Effect of γ Irradiation of Red Blood Cell Units on T-Cell Inactivation as Assessed by Limiting Dilution Analysis: Implications for Preventing Transfusion-Associated Graft-Versus-Host Disease

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Transfusion-associated graft-versus-host disease can be prevented by treating cellular blood products with γ irradiation. A wide range of γ irradiation dose levels has been used in routine practice. We used limiting dilution analysis, which measures clonable T cells, to assess the influence of 500 to 3,000 cGy of γ irradiation delivered from a 137Cs source on T cells when delivered in situ to ADSOL-preserved red blood cell (RBC) units in blood bags. In a series of experiments using RBC units irradiated within 24 hours after collection, 1,500 cGy inactivated >4 log₁₀ of T cells; however, viable T cells were detected in all experiments. With 2,000 cGy, a ≥4.7 log₁₀ decrement in T-cell growth occurred in 7 of 8 experiments. With 2,500 or 3,000 cGy, no T-cell growth (>5 log₁₀ depletion) was detected. Comparable effects were observed with ADSOL-preserved RBC units in the standard PL 146 plastic container and in the recently developed PL 2209 plastic container. T-cell inactivation, as a function of γ irradiation dose, was similar when either a 137Cs or a linear accelerator source was used. T cells isolated from ADSOL-preserved RBC units after storage for 7 and 21 days, although reduced in number as compared with a fresh unit stored for 24 hours, were viable, capable of proliferation, and susceptible to inactivation by γ irradiation. Using a sensitive in vitro assay for T-cell proliferation, we found that a γ irradiation dose of 2,500 cGy may be required to completely inactivate T cells in RBC units.

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MATERIALS AND METHODS

Blood collection. Units of whole blood were collected from consenting healthy adult volunteers into PL 146 or PL 2209 plastic containers (Baxter Healthcare, Deerfield, IL). The PL 2209 container uses a different plasticizer, which results in greater gas permeability. RBCs were prepared by centrifugation and ADSOL preservative solution (Fenwal) was added within 8 hours of phlebotomy. In a series of experiments comparing containers and irradiation sources, collected units were split before storage and irradiation to provide identical units to minimize biologic variability.

γ Irradiation. RBC units were individually irradiated in blood bags with a 137Cs source (Isomedix; Nordion, Kanata, Ontario, Canada) with doses of 500, 1,000, 1,500, 2,000, 2,500, and 3,000 cGy, sequentially, and compared with unirradiated samples (0 cGy). Successive samples were removed from each blood unit after each irradiation dose, which was delivered in a step-wise incremental fashion. All RBC units were irradiated on day 1 after collection, unless otherwise specified. In comparative studies, a Varian Clinac-18 linear accelerator was used.

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accelerator (Varian, Palo Alto, CA) was used as a radiation source. Each source delivered a dose rate of 350 cGy per minute at the internal midplane of the bag. This was confirmed at 2,500 cGy (±10%) by studies using thermoluminescent dosimeter (TLD) chips that were suspended in water-containing blood bags.

LDA. LDA specific for the growth of both CD4 and CD8 T cells was used to quantify residual functional T cells in irradiated samples. This is based on T-cell growth stimulated by polyclonal activation in the presence of excess T-cell growth factors, as we have previously described. The LDA is predicated on the single hit model in which a single cell is necessary for a positive response. This assumes that (1) immunocompetent cells are diluted to limiting doses; (2) each immunocompetent cell generates a detectable response; and that (3) all other factors, cellular and soluble, are at nonlimiting doses. This technique allows a level of detection on the order of 1 in 10⁶ mononuclear cells plated, and therefore the ability to detect a >5 log reduction in functional T cells by γ irradiation, when compared with the unmanipulated control.

Mononuclear cells (MNCs) were isolated from each irradiated sample of RBC units by centrifugation over a Ficoll-hypaque density gradient (Organon Teknika, Durham, NC) and then resuspended in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) with 5% fetal calf serum (FCS; Sigma, St Louis, MO), penicillin-streptomycin, and L-glutamine (Whittaker Bioproducts). Each sample was then plated in 6 to 8 serial threefold dilutions (10 to 20 wells per dilution) in 96-well flat-bottom plates (CoStar, Cambridge, MA). Dilutions were made based on counts of viable cells using trypan blue and a hemocytometer. The starting concentration for each dilution was chosen such that some dilutions (cells per well) were informative (ie, all the wells were not 100% positive or 100% negative). These levels had been determined in pilot studies of irradiated lymphocytes over a wide range of cell concentrations. Each 200-μL culture was supplemented with 5% human T-cell growth factor (T-CGF; Cellular Products, Inc, Buffalo, NY), 2.5 μg/mL phytohemagglutinin (PHA-M; Sigma), 10% FCS, 100 U/mL recombinant interleukin-2 (IL-2; Cellular Products), and 10⁵ irradiated (4,000 cGy) pooled allo-stimulator cells per well. The allo-stimulators were obtained from the pooled and cryopreserved buffy coats of 11 individual donors. The cultures were incubated at 37°C, 5% pCO₂ for 28 days. They were pulsed weekly with a solution of 50% FCS, 50% human T-CGF, and 500 U/mL recombinant IL-2. Assays were scored visually for T-cell growth by phase microscopy weekly for 4 weeks. A well contained at least 10 T cells in close clusters was scored as positive. Specific controls in each experiment included a preirradiation sample, to establish the efficiency of the LDA for that donor, and a negative control without added experimental cells, to establish that the allo-stimulators did not grow.

T-cell frequencies were calculated using the minimum χ² analysis, assuming Poisson distribution in plating, as described by Tassewell. Results were plotted as percent negative cultures (without T-cell growth) versus the number cells plated per culture well. Data from individual irradiated groups were calculated as frequency (f) of both experimental and control and the decreases in proliferating T cells after γ irradiation were expressed as:

\[ \log_{10} T\text{-Cell Reduction} = \log_{10} \left( \frac{f \text{ experimental}}{f \text{ control}} \right) \]

Flow cytometry. The presence of viable mature T cells in each unirradiated RBC unit was assessed by flow cytometry with a monoclonal antibody specific for CD3. Mononuclear cell fractions from preirradiation samples and those receiving 2,500 cGy were isolated (as described above), washed twice, and then resuspended in Hank's Balanced Salt Solution (Whittaker Bioproducts), 1% human serum albumin (Armour, Kankakee, IL), and 0.1% azide (Sigma). Mononuclear cells from each preirradiation RBC unit were analyzed for fluorescinated versus cell size using a FACSTAR Plus flow cytometry with a 488 nm Argon laser (Becton Dickinson, Mountain View, CA). Viable T cells were identified using a directly fluorescinated antibody to the CD3 antigen expressed on T cells (Leu-4; Becton Dickinson, San Jose, CA) and gating on cells that exclude propidium iodide (Sigma). Background staining was determined using directly fluorescinated, antimurine Thy-1.2 (Becton Dickinson) as a negative control.

RESULTS

Radiation-dose-dependent log reduction in functional T cells. A series of studies were performed in which γ irradiation was delivered in a step-wise fashion to 8 24-hour-old RBC units stored in PL 146 blood containers. Figure 1 depicts the percentage of negative cultures versus mononuclear cells plated per well from a representative experiment in which γ irradiation doses of 500, 1,000, 1,500, 2,500, and 3,000 cGy were delivered and compared with the no irradiation control (0 cGy). In unirradiated controls, T-cell growth gave a frequency of one in five mononuclear cells plated. Therefore, for every five mononuclear cells plated, one T cell proliferated. The mononuclear fraction contained 69% CD3⁺ T cells, yielding a cloning efficiency of 33% (one T cell proliferating for every three T cells plated). When irradiated with 500 cGy, the frequency of proliferating T cells decreased to 1 in 249 mononuclear cells plated. At a γ irradiation dose of 1,000 cGy, the frequency decreased to 1 in 3,847; and at 1,500 cGy, it decreased to 1 in 164,919. At 500 cGy, this represents a 1.7 log₁₀ reduction in proliferating T cells. This increases to a 2.9 log₁₀ reduction at 1,000 cGy and 4.5 log₁₀ at 1,500 cGy. At doses ≥2,500 cGy, no T-cell growth was detected. This implies frequencies of less than 1 in 10⁶, which corresponds to the LDA’s limits of detection. This represents at least a >5 log₁₀ reduction.

Data of T-cell frequency and log₁₀ reduction of T cells versus irradiation dose from RBC units collected from eight different donors and stored in the PL 146 container are seen in Table 1. These data confirm those of the representative experiment. The unirradiated control group yielded a mean T-cell growth frequency of 1 in 8 MNCs plated (range, 1:1 to 1:17). At 500 cGy, this frequency decreases to 1 in 174 MNCs plated, a 1.3 log₁₀ (SD, 0.4; median, 1.6; range, 0.8 to 1.7). At 1,000 cGy, the frequency decreases to 1 in 5,800 MNCs plated, a 2.8 log₁₀ reduction (SD, 0.1; median, 2.8; range, 2.7 to 2.9), whereas at 1,500 cGy, the frequency declines to 1 in 185,385 MNCs plated, a 4.4 log₁₀ reduction (SD, 0.5; median, 4.3; range, 2.8 to 5.7). At 2,000 cGy, T cells in one of four experiments grew at a frequency of 1 in 10³ plated MNCs. No T cells grew in the other 3 donor units; therefore, the mean frequency is less than 1 in 770,000 for the four experiments, a >4.7 log₁₀ reduction (SD, 0.2; median, >4.7; range, 4.4 to >4.9). No T-cell growth was detected at γ irradiation doses of 2,500 or 3,000 cGy in any of the experiments, a greater than 5 log₁₀ reduction (SD, 0.4; median, >5.3; range, >4.9 to >6.0, for 2,500 cGy) (SD, 0.3; median, >5.4; range, >5.2 to >6.0, for 3,000 cGy). Therefore, as the γ irradiation dose increases, the log₁₀ reduction of functional T cells increases exponentially as compared with the unirradiated controls (Fig 2).

Comparison of blood containers. Comparable inactivation of T cells was observed after irradiation of RBC units in
LDA TO ASSESS T CELLS IN IRRADIATED RBC UNITS

either of the PL 146 or the PL 2209 blood containers (Table 1 and Fig 2). These data show the same pattern of an incremental log reduction in the frequency of clonable T cells with increasing doses of irradiation and no detectable T-cell growth with 2,500 cGy. Because there was no demonstrable difference noted between the two blood containers \( (P > .05) \), these data support that there is equivalent T-cell sensitivity to \( \gamma \) irradiation in either blood container.

Comparison of \( \gamma \) irradiation sources. We conducted studies comparing two sources of \( \gamma \) irradiation used in clinical practice, \(^{137}\)Cs and linear accelerator. Donor units were split into two PL 2209 blood containers before irradiation. At equivalent radiation dose rates, comparable T-cell inactivation was obtained using these two commonly used \( \gamma \) irradiation sources. The log reduction in T cells were 1.3 and 1.0 at 500 cGy, 3.6 and 3.2 at 1,000 cGy, 5.1 and 5.0 at 1,500 cGy, and no T-cell growth at 2,500 cGy, respectively.

Effect of blood storage. The ability of \( \gamma \) irradiation to inactivate T lymphocytes in stored RBC units was studied using 7- and 21-day-old units. RBC units were stored at 1°C to 6°C in both PL 146 and PL 2209 containers for 7 days and 21 days. In 7- and 21-day-old units, viable T cells capable of proliferating were present, although at a lower frequency.
and number (Table 2) than those in the experiments using day 1 RBC units (Table 1). After 7 days of storage, viable T cells by flow cytometry were 40% to 50% CD3+, similar to samples analyzed at day 1. At day 21, the recovery of viable lymphocytes was 60% to 70%; however, the T cells could not be quantified because of cell aggregates interfering with flow cytometry. In the nonirradiated day-7 controls, the T-cell frequency in LDA was 1 in 26 to 62, whereas at day 21, the T-cell frequency decreased to 1 in 274 to 423. The data show that T cells were susceptible to inactivation by γ irradiation at both days 7 and 21, with no T-cell growth detectable after irradiation with 2,500 cGy (Table 2).

**DISCUSSION**

TA-GVHD can be prevented by the irradiation of cellular blood products; however, it is still unclear what dose of γ radiation eliminates this risk while preserving the quality of the transfused product. Currently, cellular products for infusion are irradiated over a dose range of 1,500 to 5,000 cGy. Previous recommendations of using at least 1,500 cGy are based on data showing the elimination of allogeneic reactivity in the MLC. The descriptions of third-party GVHD in immunocompromised patients who had received blood products reported to have been irradiated at up to 2,000 cGy requires reassessing the radiation dose required to inactivate T cells.

The number of viable T cells necessary to cause GVHD in the setting of allogeneic transfusion of RBCs is unknown and prospective study is precluded by the potential risks. However, the immunobiology of GVHD has been the focus of carefully performed studies in both clinical allogeneic BMT and experimental transplantation of marrow or mature lymphocytes, many of which are germane to TA-GVHD. From such studies, it is clear that mature T cells are both necessary and sufficient to cause GVHD and both CD4 and CD8 T cells have been demonstrated to be capable of causing GVHD in the laboratory. Further, depletion of T cells from allogeneic bone marrow before transplantation can eliminate GVHD, even in the setting of HLA disparity. In recipients of T-cell-depleted BMT, it has become apparent that there is a dynamic balance between infused T cells and resident T cells in the host that determines immunologic outcome: graft rejection versus GVHD versus stable tolerance. To understand the immunobiology of T-cell-depleted BMT, it is necessary to quantify very low numbers of T cells remaining in purged marrows. The detection of T cells present at levels ≤1% of the infused cells presents a problem to the quality control of clinical T-cell depletion. Assays of T-cell proliferation (mitogens and MLC) or detection of T cells by flow cytometry, which detect up to a 2 log reduction, are not sensitive in this range. The very sensitive polymerase chain reaction (PCR) technique, although capable of detecting up to a 6 log reduction (1 in 106 cells), is incapable of distinguishing viable versus nonviable cells, requiring the elimination of cells and, therefore, is not informative if cells are...
inactivated, as would be the case with irradiation. LDA, using stimuli capable of activating T cells in a polyclonal fashion, followed by expansion with excess cytokines, provide quantitative data on very low frequencies of T cells and are informative at biologically relevant levels of functional T cells (1 in $10^5$ to $10^6$). LDA is the most common method used to quantify residual T cells after T cell depletion of allogeneic marrows. The LDA provides accurate data as to the log reduction in functional T cells by comparing the depleted population with the unmanipulated starting sample. LDA can also provide a reasonable estimate of the actual number of T cells infused by determining the cloning efficiency of that individual’s T cells by correlating the T-cell content as determined by flow cytometry.

It is difficult to completely validate an in vitro estimate of a clinical entity. It is possible that the LDA does not detect as yet undescribed T-cell subset important to the generation of TA-GVHD or detects T cells that do not play a role in TA-GVHD. However, the inability to identify either phenotypic or functional characteristics of T cells capable of causing GVHD necessitates inactivating all T cells. Again, the data accumulated in clinical BMT proves instructive. When the infusion of allogeneic T cells has been carefully quantified, there is a correlation between the number of T cells infused as determined by LDA and the occurrence of GVHD. By quantitative analysis by LDA, the number of infused allogeneic T cells in the allogeneic marrow graft sufficient to cause clinically significant GVHD is in the order of a threshold of $10^5$ clonable T cells/kg. An incidence of acute GVHD of 50% in our experience infusing $5 \times 10^4$ mature donor T cells/kg into recipients of T-cell-depleted HLA disparate BMT to promote graft-versus-leukemia supports these data. The published BMT data and observations of TA-GVHD in infants with SCID would support the proposition that, in such severely immunocompromised patients, infusion of very low numbers of allogeneic T cells could cause TA-GVHD. These data do not specifically address the risk of TA-GVHD in less immunocompromised patients, infusion of very low numbers of allogeneic T cells would cause TA-GVHD. These data do not specifically address the risk of TA-GVHD in less immunocompromised patients, with known susceptibility to TA-GVHD, such as those receiving aggressive cancer chemotherapy, in whom in vivo T-cell numbers and their function vary over a wide range and fluctuate with time from treatment. Nor do these data address the dose of T cells necessary to cause TA-GVHD in the immunocompetent recipient of T cells who is immunologically unable to recognize and therefore reject them. In this latter setting, murine studies of parent into F1 would predict that a higher number of mature T cells would be required.

The LDA has previously allowed us to assess multiple log reductions in functional T cells after T-cell depletion to prevent GVHD in HLA-disparate clinical BMT. We have now investigated the ability of γ irradiation to inactivate functional T cells when delivered in situ to RBC units preserved in ADSOL and irradiated in bags. Efficacy over the range of 500 to 3,000 cGy irradiation was evaluated by quantitative assessment of functional T cells. The LDA used in these studies has been shown to detect as few as 1 in $10^5$ mononuclear cells or has shown the ability to detect a $>5$ log reduction in functional T cells by γ irradiation, as compared with previous studies using the MLC, which has a $<2$ log sensitivity.

We compared sequentially irradiated samples with the preirradiation control, in which mature T cells had been quantified by flow cytometry.

In our studies, with increasing irradiation, the frequency of T cells capable of proliferating decreased. At 1,500 cGy, T cells capable of proliferating were detected in every experiment using RBC units after 1 day of storage. There was a $4 \log_{10}$ reduction in viable T cells at 1,500 cGy (Table 1); therefore, the MLC would be expected to be noninformative at this dose. At the 2,000 cGy dose, T cells grew in only 1 of 8 experiments, which may indicate donor variability and may offer an explanation for the cases of TA-GVHD after using up to 2,000 cGy to inactivate T cells. Our studies demonstrate that no T cells were detected by LDA at radiation doses of 2,500 or 3,000 cGy, which is equivalent to a greater than 5 log reduction in frequency of clonable T cells (Fig 2). In one of the cases of TA-GVHD after the administration of putatively irradiated blood, the investigators also used LDA to evaluate T-cell inactivation after γ irradiation of purified MNCs and demonstrated a decrease in clonable T cells with increasing γ irradiation. They detected low levels of clonable T cells after 1,500 to 2,000 cGy (presented as aggregate data for 1,500 and 2,000 cGy) and no detectable T-cell growth after 3,000 cGy (2,500 cGy was not analyzed). These results are consistent with our results, as are the results of three other groups using extrapolated MLC data.

Potential variables in T-cell susceptibility to γ irradiation were also analyzed using the LDA. This included the recently developed PL 2209 blood container, which uses a different plasticizer that results in increased gas permeability, that might alter radiosensitivity of lymphocytes. Analysis of RBC units stored in the PL 146 or PL 2209 blood bags showed equivalent susceptibility of T cells to irradiation. We also compared the effects of irradiation delivered from different clinically used sources, either $^{137}$Cs or linear accelerator. We demonstrated that, at comparable dose rates, both sources were equally effective in abrogating the proliferative capability of T cells. Finally, we evaluated whether storage time may have an effect on T-cell susceptibility to γ irradiation. We show that, although storage time decreased the number of viable T cells available to proliferate, they were able to proliferate and were not resistant to inactivation by γ irradiation. Therefore, after storage for 1, 7, and 21 days, there were no functional T cells detected by LDA after 2,500 cGy of irradiation.

The combined data of these studies establishes that, with increasing dosages of γ irradiation of RBC units, there is an incremental log reduction of T cells capable of growth and that T-cell proliferation is undetectable by LDA at doses greater than or equal to 2,500 cGy. In conclusion, to assure complete inactivation of T cells to a level that is undetectable by LDA, we recommend a dose of 2,500 cGy.

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MM Pelszynski, G Moroff, NL Luban, BJ Taylor and RR Quinones