Flow Cytometric Analysis of Thymocyte Subpopulations in Mice after Whole-Body X-Irradiation

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ABSTRACT. To determined the cellular kinetics of thymocyte subpopulations in DBA1 mice after whole-body 6.8 Gy X-irradiation, they were analyzed for the expression of several cell surface antigens using flow cytometry. The results show that i) The majority of thymocytes rapidly depleted by irradiation was $CD4^+8^+$ cells. ii) radioresistant $CD4^+8^-$ and $CD4^-8^+$ survived 18–48 hr after X-irradiation were considered to be relatively mature type, since they expressed high levels of CD3 and LECAM-1. iii) CD3-positive cells were detected in $CD4^-8^-$ cells at 72 hr after irradiation.—KEY WORDS: cell surface antigen, murine thymocyte, X-irradiation.
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The thymus has been well established as the site of T cell development [21] and contains the mixture of cells on different stages of T cell development. The thymocyte acquires various cell surface antigens during proliferation and development [5]. Thymus is one of the most radiosensitive organs [8]. The depletion and regeneration of thymocytes after whole-body X-irradiation have been extensively studied with experimental animals [10, 15, 16].

We have previously reported that B220, a specific marker of pan-B cells, was expressed on both pre-apoptotic and apoptotic thymocytes of DBA1 mice early after whole-body X-irradiation [13]. However, the behavior of thymocyte subsets in the early radiation-induced apoptotic process has not been well known.

In the present study, we attempted to examine the cellular kinetics of thymocyte subpopulations in DBA1 mice at the early stage after whole-body X-irradiation, when the expression of B220 was induced. To exclude the effect of proliferation of precursor cells emigrated from the bone marrow on thymic cellularity, we employed a lethal dose of 6.8 Gy which caused a severe damage to the ability of bone marrow to repopulate the thymus [19]. Thymocyte subpopulations were analyzed by the expression of a variety of cell surface antigens using flow cytometry.

Animals: DBA1 mice were originally provided by Dr. J. Hayakawa (Institute for Experimental Animals, School of Medicine, Kanazawa University, Kanazawa, Japan) and have been bred at our laboratory [13].

X-irradiation, antibodies and flow cytometry: Female 6-wk-old mice were exposed to 6.8 Gy of whole-body X-irradiation at a dose rate of 0.46 Gy/min with a X-ray generator (Radioflex-350; Rigaku Denki Co., Ltd.) operated at 250 kV and 12.5 mA with added filtration of 0.3 mm Cu and 0.5 mm Al. Seven mice exposed to 6.8 Gy were all died within 3 weeks, suggesting the dose would be lethal. Mice were killed at various times after the irradiation, and the excised thymuses were gently teased with a forceps in Hank's balanced salt solution containing 1% fetal calf serum (HBSS-FCS) to prepare single cell suspensions. These cell suspensions were washed once with HBSS-FCS. The cells (1×10^6) were incubated for 30 min on ice in 100 μl HBSS-FCS containing the appropriate dilution of antibodies, and washed once with the same buffer. The thymocytes were stained with the following fluorescent dye-conjugated mAb: anti-CD3-fluorescein isothiocyanate (FITC) (145–2Cll, Boehringer Mannheim), anti-CD4-phycoerythrin (PE) (GK1.5, Becton Dickinson), anti-CD8-FITC (53–6.7, Becton Dickinson), anti-VLA-4-PE (PS/2, Serotec), anti-LECAM-1-FITC (MEL-14, Phermingen). Two-color flow-cytometric analysis was performed with a Cyto ACE-150 (Japan Spectroscopic Co., Ltd.) and the Cyto ACE system program version 3.04 (Japan Spectroscopic Co., Ltd.). Dead cells were routinely excluded on the basis of the forward and side light scatter. Each point and error bar in Figures are the mean and standard deviation of three to seven mice.

Statistical analysis: Statistical significance was determined by Student's *t*-test.

The total number of thymocytes rapidly decreased and reached a minimum level at 24 hr after irradiation. Reduction of the cell number concomitantly occurred in each of thymocyte subsets defined by CD4 and CD8, but the degree of reduction varied among the subsets, reflecting their radiosensitivity. Figure 1a–d show the changes in the relative proportion and in the number of each T cell subset. The percentage of $CD4^+8^+$ cells, the predominant subset in unirradiated control, rapidly decreased from 87% to 23% during 24 hr after irradiation (Figs. 1a and 2a). These findings agree well with the result obtained by other authors that $CD4^+8^+$ thymocytes are most susceptible to radiation-induced apoptosis [6, 25]. In striking contrast to $CD4^+8^+$, a remarkable proportional increase in $CD4^+8^-$ was observed from 18 hr after irradiation (Fig. 1b), suggesting that the radioresistant cells survived exposure to 6.8 Gy. The similar results have been reported by Williams *et al.* [26] and Huiskamp and van Ewijk [10]. A small but significant increase in the percentage of $CD4^-8^+$ was also observed at 18 hr after irradiation (Fig. 1c). On the other hand, the percentage of immature $CD4^-8^-$ thymocytes increased remarkably from 48 hr after irradiation and reached 49.4%, a maximum value, at 72 hr after irradiation (Fig. 1d). Figure 2a shows the changes in the cytograms of thymocyte subsets as defined by CD4 and CD8 expression. It is of interest to

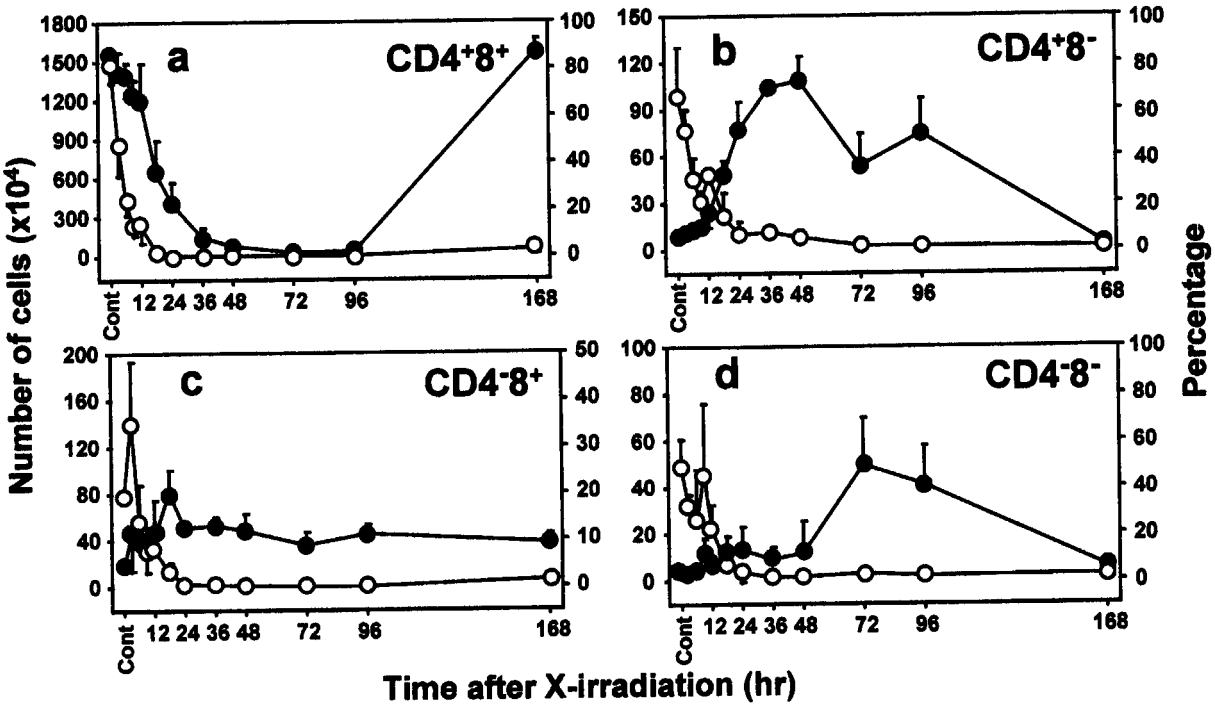

Fig. 1. The change in the number (○) and the proportion (●) of CD4/8 thymocyte subpopulations after 6.8 Gy whole-body X-irradiation. (a) $CD4^+8^+$, (b) $CD4^+8^-$, (c) $CD4^-8^+$, (d) $CD4^-8^-$. Each point is the mean ± SD as calculated from 3 to 7 mice.

note that the increase in the fraction of the cells expressing reduced levels of CD4 and CD8, a minor fraction in the unirradiated control (Fig. 2a), was observed in $CD4^+8^+$ population 6 and 18 hr after irradiation.

We have previously observed that a dramatic reduction of the cell number accompanied some cellular changes characteristic for apoptosis, namely, decrease in cell size as well as DNA content per cell and the expression of B220, an isoform of murine CD45 molecule [13]. Therefore, the cause of reduction in radiosensitive cells can be ascribed to radiation-induced apoptosis. The proportion of $CD4^+8^+$ cells increased with decrease in that of $CD4^-8^-$ cells at 96–168 hr after irradiation. From 96 to 168 hr after irradiation, the number of $CD4^+8^+$ cells increased from 1.06×10^4 to 64.75×10^4, while the number of $CD4^-8^-$ cells remained unchanged. The increased number of $CD4^+8^+$ cells may be differentiated from $CD4^-8^-$ subset [3, 14].

CD3 is a useful marker of the maturation stage of thymocytes and highly expressed on the cell surface of mature $CD4^+8^-$ and $CD4^-8^+$ subsets [23]. The changes in the expression level of CD3 and in the relative proportion of $CD3^+$ cells after irradiation are shown in Fig. 2b and Fig. 3, respectively. Apparent increases in the percentage of $CD4^+8^-$ and $CD4^-8^+$ subsets were closely related with the increase in the percentage of $CD3^+$ cells (Fig. 1b and c). This suggests that the majority of radioresistant $CD4^+8^-$ (and probably $CD4^-8^+$) cells survived exposure to 6.8 Gy expressed CD3 on their surface. At 72 hr after irradiation, more than 80% of thymocytes would be positive for CD3, and about half of them were $CD4^-8^-$. These results show that at least 30% of $CD4^-8^-$ thymocytes were positive for CD3 antigen. Ayukawa *et al.* reported that 22% of $CD4^-8^-$ were $CD3^+$ on day 8 after 800 rad irradiation [2]. $CD3^+CD4^-8^-$ thymocytes are known to be a minor population usually detected in thymus from adult mice [4, 12, 22]. They contain both α/β and γ/δ TCR^+ cells [4, 12, 16, 24]. However, relatively little is known about the *in vivo* function and the differentiation pathway of $CD3^+CD4^-8^-$ cells [7]. Whether $CD3^+CD4^-8^-$ cells within $CD4^-8^-$ subset can be the precursor of $CD3^+CD4^+8^+$ cells, mature $CD4^+8^-$ and $CD4^-8^+$ cells remains to be determined.

LECAM-1 is a receptor involved in the adherence of T cells to endothelium of blood vessel [11, 20]. On the other hand, VLA-4 is a member of the integrin superfamily of cell adhesion molecules [18]. The expression level of LECAM-1 increases with maturation of thymocytes [11, 20], while that of VLA-4 decreases [18]. These markers, therefore, serve as parameters for assessing thymocyte maturation. In unirradiated control, 40% of thymocytes expressed LECAM-1 at low level (Fig. 2c). The level increased gradually with time after irradiation. At 72 hr after irradiation, more than 70% of thymocytes were positive for LECAM-1, and about half of them were $CD4^-8^-$. These results suggest that at least 20% of $CD4^-8^-$ thymocytes were $CD3^+LECAM^-1^+$. This is the first report concerning the change of LECAM-1 expression on thymocytes after irradiation. Remarkable change in the expression level of VLA-4, as reported by Sawada *et al.* [18], was not observed

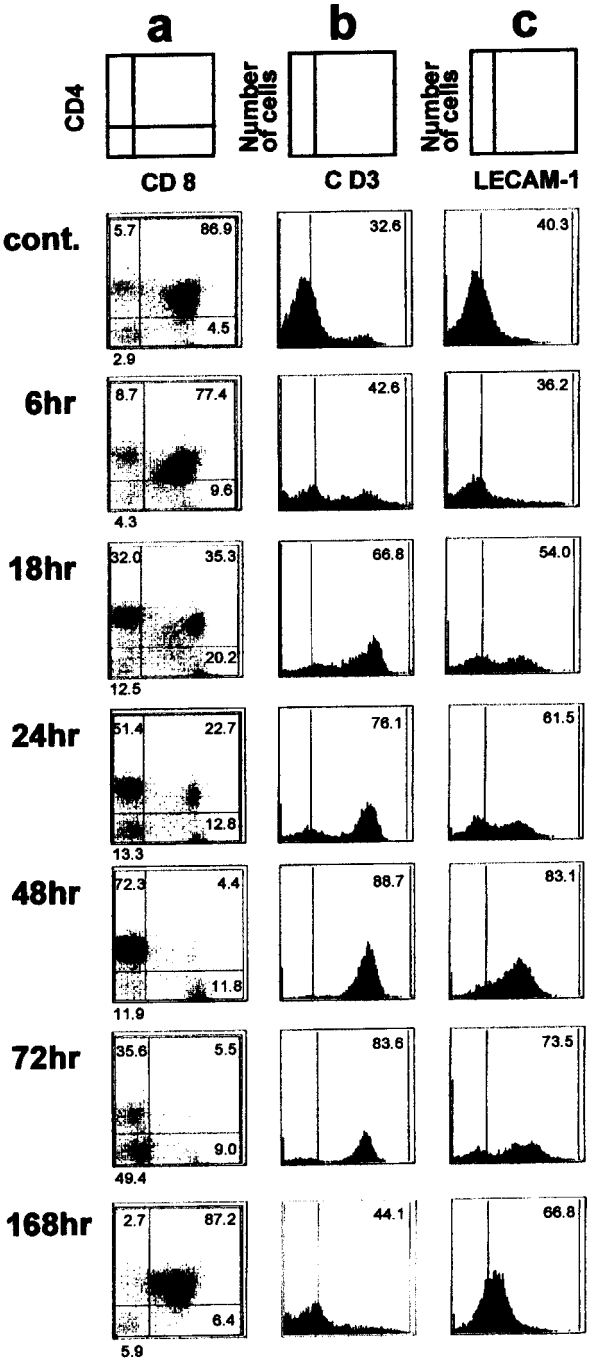

Fig. 2. The expression patterns of CD4/CD8, CD3 and LECAM-1 at various times after 6.8 Gy whole-body X-irradiation. Thymocytes were analyzed for CD4 and CD8 expression (a), CD3 (b) or LECAM-1 (c). (a) the upper left, upper right, lower left and lower right areas represent $CD4^+8^-$, $CD4^+8^+$, $CD4^-8^-$ and $CD4^-8^+$, respectively. (b)(c) The vertical line in each panel indicates the upper limit of the autofluorescence intensity from unstained cells. In each panel, 10,000 cells were analyzed. The numerical values show the mean percentage as calculated from 3 to 7 mice.

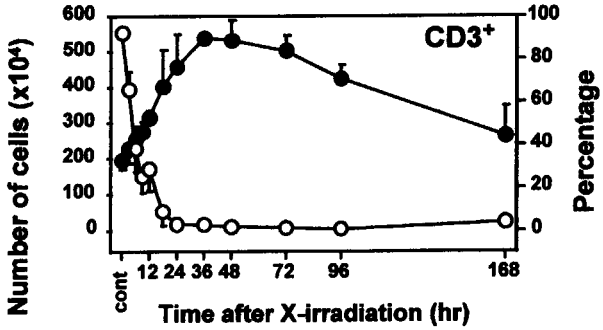

Fig. 3. The change in the number (○) and the proportion (●) of CD3 positive thymocytes after 6.8 Gy whole-body X-irradiation. Each point is the mean ± SD as calculated from 3 to 7 mice.

(data not shown).

In conclusion, we confirmed the early changes in the thymocyte subsets in mice after whole-body 6.8 Gy X-irradiation and observed that $CD4^+8^+$ was the most radiosensitive subset depleted within 24 hr after irradiation, while $CD4^+8^-$ and $CD4^-8^+$ were more radioresistant than $CD4^+8^+$. The dramatical reduction of $CD4^+8^+$ can be attributed to apoptosis within 24 hr after irradiation, when the expression of B220 increased on the subset [13]. Most of $CD4^+8^-$ and $CD4^-8^+$ cells survived exposure to 6.8 Gy were positive for CD3 and LECAM-1. Therefore, these cells were considered to be mature type and to migrate to peripheral lymph organs. The increased proportion of $CD3^+CD4^-8^-$ cells, a minor subpopulation in the normal thymus, was detected from 48 hr after irradiation. A further study is necessary to determine the origin and the fate of $CD3^+CD4^-8^-$ cells.

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