

LOW DOSE RADIOSENSITIVITY OF ALLOIMMUNE  
CYTOTOXIC T CELLS\*

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The effects of ionizing radiation on the immune system relate primarily to the radiosensitivity of the small lymphocyte. Within the past several years, as our concepts of cell-cell interactions have evolved with emphasis on functional subsets of lymphocytes, it has become clear that subpopulations differ in their radiosensitivity (1). Further, the lymphocyte is unique, in that radiation-induced cell death occurs not only at the time of cell division but also immediately after exposure. This latter type of injury is called interphase cell death (1).

The purpose of the present study is twofold: (a) to determine the radiosensitivity of the cells which express cytotoxic effector activity with particular emphasis on very low dose (10–25 rad) gamma irradiation, and (b) to determine the Lyt phenotype and proportion of radiosensitive cells susceptible to interphase death.

Materials and Methods

*Mice.* Female C57BL/6, B10, B10.A, BALB/c, BALB/c-H-2<sup>b</sup>, BALB/c-H-2<sup>g</sup>, BALB/c-H-2<sup>k</sup>, CBA, SJL, and DBA/2 mice, 8–12 wk of age, were obtained from our animal colonies at the University of New Mexico.

*Cell Lines.* The MPC-11 (H-2<sup>d</sup>) BALB/c plasmacytoma and EL-4 (H-2<sup>b</sup>) C57BL/6 lymphoma cell lines were used as the target cells in cytotoxicity assays. These lines were maintained by serial passage in tissue culture.

*Preparation of Spleen Cells.* Spleen cells were prepared as previously described (2). Briefly, single-cell suspensions were made, and erythrocytes lysed from the stimulator populations with ammonium chloride. Erythrocytes were not removed from the responder populations. All cells were washed twice in balanced salt solution (BSS) before culturing.

*In Vitro Generation of Cytotoxic T Lymphocytes (CTL).* The culture medium was Dulbecco's minimal essential medium (DME) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and  $5 \times 10^{-5}$  M 2-mercaptoethanol. Splenic lymphocytes ( $2 \times 10^6$ /ml) were cultured with lethally irradiated (2,500 rad) stimulator spleen cells ( $4 \times 10^5$ /ml) in DME culture medium. The cultures were established in Sterilin trays (type 306V, Sterilin Limited, Middlesex, England) using 4 ml of the cell mixture per well. Cultures were maintained at 37°C for 5 d with 7% CO<sub>2</sub>.

*CTL Assay.* Cytotoxic responses were measured in a short-term <sup>51</sup>Cr release assay.  $5 \times 10^6$  target cells were mixed with 100 µCi Na<sub>2</sub>CrO<sub>4</sub> (1 mCi/ml, Amersham Corp., Arlington Heights, IL) in 0.25 ml FCS. The cells were incubated at 37°C for 1 h. The labeled cells were washed three times in BSS and resuspended in DME culture medium at  $2.5 \times 10^5$ /ml.

Quantitative measurements of CTL activity were performed by the method of Cerottini and

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Brunner (3). Briefly, cultured responder cells were harvested, washed, resuspended in DME ( $5 \times 10^6$ ,  $2.5 \times 10^6$ , or  $1.25 \times 10^6$ /ml), and 100  $\mu$ l were plated in triplicate in V-bottomed microtiter plates (Linbro Scientific Co., Hamden, CT). 100  $\mu$ l of target cells was subsequently added to each well. The effector/target (E/T) ratios were 20:1, 10:1, and 5:1.

After a 4-h incubation at 37°C, radioactivity was determined by sampling 0.1 ml of the supernatant from each well and counting in a Nuclear Chicago (Chicago, Il) gamma counter. Percent specific  $^{51}\text{Cr}$ -release was calculated as:  $([\text{experimental release} - \text{spontaneous release}] / [\text{maximum release} - \text{spontaneous release}]) \times 100$ . Maximum release was determined by detergent lysis of target cells, and spontaneous release was determined by culturing target cells alone in DME. The percent specific lysis of the triplicate cultures varied from the mean by  $\leq 8\%$ .

**Irradiation.** Stimulator cells were exposed to 2,500 rad using a General Electric Maximar 250III x-ray machine (General Electric Co., Wilmington, MA). Irradiation of responder cells was performed on day 4 of the allosensitization unless otherwise noted. Responder cells received 0, 10, 25, 50, 75, 100, or 200 rad of x rays administered at a dose rate of 70 rad/min.

**Fluorescent Staining.** The phenotype of the CTL surviving irradiation was determined by staining with the monoclonal antibodies (MAb) anti-Thy-1.2, Lyt-1, or Lyt-2. Briefly,  $5 \times 10^5$  CTL were dispensed into 10  $\times$  75-mm glass tubes in 25- $\mu$ l vol. Optimal dilutions of the MAb were incubated with the cells for 20 min at 4°C. The cells were washed twice with cold BSS, and a fluorescent second-step goat-anti-rat Ig serum was added. Staining was carried out for 20 min at 4°C. Finally, the cells were washed twice with cold BSS and resuspended at  $2 \times 10^5$ /ml for analysis by flow cytometry.

The MAb were those derived by Ledbetter and Herzenberg (4) and have been described previously. The fluorescent goat-anti-rat immunoglobulin (Ig) was purchased from Meloy Laboratories Inc. (Springfield, VA). It reacts with the MAb described above, does not react with thymocytes or peripheral T cells, and strongly reacts with mouse B cells. The anti-B cell activity was of no consequence in these studies because the viable alloimmune CTL populations were 95–98% T cells.

**Flow Cytometry Analysis.** All flow analysis was performed on a fluorescence-activated cell sorter (FACS) (FACS III; B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA) as previously described (5). The 488-nm line of the laser was used for fluorescence excitation, and the FACS was calibrated daily using glutaraldehyde-fixed chicken erythrocytes as a standard. Amplifier and gain settings were set to bring all viable control cells (unstained and cells with second step only) on scale. These control populations were represented in the fluorescence channels 128–142. All specifically stained positive cells were located in channels  $>142$ . Dead cells were “gated” out on the basis of their characteristic low light scatter profile (6). Routinely,  $1 \times 10^4$  cells were analyzed for each test.

We determined the percentage of Lyt-1<sup>+</sup>, Lyt-2<sup>+</sup>, and Lyt-1,2<sup>+</sup> cells in the following way. First, Thy-1.2<sup>+</sup> cells accounted for 95–98% of the viable cells recovered from the cultures. Second, dual Lyt-1 and Lyt-2 staining marked 90–95% of the viable cells recovered from the cultures. Thus, at least 95% of the Thy-1.2<sup>+</sup> cells expressed Lyt markers. By comparing the percent of cells staining positive with MAb anti-Lyt-1 (Lyt-1<sup>+</sup> and Lyt-1,2<sup>+</sup> cells) or MAb anti-Lyt-2 (Lyt-2<sup>+</sup> and Lyt-1,2<sup>+</sup> cells) with the total number of Thy 1.2<sup>+</sup> cells, a determination of individual Lyt subsets was made.

**Bromo-Deoxyuridine (BUdR) and Light Suicide.** The methods used for BUdR and light suicide were essentially those described by Janeway et al. (7). Freshly prepared BUdR (ICN Nutritional Biochemicals, Cleveland, OH) was added to the cell cultures at a final concentration of  $3 \times 10^{-6}$  M on day 4. 24 h later, the cultures were placed 1 cm from a 40-W fluorescent lamp and exposed to the light for 90 min. The concentration of BUdR, the 24-h pulse time, and a single 90-min light treatment are sufficient to eliminate a majority of cells proliferating to a stimulus. For example, we have found that when an allosensitized culture is pulsed on day 2 with BUdR and illuminated on day 3, there is an 80% reduction in CTL activity on day 5.

**Calculation of Lytic Units.** We define lytic units in this report as the percent viable cells recovered from the irradiated cultures multiplied by the percent specific lysis observed in the CTL assay. For comparisons, the data have been normalized such that the quantity (percent viable cells  $\times$  percent specific lysis) for the control (0 rad) cultures is equal to 100 lytic units.

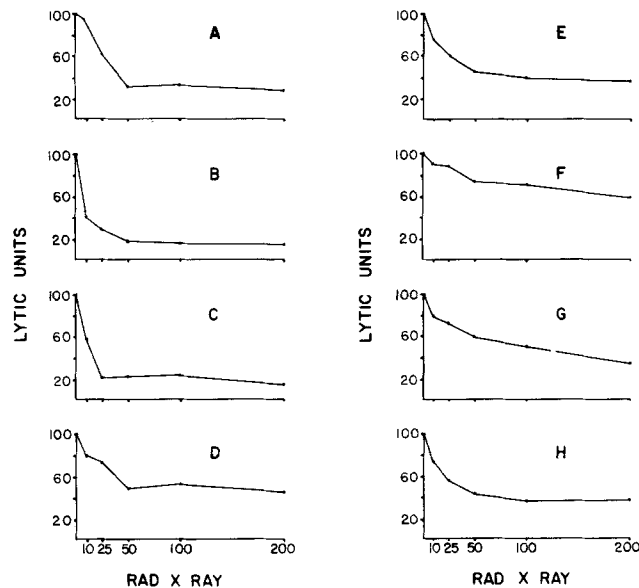


FIG. 1. Low dose x-ray sensitivity of alloimmune CTL. Lytic units of cytotoxic activity are plotted against different doses of x ray administered 24 h before CTL assay. The assays were performed at E/T ratios of 20:1, 10:1, and 5:1. The effect of radiation on the CTL populations was evident at all effector cell concentrations, thus only the 20:1 data is shown. The panels are as follows: (A) C57BL/6 anti-BALB/c; (B) B10 anti-BALB/c; (C) B10.A anti-BALB/c; (D) BALB/c-*H-2<sup>k</sup>* anti-BALB/c; (E) BALB/c-*H-2<sup>k</sup>* anti-BALB/c; (F) BALB/c-*H-2<sup>b</sup>* anti-BALB/c; (G) CBA anti-BALB/c; (H) SJL anti-BALB/c.

### Results and Discussion

**Low Dose X-Ray Sensitivity of CTL.** The data shown in Fig. 1 are representative of many experiments examining the effects of low doses of radiation on CTL function. Each graph is based on the following experimental protocol. Allogeneic cultures are established on day 0. On day 4, the cultures are harvested and divided into six groups. Each group receives the indicated dose of radiation, 0, 10, 25, 50, 100, or 200 rad. The groups are maintained in culture until day 5. On day 5, the number and percentage of viable cells is determined. All groups are then adjusted to contain the same number of viable cells per ml and subsequently used as effector cells in the CTL assay.

Several conclusions can be drawn from Fig. 1. There is a distinct and precipitous decrease in the number of lytic units as a result of exposure to 10–25 rad to the CTL. This effect plateaus when the x-ray dose is >100 rad. Actually, the number of lytic units remains fairly constant between 200 and 1,500 rad (high dose data not shown). Thus, the curves have two components and demonstrate that radiosensitive and radioresistant cells are present in the CTL population. This conclusion is based on the observation that when a constant number of viable cells from the allosensitization cultures are used in CTL assays, the percent specific lysis always remains the same, regardless of whether the cells have received 0 or 200 rad. We interpret this to mean that irradiation of the sensitized cells kills CTL. If this were not the case, then adjusting the cell concentration of each irradiated group to a constant number of viable cells per ml would enrich for CTL activity. This does not occur. Thus, irradiation must kill CTL. In essence, the data presented really represent survival curves. The curves were derived by multiplying a constant (specific lysis) by the

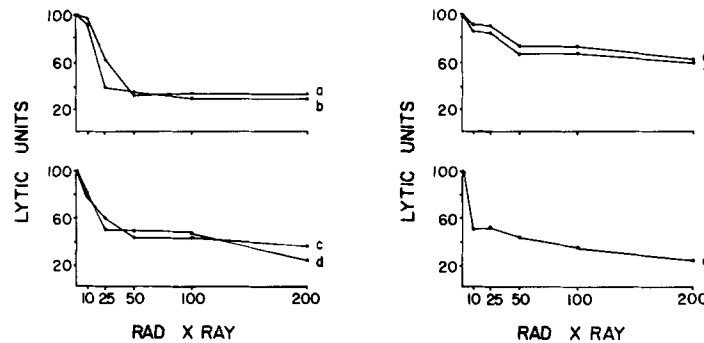


FIG. 2. Low dose x-ray sensitivity of alloimmune CTL using different allosensitizing cells. Data are presented for the 20:1 E/T ratio. The panels are as follows: (a) C57BL/6 anti-BALB/c; (b) C57BL/6 anti-DBA/2; (c) BALB/c-*H-2<sup>k</sup>* anti-BALB/c; (d) BALB/c-*H-2<sup>k</sup>* anti-DBA/2; (e) BALB/c-*H-2<sup>b</sup>* anti-DBA/2; (f) BALB/c-*H-2<sup>b</sup>* anti-BALB/c; (g) DBA/2 anti-C57BL/6.

present cell viability after irradiation. The specific lysis component is really a control to show that the surviving cells indeed express a constant CTL activity.

It is clear from Fig. 1 that all the strains tested exhibited radiation-sensitive and -resistant CTL; however, variations also exist. For example, the C57BL/6, B10, and B10.A mice show a pronounced response to 25 rad with ~40% (C57BL/6) to 80% (B10.A) of their CTL being radiosensitive. However, this radiosensitivity does not appear to follow any obvious genetic pattern. For example, C57BL/6, B10, and BALB/c-*H-2<sup>b</sup>* are all of the *H-2<sup>b</sup>* haplotype, yet the responses to low dose radiation are quite different. The same observation can be made for BALB/c-*H-2<sup>k</sup>* and CBA CTL. On the other hand, the BALB/c *H-2* congenics, which possess different *H-2* types on the same genetic background, also show much variation in response to low dose radiation.

The variation in the radiosensitive CTL populations from different responder strains is not dependent upon the efficiency of the allosensitization. Thus, the pronounced effect of 25 rad on C57BL/6 CTL as compared with BALB/c-*H-2<sup>b</sup>* CTL is not reflected in a difference in the ability of the cells to kill targets. All the CTL in this study effected ~80–90% specific lysis at E/T ratios of 20:1. Therefore, it appears that genuine differences exist between strains in the generation of radiosensitive CTL. Further, radiosensitive CTL are not restricted to allosensitizations using BALB/c stimulator cells. A comparison of BALB/c, DBA/2, and C57BL/6 stimulator cells cultured with different responder cell types is shown in Fig. 2. Clearly, the radiosensitive CTL populations are generated in each case. Thus, we conclude that radiosensitive CTL are a general component of cytotoxic allogeneic responses.

*Reproductive vs. Interphase Cell Death.* There are two main possibilities to account for the extremely radiosensitive CTL. These cells may be active in some portion of the cell cycle and hence sensitive to x ray, or they may be subject to interphase death (1). Our approach to distinguish between these possibilities is based on BUdR and light suicide experiments. If the cells are radiosensitive because they are cycling, then BUdR and light should abolish CTL activity. On the other hand, BUdR treatment should have no effect if the radiosensitive cells are not cycling. Shown in Fig. 3 are the results of control and BUdR-treated cultures. It should be noted that the time course for these experiments has been changed. BUdR was added to the cultures on day 4,

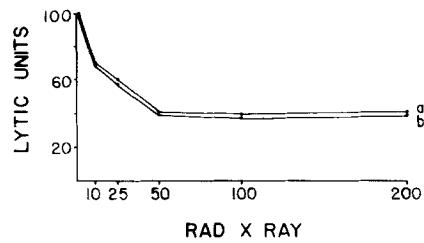


FIG. 3. Effect of BUdR and light treatment on radiosensitivity of alloimmune CTL. The BUdR pulse was given on day 4 to determine if the radiosensitive cells were cycling at this time. Fluorescent light exposure and x-irradiation were performed on day 5, and the CTL assay was done on day 6. Data are presented for the 20:1 E/T ratio. Line a represents the BUdR-treated CTL, and line b represents the control CTL. Both were exposed to irradiation.

TABLE I  
*Lyt Phenotype of Thy-1.2-positive Alloimmune CTL after x-irradiation*

Rad*	Percent Lyt-1 <sup>+</sup> cells‡	Percent Lyt-2 <sup>+</sup> cells	Percent Lyt-1,2 <sup>+</sup> cells
0	37	11	52
25	50	12	38
50	50	20	30
100	52	19	29

\* Data are based on the viable cells recovered from the C57BL/6 anti-BALB/c allosensitization. Similar data were obtained with the B10 and B10.A responder CTL populations. Mice on the black background were used because they show the most pronounced effect of low dose irradiation.

‡ The proportion of Lyt subsets was determined by comparing the number of cells staining positive with MAb anti-Lyt-1 or anti-Lyt-2 to the total number of MAb Thy-1.2-positive cells. For example, the subset Lyt-1 was determined by subtracting the number of Lyt-2<sup>+</sup> cells (Lyt-2<sup>+</sup> and Lyt-1,2<sup>+</sup>) from the number of Thy-1.2<sup>+</sup> cells.

fluorescent and x-irradiations performed on day 5, and CTL activity assayed on day 6. In short, there are no detectable differences between the control and BUdR groups. Thus, we conclude that the radiosensitive cells undergo radiation-induced interphase death.

*Lyt Phenotype of Radiosensitive CTL.* The next question we addressed was the identification of the CTL population that appears to be particularly susceptible to interphase death. Our efforts were directed toward a study of the Lyt phenotypes because virtually all the viable cells from the allosensitization procedure were T cells, as defined by MAb anti-Thy-1.2 staining. The results are presented in Table I. We have found that increasing doses of radiation result in a reduction of the Lyt-1,2<sup>+</sup> lymphocyte subset. As a consequence, the Lyt-1<sup>+</sup> and Lyt-2<sup>+</sup> cells constitute a greater portion of the residual lymphocyte population. Thus, we can make two conclusions: (a) Lyt-1,2<sup>+</sup> cells are extremely radiosensitive; (b) Lyt-1,2<sup>+</sup> cells must express cytotoxic activity because their elimination by low dose x ray results in a decrease of lytic units. However, the question arises as to whether only Lyt-1,2<sup>+</sup> cells have been killed. If one examines the data in Fig. 1, it is evident that some CTL populations exhibit >50% reduction in the number of lytic units after low dose irradiation. This means, for example, that exposure to 25 rad has resulted in >50% cell death among these CTL populations. Thus, the degree of cell death does not correlate to the quantitative changes in the Lyt subsets seen in Table I. We interpret this to mean that each Lyt cell subset has radiosensitive members, but the Lyt-1,2<sup>+</sup> subset contains proportionally many more radiosensitive CTL.

The use of Lyt phenotypes has been a useful marker for defining functional T cell subsets. However, it appears that exceptions exist to the general classification of T cells as Lyt-1,2<sup>+</sup> precursors, which give rise to Lyt-1<sup>+</sup> helper cells that can proliferate to I region antigens, and Lyt-2<sup>+</sup> cells, which may be cytotoxic to K/D antigens or express suppressor function (8-11). Thus, cytotoxic Lyt-1,2<sup>+</sup> cells are not without precedent. The interesting question will be whether or not radiosensitive CTL are generated across specific intra-H-2 responder and stimulator differences.

In summary, we have described a phenomenon of very low dose radiation-sensitive effector cells. These observations may point to the existence of additional subsets of cytotoxic T cells that share Lyt phenotypic markers, but are separable at this time by differential x-ray sensitivity.

### Summary

Low dose radiosensitivity of in vitro generated alloimmune murine cytotoxic T lymphocytes (CTL) was studied. It appears that a subset of CTL exists that can be killed with 10-25 rad of x rays. These radiosensitive CTL are Lyt-1,2<sup>+</sup> T lymphocytes. Analyses of cytotoxicity by chromium release assays indicate that the radiosensitive CTL are present in responder spleen cell cultures from all strains of mice tested. The generation of these effector cells is most pronounced in animals of the C57BL background. The mechanism of low dose radiosensitivity appears to be interphase death.

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