Determination of *In Vivo* and *In Vitro* Radiosensitivity of Mouse Splenic T-lymphocytes Using a T-cell Cloning Technique

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Abstract : Recently the authors established a method for culturing mouse splenic T-lymphocytes with T-cell growth factor (TCGF) and feeder cells *in vitro*. Using this method, T-lymphocytes grow for approximately 14 days with population doubling times of 27-29 hr; cloning efficiencies (CEs) of mouse spleen cells ranged from three to twelve percent. Using this colony forming assay, *in vivo* and *in vitro* radiosensitivity of mouse splenic T-lymphocytes in the G₀ phase and *in vitro* radiosensitivity of proliferating T-lymphocytes (cycling T-lymphocytes) were examined. For *in vitro* irradiation, the dose-survival curve of T-lymphocytes in G₀ phase gave a D₀ value of 0.99 Gy and a D_q value of 0.87 Gy and that of cycling T-lymphocytes gave a D₀ value of 1.04 Gy and a D_q value of 0.19 Gy. For *in vivo* irradiation, the dose-survival curve of T-lymphocytes gave a D₀ value of 1.01 Gy and a D_q value of 0.73 Gy. These results suggest that the recovering activity from sublethal damage of G₀ T-lymphocytes was more effective than that of cycling T-lymphocytes. Furthermore, this colony forming assay system appears to be very useful for screening the effects of *in vivo* exposure to toxic and/or mutagenic agents and for comparing the effects of *in vivo* exposure with those of *in vitro* exposure to toxic agents as well as radiation.

Key words : radiosensitivity, X-irradiation, T-lymphocyte, clonal assay, *in vivo*.

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Introduction

Human lymphocyte clonal assay systems have recently been developed for quantification and study of the effects of mutagens (Albertini *et al.*, 1985; Morley *et al.*, 1985). However, the effects of toxic agents and mutagens for active *in vivo* exposure cannot be examined using only these systems, and it is very difficult to compare the effects of *in vivo* exposure with those of *in vitro* exposure of such agents. Therefore, the development of a similar assay system in an experimental animal such as mice is necessary and will provide important information for a number of fields. Mouse lymphocyte clonal assay systems have been reported by Jones *et al.* (1985), Inamizu *et al.* (1986) and Dempsey & Morley (1986). Modifying their assay systems, the authors of this report established a mouse T-cell clonal assay system that is simpler and more useful.

The lymphatic system is one of the most radiosensitive organs and the radiosensitivity of lymphocytes has been examined by various authors including Anderson *et al.* (1977) and Sprent *et al.* (1974). Many methods such as the incorporation of tritiated thymidine in

alloantigen activated T-lymphocytes (Sprent *et al.*, 1974), the number of splenic T-lymphocytes identified by a immuno-fluorescence technique (Kataoka & Sado, 1975), PHA and Con-A response of T-lymphocytes (Anderson *et al.*, 1977), the plaque forming cell (PFC) response, (Carlson & Lubet, 1976; Sado *et al.*, 1985), and cell-mediated lympholysis response (Sado *et al.*, 1985) are used for analysing lymphocyte radiosensitivity. However, these methods cannot evaluate the clonogenic ability of mouse lymphocytes as radiation effects. Our newly established method is very simple and easy for analysing the effects of both *in vivo* and *in vitro* irradiation on clonogenic ability. In the present study, the difference in T-lymphocyte radiosensitivity between the G₀ phase and exponential phase *in vitro*, and between *in vivo* and *in vitro* irradiation, is evaluated by our colony forming assay method.

Materials and Method

Animals

Wistar male rats weighing 200 g were used for the preparation of T-cell growth factor (TCGF). C3H/He/Slc female mice were used at the age of 12–20 weeks for the radiosensitivity studies.

Preparation of T-cell growth factor

For TCGF preparation the authors modified the method of Gillis *et al.* (1978). The spleen cells of rats were hemolyzed in Gey's solution replacing NaCl with NH₄Cl and suspended in Hanks' balanced salt solution (HBSS). These suspended cells (2×10^6 cells/ml) were cultured in the culture medium [i.e., RPMI 1640 medium supplemented with 10% heat-inactivated (56°C, 30 min.) fetal bovine serum (Gibco), 20 mM Hepes, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin] containing 50 µM of 2-mercaptoethanol with Concanavalin-A (Con-A, 10 µg/ml, Sigma) and PMA (phorbol 12-myristate 13-acetate, 10 ng/ml, Sigma) at 37°C in a 5% CO₂ humidified atmosphere for 40 hours. The supernatant was used as TCGF. The activity of TCGF was tested by cloning efficiency (CE) of mouse spleen cells using a colony forming assay system (described below). The optimal concentration of TCGF in the growth medium (described below) for the colony forming assay was determined to be 20–25 percent.

Feeder cells

A lymphoblastoid cell line (LCL-TK6), a subclone of WI-L2, was a gift from Dr. J. J. McCormick, Michigan State University. LCL-TK6 cells were maintained at densities between 10^5 and 10^6 cells per ml in the culture medium. They received 55 Gy ⁶⁰Co γ-irradiation to prepare X-TK6 cells for use as feeder cells and were either immediately used or frozen at 2×10^7 cells/vial.

Mouse T-lymphocyte cultures

The spleens of C3H mice were placed in HBSS and teased to release all of the spleen cells and then filtered through a stainless steel mesh. The spleen cells were hemolyzed in Gey's solution replacing NaCl with NH₄Cl at 4°C for 5 min to remove red blood cells. After washing twice in RPMI 1640 medium, the spleen cells were cultured in the growth medium (culture medium supplemented with 20–25% TCGF and 1×10^5 cells/ml X-TK6) at 37°C in a 5% CO₂ humidified atmosphere. As mass culture, the spleen cells were subcultured every 3–4 days to maintain exponential growth at a cell density of less than 10^6 cells/ml.

Colony forming assay

The same spleen cells used for mass culture were seeded to each well of 96-well microtiter plates (flat bottom, Corning) in 0.20 ml of growth medium at appropriate cell densities (10–100 cells/ml). After a 5-day interval, 0.1 ml growth medium was fed to each well. After 11–12 days of culture at 37°C in a 5% CO₂ humidified atmosphere, the presence of colonies was determined using an inverted microscope. The cloning efficiency of mouse spleen cells was calculated for each plate assuming a Poisson distribution of cells with the ability to produce clonal outgrowth in wells:

$$CE = \frac{-\ln(\text{empty wells/total wells})}{\text{number of cells seeded/well}}.$$

Irradiation

X-rays were delivered from an X-ray machine (Toshiba Corp.) operated at 250 kVp 12 mA with a filtration by 0.5 mm Cu plus 1.0 mm Al, and a dose rate of 0.42 Gy/min.

1) *In vivo* irradiation: The mice were given whole body irradiation with 1 Gy – 4 Gy X-rays. 2) *In vitro* irradiation: The spleen cells were irradiated in the culture medium a) immediately after preparation (unstimulated cells), and b) after 3–4 days culture in the growth medium (stimulated cells). Immediately after all irradiation, the spleen cells were resuspended in a new growth medium and seeded to 96-well microtiter plates for colony forming assay. The cell survival was calculated from the cloning efficiencies of the irradiated T-lymphocytes by comparing them to that of the nonirradiated controls. The surviving fraction was plotted as a fraction of the dose of X-rays, and the regression line and the values of D₀ and D_q were obtained by the least square method.

Fluorescence staining and flow cytometry analysis

The phenotype of growing T-lymphocytes was determined by staining with monoclonal antibodies (MAB); anti-Thy1.2, anti-Ly1.1, and anti-Ly2.1 (Meiji Institute of Health Science) and goat anti-mouse IgG-FITC (Tago Inc.) as fluorescein-conjugates. The fluorescent staining method used in this experiment followed the method of Spellman & Anderson (1982). Briefly, 2×10^5 growing T-lymphocytes were dispensed into a 4 ml plastic tube

Table 1. Expression of T-cell differentiation antigen on growing splenic lymphocytes cultured with TCGF and feeder cells

Exp. No.	Cell surface antigen (%)		
	Thy1,2	Ly1,1	Ly2,1
1	96	6	93
2	98	8	92
3	99	2	97
4	99	3	95
5	98	1	96
Mean $\pm$ SD	98 $\pm$ 1	4 $\pm$ 3	95 $\pm$ 2

Flow cytometry analysis was performed at day 10 in mass culture.

Table 2. Phenotype of T-cell differentiation antigen

Phenotype	Ly1,1+	Ly1,1-	Ly1,1+	Ly1,1-
	Ly2,1-	Ly2,1+	Ly2,1+	Ly2,1-
Number of colonies	46	25	1	0

T-cells recovered from clonal assay were studied.

Fig. 1. Growth profile of splenic T-lymphocytes of C3H mice stimulated by TCGF and feeder cells *in vitro*. The open circles indicate splitting of the culture to lower cell density and refeeding by complete change of the growth medium. **Fig. 2.** Growth profile of splenic T-lymphocytes of C3H mice stimulated by TCGF and feeder cells *in vitro*. X-axis indicates days after separation of spleen cells. Y-axis indicates population doublings. Exp. 1: (○), Exp. 2: (●), Exp. 3: (□)

Population doubling time: 28.2 ± 1.2 hr

and washed twice in cold phosphate buffered saline (PBS). These cells were incubated with optimal dilutions of MAB for 40 min at 4°C. The cells were washed twice in cold PBS and optimal diluted goat anti-mouse IgG-FITC was added. Staining was carried out for 40 min at 4°C. Finally, the cells were washed three times and resuspended at about 1×10^5 cells/ml for analysis by flow cytometer (EPICS5 MDADS system; Coulter Electronics Inc.).

Results

Mouse T-lymphocyte culture

Growth profiles of mouse splenic T-lymphocytes stimulated with TCGF and feeder cells are shown in Figs. 1 and 2. The exponential growth pattern was repeated from day 2 or 3 to day 12 or 13. Thus, T-lymphocytes grew for approximately 14 days with population doubling times of 27–29 hr.

The relationship between cloning efficiency of unstimulated mouse spleen cells and final concentration of TCGF in the growth medium is shown in Fig. 3. The cloning efficiency of unstimulated mouse spleen cells was dependent on TCGF concentration and the cloning

Fig. 3. The effect of TCGF on cloning efficiency of spleen cells of C3H mice. ●: cells treated in the G₀ phase, ○: cycling cells. Sample size is 4 to 5 per point. Vertical bars represent range of geometric mean $\pm$ SD.

efficiency showed maximum (3–4%) at 20–25 percent of TCGF. Thus, 20–25% TCGF in the growth medium was used as the final concentration in all experiments. The cloning efficiency of growing mouse spleen cells, however, was higher (7–12%) than that of the unstimulated cells.

Analysis of T-lymphocytes with MAB against T-lymphocyte differentiation antigens (at day 10 in mass cultures) is shown in Table 1. The proportion of growing cells expressing Thy 1,2 antigen was more than 98 percent. The proportion of growing cells expressing Ly2,1 antigen was more than 95 percent and much higher than cells expressing Lyl,1 antigen (about 4 percent). LCL-TK6 cells and X-TK6 cells as feeder cells did not have T-lymphocyte surface markers.

Fig. 4. Dose-survival curves for splenic T-lymphocytes of C3H mice irradiated *in vitro*. Test cells were exposed to graded doses of x-rays *in vitro* immediately after preparation (●: cells in the G₀ phase) and after culturing for 3–4 days (○: cycling cells). Sample size is 5 per point. Vertical bars represent range of geometric mean $\pm$ SD.

● : D₀: 0.99, D_q: 0.87 (Gy)
○ : D₀: 1.04, D_q: 0.19 (Gy)

Fig. 5. Dose-survival curves for splenic T-lymphocytes of C3H mice irradiated *in vivo* and *in vitro*. Test cells exposed to graded doses of X-rays *in vitro* (immediately after preparation, G₀ phase, ●) and *in vivo* (whole body irradiation, ○). Sample size is 5 per point. Vertical bars represent range of geometric mean $\pm$ SD.

● : D₀: 0.99, D_q: 0.87 (Gy)
○ : D₀: 1.01, D_q: 0.73 (Gy)

Seventy-two colonies isolated in clonal assays were studied for surface marker phenotypes. The cumulative results are summarized in Table 2. Forty-six of the 72 colonies showed a Ly1,1+, Ly2,1- phenotype and 25 colonies showed a Ly1,1-, Ly2,1+ phenotype while only one was Ly1,1+, Ly2,1+.

Radiosensitivity of mouse splenic T-lymphocytes

The dose-survival curve of mouse splenic T-lymphocytes irradiated *in vitro* is shown in Fig. 4. For *in vitro* irradiation the dose-survival curve of unstimulated T-lymphocytes gave a D_0 value of 0.99 Gy and a D_q value of 0.87 Gy and that of stimulated T-lymphocytes gave a D_0 value of 1.04 Gy and a D_q value of 0.19 Gy. Thus, the D_0 value of unstimulated T-lymphocytes was almost equal to that of stimulated T-lymphocytes, but the D_q value of unstimulated T-lymphocytes was significantly higher than that of stimulated T-lymphocytes. The dose-survival curve of mouse splenic T-lymphocytes irradiated *in vivo* is shown in Fig. 5. The dose-survival curve of T-lymphocytes gave a D_0 value of 1.01 Gy and a D_q value of 0.73 Gy. Accordingly, the D_0 value of T-lymphocytes irradiated *in vivo* was almost equal to that of unstimulated T-lymphocytes irradiated *in vitro*, and the D_q value of T-lymphocytes irradiated *in vivo* did not differ much from that of unstimulated T-lymphocytes irradiated *in vitro*.

Discussion

Mouse T-lymphocyte culture

Long-term culture of cytotoxic T-lymphocyte with allogeneic antigen and TCGF has been reported (Gillis & Smith, 1977; Boehmer & Haas, 1981). The X-TK6 cells used as feeder cells in our T-lymphocyte culture method were immunologically not allogeneic but xenogeneic stimulators. Thus, this culture method is a modification of the culture methods of cytotoxic T-lymphocyte with allogeneic antigen and TCGF. As shown in Tables 1 and 2, it is clear that the proliferative mouse splenic cells using our culture method were T-lymphocytes. In a mixed lymphocyte culture, responding lymphocytes acted as cytotoxic T-lymphocyte and most of them expressed Ly2,3 antigen (Cantor & Boyse, 1975; Wagner & Rollinghoff, 1978). In the mass culture of our method, most of the proliferative T-lymphocytes expressed Ly-2 antigen. This is in agreement with the results reported for mixed lymphocyte cultures. However, the proportion of phenotype in the mass culture differed from that in the clonal culture. Several T-lymphocyte clones expressing Ly-1 antigen were derived by the limiting dilution technique (Glasebrook & Fitch, 1980). The difference in proportion appears to have been caused by the following culture conditions: T-lymphocyte density, contact with feeder cells and cell-to-cell interactions between T-lymphocytes.

There are a few reports on cloning efficiency of mouse splenic T-lymphocytes estimated by colony forming assay with microtiter plates. Inamizu *et al.* (1986) and Jones *et al.*

(1985) reported that the frequency of 6-thioguanine-resistant T-lymphocytes in mice could be estimated by colony forming assay. In their study, the presence of colonies was determined by the uptake of ^3H -thymidine, while in our investigation, the presence of colonies was determined using an inverted microscope. Dempsey & Morley, (1986) also used the microscopic technique but their method slightly differs from our culture method. Our assay method is simpler, easier and more exact than those methods.

As shown in Figs. 1 and 2, T-lymphocytes grow exponentially from day 2–3 in mass culture. It is assumed that T-lymphocytes at day 3–4 (stimulated cells) are cycling cells. Gerber *et al.* (1986) examined the proportion of cells in the G_1 and/or G_0 phase among unstimulated mouse spleen cells at 24 hrs after separation of spleen cells. By inference from their results, it appears that immediately after separation most unstimulated T-lymphocytes remain in G_0 phase.

Radiosensitivity

As shown in Fig. 3, the dose-survival curve of T-lymphocytes has a shoulder and a slope. For *in vitro* irradiation, the D_0 value of T-lymphocytes irradiated in G_0 phase is almost equal to that of cycling T-lymphocytes, but the D_q value of T-lymphocytes irradiated in G_0 phase is higher than that of cycling T-lymphocytes.

Tatsugawa *et al.* (1987) examined *in vitro* radiosensitivity of human T-lymphocytes by colony forming assay using PHA and recombinant interleukin-2 and reported that the dose-survival curve of T-lymphocytes had a broad shoulder. Woods & Lowenthal (1984) compared dose responses of fresh nonproliferating and proliferating human lymphocytes three days after PHA stimulation and concluded that the shoulder of the dose-survival curve of the growing cells had greatly diminished. Our results are consistent with their results although their reports are on human T-lymphocytes. These results suggest that the recovering activity from sublethal damage of G_0 T-lymphocytes is more effective than that of cycling T-lymphocytes. It is necessary to perform experiments on fractional irradiation to clarify this hypothesis.

As shown in Fig. 4, the dose-survival curve of G_0 T-lymphocytes irradiated *in vivo* is very similar to that of G_0 T-lymphocytes *in vitro*. Variation of conditions such as *in vivo* or *in vitro* irradiation does not appear to affect very much the radiosensitivity of T-lymphocytes in this experimental system. Kataoka & Sado (1975) reported that a dose-survival curve of T-lymphocytes with a D_0 value of 1.95 Gy and a D_q value of 1.85 Gy was obtained by a number of T-lymphocytes in the spleens of C3H mice at day 3 after whole body irradiation. Sado *et al.* (1985) obtained a dose-survival curve with a D_0 value of 0.57 Gy and D_q value of 1.52 Gy in PFC response of C3H mice at day 3 after whole body irradiation and a D_0 value of 1.75 Gy and D_q value of 0.95 Gy in cell-mediated cytotoxic response of C3H mice at 24 hr after whole body irradiation. Their results for D_0 and D_q differ from our data. However, the results of the present experiment are partially consistent with the results of Kataoka *et al.* and Sado *et al.* from the point of view that the dose-survival curve of lympho-

cytes at *in vivo* irradiation has a shoulder. For *in vivo* exposure to X-rays, the number of T-lymphocytes in the spleens begins to recover from 2–3 days after irradiation (Anderson *et al.*, 1977). Our *in vitro* assay system can reproduce *in vivo* conditions of T-lymphocyte re-growth and provide the means for examining radiosensitivity through clonogenic ability as the end point of radiation effects.

Thus, our colony forming assay system appears to be very useful for screening the effects of *in vivo* exposure to toxic and/or mutagenic agents and for comparing the effects of *in vivo* exposure with those of *in vitro* exposure of toxic agents as well as radiation.

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クローニングテクニックを用いたマウス脾臓 T-リンパ球の放射線感受性

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要 旨: 今回我々は, TCGF (T-Cell Growth Factor, ラット脾細胞 Con-A 刺激上清) と刺激細胞 (致死量の γ 線を曝露したヒト B リンパ球芽球様細胞) を用いマウス脾臓 T-リンパ球の長期培養法を確立した. この培養条件下での T-リンパ球の集団倍加時間は 27-29 時間で, 約 2 週間の培養が可能であり, マイクロプレート法によるコロニー形成率は 3-12% であった. そこで, マイクロプレート法による生存率解析を用い, マウス脾臓 T-リンパ球の放射線感受性を *in vivo*, *in vitro* 曝露および G_0 期, 対数増殖期の細胞群において比較検討を行った. その結果, G_0 期の T-リンパ球は対数増殖期のそれに比して亜致死障害よりの回復力が強い可能性が示唆された. またこの測定系では, *in vivo*, *in vitro* 曝露において T-リンパ球の放射線感受性にほとんど差は見られなかった. このように, この測定系は物理的要因や毒性および変異原性物質の *in vivo*, *in vitro* 曝露による影響を比較し, スクリーニングする方法として有用である可能性が考えられた.

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