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Sequential appearance of T-cell receptor $\gamma\delta$- and $\alpha\beta$-bearing intestinal intra-epithelial lymphocytes in mice after irradiation

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SUMMARY

We have previously reported that T-cell receptor (TcR) $\gamma\delta$-bearing T cells precede TcR $\alpha\beta$-bearing T cells in appearance in the thymus after whole-body irradiation. In the present study, the kinetics of appearance of intestinal intra-epithelial lymphocytes (IEL) was examined in mice after whole-body irradiation with a lethal dose of 9·5 Gy or with a sublethal dose of 6 Gy. The number of CD3$^+$ IEL decreased to the lowest value 4 days after irradiation with 9·5 Gy, and thereafter increased to half as many as the normal level by day 7. Thy-1$^+$TcR$\alpha\beta^-$ IEL and Thy$^-$TcR$\alpha\beta^-$ IEL recovered considerably by day 7 after the irradiation, whereas Thy-1$^+$TcR$\alpha\beta^+$ IEL and Thy-1$^+$TcR$\alpha\beta^+$ IEL hardly recovered at this stage. All mice died within 12 days after irradiation with a lethal dose of 9·5 Gy. On the other hand, when irradiation dose was decreased to 6 Gy, all mice survived beyond 40 days after irradiation. The number of CD3$^+$ IEL recovered to the normal level by 10 days after irradiation with 6 Gy. Consistently with the results in mice irradiated with a lethal dose, the first cells to increase in IEL of mice irradiated with a sublethal dose were TcR$\gamma\delta^+$ IEL expressing Thy-1 antigen. The number of Thy-1$^+$TcR$\gamma\delta^+$ IEL increased to approximately two-fold as many as that in normal mice by day 10, while TcR$\alpha\beta^+$ IEL began to increase in number from day 20 after irradiation and recovered to the normal level by day 40 after irradiation. Thus, sequential appearance of TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ IEL was evident after irradiation, similar to that seen in the thymus after irradiation. The IEL on day 10 after a sublethal irradiation, which is composed mainly of Thy-1$^+$TcR $\gamma\delta^+$ IEL, exhibited a strong cytolytic activity against P815 in the presence of anti-CD3 mAb, suggesting that the early appearing Thy-1$^+$TcR$\gamma\delta^+$ IEL may play important roles in epithelial immunity at an early stage after irradiation.

INTRODUCTION

T cells recognize foreign antigens via their T-cell-antigen receptor (TcR). At least two kinds of TcR have been identified in mice. One is a heterodimer of α and β chains which recognizes nominal antigens in the context of self-major histocompatibility complex (MHC) gene products (reviewed in ref. 1) and another is a heterodimer of γ and δ chains, some of which are specialized to recognize mycobacterial antigens including 65 kdalton (kD) heat shock protein.[2–6] During T-cell differentiation in the foetal thymus, TcR$\gamma\delta$-bearing thymocytes appear first, followed by TcR$\alpha\beta$-bearing thymocytes.[7] TcR$\gamma\delta$ represents the first CD3-associated TcR in ontogeny and perhaps TcR$\gamma\delta$-bearing T cells precedes TcR$\alpha\beta$-bearing T cells in evolution.

We have recently reported that sequential appearance of TcR$\gamma\delta$- and then TcR$\alpha\beta$-bearing thymocytes can be observed in the adult thymus of mice after irradiation with various doses of 6, 8 and 9·5 Gy.[8–11] The 'radioresistant T-cell precursors' in the thymus of irradiated mice appear to differentiate in a synchronous manner, similar to that seen in the foetal development.[7] The first cells to repopulate the thymus on day 7 after irradiation were CD4$^-$CD8$^-$ thymocytes expressing a high amount of TcRγ and δ chain gene messages.[8–10] From day 7 to day 14, TcR$\alpha\beta$-bearing thymocytes increased to a predominant level in the thymus. Thus, an ordered appearance of TcR$\gamma\delta$- and TcR$\alpha\beta$-T cells occurs in the adult thymus after irradiation.

Intestinal intra-epithelial lymphocytes (IEL) represent a unique lymphoid population with expression of CD8 and with the ability to exhibit non-MHC-restricted cytolytic activity.[12–16] The IEL consist of heterogeneous populations such as Thy-1$^-$ IEL and Thy-1$^+$ IEL, TcR$\alpha\beta^+$ IEL and TcR$\gamma\delta^+$ IEL.[12–16] Recently studies with athymic nude mice, scid mice, and

Abbreviations: GALT, gut-associated lymphoid tissues; IEL, intra-epithelial lymphocytes; MHC, major histocompatibility complex; MLN, mesenteric lymph node; PP, Peyer's patch; TcR, T-cell receptor.

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mice thymectomized, irradiated and repopulated with T-cell-depleted bone marrow cells have revealed that a significant fraction of TcR$\gamma\delta^+$ IEL can develop along an extrathymic pathway.[17 20]

In the present study, to determine whether an ordered appearance of TcR$\gamma\delta^+$ and TcR$\alpha\beta^+$ T cells is observed in IEL after irradiation, we examined the kinetics of appearance of IEL after irradiation with a lethal dose (9·5 Gy) or with a sublethal dose (6 Gy). Our data indicate that the TcR$\gamma\delta^+$ IEL precede the TcR$\alpha\beta^+$ IEL in appearance after irradiation and that the IEL at the early stage after irradiation exhibited a significantly higher level of anti-CD3-redirected cytotoxic activity. The implications of these findings for development and roles of the early appearing TcR$\gamma\delta^+$ IEL were discussed.

MATERIAL AND METHODS

Mice

Female AKR/J Sea at 6 weeks of age were purchased from Seiwa Experimental Animal Institute (Fukuoka) and bred under specific pathogen free condition in Animal Facility in Kyushu University. Each group of experiments consists of five mice at 8 weeks of age.

Irradiation

Whole-body irradiation with 9·5 Gy or 6 Gy was performed with a ^{60}Co γ-ray irradiator (model RE100 1: Toshiba, Tokyo) at a rate of 0·75 Gy/min.

Purification of IEL

Mice were killed by heart puncture under anaesthesia with ether and small intestine, Peyer's patch (PP) and mesenteric lymph node (MLN) were removed from the mice. IEL were isolated according to a modification of a previously described procedure.[21] Briefly, the small intestine was cut into 5-mm pieces and stirred at 37° in medium 199 (Gibco, Grand Island, NY) with the addition of 1 mM dithioerythritol (Sigma Chemical Co., St Louis, MO). A mixture of lymphocytes and epithelial cells were then centrifuged through a 30/67·5% Percol gradient. IEL at the interface were collected. Single-cell suspensions of PP cells and MLN cells were prepared in Hanks' balanced salt solution.

Flow cytofluorometric (FCM) analysis

Freshly isolated IEL, PP cells and MLN cells were stained with monoclonal antibodies (mAb) as follows: anti-TcR β chain (H57-597, kindly provided by Dr R. Kubo, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO), anti-ε chain of CD3 (145-2C11, kindly provided by Dr J. A. Bluestone, University of Chicago, IL), anti-TcR δ chain (GL3, purchased by Phamingen, San Diego, CA), biotin-conjugated anti-Thy-1·1 (Meiji, Tokyo), biotin-anti-Lyt-2 (CD8α), and phycoerythrin (PE)-anti L3T4 (CD4, Becton Dickinson, Oxnard, CA). PE-streptavidin and streptavidin-DuoCHROM were purchased from Becton Dickinson. Anti-Lyt-3.1 (CD8β) mAb were obtained from Cedarlane Laboratories (Westbury, NY). For triple-colour analysis, cells were stained with FITC-anti-CD3 mAb, PE-anti-CD4 mAb and biotin-anti-CD8 mAb before addition of strevtavidin-DuoCHROM. The stained samples were analysed with a single-beam flow cytometer, FACScan (Becton Dickinson). Forward and side angle light scatter were used to exclude dead and aggregated cells. The data were analysed with the Consort 30 Research Software in the case of double-colour analysis and with FACScan Research Software in the case of triple-colour analysis. The results were presented as fluorescence histograms with the relative number of cells on linear scale plotted vs. the relative fluorescence intensity on a logarithmic scale, or with the relative fluorescence intensity on the both in arbitrary units. Absolute numbers per organ of the respective lymphocytes were determined by single histogram for some lymphocyte markers in the total cell population.

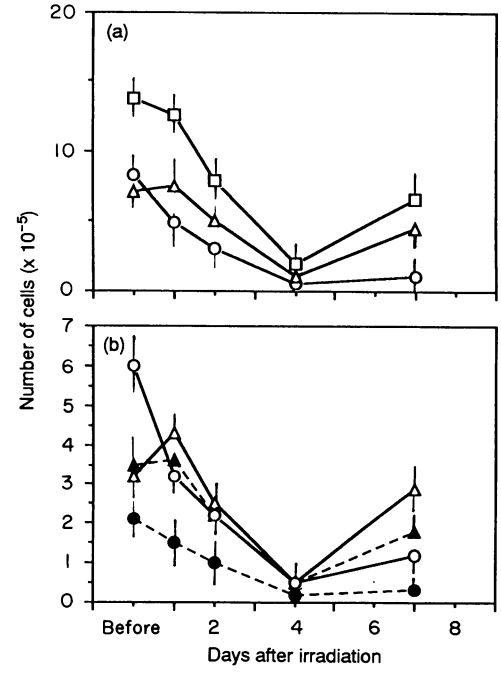

Figure 1. Cell kinetics of IEL subpopulations after irradiation with 9·5 Gy. Each point and bar represents mean value and standard deviation of five mice calculated as total cell counts × percentage of the corresponding cell population. (a) CD3$^+$ IEL (□), TcR$\alpha\beta^+$ IEL (○), TcR$\alpha\beta^-$ IEL (△) and (b) Thy-1$^+$ TcR$\alpha\beta^+$ IEL (○), Thy-1$^-$ TcR$\alpha\beta^+$ IEL (●), Thy-1$^+$ TcR$\alpha\beta^-$ IEL (△), Thy-1$^-$TcR$\alpha\beta^-$ IEL (▲).

Redirected cytotoxicity assay

IEL were assayed for cytotoxic activity against Fc receptor$^+$ DBA/2 mastocytoma P815 cells. Freshly isolated IEL ($2{\cdot}5 \times 10^5$ or $1{\cdot}25 \times 10^5$ cells per well) were incubated in 96-well round bottom microtitre plates for 4 hr at 37° with ^{51}Cr-labelled P815 target cells (5×10^3) that had been previously incubated with 2 μg/ml of anti-CD3 mAb. Per cent specific lysis was calculated as follows:

$$\frac{\text{c.p.m. released with effector} - \text{c.p.m. released alone}}{\text{c.p.m. released by detergent} - \text{c.p.m. released alone}} \times 100.$$

Statistical analysis

The statistical significance of the data was determined by the Student's t-test. A P-value of less than 0·05 was taken as significant.

RESULTS

Kinetics of IEL after irradiation with a lethal dose of 9·5 Gy

To investigate the cell kinetics of IEL after irradiation, the

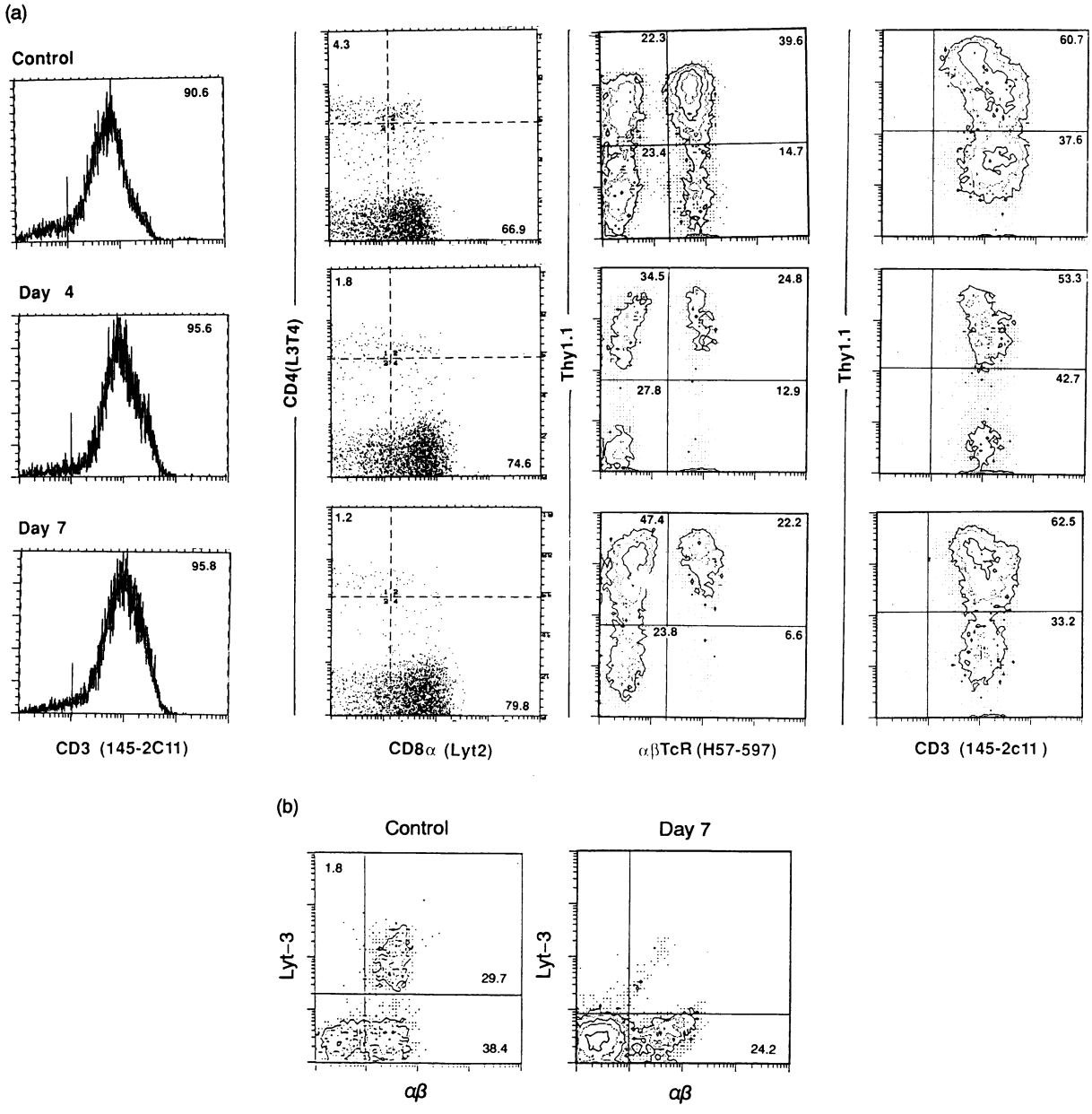

Figure 2. Flow cytometric analysis of cell surface markers on mesenteric lymph node cells and intestinal intra-epithelial lymphocytes. (a) Intestinal intra-epithelial lymphocytes in untreated control mice and in mice irradiated with 9·5 Gy 4 days previously or 7 days previously were stained with FITC-anti-CD3 mAb(145-2C11), PE-anti-CD4 mAb and biotin-anti-CD8 mAb before addition of streptavidin-DuoCHROME, with FITC-anti-TcRαβ mAb (H57-597) and biotin-Thy-1.1 mAb plus streptavidin-PE or with FITC-anti-CD3 mAb and biotin-Thy-1.1 mAb plus streptavidin-PE. (b) Intestinal intra-epithelial lymphocytes from untreated control mice and those from mice irradiated with 9·5 Gy 7 days previously were stained with FITC-anti-TcRαβ mAb and biotin-Lyt-3.1 mAb plus streptavidin-PE.

numbers of $CD3^+$ IEL were counted after irradiation of a sublethal dose of 9·5 Gy. As shown in Fig. 1a, the irradiation led to a severe reduction in the numbers of $CD3^+$ IEL by day 4. Thereafter, the number of the IEL increased gradually, whereas the number of $CD3^+$ cells in MLN and PP remained at the lower levels (data not shown).

To characterize the cell subpopulations in IEL after irradiation, we examined the FCM analysis on the IEL after irradiation with a lethal dose of 9·5 Gy. The proportion of $CD3^+CD4^-CD8^+$ cells in IEL increased on day 4 and on day 7 after irradiation. $TcR\alpha\beta^-$ cells, most of which bore CD3, gradually increased in proportion by day 7, whereas the $CD3^+TcR\alpha\beta^+$ cells decreased by day 7 after irradiation (Fig. 2a). On the basis of expression of CD8 α and β chains, $TcR\alpha\beta^+CD8^+$ IEL are further divided into two subsets; $TcR\alpha\beta^+$ IEL bearing CD8α homodimer and $TcR\alpha\beta^+$ IEL bearing CD8 α and β heterodimer.[12-16] Two-colour staining with anti-TcRαβ mAb and anti-Lyt-3.1 mAb revealed that most of $TcR\alpha\beta^+$ IEL on day 7 after irradiation expressed CD8 α homodimer (Fig. 2b). As summarized in Fig. 1b, within the $CD3^+$ IEL, $Thy\text{-}1^+TcR\alpha\beta^-$ IEL and $Thy\text{-}1^-TcR\alpha\beta^-$ IEL, both of which presumably bear $TcR\gamma\delta^+$, recovered remarkably, while

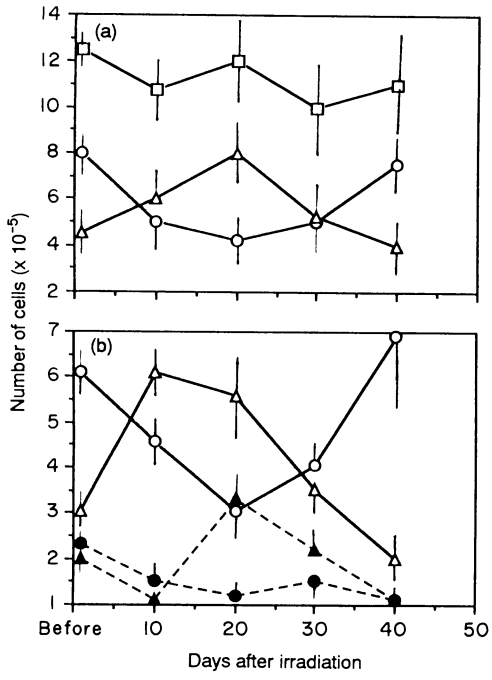

Figure 3. Cell kinetics of IEL subpopulations after irradiation with 6 Gy. Each point represents mean value of five mice calculated as total cell counts × percentage of the corresponding cell population. (a) $CD3^+$ IEL (□), $TcR\alpha\beta^+$ IEL (○), $TcR\gamma\delta^+$ IEL (△) and (b) Thy-1^+ $TcR\alpha\beta^+$ IEL (○), Thy-1^- $TcR\alpha\beta^+$ IEL (●), Thy-$1^+TcR\gamma\delta^+$ IEL (△), Thy-1^- $TcR\gamma\delta^+$ IEL (▲).

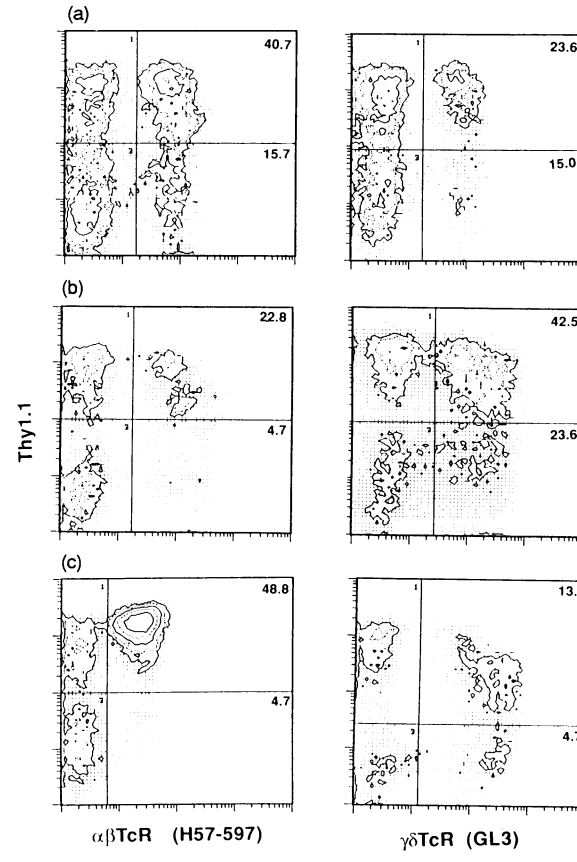

Figure 4. Intestinal intra-epithelial lymphocytes in untreated control mice and in mice irradiated with 6 Gy 20 days previously or 40 days previously were stained with FITC-anti-$TcR\alpha\beta$ mAb (H57-597) and biotin-Thy-1.1 mAb plus streptavidin-PE or with FITC-anti-$TcR\gamma\delta$ mAb (GL3) and biotin-Thy-1.1 mAb plus streptavidin-PE.

only a marginal increase in the numbers of Thy-1^- $TcR\alpha\beta^+$ IEL and Thy-$1^+TcR\alpha\beta^+$ IEL was observed after irradiation with a lethal dose of 9·5 Gy.

Kinetics of IEL after irradiation with a sublethal dose of 6 Gy

Since all mice irradiated with 9·5 Gy died within 12 days, it was impossible to examine the kinetics of IEL over 10 days. To investigate the kinetics of appearance of $CD3^+$ IEL at a relatively late stage after irradiation, we examined the fate of the IEL in mice irradiated with a sublethal dose of 6 Gy. The number of $CD3^+$ IEL already recovered to the same level of normal mice on day 10 after irradiation (Fig. 3a), whereas the numbers of $CD3^+$ cells in MLN and PP remained at the lower level on day 10 after irradiation. It took approximately 40 days for the MLN cells and PP cells to recover to the normal level after irradiation (data not shown).

We further characterized the cell populations in the IEL recovering after irradiation by FCM analysis. As shown in Fig. 4, the proportion of Thy-$1^+TcR\gamma\delta^+$ IEL increased on day 20 after irradiation, whereas IEL on day 40 after irradiation consisted of a large proportion of Thy-$1^+TcR\alpha\beta^+$ cells. The kinetics of absolute numbers of IEL subpopulations was shown in Fig. 3b. On day 10 after irradiation, most IEL were of Thy-$1^+TcR\gamma\delta^+$ in phenotype. The number of Thy-$1^-TcR\gamma\delta^+$ IEL increased to the maximum level on day 20 after irradiation. The number of Thy-$1^+TcR\alpha\beta^+$ IEL gradually increased and reached the normal level by day 40 after irradiation and conversely $TcR\gamma\delta^+$ IEL decreased in number at this stage. Thus, the $TcR\gamma\delta^+$ IEL recovered first, followed by an increase in $TcR\alpha\beta^+$ IEL after irradiation with a sublethal dose of 6 Gy.

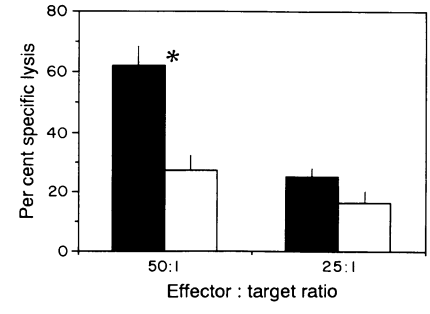

Figure 5. Anti-CD3-redirected cytolytic activity of IEL against P815 mastocytoma: IEL from untreated control mice (□) and IEL on day 10 after irradiation with 6 Gy (■). ^{51}Cr-labelled P815 cells were used as target cells. Each point and vertical bar represents the mean value and standard error of triplicate samples. * Significant from control; $P < 0.001$.

Redirected cytotoxicity of IEL recovering after irradiation

IEL are known to exhibit non-MHC-restricted cytolytic activity.[12 16] To compare the activity of IEL before irradiation and after irradiation, the cytolytic activity of IEL against P815 in the presence of anti-CD3 mAb was examined. As shown in Fig. 5, the IEL on day 10 after irradiation with 6 Gy, most of which were Thy-1$^+$TcR$\gamma\delta^+$ cells, showed a significantly higher level of the cytolytic activity than that in normal mice ($P < 0{\cdot}001$). These results indicated that the early-appearing IEL might be in an activated state.

DISCUSSION

We have obtained evidence for an ordered appearance of TcR$\gamma\delta^+$ IEL and TcR$\alpha\beta^+$ IEL after irradiation. In the thymus of mice irradiated with a lethal dose (9·5 Gy), only one wave for regeneration of intrathymic radioresistant T-cell precursors was observed from day 4 to day 14 after irradiation (data not shown). Although an increase in the numbers of CD3$^+$ cells in PP cells or MLN was not evident until day 12 after irradiation, the number of CD3$^+$ IEL, especially TcR$\gamma\delta^+$ IEL, increased from day 4 to 7 after a lethal dose of irradiation (Fig. 1). When the radiation dose was reduced to 6 Gy, thymus cells regenerated in two waves after irradiation. The first wave from day 4 to day 14 is the regeneration of radioresistant intrathymic T-cell precursors. The second one from day 20 to day 30 is thought to be due to the regeneration of T-cell precursors repopulating from the bone marrow.[22 25] In PP and MLN of mice irradiated with a sublethal dose, it took approximately 40 days for the cells to recover to the normal level (data not shown). On the other hand, the number of CD3$^+$ IEL recovered rapidly to the normal level by day 10 after irradiation. The rapid recovery of the number of CD3$^+$ IEL is ascribed to the increase in TcR$\gamma\delta^+$ IEL (Fig. 3). The cell kinetics of TcR$\alpha\beta^+$ IEL after irradiation showed much the same pattern as that seen in PP and MLN (data not shown).

Several possibilities can be raised to explain the finding that TcR$\gamma\delta^+$ IEL precede TcR$\alpha\beta^+$ IEL in appearance after irradiation. The first is that the early-appearing TcR$\gamma\delta^+$ thymocytes derived from radioresistant intrathymic T-cell precursors may migrate from the thymus faster than the TcR$\alpha\beta^+$ thymocytes. However, only a few, if any, TcR$\gamma\delta^+$ T cells were detected in the peripheral lymphoid tissues at the early stage after irradiation (data not shown). The studies with irradiated mice repopulated with bone marrow cells revealed that it took more than 14 days for radioresistant intrathymic T-cell precursors to maturate and migrate from the thymus to the peripheral lymphoid organs.[26] The TcR$\gamma\delta^+$ IEL already increased in number on day 7 after a lethal dose of irradiation, when the thymocytes began to increase in number in these mice (Fig. 1). Taken together, it is unlikely that the TcR$\gamma\delta^+$ T cells migrate from the thymus at the early stage after irradiation. The second possibility is that radioresistant T-cell precursors of TcR$\gamma\delta^+$ T cells present in IEL may regenerate locally at the intestine outside the thymus. The studies with athymic nude mice and mice thymectomized, irradiated and repopulated with foetal liver cells reveal that a major population of TcR$\gamma\delta^+$ IEL develop along an extrathymic pathway.[18] Recently, Guy-Grand *et al.* have found that IEL contain mRNA for the RAG-1 which is required for TcR gene rearrangement, suggesting that TcR gene rearrangement may occur in gut microenvironment.[19] It would be thus most possible that TcR$\gamma\delta^+$ IEL differentiating locally at the intestine may appear at the early stage after irradiation.

TcR$\alpha\beta^+$ IEL bearing CD8α homodimer have been reported to be detected in nude mice, scid mice and mice thymectomized, irradiated and repopulated with T-cell-depleted bone marrow cells.[19] The forbidden T-cell clones bearing TcR$\alpha\beta$ capable of recognizing the self-superantigens, which are normally deleted in the thymus, are detected in the IEL bearing CD8 α homodimer.[27] These results suggested that TcR$\alpha\beta^+$ IEL bearing CD8 α homodimer can be considered as a thymus-independent population. In our results, the IEL on day 7 after irradiation with 9·5 Gy, in which an appreciable level of TcR$\alpha\beta^+$ IEL were present, expressed exclusively CD8 α homodimer, indicating that the early-appearing TcR$\alpha\beta^+$ IEL also develop along an extrathymic pathway, as suggested similarly with TcR$\gamma\delta^+$ IEL.

On the basis of expression of Thy-1 antigen, the IEL are reported to be divided into two groups, Thy-1$^+$ and Thy-1$^-$.[12 16] Thy-1$^-$ IEL are known to express exclusively CD8 with α chain homodimer, suggesting that the Thy-1$^-$ population may be a thymus-independent population.[19] Our data presented here that most of the early-appearing IEL after irradiation are Thy-1$^+$ IEL argue against the assumption that the early-appearing IEL after irradiation develop along an extrathymic pathway. However, the study with athymic radiation chimeras has suggested that Thy-1$^+$ IEL can develop extrathymically.[20] Furthermore, the study with germ-free mice has demonstrated that freshly isolated IEL from germ-free mice display neither Thy-1 antigen nor lytic activity but the acclimation of germ-free mice to non-sterile conditions results in the generation of Thy-1$^+$ IEL and induction of lytic activity.[28] It would thus appear that the appearance of Thy-1$^+$ IEL may be dependent on the presence of external stimuli. Further analysis with IEL in thymectomized and irradiated mice should be required to elucidate the lineage of the early-appearing IEL after irradiation.

The early-appearing Thy-1$^+$ IEL exhibited a strong cytolytic activity in the presence of anti-CD3 mAb, suggesting that these IEL may be in activated state after irradiation. A supralethal dose of whole-body irradiation is known to cause some damage to the central nerve system, gut mucosal cells and haematopoietic cells.[29 31] Studies using germ-free, monoassociated and conventionalized mice reveal that severe infections with indigenous enteric Gram-negative bacteria translocating from gastrointestinal tract is a leading cause of the radiation-induced lethality.[32 34] Indigenous infection with enteric Gram-negative bacteria are thought to be mainly due to the defect of the first defence mechanism mediated by the haematopoietic cells in the gut-associated lymphoid tissue.[31] In mice irradiated with 6 Gy, the early-appearing IEL which display an increased level of cytolytic activity may contribute to the first line of host defence against invasion of enteric bacteria.

In conclusion, we have obtained evidence that an ordered appearance of TcR$\gamma\delta^+$ IEL and TcR$\alpha\beta^+$ IEL occur after irradiation. The early-appearing TcR$\gamma\delta^+$ IEL may participate in immune surveillance at the first line of host-defence in the epithelium against invasion of enteric bacteria after irradiation.

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