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Cytotoxic activity against tumour cells mediated by intermediate TCR cells in the liver and spleen

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SUMMARY

Morphological and phenotypic characterization in previous studies has indicated that intermediate (int) T-cell receptor (TCR) cells or T natural killer (T_{NK}) cells may stand at an intermediate position between NK cells and high TCR cells of thymic origin in phylogenetic development. In this study, a functional study on cytotoxic activity against various tumour targets was performed in each purified subset. When a negative selection method entailing *in vivo* injection of anti-asialo GM_1 antibody or anti-interleukin (IL)-2Rβ monoclonal antibody (mAb) was applied, IL-2Rβ^+ CD3$^-$ NK cells were found to have the highest NK activity while IL-2Rβ^+ int CD3 (or TCR) cells had a lower level of the NK activity. High CD3 cells (freshly isolated) did not have any such activity. Sorting experiments further revealed that the NK function mediated by int CD3 cells was augmented when they were exposed to anti-CD3 mAb, anti-TCR$\alpha\beta$, or anti-TCR$\gamma\delta$ mAb. This phenomenon was not observed in NK cells and high CD3 cells. More importantly, when anti-CD3 mAb (or anti-TCR mAb) was added to the assay culture, int CD3 cells became cytotoxic against even NK-resistant tumour (FcγR$^-$, Fas$^+$) targets. Liver mononuclear cells or int CD3 cells exposed to anti-CD3 mAb for 6 hr showed an elevated level of perforin in their cytoplasms. The present results suggest that int CD3 cells are usually non-cytotoxic against various tumours but become functional after being stimulated via the TCR–CD3 complex.

INTRODUCTION

It is well established that high T-cell receptor (TCR) cells (i.e. conventional T cells) are generated by the mainstream of T-cell differentiation in the thymus.[1,2] Since this pathway efficiently eliminates self-reactive forbidden clones at a double-positive (DP) CD4$^+$ CD8$^+$ stage, the resulting T cells comprise clones which recognize foreign antigens in the context of self-major histocompatibility complex (MHC) antigens.[3,4] On the other hand, it was recently demonstrated that intermediate (int) TCR cells are generated by extrathymic pathways in the liver,[5,7] as well as a thymic alternative pathway.[8–14] Int TCR cells carry many properties which are more primordial than those of high TCR cells. For example, they have intermediate levels of TCR (and CD3), contain self-reactive clones,[15] comprise double-negative (DN) CD4$^-$ CD8$^-$ cells as well as single-positive cells,[16,17] and constitutively express interleukin (IL)-2Rβ and natural killer (NK) 1.1 (if not all) antigens.[18] In this regard, some other investigators termed them NK1.1$^+$ T cells of T_{NK} cells.[8–14] Some of these properties, including the morphology of granular lymphocytes, are similar to those of NK cells.

Despite the above-mentioned phenotypic characterization, functional characterization for int TCR cells is limited. Arase *et al.* reported that T_{NK} cells isolated from the thymus mediate autoreactivity against self DP thymocytes.[19] Hashimoto *et al.* revealed that anti-tumour activity of int TCR cells was highly augmented by an *in vivo* injection of interleukin-12 (IL-12).[20] In the present study, we attempted to determine how NK cells, int TCR cells, and high TCR cells mediated cytotoxicity against NK-sensitive and NK-resistant targets. Although int TCR cells had a lower cytotoxicity against a NK-sensitive target, YAC-1, than did NK cells, they were able to mediate potent cytotoxicity against NK-sensitive and NK-resistant tumour cells in the presence of anti-CD3 mAb or anti-TCR monoclonal antibody (mAb). Such phenomenon was not seen in NK cells and high TCR cells. Concerning their responses to IL-2, IL-12, and other cytokines for the augmentation of cytotoxicity,[20] int TCR cells seem to be one of the important effector cells for tumour immunity. It is also speculated that int CD3 cells exert the perforin system through the recognition between the TCR–CD3 complex/target molecules.

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Abbreviations: DP, double-positive; int, intermediate; DN, double-negative; MNC, mononuclear cells; IL-2Rβ, IL-2R β-chain.

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MATERIALS AND METHODS

Mice

C3H/He mice were originally purchased from the Jackson Laboratory, Tokyo, and were maintained in the animal facilities of Niigata University under specific pathogen-free conditions. These mice were used at the age of 8 weeks.

Injection of anti-asialo GM_1 antibody or anti-IL-2Rβ mAb in vivo

To eliminate NK cells or a mixture of NK cells and int TCR cells, a single *in vivo* injection of anti-asialo GM_1 antibody (200 μg/mouse)[21,22] or anti-IL-2Rβ mAb (100 μg/mouse)[23] was performed, respectively. Since the maximum depletion of corresponding subsets was seen on days 3 to 5, mice were killed during this period for cytotoxic assay.

Cell preparation

Hepatic mononuclear cells (MNC) were prepared as previously described.[22] Briefly, mice anaesthetized with ether were killed by total bleeding from the incised axillary artery and veins. The liver was removed, cut into small pieces with scissors, pressed through 200-gauge stainless steel mesh, and suspended in Eagle's minimal essential medium (MEM) containing 5 mM HEPES (Nissui Pharmaceutical Co., Tokyo) and 2% heat-inactivated new-born calf serum. After being washed with the medium once, the cell pellet was resuspended in the medium. MNC were isolated from parenchymal hepatocytes, the nuclei of hepatocytes, and Kupffer cells by the Percoll (35% Percoll containing 100 U/ml heparin) gradient method.[24]

Splenocytes were obtained by pressing the spleen through 200-gauge steel mesh, and erythrocytes were disrupted by 0.83% NH_4Cl–Tris buffer (0·17 M, pH 7·6).

Immunofluorescence tests

The surface phenotype of cells was analysed using mAbs in conjunction with a two-colour immunofluorescence test.[22] Fluoroscein isothiocyanate (FITC)- or biotin-conjugated anti-CD3 (145-2C11), anti-TCRαβ (H57-597), anti-TCRγδ (GL3), anti-IL-2Rβ (TM-β1), and anti-NK1.1 (PK136) mAbs were obtained from PharMingen, San Diego, CA. Biotin-conjugated reagents were developed with phycoerythrin (PE)-conjugated avidin (Caltag Laboratories, San Francisco, CA). The fluorescence-positive cells were analysed with a fluorescence activated cell sorter (FACScan; Becton-Dickinson, Mountain View, CA).

Cytotoxicity assay

Cytotoxicity was examined by a ^{51}Cr-specific release assay with an incubation time of 6 hr, as previously described.[24] YAC-1 cells are a target for NK-sensitive cytotoxicity. EL-4 (B6 origin), FM3A and MH134 cell lines (C3H/He origin) were used as target cells for NK-resistant cytotoxicity. They are all $FcγR^-$, estimated by 2.4G2 mAb (PharMingen). However, YAC-1 cells were Fas^{low} while other tumours were Fas^+, estimated by anti-Fas mAb (kindly provided by S. Nagata, Osaka University School of Medicine, Osaka). Target cells labelled with sodium chromate (Amersham International, Arlington Heights, IL) were used, whereas effector cells were either hepatic MNC or splenic lymphocytes isolated from normal or treated mice. Percent cytotoxicity was produced at the indicated target-to-effector ratios and in triplicate cultures.

In some experiments, unconjugated mAbs against IL-2Rβ, CD2, CD3, TCRαβ, and TCRγδ (PharMingen) were added to the cytotoxic assay culture at a concentration of 1 μg/ml.

Cell sorting

To directly compare the magnitude of cytotoxicity, various cell fractions were isolated by FACStar II Plus (Becton Dickinson Co., Mountain View, CA). The sorted fractions included $CD3^-$ IL-$2Rβ^+$ NK cells, IL-$2Rβ^+$ int CD3 cells, IL-$2Rβ^-$ high CD3 cells, and int TCRαβ cells, high TCRαβ cells, and $TCRγδ^+$ cells. Pre-staining for the sorting was the same as described above (immunofluorescence tests).

Immunoperoxidase staining for perforin

Cytospin preparations of sorted fractions were air-dried. The preparations were then fixed in cold acetone (4 min) and 4% paraformaldehyde in phosphate-buffered saline (PBS) (1 min).[26] They were then washed with 50 mM Tris–HCl (0·17 M, pH 7·6), and treated with 0·5% periodic acid (10 min) and 0·3% H_2O_2 in methanol (15 min). They were blocked with Ca^{2+}-free PBS containing 5% bovine serum albumin (BSA) and 0·02% NaN_3 (30 min), and incubated with rat anti-mouse perforin mAb (P1-8; kindly provided by Dr K. Okumura, Tokyo, Japan) (60 min). To visualize Ab binding, the preparations were washed with Ca^{2+}- and Mg^{2+}-free PBS, incubated with biotinylated goat anti-rat immunoglobulin G (IgG) (Sigma Chemical Co., St Louis, MO) (60 min), washed again with Ca^{2+}- and Mg^{2+}-free PBS, incubated with extravidin peroxidase (Sigma Chemical Co.) (30 min), and washed with Ca^{2+}-free PBS before developing with a substrate mixture kit (Bio-Makor, Rehovot, Israel) to generate the red colour. Cells were counter-stained with hematoxylin.

Statistical analysis

Statistical significance was analysed by Student's *t*-test.

RESULTS

Phenotypic characterization of int TCR cells in the liver and spleen

Prior to functional characterizations of int TCR cells, phenotypic characterization was carried out (Fig. 1). Since int TCR cells constitutively express IL-2Rβ,[18] two-colour staining for CD3 and IL-2Rβ was performed. In the liver and spleen of control mice, int TCR cells were identified as CD3-int^+ IL-$2Rβ^+$. These int CD3 cells were more abundant in the liver than in the spleen. Similarly, NK cells were identified as $CD3^-$ IL-$2Rβ^+$, while high TCR cells of thymic origin were identified as CD3-$high^+$ IL-$2Rβ^-$.

It was previously reported that NK cells express asialo GM_1 antigens while int TCR cells almost lack the expression of such antigens.[22] On the other hand, both NK cells and int TCR cells express IL-2Rβ. To eliminate either NK cells alone or both NK and int TCR cells, *in vivo* treatment of mice with anti-asialo GM_1 antibody or anti-IL-2Rβ mAb (TM-β1) was performed (Fig. 1, centre and right columns). Mice were examined on day 3 after each treatment. As expected, almost all NK cells were eliminated by the treatment of anti-asialo GM_1 antibody, and

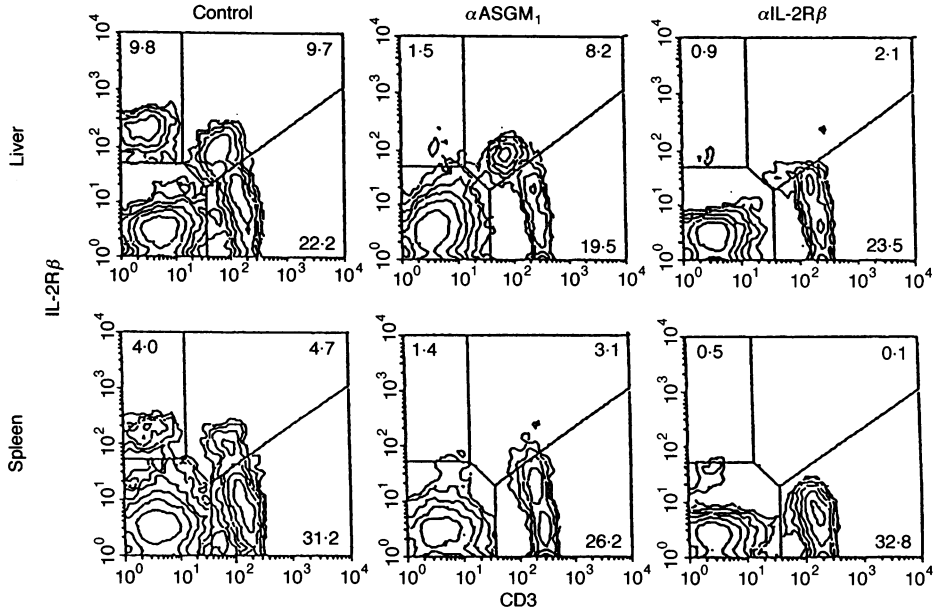


Figure 1. Elimination of NK cells, or a mixture of NK cells and int CD3 cells by the *in vivo* injection of anti-asialo GM_1 antibody ($\alpha ASGM_1$) or anti-IL-2Rβ mAb (TM-β1) (αIL-2Rβ). Hepatic and splenic MNC were obtained from each group of control or treated mice (on day 3). Two-colour staining for CD3 and IL-2Rβ was performed. Numbers in the figure represent the percentages of fluorescence-positive cells in corresponding areas.

both NK cells and int TCR cells were eliminated by the injection of anti-IL-2Rβ mAb. Some int TCR cells in the spleen seemed to be somewhat sensitive to the treatment of anti-asialo GM_1 antibody.

Cytotoxicity against NK-sensitive and NK-resistant targets mediated by hepatic and splenic MNC

Functional assay was then performed by using hepatic and splenic MNC which were obtained from mice with or without the treatment of antibodies (Fig. 2). Both hepatic and splenic MNC in control mice mediated potent cytotoxicity against a NK-sensitive target, YAC-1, but such mediation was completely absent against the NK-resistant target, EL-4. Hepatic MNC, in which both NK cells and int TCR cells are abundant, were found to have higher cytotoxicity against YAC-1 than did splenic MNC. When MNC were isolated from mice pretreated with anti-asialo GM_1 antibody (on day 3), more than 60% of the cytotoxicity of MNC against YAC-1 was eliminated in the liver and spleen. On the other hand, the pretreatment with anti-IL-2Rβ mAb was found to eliminate almost all such cytotoxicity. This raises the possibility that some NK activity is also mediated by int TCR cells as is the case with NK cells.

Cell separation experiment for the analysis of lymphocyte subsets in the cytotoxicity

To more definitely identify the lymphocyte subsets which mediate the cytotoxicity, various subsets were purified from liver MNC by a cell sorter after staining for CD3 and IL-2Rβ (Fig. 3a). The purity of all sorted NK cells, int CD3 cells, and high CD3 cells was beyond 98%. It was demonstrated that NK cells and int CD3 cells mediated the cytotoxicity against YAC-1 target, whereas high CD3 cells did not at all (Fig. 3b). One difficulty to estimate was a result that sorted int CD3 cells showed higher cytotoxicity than did sorted NK cells. As shown in Fig. 2, more than 60% cytotoxicity was mediated by asialo GM_1^+ NK cells. Therefore, it raises the possibility that the cytotoxicity of int CD3 cells was augmented during the sorting procedure applied here.

A similar phenomenon was observed when NK-resistant syngeneic targets, FM3A and MH134 cells, were used (Fig. 3c). Sorted int CD3 cells could mediate cytotoxicity even against syngeneic tumours. In contrast to the case of NK-sensitive target, sorted NK cells could not mediate the cytotoxicity against these NK-resistant targets. In other words, the self-reactivity

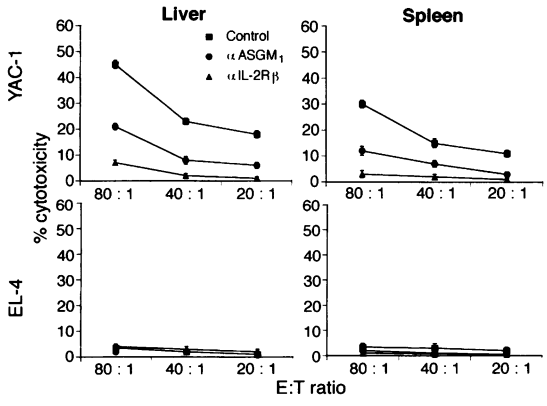
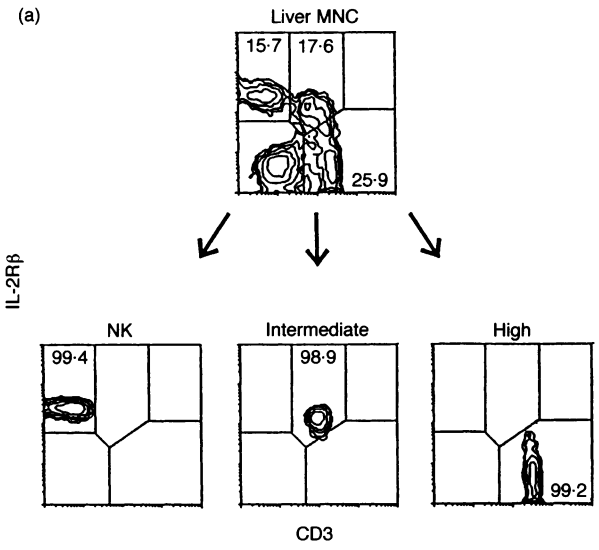


Figure 2. Cytotoxic activity of hepatic and splenic MNC isolated from control and treated mice. Mice treated with anti-asialo GM_1 antibody and with anti-IL-2Rβ mAb were used on day 3. Cytotoxic activity of hepatic and splenic MNC was examined against NK-sensitive target, YAC-1, and NK-resistant target, EL-4, for 6-hr analysis.



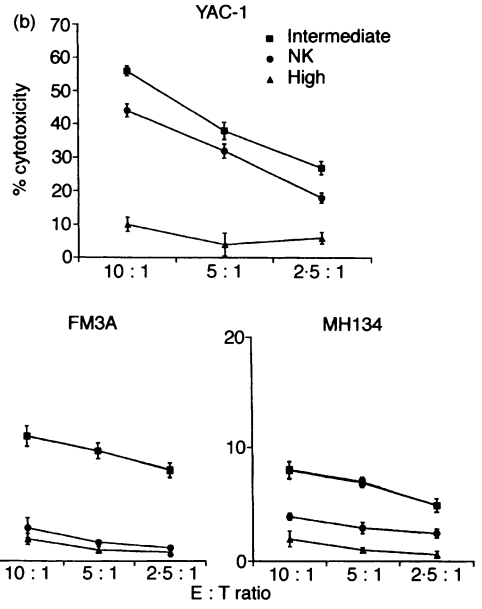


Figure 3. A comparison of cytotoxicity against various targets between sorted NK, int TCR, and high TCR cells from the liver. (a) Purity after sorting; (b) cytotoxic activity against YAC-1, (c) cytotoxicity against FM3A and MH134. The purity of sorted fractions for NK, int TCR, and high TCR cells was >98%. Unexpectedly, sorted int TCR cells showed the highest cytotoxicity against the NK-sensitive target, YAC-1 cells. Only int TCR cells had cytotoxicity against the NK-resistant targets, FM3A and MH134.

against syngeneic tumours is the event for a restricted population of int TCR cells.

Cytotoxicity against NK-sensitive and NK-resistant targets mediated by int CD3 cells in the presence of anti-CD3 mAb

Since int CD3 cells were exposed to anti-CD3 mAb during the sorting procedure, it was examined whether the addition of

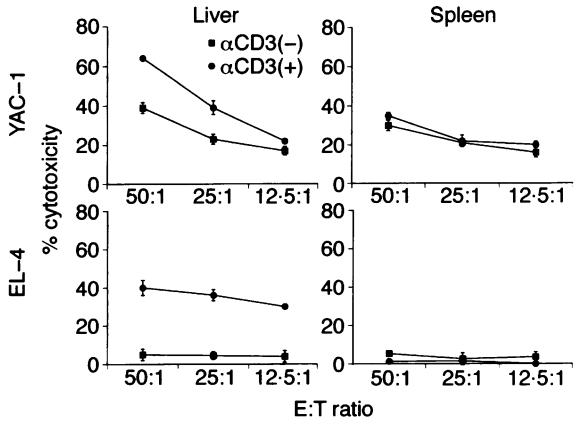


Figure 4. Anti-CD3 mAb augmented the cytotoxicity of liver MNC against YAC-1 and EL-4 targets. Hepatic MNC, which contained a large proportion of int CD3 cells, showed the anti-CD3 mAb-mediated cytotoxicity in both targets. Anti-CD3 mAb was added to the assay culture (6-hr analysis) at a concentration of 1 μg/ml.

anti-CD3 mAb to the assay system augmented the cytotoxicity against NK-sensitive target (YAC-1) and NK-resistant target (allogeneic tumour) (EL-4) in a 6-hr incubation (Fig. 4). It was demonstrated that hepatic MNC, which contain the highest level of int CD3 cells, were augmented by the addition of anti-CD3 mAb in terms of both cytotoxicity. Such augmentation for splenic MNC was minimal.

In this experiment, we used two NK-resistant targets, MH134 hepatoma cells and FM3A mammary carcinoma cells (syngeneic origin of C3H/He mice) (Fig. 5). The object of this experiment was to examine whether int CD3 cells in C3H/He mice mediate cytotoxicity against syngeneic tumour cells. When whole MNC in the liver and spleen were used in the absence of anti-CD3 mAb, the cytotoxicity was minimal, even in the liver (Fig. 5a). It is revealed that the cytotoxicity against NK-resistant syngeneic tumours were usually absent even in int CD3 cells. However, prominent cytotoxicity against both targets emerged in the presence of anti-CD3 mAb in the liver.

Since NK cells do not carry the TCR–CD3 complex, they were not related to the cytotoxicity in the presence of anti-CD3 mAb. To confirm that the above-mentioned cytotoxicity was mediated by int CD3 cells, hepatic and splenic MNC were isolated from mice pretreated with anti-IL-2Rβ mAb 3 days before killing (Fig. 5b). These MNC did not mediate the cytotoxicity against NK-resistant targets any more.

mAbs against TCR–CD3 complex augment NK cytotoxicity for int CD3 cells

We then investigated what types of mAbs were able to augment the cytotoxicity against the FM3A and EL-4 targets (Fig. 6). First, effector cells were purified by the cell sorter after two-colour staining for TCRαβ and TCRγδ (Fig. 6a). Sorted TCRγδ$^+$ cells, and sorted int TCRαβ$^+$ cells, but not sorted high TCRαβ$^+$ cells, mediated the cytotoxicity against both FM3A and EL-4 (Fig. 6b). Then, effector cells were hepatic MNC obtained from control mice (Fig. 6c). mAbs against IL-2Rβ, CD2, TCRαβ, and TCRγδ were used at a concentration of 1 μg/ml. Anti-TCRγδ and anti-TCRαβ mAbs augmented the

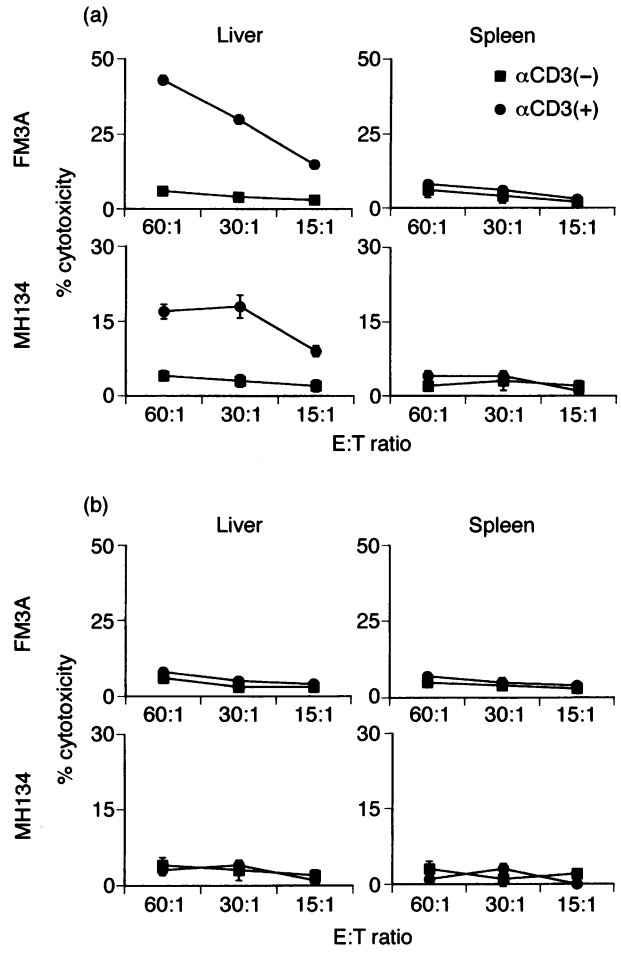


Figure 5. Anti-CD3 mAb-mediated cytotoxicity against NK-resistant tumour targets, FM3A and MH134, by hepatic MNC. (a) Whole hepatic MNC; (b) IL-2Rβ^+ cell-depleted hepatic MNC. In the presence of anti-CD3 mAb (1 μg/ml), hepatic MNC, in which a large proportion of IL-2Rβ^+ int TCR cells were present, mediated the cytotoxicity against the NK-resistant targets. This cytotoxicity was eliminated when IL-2Rβ^+ cell-depleted hepatic MNC were used.

cytotoxicity ($P < 0{\cdot}05$), although the magnitude of augmentation varied. Anti-CD2 and anti-IL-2Rβ mAbs did not show such augmentation in both targets. Reflecting the approximately 25% level of $\gamma\delta$ T cells among int CD3 cells in the liver, a significant augmentation seemed to be induced by anti-TCR$\gamma\delta$ mAb as well. All mAbs against the TCR–CD3 complex seemed to be capable of efficient augmentation.

Perforin induction of liver MNC incubated with anti-CD3 mAb

Perforin induction of liver MNC incubated with anti-CD3 mAb raised the possibility that the addition of anti-CD3 mAb into the assay culture induced the cross-linking of TCR–CD3 complex on the cell surface and resulted in the increase in the level of killing device, such as perforin. This possibility was examined by using hepatic and splenic MNC (Fig. 7). MNC were incubated for 6 hr in the presence or absence of anti-CD3 mAb and the proportion of perforin$^+$ cells was enumerated in the cell smears stained with histochemistry. A prominent increase in the proportion of perforin$^+$ cells was seen in liver MNC cultured with anti-CD3 mAb. The picture of perforin$^+$ cells are represented in the right-hand column. The sorted int CD3 cells were also confirmed to express a higher level of perforin after 6 hr culture (data not shown).

DISCUSSION

In this study, we demonstrated that int TCR cells mediated some NK-like cytotoxicity against the YAC-1 target, as was the case with NK cells. This NK-like cytotoxicity mediated by int TCR cells was lower than that by NK cells, as shown by the results of a negative selection method for lymphocyte subsets. In the course of subsequent experiments for cell sorting, it was revealed that the NK-like activity of int TCR cells could be augmented when exposed with anti-CD3 mAb. Other mAbs against TCR$\alpha\beta$ and TCR$\gamma\delta$ showed a similar effect. Primarily, int TCR cells as well as NK cells, do not have cytotoxicity against NK-resistant targets. However, only int TCR cells became cytotoxic against such NK-resistant tumours after treatment with anti-CD3 mAb or anti-TCR mAb. These results raise the possibility that int TCR cells are important effector cells for cancer immunity under certain conditions.

In earlier studies, several investigators reported a phenomenon in which some effector cells had anti-CD3 or anti-TCR$\alpha\beta$ mAb-mediated cytotoxicity against NK targets.[27–29] At that time, a concept of int TCR cells did not yet exist. In this study, we demonstrated that int TCR cells are such effector cells because neither NK cells nor high TCR cells mediated such cytotoxicity, at least under resting conditions. In this study, we used NK-resistant targets which lack the expression of FcγRIIb and FcRIII, identified by the 2.4G2 mAb. In this regard, reverse antibody-dependent cell-mediated cytotoxicity (ADCC) was not possibly concerned with the present phenomenon. It is speculated that clustering of TCR–CD3 complex on the surface by anti-CD3 mAb (and anti-TCR mAb) accelerated the recognition of some target molecules on tumour cells. It is also speculated that int CD3 cells exert the perforin system through the recognition between the TCR–CD3 complex/target molecules. If this is the case, it raises the possibility that some tumour-associated antigens are presented by monomorphic MHC antigens. In contrast to conventional T cells (i.e. high TCR cells), cumulative data suggest that int TCR cells or T_{NK} cells recognize self-antigens by non-polymorphic MHC antigens such as CD1 and TL.[30–34]

An additional mechanism was raised from the data of the histochemical staining of perforin in this study. Without the existence of target cells in the culture, anti-CD3 mAb could increase the perforin level of liver MNC or int CD3 cells. Perforin is known to be associated with the killing of T cells (e.g. cytotoxic T lymphocytes) as well as NK cells.[35,36] Perforin was found to be also associated with the killing of int TCR cells. In a previous study,[18] we reported that the perforin level in the cytoplasms was greater in NK cells than int CD3 cells. However, the *in vivo* treatment with IL-12 into mice increased the perforin level in the cytoplasms of int CD3 cells. It is concluded that the perforin level of int CD3 cells always awaits an increase by some stimuli.

In a recent study, we have found that the cytotoxicity against non-malignant self-cells by int TCR cells are mainly mediated by means of Fas ligand (FasL)/Fas molecules. In that study, thymocytes of syngeneic B6 origin are a target, while

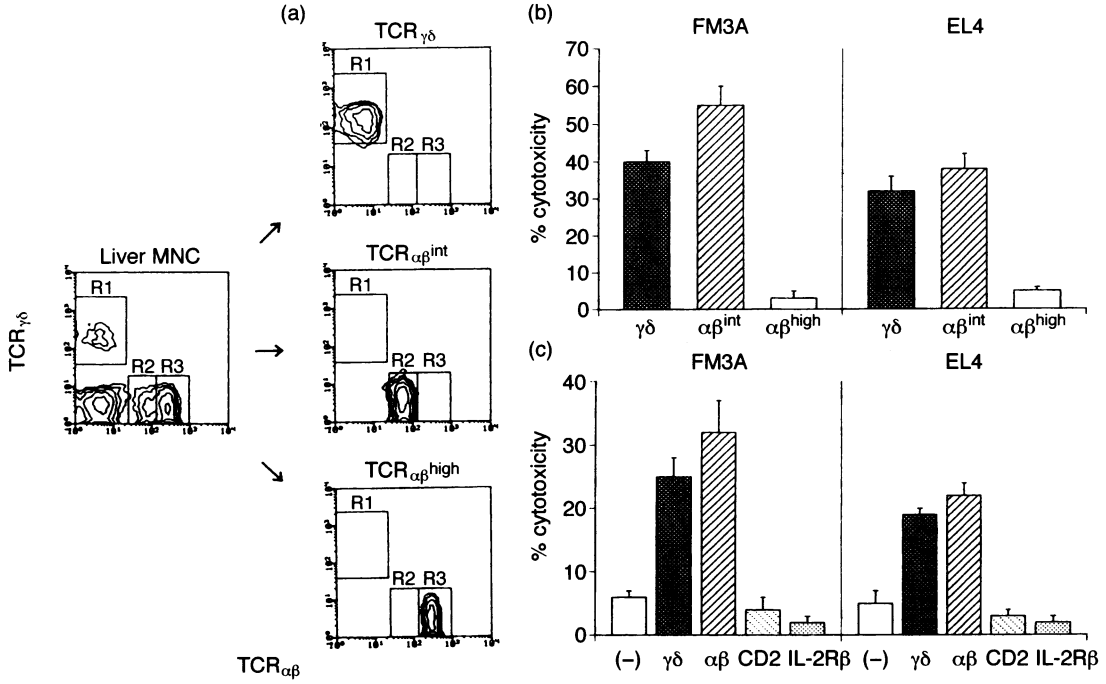


Figure 6. Analysis of mAbs which augmented the cytotoxicity. (a) Sorting of TCR$\gamma\delta^+$ cells, int TCR$\alpha\beta^+$ cells and high TCR$\alpha\beta^+$ cells. (b) The cytotoxicity of the sorted fractions of TCR$\gamma\delta^+$ cells and int TCR$\alpha\beta^+$ cells (E/T ratio = 30 : 1). (c) Addition of mAbs into the assay culture (effector cells were whole liver MNC, E/T ratio = 50 : 1). Various mAbs were added to the assay culture (6-hr analysis) of the cytotoxocity against FM3A and EL-4 targets at a concentration of 1 μg/ml in experiments shown in (c). Stimuli via the TCR–CD3 complex were able to augment the cytotoxicity.

sorted int TCR cells of B6 origin are effectors. Fas$^-$ thymocytes (from B6-*lpr/lpr* mice) of FasL$^-$ int TCR cells (from B6-*gld/gld* mice) could not mediate such autologous killing (T. Moroda *et al.*, manuscript in preparation). Int TCR cells exert their killing against self-cells by means of the FasL/Fas system.

At present, we have not known yet how the cytotoxicity against tumour cells by int TCR cells is mediated in detail. Since tumour targets (except Fas^{low} YAC-1 cells) used in this study were Fas$^+$, there is a possibility that the mechanism of FasL/Fas pathway was also responsible for the killing in the present experimental protocol. Namely, both the perforin system and FasL/Fas system are candidates for the mechanisms in the killing of int TCR cells.

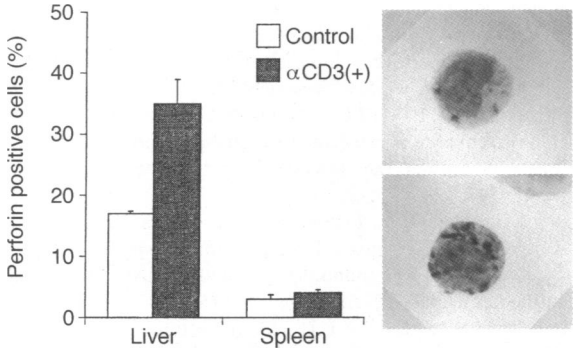


Figure 7. Increase in the proportion of perforin$^+$ cells in hepatic MNC by the culturing with anti-CD3 mAb. Hepatic and splenic MNC (1×10^6/ml) were incubated in a 24-well flat bottomed microculture plate for 6 hr at 37° in a CO_2-incubator, in the presence or absence of anti-CD3 mAb (αCD3) (1 μg/ml). Perforin$^+$ cells are represented in the right column.

Under resting conditions, high TCR cells mediated neither the cytotoxicity against NK-sensitive targets nor that against NK-resistant targets. However, after activation with some T-cell mitogens (e.g. phytohaemagglutinin and concanavalin A), they acquired some cytotoxicity against NK-sensitive targets, and such cytotoxicity was augmented in the presence of anti-CD3 mAb (our unpublished observation). These results are compatible with the earlier report that the NK-like cytotoxicity of some CTL clones was augmented by anti-CD3 mAb.[27–29]

In this study, we used mouse materials to identify extrathymic T cells (i.e. int TCR cells). However, our recent study revealed that one of the counterparts for human extrathymic T cells is CD57$^+$ T cells.[37] This result is compatible with an earlier report that CD57$^+$ T cells (i.e. Leu-7$^+$ T cells) are effector cells against tumour targets in the presence of anti-CD3 mAb.[28]

It has been established that int TCR cells always contain self-reactive forbidden clones.[6,15] This is true for both int TCR cells which are generated by extrathymic pathways or by an alternative intrathymic pathway. Concerning their reactivity to immobilized anti-Vβ mAbs against forbidden clones (e.g. Vβ3$^+$ and Vβ11$^+$ cells in C3H/He mice with Mls-1^{b}2^a), they are at anergic states under resting conditions.[38,39] Thus, they responded to such the immobilized mAbs against forbidden Vβ^+ cells only in the presence of IL-2 (5 U/ml). The present functional study also supports this concept; namely, int TCR cells mediated the cytotoxicity against NK-resistant targets only in the presence of anti-CD3 mAb or anti-TCR mAb.

In this study, anti-TCR$\gamma\delta$ mAb was also found to mediate

the augmentation. This result might be due to the fact that one-quarter of the hepatic MNC used in this experiment were $\gamma\delta$ T cells.[40] This result was confirmed by using sorted $\gamma\delta$ T cells in the present study. On the other hand, the proportion of $\gamma\delta$ T cells in human peripheral blood is not so high ($< 5\%$). This situation explains a new finding for anti-TCR$\gamma\delta$ mAb. The effect of anti-IL-2Rβ and anti-CD2 mAbs on the augmentation was not observed.

In conjunction with a recent report on the cytotoxicity *in vivo* against tumour cells mediated by int TCR cells,[20] autoreactivity of int TCR cells may be an important effector function for tumour immunity. This speculation seems to be reasonable, because the majority of tumour-infiltrating lymphocytes (TIL) consist not only of NK cells but also of int TCR cells.[41] The level of high TCR cells among TIL is lower than the levels of NK cells and int TCR cells at the sites of various tumours. Further *in vivo* studies on int TCR cells in tumour immunity should be carried out.

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