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Autologous killing by a population of intermediate T-cell receptor cells and its NK1.1$^+$ and NK1.1$^-$ subsets, using Fas ligand/Fas molecules

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SUMMARY

Self-reactive clones, estimated by anti-Vβ monoclonal antibodies (mAb) in conjunction with the Mls system, are confined to a population of intermediate (int) T-cell receptor (TCR) (or CD3) cells (i.e. TCR^{int} cells), but are not found among TCR^{high} cells. The next questions to be answered are whether autologous killing is confined to TCR^{int} cells and how such killing is mediated. In this study, ^{51}Cr-labelled thymocytes of syngeneic or allogeneic origin were used as target cells (4-hr assay). When liver and splenic mononuclear cells (MNC) obtained from B6 mice were used as effector cells, prominent autologous killing was seen in liver MNC, but not splenic MNC. Such killing was not seen when thymocytes from B6-*lpr/lpr* mice (i.e. Fas$^-$) were used as target cells, nor when liver MNC from MRL-*gld/gld* mice (i.e. Fas ligand$^-$) were used as effector cells (target thymocytes of MRL-+/+ mice). Cell separation experiments using a cell sorter revealed that autologous killing was mediated for the most part by CD3^{int} cells, while allogeneic killing was mediated entirely by natural killer (NK) cells, TCR^{int} cells and TCR^{high} cells. Among CD3^{int} cells, the NK1.1$^+$ subset (i.e. NK1.1$^+$ T cells) manifested a higher level of autologous killing than did the NK1.1$^-$ subset. Consistent with the results of a functional assay, it was found by reverse-transcription–polymerase chain reaction (RT-PCR) assay that CD3^{int} cells among liver MNC showed the expression of Fas ligand mRNA, while thymocytes expressed Fas mRNA. When class I major histocompatibility complex (MHC)$^-$ thymocytes (from β_2-microglobulin-deficient mice) were used as target cells, NK cells, but not CD3^{int} cells, showed potent cytotoxicity. These results suggest that autologous killing is a major function of TCR^{int} cells with self-reactivity, and that such killing is mediated by means of Fas ligand/Fas molecules.

INTRODUCTION

Recent studies have revealed that there are two major types of T cells that appear in the peripheral immune organs, namely, interleukin-2 receptor (IL-2R)$\alpha^-\beta^-$ high T-cell receptor (TCR) cells and IL-2R$\alpha^-\beta^+$ intermediate (int) TCR cells (i.e. TCR^{int} cells).[1,2] The former cells are generated through the mainstream of T-cell differentiation in the thymus (i.e. conventional T cells), whereas the latter are generated through the extrathymic pathway in the liver and an alternative intrathymic pathway. In addition to the distinct density of the TCR–CD3 complex, TCR^{high} cells and TCR^{int} cells are distinguished from each other by other properties. For example, TCR^{high} cells with the IL-2R$\alpha^-\beta^-$ phenotype under resting conditions acquire a high affinity IL-2R (i.e. IL-2R$\alpha^+\beta^+$) after antigenic stimulation.[3] In contrast, TCR^{int} cells remain at a level of intermediate affinity IL-2R (i.e. IL-2R$\alpha^-\beta^+$) *in vivo* even after stimulation. TCR^{int} cells are also unique with respect to the expression of NK1.1 alloantigens.[4–6] Approximately half of them express this antigen (i.e. NK1.1$^+$ T cells or TNK cells), whereas the other half lack its expression. In other words, TCR^{int} cells comprise of both NK1.1$^+$ and NK1.1$^-$ subsets.

Another important property ascribed to TCR^{int} cells appears to be the composition of self-reactive forbidden clones.[7,8] Although the elimination of self-reactive clones by mainstream T-cell differentiation is complete, the pathways of TCR^{int} cell differentiation (i.e. the extrathymic pathway and an alternative intrathymic pathway) consistently produce self-reactive clones. If this is true, questions arise as to whether autologous killing due to self-reactivity is confined to TCR^{int} cells and how such killing is mediated. The major purpose of this study was to determine what types of lymphocytes are associated with autologous killing, especially against rapidly proliferating normal cells. For this purpose, various effector cells, including TCR^{int} cells and the NK1.1$^+$ and NK1.1$^-$ subsets, were purified by a cell sorter and thymocytes of

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Abbreviations: int, intermediate; MNC, mononuclear cells; RT, reverse transcriptase.

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syngeneic or allogeneic origin were used as target cells. It was demonstrated that, in contrast to allogeneic killing, syngeneic killing was mediated for the most part by TCR^{int} cells, and that such killing was mediated by Fas ligand/Fas molecules. The present results may be very important for understanding autoreactive T cells and their involvement in autoimmune diseases and chronic graft-versus-host (GVH) disease.

MATERIALS AND METHODS

Mice

C57BL/6 (B6), C3H/He, B6-*lpr/lpr*, MRL-+/+ and MRL-*gld/gld* mice, which were originally purchased from Charles River Japan Inc. (Kanagawa, Japan), were used at the ages of 8–12 weeks. β_2-microglobulin-deficient mice[9] and class II major histocompatibility complex (MHC) knockout mice[10] were also used. These mice were maintained at the animal facility of Niigata University under specific pathogen-free conditions.

Cell preparations

Mice anaesthetized with ether were killed after complete exsanguination through incised axillary arteries and veins. Specimens from the liver, spleen, thymus and lymph nodes were removed and kept in phosphate-buffered saline (PBS) (pH 7.2) on ice until cell preparation.

To obtain liver mononuclear cells (MNC), the liver obtained from one mouse was cut into small pieces with scissors, pressed through a 200-gauge stainless steel mesh, and then suspended in 40 ml of Eagle's minimum essential medium (MEM) supplemented with 5 mM HEPES (Nissui Pharmaceutical Co., Tokyo, Japan) and 2% heat-inactivated new-born calf serum (NCS). After washing once with medium, the cells were fractionated by centrifugation in 15 ml of 35% Percoll solution containing 100 IU/ml heparin for 15 min at 2000 r.p.m.[11] The pellet of cells containing red blood cells (RBC) was resuspended and washed with the medium. To deplete RBC, ammonium chloride (155 mM NH_4Cl + 10 mM $KHCO_3$ + 1 mM EDTA-Na)/Tris buffer solution was added and the mixture was kept on ice for 5 min. After being washed twice with the medium, liver MNC were suspended in 1 ml of medium and the number of cells was counted.

Spleen cells and thymocytes were obtained by forcing each organ through a 200-gauge stainless steel mesh. To deplete RBC, spleen cells were lysed with 0.83% ammonium chloride–Tris buffer and washed twice with the medium.

Immunofluorescence tests

The surface phenotypes of cells were analysed using monoclonal antibodies (mAb) in conjunction with a two- or three-colour immunofluorescence test.[1] The mAb used included fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or biotin-conjugated reagents of anti-CD3 (145-2C11), anti-NK1.1 (PK136) and anti-IL-2R β-chain (TM-β1) mAb (PharMingen Co., San Diego, CA). Biotin-conjugated reagents were developed with PE- or Red 613-conjugated streptavidin (Becton Dickinson, Mountain View, CA). The fluorescence-positive cells were analysed with a FACScan using LYSIS II software (Becton Dickinson).

Cell sorting

MNC obtained from the liver were stained with FITC-conjugated anti-CD3 mAb and biotin-conjugated anti-IL-2Rβ or NK1.1 mAb followed by PE-conjugated streptavidin, and sorted by FACS Vantage (Becton Dickinson). Cell fractions were harvested, washed, and used for cytotoxicity assay or polymerase chain reaction (PCR) analysis.

Cytotoxicity assay

Cytotoxicity was examined by a specific ^{51}Cr-release assay with an incubation time of 4 hr, as described previously.[7] Thymocytes of syngeneic or allogeneic origin labelled with sodium chromate (^{51}Cr) (Amersham International, Arlington Heights, IL) were used, and effector cells were either hepatic MNC, splenic MNC, or purified fractions. Percentage cytotoxicity was determined by using 2×10^4 labelled thymocytes at the indicated ratios of target to effector cells in triplicate cultures. ^{51}Cr-labelling for thymocytes was done for 1 hr. During a 4-hr incubation assay, spontaneous chromium release ranged from 15% to 20%. However, this release was eliminated by calculation.

Reverse-transcription–PCR (RT-PCR)

Total RNA was extracted from MNC by the acid quanidium thiocyanate–phenol–chloroform method. cDNA was synthesized using M-MLV transcriptase (Takara Shuzo, Tokyo, Japan) and random hexamer primer (Takara Shuzo). PCR amplification of synthesized cDNA was conducted using 1 μg of cDNA added to a reaction mixture containing 50 μM KCl, 10 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1 mM of each primer, and 2.5 U Taq polymerase (Takara Shuzo) in a reaction volume of 50 μl. Each sample was overlaid with light mineral oil (Sigma Chemical Co., St Louis, MO) and incubated in a DNA thermal cycler (Perkin Elmer Co., Norwalk, CT) for 35 cycles. Each cycle consisted of 50 seconds at 94°, 30 seconds at 55°, and 2 min at 72°.

The following primers were used.[12,13] β-actin:
5′-CGTGACATCAAAGAGAAGCTGGTGC,
3′-GCTCAGGAGGAGCAATGATCTTGAT; FAS:
5′-TTGCTGTCAACCATGCCAAC,
3′-CACAGTGTTCACAGCCAGGA; Fas ligand:
5′-ATGGTTCTGGTGGCTCTGGT,
3′-GTTTAGGGGCTGGTTGTTGC.

RESULTS

Cytotoxicity against syngeneic or allogeneic thymocytes by MNC obtained from the liver and spleen

B6 mice were used to obtain MNC from the liver and spleen. To determine whether these MNC exerted cytotoxicity against syngeneic or allogeneic cells, thymocytes isolated from B6 ($H\text{-}2^b$), C3H/He ($H\text{-}2^k$) and B6-*lpr/lpr* mice were used as targets (Fig. 1). Effector and target cells were allowed to interact at the indicated ratios for 4 hr. In the case of syngeneic combination, killing was seen in only liver MNC. A similar pattern of killing was also produced by MNC in the liver even when allogeneic thymocytes were used, although the magnitude was lower.

In the final portion of this experiment, we used thymocytes from B6-*lpr/lpr* mice as a target. This is a syngeneic combination but these thymocytes lack the expression of normal Fas molecules on the surface. Interestingly, no killing by either liver or splenic MNC was observed in this combination.

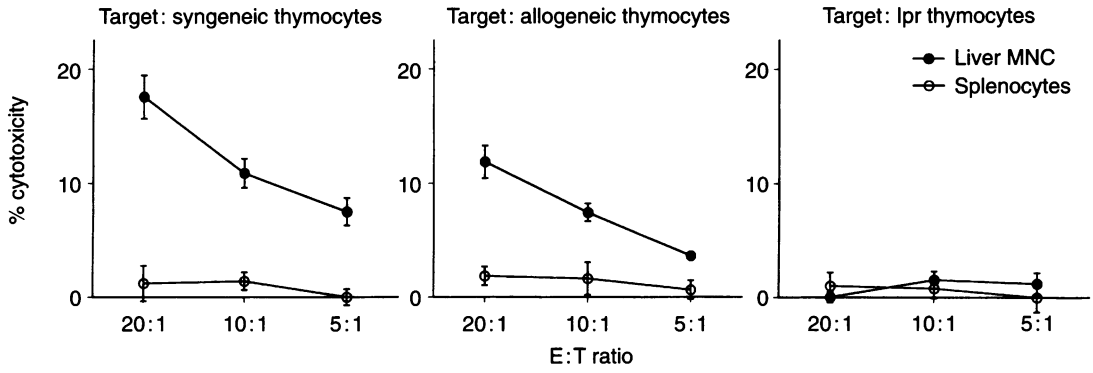

Figure 1. Cytotoxicity against thymocytes of syngeneic or allogeneic origin by liver and splenic MNC and the failure of autologous killing against Fas$^-$ target. Effector (E) cells were isolated from the liver and spleen of B6 mice (H-2^b). Target (T) cells were either of syngeneic origin (B6) or allogeneic origin (C3H/He, H-2^K). ^{51}Cr-labelled targets were allowed to interact with effectors for 4 hr. Mean and 1 SD of three experiments are represented. Liver MNC were efficient cells for both syngeneic and allogeneic targets. The Fas$^-$ target, i.e. thymocytes from B6-*lpr/lpr* mice, was not sensitive to syngeneic effectors.

Phenotypic characterization of MNC in the liver, spleen and thymus of B6 mice, the cells used in Fig. 1, is represented in Fig. 2. In this experiment, two-colour staining for CD3 and IL-2Rβ and that for CD3 and NK1.1 were performed simultaneously. As already shown, MNC in the liver comprised a large proportion of IL-2Rβ^+ CD3^{int} cells as well as CD3$^-$IL-2Rβ^+ NK cells. By staining for CD3 and NK1.1, it was obvious that CD3$^+$ NK1.1$^+$ cells (i.e. NK1.1$^+$ T cells) belonged to CD3^{int} cells (note the intensity of CD3). Concerning the percentages of NK1.1$^+$ T cells, 67% of CD3^{int} cells were of the NK1.1$^+$ subset and the rest were of the NK1.1$^-$ subset. It was also confirmed that the ratios of the NK1.1$^+$ subset among CD3^{int} cells were quite low in the spleen (19%) and thymus (32%).

Absence of autologous killing by liver MNC in MRL-*gld/gld* mice

Since autologous killing was not seen when thymocytes were obtained from B6-*lpr/lpr* mice (deficiency of normal Fas antigens),[14] it was speculated that autologous killing is mediated by Fas ligand/Fas molecules. To confirm this notion, effector cells of liver MNC were prepared from MRL-*gld/gld* mice (deficiency of normal Fas ligand)[15] (Fig. 3). In this experiment, target cells were thymocytes of MRL-+/+ mice. Effector cells of liver MNC from MRL-+/+ mice were also used as a normal control. It was demonstrated clearly that effector cells that lack Fas ligand were not able to mediate autologous killing at all. In contrast, normal effector cells mediated autologous killing.

TCR^{int} cells as the major effector cells for autologous killing

Since the prominent cytotoxicity against syngeneic or allogeneic target cells was mainly mediated by liver MNC, a further characterization of effector cells against these targets was conducted (Fig. 4). Lymphocyte subsets, CD3$^-$ IL-2Rβ^+ (NK cells), CD3^{int} IL-2Rβ^+ (TCR^{int} cells) and CD3^{high} IL-2Rβ^- (TCR^{high} cells), were purified from liver MNC of B6 mice by a cell sorter after two-colour staining for CD3 and IL-2Rβ (Fig. 4a). The purity of each fraction was 98% or more. The target cells were syngeneic (B6) and allogeneic (C3H/He) thymocytes. A very interesting result was forthcoming: TCR^{int} cells mediated the most potent activity of the autologous killing (Fig. 4b). NK cells also mediated cytotoxicity, whereas CD3^{high} cells did not do so at all. On the other hand, all lymphocyte subsets were able to mediate allogeneic killing, showing a different magnitude (i.e. NK cells > CD3^{int} cells > CD3^{high} cells). This experiment was repeated three times and consistent results were obtained (data not shown).

Comparison of the potency of autologous cytotoxicity between NK1.1$^+$ and NK1.1$^-$ subsets among CD3^{int} cells

NK1.1$^+$ T cells (TNK cells) are known to exist in various immune organs, and such NK1.1$^+$ T cells have been found within a population of CD3^{int} cells.[1] We investigated which subset of CD3^{int} cells, i.e. CD3^{int} NK1.1$^+$ or CD3^{int} NK1.1$^-$, comprised the major effector cells against a syngeneic target (Fig. 5). Each subset was purified by cell sorter after two-colour staining for CD3 and NK1.1 (Fig. 5a). The purity was 96% or more. The activity of autologous killing was then compared (Fig. 5b). Major cytotoxicity was seen in the CD3^{int} NK1.1$^+$ subset. However, this was not an 'all or nothing phenomenon,' because some autologous killing was also seen in the CD3^{int} NK1.1$^-$ subset.

Expression of mRNA of Fas and Fas ligand molecules by whole liver MNC and the CD3^{int} cells among them

The experiments outlined thus far revealed that autologous killing was mediated through the interaction of Fas ligand/Fas molecules. In the experiment reported in this section, the expression levels of Fas and Fas ligand in various lymphocyte subsets were compared by the RT-PCR method (Fig. 6). By using MNC from the liver, thymus and spleen, a unique pattern of expression of Fas and Fas ligand was produced (Fig. 6a). Thus, both liver MNC and thymocytes were found to be Fas mRNA$^+$, while only liver MNC were Fas ligand mRNA$^+$. These findings are compatible with the notion that Fas ligand$^+$ liver MNC mediate autologous killing against Fas$^+$ thymocytes.

Liver MNC were purified further into various subsets by cell sorter, namely CD3^{int} IL-2Rβ^+, CD3^{high} IL-2Rβ^- and

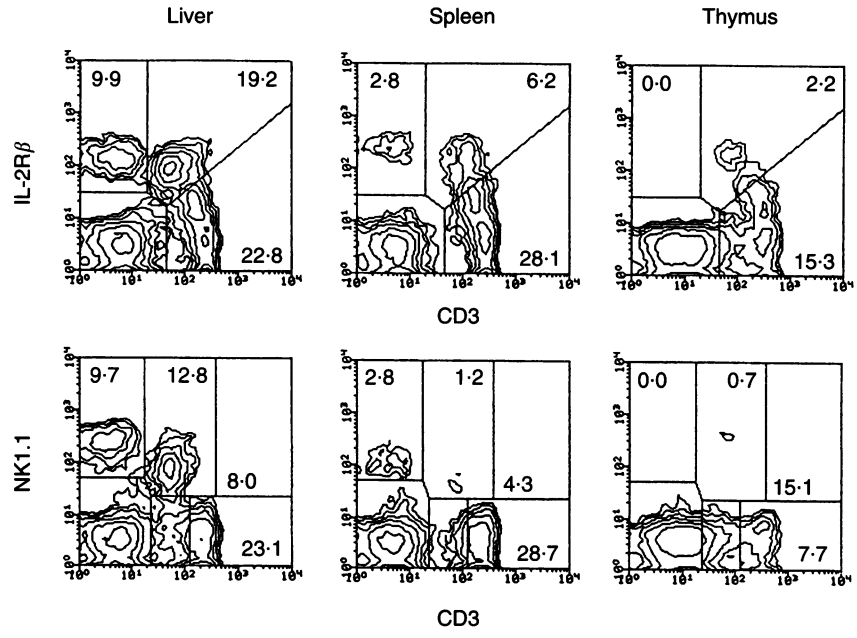

Figure 2. Phenotypic characterization of MNC isolated from the liver, spleen and thymus. B6 mice at the age of 8 weeks were used to obtain MNC from various organs. Two-colour staining for CD3 and IL-2Rβ and that for CD3 and NK1.1 were performed. Numbers in the figure represent the percentages of fluorescein-positive cells in the corresponding area. Representative results of three experiments are shown. This figure suggests that IL-2Rβ^+ CD3^{int} cells comprised both NK1.1$^+$ and NK1.1$^-$ subsets.

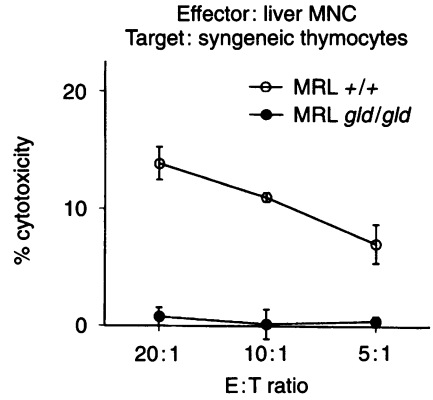

Figure 3. Lack of effector function of autologous killing by Fas ligand$^-$ liver MNC. Effector cells were liver MNC isolated from either MRL-*gld/gld* (Fas ligand$^-$) or MRL-+/+ mice. Target cells were thymocytes in MRL-+/+ mice. A 4-hr assay was carried out at the indicated effector to target (E:T) ratios. Mean and 1 SD of three experiments are represented. Fas ligand$^-$ liver MNC completely lacked the effector function.

CD3$^-$ IL-2Rβ^+ (NK) cells, and RT-PCR analysis was conducted (Fig. 6b). CD3^{int} IL-2Rβ^+ cells were both Fas mRNA$^+$ and Fas ligand mRNA$^+$, whereas CD3$^-$ IL-2Rβ^+ (NK) cells were only Fas ligand mRNA$^+$. CD3^{high} IL-2Rβ^- cells showed a faint peak of Fas mRNA.

CD3^{int} IL-2Rβ^+ cells were separated further into NK1.1$^+$ and NK1.1$^-$ subsets by cell sorter and were examined for expression of Fas and Fas ligand mRNA. It was found that both NK1.1$^+$ and NK1.1$^-$ subsets were Fas mRNA$^+$ and Fas ligand mRNA$^+$ (data not shown).

Decreased cytotoxicity of CD3^{int} cells against class I MHC$^-$ thymocytes

As shown previously,[16] the pretreatment of CD3^{int} cells with anti-TCR (or CD3) mAb increased the perforin level in the cytoplasm and resulted in an elevation (up to 30%) of the cytotoxicity. The existence of this effect raised the possibility that TCR^{int} (or CD3^{int}) cells used their own TCR–CD3 complex to recognize syngeneic or allogeneic MHC antigens expressed on the target cells. To investigate this possibility, we used thymocytes obtained from class I MHC knockout mice and class II MHC knockout mice (Fig. 7). The effector cells were liver MNC from B6 mice. Decreased cytotoxicity was seen against the thymocytes of class I MHC knockout mice, while the cytotoxicity against the thymocytes of class II MHC knockout mice was comparable to that against the thymocytes in normal mice (Fig. 7a).

We then examined which lymphocyte subsets mediated the remaining cytotoxicity against the thymocytes of class I MHC knockout mice (Fig. 7b). The cell separation was conducted as in Fig. 4. Repeated experiments showed that the highest cytotoxicity was produced by NK cells, while some cytotoxicity was also seen in CD3^{int} cells. CD3^{high} cells did not have such activity. The inverted order of the magnitude of cytotoxicity against the thymocytes of class I MHC knockout mice (i.e. NK cells > CD3^{int} cells) was noteworthy.

DISCUSSION

In the present study, we have demonstrated that autologous killing is mediated mainly by TCR^{int} cells and their NK1.1$^+$ subset, and that all such killing is mediated by Fas ligand/Fas molecules. In a previous study, we reported that self-reactive

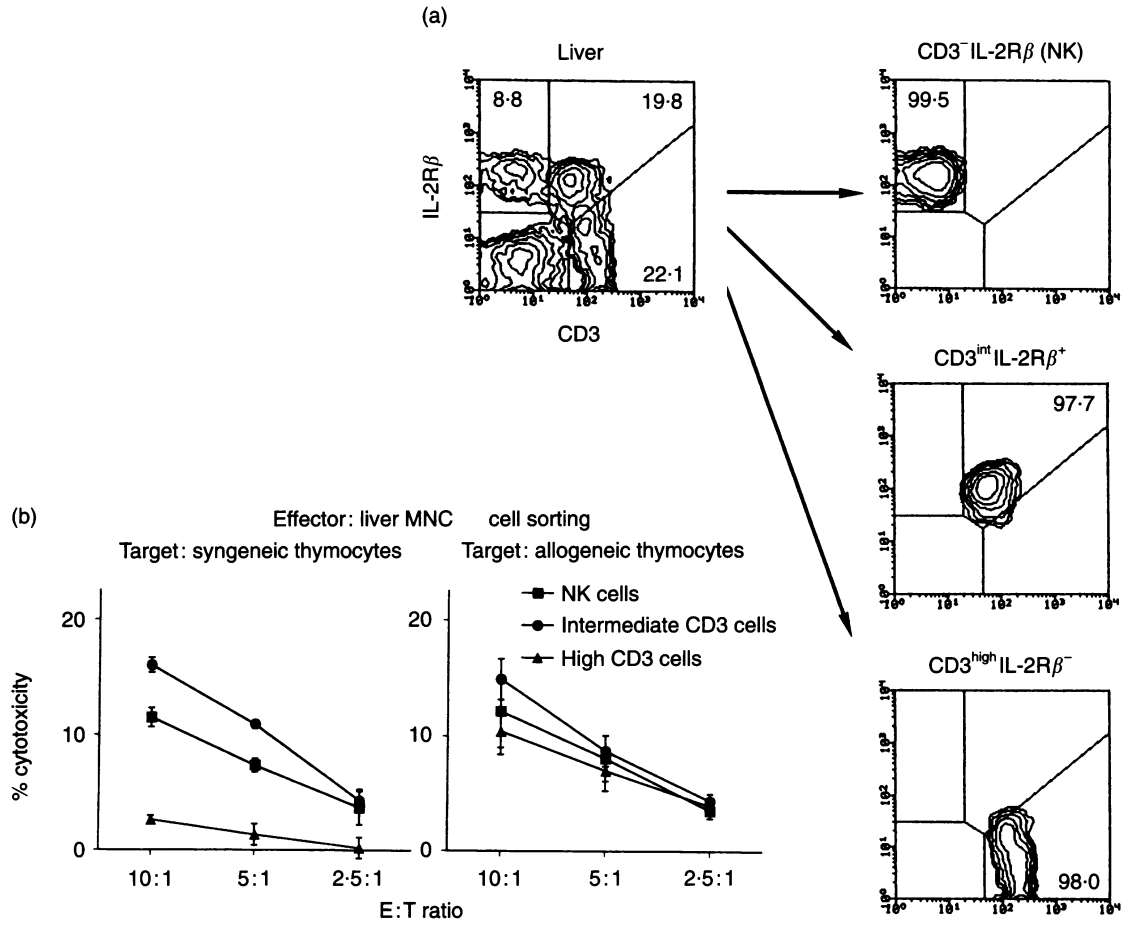

Figure 4. A comparison of the potency of autologous and allogeneic killing among purified populations of lymphocytes. (a). Cell sorting and phenotypic characterization. (b) Autologous and allogeneic killing by sorted fractions. Cell preparation of liver MNC was performed by a cell sorter after two-colour staining for CD3 and IL-2Rβ. Mean and 1 SD of three experiments are represented. Autologous killing was mediated by $CD3^{int}$ cells and NK cells, although allogeneic killing was seen in NK cells, $CD3^{int}$ cells and $CD3^{high}$ cells.

T-cell clones, estimated by anti-Vβ mAb in conjunction with the Mls system, are confined to a population of TCR^{int} cells.[7,17] More precisely, self-reactive forbidden clones are generated by the pathway of TCR^{int} cell differentiation, including the extrathymic pathway and an alternative intrathymic pathway. On the other hand, the negative selection of self-reactive clones by the mainstream pathway in the thymus is complete. Taken together, these facts indicate that self-reactivity is a major event for TCR^{int} cells in terms of both phenotypic and functional characterization.

The autologous killing mediated by TCR^{int} cells, but not by TCR^{high} cells, was extremely striking when compared with allogeneic killing. Such restriction was not seen in the case of allogeneic killing, i.e. all NK cells, TCR^{int} cells and TCR^{high} cells were able to mediate the killing. The magnitude of allogeneic killing of NK cells was the highest among them.

It is well known that the killing of cytotoxic T cells as well as of NK cells is mediated by both the Fas ligand/Fas molecules and the perforin system.[18,19] In the case of TCR^{int} cells, the preforin level in their cytoplasm is much lower than that of NK cells.[1] The present study revealed that the expression levels of Fas ligand mRNA as well as of Fas mRNA were quite high in TCR^{int} cells in the liver. This situation is compatible with the conclusion that the high level of autologous killing in TCR^{int} cells is mediated by Fas ligand/Fas molecules, but not by perforin.

According to the data from the present study, the cytotoxicity mediated by TCR^{int} cells seemed to include both class I MHC-restricted and non-MHC-restricted cytotoxicities. Thus, the level of cytotoxicity against normal class I^+ thymocytes was comparable to that against class II^- (I^+) thymocytes (data not shown). On the other hand, the level of cytotoxicity against class I^- thymocytes was consistently low (but not nil).

When tumour cell lines were used, only NK cells exerted the cytotoxicity.[20] In other words, freshly isolated TCR^{int} cells did not have cytotoxicity against tumour cells (even if they were Fas^+). However, if TCR^{int} cells were preincubated with anti-TCR or anti-CD3 mAb[20] or IL-12, they acquired perforin in the cytoplasm and exerted cytotoxicity against tumour cells. It is presumed that the mechanisms involved in the cytotoxocity against syngeneic thymocytes and tumour cells are somewhat different.

In this study, we applied thymocyte targets of syngeneic or allogeneic origin. This idea comes from the experiments by Arase *et al.*, in which they used double-positive (DP) $CD4^+$ $CD8^+$ thymocytes as a target for $NK1.1^+$ T cells in

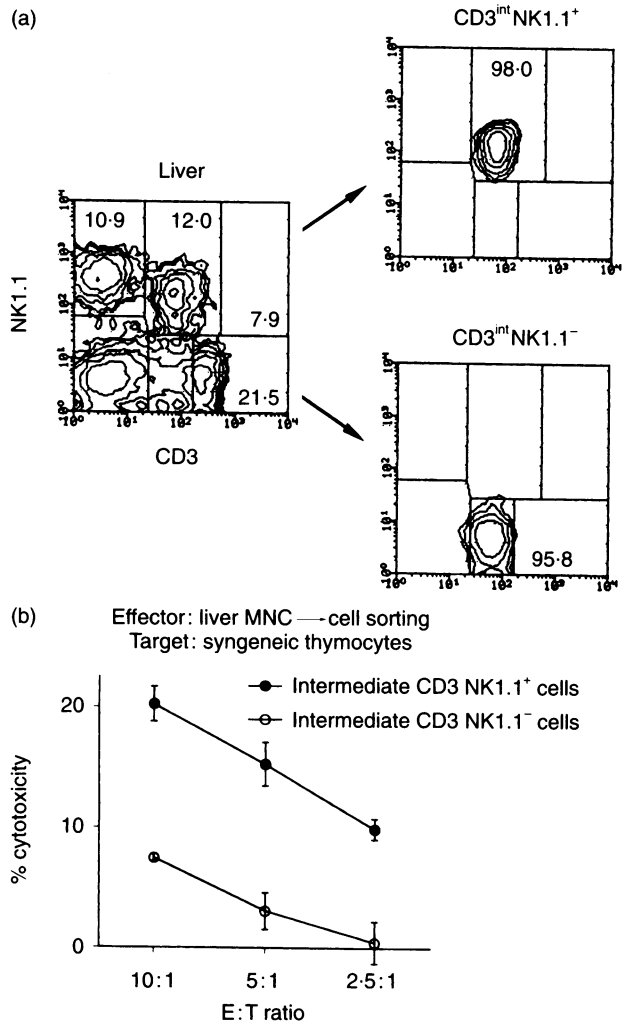

Figure 5. A comparison of the potency of autologous killing between NK1.1$^+$ and NK1.1$^-$ subsets among CD3^{int} cells. (a) Cell sorting and phenotypic characterization. (b) Autologous killing of NK1.1$^+$ and NK1.1$^-$ subsets. NK1.1$^+$ and NK1.1$^-$ subsets were purified from liver MNC of B6 mice by a cell sorter after two-colour staining for CD3 and NK1.1. Mean and 1 SD of three experiments are represented. Autologous killing by the NK1.1$^+$ subset was higher than that by the NK1.1$^-$ subset.

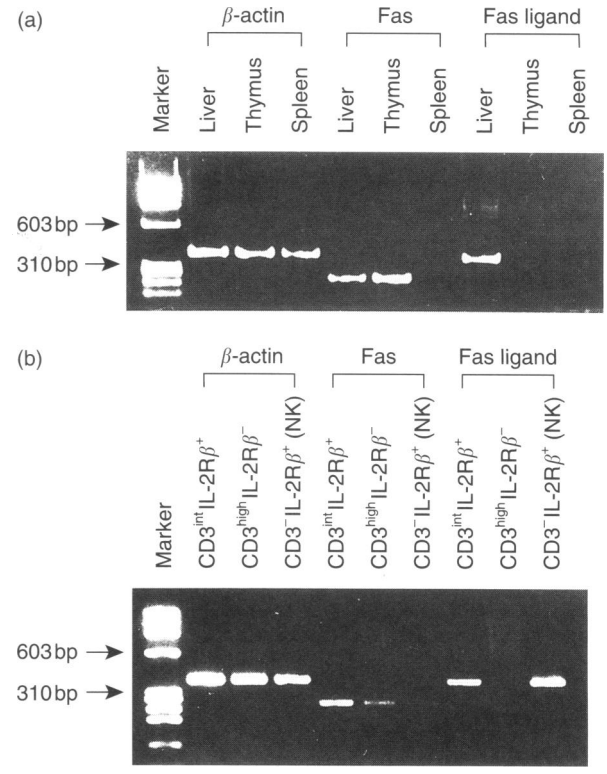

Figure 6. Expression of Fas and Fas ligand mRNA by whole MNC (a) and lymphocyte subsets (b) yielded by the liver, thymus and spleen. B6 mice at the age of 8 weeks were used to obtain MNC from various organs. Liver MNC were further purified into lymphocyte subsets, CD3^{int} IL-2Rβ^+, CD3^{high} IL-2Rβ^- and CD3$^-$ IL-2Rβ^+ (NK) cells, by cell sorter after two-colour staining for CD3 and IL-2Rβ. Total RNA was then extracted from each cell population for RT-PCR analysis of mRNA of β-action, Fas and Fas ligand. Thymocytes (targets in this study) were Fas mRNA$^+$, while liver MNC and CD3^{int} cells (effector cells) were Fas ligand mRNA$^+$. NK cells were Fas ligand mRNA$^+$ but Fas mRNA$^-$ at this amplification level.

the thymus.[8] We now know that ^{51}Cr-labelled whole thymocytes are useful targets for both syngeneic and allogeneic killing. Non-specific killing was not serious during this incubation time, as shown by the results of thymocytes from B6-*lpr/lpr* mice (i.e. Fas$^-$). It is speculated that many rapidly proliferating self-cells become targets of TCR^{int} cells. Thus, it was reported that enterocytes become a target for intraepithelial lymphocytes (i.e. extrathymic T cells in the intestine).[21] The low cytotoxicity against self-thymocytes in this study does not necessarily mean a low function of autologous killing by TCR^{int} cells. This was due to a short incubation time (4 hr) for the assay, because TCR^{int} cells could exert more potent cytotoxicity if the time was prolonged. However, thymocytes themselves have a nature to evoke apoptosis *in vitro* at 37°. In this regard, we applied this condition to suppress the increase of spontaneous ^{51}Cr-release. In contrast to cultured cells with low viability, the rate of such spontaneous release from thymocytes was consistent. As a result, the deviation of cytotoxicity from experiments to experiments was not high.

Recently we have experienced that ^{51}Cr-labelling for rapidly proliferating self-cells (i.e. non-tumour target), including hepatocytes, enterocytes and concanavalin A-activated lymphoblasts, is very difficult because of a high level of spontaneous ^{51}Cr-release. On the other hand, established tumour cells do not have such a high level of spontaneous ^{51}Cr-release. We finally selected thymocytes as a non-tumour target.

A similar population of NK1.1$^+$ T cells in the thymus manifested killing against syngeneic CD4$^+$ CD8$^+$ thymocytes.[8] However, these NK1.1$^+$ T cells in the thymus required a 16-hr incubation to induce killing. In the case of liver MNC or NK1.1$^+$ T cells isolated from liver MNC, more potent cytotoxicity was observed, because a 4-hr incubation was sufficient to induce significant killing. As shown previously,[22,23] lymphoma cells, hepatoma cells, fibrosarcoma cells, etc., become targets of killing by TCR^{int} cells in the liver. We recently reported that mice with liver chirrosis lack functional TCR^{int} cells and permit the onset of hepatoma with ageing.[16] It is known that hepatocytes (and hepatoma) express both CD1 antigens[24] and

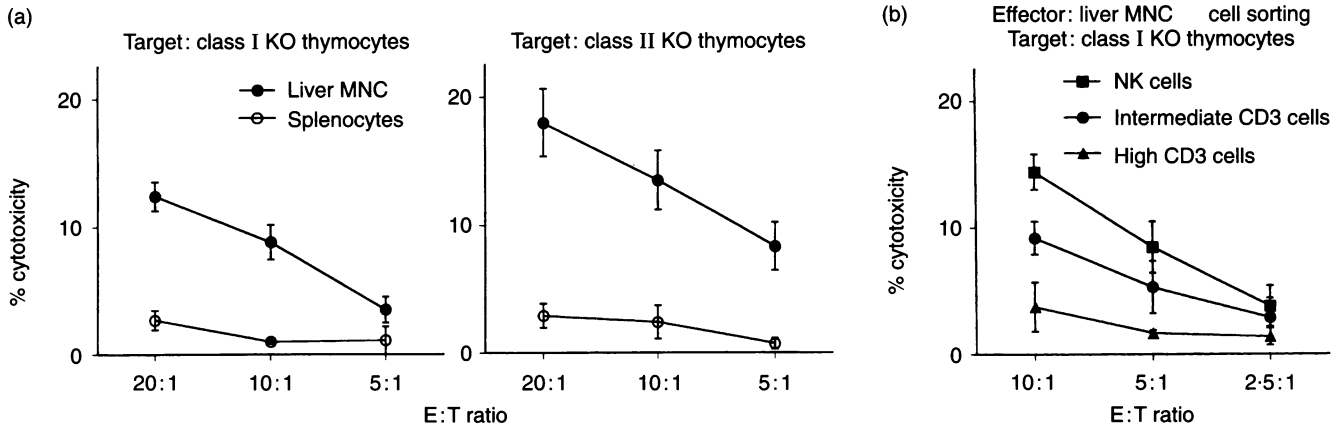

Figure 7. Cytotoxic activity of whole liver MNC and the purified fractions against class I MHC$^-$ and class II MHC$^-$ thymocytes. (a) Cytotoxicity of whole liver MNC. (b) Cytotoxicity of the purified fractions of liver MNC. In this experiment, target thymocytes were obtained from class I MHC knockout mice and class II MHC knockout mice of B6 background. Whole liver MNC and the purified fractions were obtained from B6 mice. Mean and 1 SD of three experiments are represented. The cytotoxicity of liver MNC against class I MHC$^-$ target decreased, whereas that against class II MHC$^-$ target was unchanged. In the case of class I MHC$^-$ target, NK cells had the highest cytotoxicity.

Fas antigens[25] on the surface. It is presumed that TCR^{int} cells in the liver are extremely important not only for the regulation of rapidly proliferating cells but also for the surveillance of malignant cells.

Among TCR^{int} cells, the NK1.1$^+$ subset was found to achieve more efficient autologous killing than the NK1.1$^-$ subset. Some of our results in the liver are quite compatible with the results of NK1.1$^+$ T cells in the thymus reported by Arase *et al.*[8] However, they did not compare such cytotoxicity with those of other populations. We also extended this study to compare allogeneic killing with autologous killing by using various purified populations.

The augmentation of autologous killing of CD3^{int} cells by anti-CD3 mAb has been reported previously.[16] Although we did not mention the effect of anti-CD3 mAb on the thymocyte killing, the pretreatment of cells with anti-CD3 mAb augmented the cytotoxicity up to 20% (T. Kawamura, manuscript submitted for publication). Therefore, the cytotoxicity of CD3^{int} cells, but not other types of cells, represented here was slightly higher than that by freshly isolated cells. This phenomenon also indicates that the interaction of the TCR–CD3 complex with MHC antigens might be very important for the incubation of autologous killing. In this regard, it is speculated that some monomorphic MHC (e.g. CD1b) might be associated with the recognation of TCR^{int} cells or NK1.1$^+$ T cells, if not all.[26–28] To investigate this possibility, we also used class I MHC$^-$ thymocytes and class II MHC$^-$ thymocytes. Interestingly, whole liver MNC and purified CD3^{int} cells from the liver showed a decreased level of cytotoxicity against the class I MHC$^-$ target. In this case, NK cells mediated much more cytotoxicity. This result is compatible with previous reports that NK cells exert their killing of MHC$^-$ targets.[29,30] In any case, CD3^{int} cells possibly use their TCR–CD3 complex to recognize self-antigens in the context of monomorphic (or polymorphic) MHC. The remaining cytotoxicity against class I MHC$^-$ targets suggests that CD3^{int} cells have some non-MHC restricted cytotoxicity as well.

In a recent study,[17] BALB/c mice that fell victim to autoimmune-like graft versus host disease (GVHD) after syngeneic bone marrow transplantation were found to have an expansion of self-reactive forbidden clones (Vβ11$^+$ cells) in the target organs. All such forbidden clones were estimated to be TCR^{int} cells. It is therefore conceivable that the autoreactivity of TCR^{int} cells is functional *in vivo* under certain conditions.

Although the NK1.1$^-$ subset of TCR^{int} cells had a lower cytotoxicity, we could not neglect the existence of this population. Thus, the NK1.1$^-$ subset of TCR^{int} cells is a major population of B6-*nu/nu* mice and autoimmune B6-*lpr/lpr* mice (T. Moroda *et al.*, unpublished observations).

Concerning the primitive features of TCR^{int} cells in terms of MHC recognition, phenotype and morphology, it is speculated that TCR^{int} cells may develop earlier than thymus-derived T cells in phylogeny.[31] The present results of autologous killing by TCR^{int} cells show the existence of another primitive feature of this population.

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