCR cells as well as the subsequent decrease in the number of intermediate TCR cells. At present, we do not know whether the factors which induced the activation of extrathymic T cells and thymic atrophy are the same or not.

Finally, in the present study we surveyed the intermediate TCR cells by using mainly CD3 markers. This was due to the predominance of intermediate TCR- $\alpha\beta$ cells in the liver. This was even true in the case of mice with malignancies (24). We have not yet directly determined whether the cytotoxicity against syngeneic tumor cells is mediated by intermediate TCR cells or not. These subjects, including the question as to what T cell clones among intermediate TCR cells in the liver are actually associated with specific tumor-immunity, are under investigation. The activation of extrathymic T cells occurred only during the initial period after tumor inoculation. If we succeed in continuously activating the extrathymic pathway of T cell differentiation during the entire period of tumor burden, it may lead to the development of an appropriate cancer therapy. The extrathymic pathway is now known to be activated by bacterial stimulation (3), estrogen administration (20) and lymphotoxin injection (11). Therefore, the continuous activation of the extrathymic pathway of T cell differentiation will be examined in future studies.

This work was supported by a grant-in-aid for Scientific Research and Cancer Research from the Ministry of Education, Science and Culture, and a grant from the Medical Research Foundation of Niigata University, Japan.

Received 9 December 1992; and accepted 22 December 1992

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