Third-Party–Mediated Graft Rejection and Graft-Versus-Host Disease After T-Cell–Depleted Bone Marrow Transplantation, as Demonstrated by Hypervariable DNA Probes and HLA-DR Polymorphism


Graft rejection after marrow transplantation is generally thought to be mediated by alloreactive immune effector cells of host origin. Transfused blood products also contain immune cells capable of alloreactivity against both donor graft and host. To reduce the risk of transfusion-associated graft-versus-host disease (GVHD) and graft rejection, standard procedure is to irradiate all blood products with at least 1,500 rad before transfusion. We report a patient with chronic myelogenous leukemia who developed graft rejection and GVHD after receiving a T-cell–depleted transplant from a serologically HLA-A, B, DR/DQ matched and mixed lymphocyte culture (MLC) nonreactive unrelated donor. Cytogenetic analysis of marrow cells collected at the time of graft rejection revealed a PH1-negative female karyotype that was not consistent with donor cells. Use of specific minisatellite DNA probes (YNH 24, H-RAS, and 3′ HVR) revealed the exclusive presence of third-party (neither donor nor recipient) restriction-fragment–length polymorphisms (RFLP) in both peripheral blood and marrow cells of host origin. Transfused blood products also contain immune cells capable of alloreactivity against both donor graft and host antigens. To protect patients against potentially alloreactive cells capable of producing GVHD, irradiation of marrow transplant recipients has become standard procedure. This dose of radiation was selected empirically, largely by extrapolation from in vitro mixed lymphocyte cultures (MLCs) and mitogen-driven lymphocyte proliferation assays, as well as by observed clinical experience. However, neither the frequency of lymphocytes that survive irradiation at 1,500 rad nor the number of immune cells necessary to mediate graft rejection or GVHD has been well established. Furthermore, there is no evidence that levels of irradiation sufficient to prevent transfusion-related (third-party–mediated) GVHD will also abrogate the risk of transfused cells causing graft rejection. The paucity of prior reports of third-party–mediated graft rejection may be because methods historically used to document engraftment, such as karyotype and HLA serotype analyses, cannot demonstrate the origin of engrafted cells in all cases.

In this study we report a patient who, despite prior irradiation of all blood products with 2,000 rad, developed graft rejection and GVHD approximately 3 weeks after receiving a bone marrow transplant from a serologically HLA-matched unrelated donor. Restriction-fragment–length polymorphism (RFLP) analysis of marrow and peripheral-blood cells at the time of graft rejection, using multiple hypervariable DNA probes, demonstrated a persistent pattern (on two separate occasions) that was neither that of the donor nor the host, indicative of third-party engraftment. This finding was confirmed with DNA-based HLA typing, using polymerase-chain reaction (PCR) for gene amplification and oligonucleotide probe hybridization, by which peripheral-blood cells at the time of rejection were identified to be of a third-party HLA-DR type. To estimate the frequency of T lymphocytes able to proliferate after irradiation and thus potentially to be capable of contributing to GVHD or graft rejection, we performed limiting dilution analysis of the response to T-cell mitogen. The results demonstrated a 5- to 6-log reduction in T-cell frequency at the time of engraftment.
doses of 1,500 to 2,000 rad as compared with unirradiated controls, indicating that a small percentage of T cells in blood products survive irradiation at these doses. Assuming that T cells are a component of this third-party-mediated immune response, these data have potential implications regarding present blood-product irradiation guidelines, as well as the number of functional immune cells capable of mediating graft rejection and GVHD.

CASE REPORT
The patient was a 34-year-old woman with Philadelphia chromosome-positive chronic myelogenous leukemia in the chronic phase who received a T-cell–depleted marrow transplant from an unrelated female donor who was serologically HLA-A, B, DR/DQ, and MLC matched. Conditioning was with high-dose Ara-C, cyclophosphamide, fractionated total-body irradiation (14 Gy), and methylprednisolone according to a previously described protocol. Random donor platelets and red cells were irradiated with 2,000 rad using a Gammacell 1000 Cesium Irradiator (Atomic Energy of Canada Ltd, Kanata, Ontario) and given to the patient as needed to maintain platelet count over 20,000/μL and hematocrit over 30. On day 12 posttransplant there was a brief rise in total white-cell count to 1,000/mm³ with a demonstrable increment of 50 to 100 granulocytes, followed by an abrupt decline to nadir levels (Fig 1). A bone marrow exam on the 17th posttransplant day revealed a hypoplastic marrow, consistent with a diagnosis of graft rejection, with no evidence of granulocytic or erythroid reconstitution. Over the next few days the patient developed watery diarrhea and a diffuse maculopapular rash (Fig 1). A skin biopsy revealed Grade II GVHD. Day 17 marrow karyotype analysis revealed a Philadelphia chromosome-negative female karyotype that was not of donor origin. To define the origin of cells present at the time of graft rejection, day 17 and day 20 marrow and blood specimens were examined by RFLP analysis (first and second RFLPs in Fig 1), as described below. Following documentation of graft rejection, the patient was reconditioned and received a second marrow transplant from an unrelated male donor who was HLA-A, B matched but mismatched at one DR/DQ locus. Conditioning was with nitrogen mustard, cyclophosphamide, methylprednisolone, and total lymphoid irradiation (2 Gy). Despite prompt engraftment, the patient ultimately expired from sepsis on the 96th hospital day. Autopsy was not performed.

MATERIALS AND METHODS
Cytogenetic analysis. Chromosome studies were done using a direct technique for processing bone marrow aspirates. Slide preparations were stained by quinacrine mustard with fluorescence (QFQ banding). In each case, at least 19 metaphases were examined microscopically for chromosomal aberrations and QFQ-banding polymorphisms. At least three karyotypes were prepared for each case.

RFLP analysis. High molecular weight DNA was extracted from EDTA-anticoagulated blood specimens and digested to completion with either Taq I or Pst I. The resulting DNA was fractionated on 0.8% agarose gels, blotted onto magnagraph (MSI), and subjected to Southern blot analysis.

The DNA probes were labeled by random priming (Amersham, Arlington Heights, IL) to a specific activity of 1 to 3 x 10⁶ CPM/μg. Following an overnight hybridization at 42°C (0.02 g/dL Ficoll, 0.02 g/dL polyvinylpyrrolidone, 0.02 g/dL bovine serum albumin, 0.5% sodium dodecyl sulfate [SDS], 5 x standard saline citrate [SSC] [1 x SSC is 0.05 mol/L NaCl, 0.015 mol/L sodium citrate], 0.02 mol/L NaPO₄ [pH 6.5], 0.02% dextran sulfate, and 50% formamide), all filters were washed four times in 2 x SSC/0.1% SDS for 15 minutes at 60°C and then in 0.2 x SSC/0.1% SDS for 30 minutes at 60°C. The washed filters were then subjected to autoradiography using XAR-5 film (Kodak, Rochester, NY) in the presence of intensifying screens (DuPont Lightning-Plus, Wilmington, DE). When necessary, the radioactive filters were washed free of probe with 0.4 mol/L NaOH for 5 minutes and then neutralized in 2 x SSC/0.2 mol/L Tris-HCl (pH 7.5) before the next hybridization.

![Fig 1](https://example.com/fig1.png)

**Fig 1.** Outline of clinical course for the first 30 days posttransplant. White blood cell (WBC) count and temperature curve are expressed on the ordinates. Frequency and number of blood-product transfusions are indicated by arrows across the top of the figure. Time course of rash and diarrhea occurring contemporaneously with biopsy-proven Grade II skin GVHD is noted by shaded area. A second marrow transplant (denoted by arrow on the abscissa) was given to the patient on day 31. ANC, absolute neutrophil count; RBC, red blood cell; platelet TX, platelet transfusion.
The DNA probes used in this study included (1) H-ras (R. Weinberg, Whitehead Institute for Biomedical Research) (2) YNH-2A (R. Weinberg, Whitehead Institute for Biomedical Research) and (3) 3' HVR (D.J. Weatherall, Oxford University, England). These probes identify DNA hypervariable regions that are detected following digestion by a number of restriction enzymes, including Pst I and Taq I.

**Analysis of HLA-DR sequence polymorphism.** Genetic analysis of HLA-DR polymorphism was performed by sequence-specific oligonucleotide-probe hybridization (SSOPH) as described previously, except that the cycles for PCR were 15 seconds at 98°C, 1 minute at 55°C, and 15 seconds at 72°C.

Briefly, DNA was isolated from cryopreserved peripheral-blood lymphocytes. The exon encoding the first domain of HLA-DR chains was amplified in vitro using the PCR, and amplified DNA was applied to Gene Screen Plus membranes (DuPont, Boston, MA) and hybridized with 32P-oligonucleotide probes under conditions that resolve single-base-pair mismatches. Each hybridization included amplified DNA controls from the core panel cell of the Tenth International Histocompatibility Workshop, whose names are followed by their DR alleles: WT100-DR1, AMAI-DR2a, WIR-DR2b, RML-DR2c, STEIN-DR3, YAR-DR4b, WT51-DR4a, JHAF-DR4c, PEI17-DR4d, SWEID-DR5, WDV-DR6a, EMJ-DR6c, TEM-DR6b, AMALA-DR6d, LBUF-DR7, MADURA-DR8, DKB-DR9, and BM16-DR12. The 23 oligonucleotide probes used detected all the above HLA-DR sequences as well as DR52a, 52b, and 52c. The exact hybridization and wash conditions, as well as the sequences of the probes, will be published separately.

**Limiting dilution analysis (LDA) of T-cell proliferation.** LDA assays for estimating the frequency of proliferating T cells following polyclonal activation were performed as follows: A microwell was used for cells given 0 or 500 rad. Eight serial twofold dilutions of responder peripheral-blood cells from normal volunteers or random buffy-coat cells (100 μL/well) were carried out in U-bottomed 96-well microplates (24 wells per dilution). Complete Dulbecco’s modified Eagles medium (C-DMEM) supplemented with 8% human AB plasma was used as diluent and culture medium. Lymphocyte conditioned medium (LCM) was prepared by costimulation of buffy-coat cells with phytohemagglutinin (PHA) and irradiated allogeneic peripheral-blood lymphocytes (PBLs) for 24 to 48 hours. Each batch of LCM was titrated to determine the level of interleukin-2 activity. Residual PHA in the LCM was sufficient to activate polyclonally T cells in the responder blood samples. Irradiated (4,000 rad) PBL, obtained from randomly selected buffy coats after Sepacell (Sepratech Corporation, Oklahoma City, OK) density-gradient separation, were used as stimulators and suspended at a concentration of 1 x 10^6/mL in 2 x LCM to all microtiter wells; 100μL of the suspension was distributed to each well. Control wells for microplates contained 4,000-rad-irradiated stimulator cells in LCM (N = 48 wells) but no responder cells.

A macrowell assay was used for cells given 1,500-3,000 rad because of the large number of cells used. Five serial dilutions of responder cells were performed in 24-well macromotes (12 wells per dilution). One mL of 2 x LCM was added to each macrowell to bring the total volume up to 2 mL per well. Stimulator cells were not added to the macrowells. Control wells were set up in the same way as in the LDA assay. For experimental wells, the initial concentration of cells in each assay was adjusted according to the anticipated frequency of response. The plates were incubated at 37°C in a humidified mixture of air and CO₂ for 11 to 14 days. Half the medium in each well was replaced with fresh LCM (1 x) at 4- to 5-day intervals.

Tritiated thymidine (\(^{3}H\)-Tdr) was added to each microwell (1 μCi/200 μL) for the last 6 to 18 hours of incubation. Each macrowell, 200-μL aliquots of the cell suspension were placed in duplicate microwells and labeled with \(^{3}H\-Tdr.\) After incubation, the labeled cells were collected onto filter paper, dried, and placed in vials containing liquid scintillation cocktail. The amount of \(^{3}H\-Tdr\) incorporated by the cells in each well was measured. Individual wells were scored as positive for proliferation when the cpm of incorporated \(^{3}H\-Tdr\) exceeded the mean cpm for the respective control wells by at least 3 SD. Minimal estimates of the frequency of proliferating T lymphocytes were made by chi-square minimization according to the method of Taswell.

**Immunofluorescent assays.** Analysis of cell-surface antigens on activated and expanded cells collected from LDA assay-culture wells was done by immunofluorescent flow cytometry using standard methods. All reagents were obtained from Becton-Dickinson (Mountain View, CA). The labeled cells were analyzed on a FACS Analyzer (Becton-Dickinson) equipped with a FACSLite laser and Consort 30 computer support.

**RESULTS**

**Cytogenetic studies.** Cytogenetic analysis of recipient marrow cells collected before transplantation revealed a 46,XX,(t;9;22)(q34;q11) karyotype (Fig 2A). Marrow cells subsequently collected at the time of graft rejection demonstrated a 46,XX karyotype (Fig 2B). Chromosome-3 polymorphisms were employed to determine the origin of the cells at the time of graft rejection. Analysis of these polymorphisms revealed that the cells found in the patient after transplant were not those of the donor (Fig 2C). The specific chromosome-3 polymorphism identified in the posttransplant karyotype (day 17 and day 20) is common, found in approximately 50% of normal subjects, and therefore did not distinguish recipient cells from other posttransplant engrafted cells.

**RFLP analysis.** RFLP analysis, using multiple hypervariant minisatellite DNA probes, was performed on peripheral-blood and marrow cells (Fig 3A and B). Recipient and donor DNA were initially examined with each probe using previously stored marrow (Fig 3A and B) and peripheral blood (not shown). DNA digests revealed distinct RFLP patterns that were homozygous for donor and recipient. Marrow and peripheral blood collected at day 17 revealed a third distinct pattern that was neither that of the host nor the donor (labeled “Post-Trans” in Fig 3A and B). This third pattern was evident with each of the probes. The RFLP analysis was repeated with marrow and blood samples obtained three days later (day 20 posttransplant) and showed persistence of this unique third-party banding pattern (data not shown).

**DNA typing of HLA-DR.** To confirm third-party engraftment and to retrospectively analyze the extent of HLA class II matching between donor and recipient, HLA-DR genes were polymerase chain reaction (PCR) amplified and alleles analyzed by sequence-specific oligonucleotide-probe hybridizations (Fig 4). The first panel shows the results with a DR7-specific probe. In the right column, the fourth position from the top shows hybridization to a DR7-homozygous cell line. The bottom of the right column is one of the same posttransplant samples used in the previous RFLP analysis and is positive with the DR7 probe. A second posttransplant sample (fourth from the bottom in the right column), prepared independently (obtained on two separate occasions), also hybridizes with this probe, indicating that the posttransplant PBLs from the patient are DR7. Neither
Fig 2. Cytogenetic analysis of marrow cells. (A) Pretransplant study showed a 46,XX,t(9;22)(q34;q11) karyotype. (B) Posttransplant (day 17) cytogenetics showed a 46,XX (Ph1-negative) karyotype. (C) Chromosome-3 polymorphisms showed that posttransplant (day 17) cells were not of donor origin, but could not distinguish between these cells and those of the recipient.
the pretransplant patient samples nor the donor samples are DR7 (second and third position from bottom, right column). The third and fourth panels (probes DE70 and H33) show hybridization with DRw6 and DR4 probes, respectively. Both pretransplant patient and donor samples hybridize with these probes. There is no evidence of DR4 or DRw6 genes in posttransplant PBLs. Finally, panels five and six (FL37 and E28 probes) identify a difference in the DR52 alleles between pretransplant patient and donor samples (52a in patient v 52c in the donor).

Limiting dilution analysis. Limiting dilution analysis was performed to quantify the number of functional T cells in normal peripheral blood that were able to respond polyclonally to mitogen after irradiation. Figure 5 summarizes the results of these studies. Data are presented as the log^0 reduction in responding T cells as a function of a given radiation dose. The frequency of T cells responding to PHA in unirradiated PBLs was 1/2.6 cells (range, 1/1.4 to 1/6.2; mean of eight experiments). That is, 38.5%, or 385 of every 10,000 PBLs, were PHA-responsive. Frequency estimates at 500 rad revealed an approximate 3-log reduction as compared with unirradiated controls (average frequency, 1/1,851; range, 1/1,128 to 1/7,466). The results at 1,500 rad and 2,000 rad were combined. Irradiation at these doses reduced the frequency of functional T cells by a mean of 5.7 logs (average frequency, 1/1,272,000; range, 1/852,000 to 1/2,242,000). So far we have not detected any significant response after 3,000 rad of irradiation, using cell doses as high as 4 x 10^6 per macrowell; this suggests a reduction of more than 6 logs. It should be noted that we have observed on rare occasion a complete loss of T-cell response with a radiation dose as low as 1,000 rad. This suggests extensive variability in the radiosensitivity of PBLs from different individuals.

Flow-cytometric analysis of the cells obtained from LDA culture wells showed that CD3+ T lymphocytes were the predominant responding cell population. Eighty-eight percent of the viable cells taken from wells seeded with unirradiated PBLs were CD3+, and 94% expressed the T-cell markers CD4 and/or CD8 (average of three experiments). Responding cells after 1,500 to 2,000 rad were 76% CD3+, and 87% carried the CD4 and/or CD8 molecules (three determinations). The average percentage from two experiments using PBLs given 500 rad was 78% CD3+, with 64% expressing CD4 and/or CD8. Irradiated and unirradiated cultures contained a small percentage of NKH1+ cells (5% to 13%), 80 to 95% of which coexpressed CD3. Leu M3+ monocytes also were a minor population (1% to 4%). These results indicated that T cells could survive and respond to polyclonal activation in the presence of exogenous growth factors even after 2,000 rad of irradiation. Similar results were obtained in one experiment using alloantigen (MLC reactivity) instead of mitogen as the T-cell stimulus (data not shown).

DISCUSSION

Graft rejection after marrow transplantation is generally thought to be mediated by host-derived alloreactive
immune-effector cells. Experimental work in murine models\textsuperscript{19} as well as clinical studies of T-cell–depleted BMT\textsuperscript{21-25} have provided evidence that failure of engraftment is in part due to host-derived T cells. Natural killer (NK) cells of host origin have also been implicated as mediators of graft rejection\textsuperscript{21,22}; however, third-party cells have not usually been considered to be potential effectors of rejection\textsuperscript{26,27}.

Graft rejection in T-depleted allogeneic marrow transplantation clinically has been characterized by the development of profound pancytopenia in association with a
transient peripheral lymphocytosis, occasionally with concomitant marrow lymphocyte infiltration.20,27 The lymphocytosis typically resolves in several days, after which time karyotype or HLA serotype analysis invariably reveals only cells of host origin. The clinical course of the patient reported here is consistent with these prior reports. However, in this patient both RFLP analysis and DNA-based HLA typing at the time of graft rejection revealed unequivocally only cells of a third-party origin. The persistence of these cells on repeat analyses several days later suggested that this third-party–derived population contained immune-effector cells that were the mediators of the graft rejection in this patient. The concomitant presence of graft rejection and GVHD in this patient further supports the concept of a third-party–mediated process, as these are typically mutually exclusive events.28

Traditional methods of documenting donor marrow engraftment, such as karyotype analysis and HLA typing, have inherent limitations,9,10 especially in T-cell–depleted marrow transplants, where mixed chimerism is more prevalent.11 Third-party cells might not be detected using these methods, as illustrated by the shortcoming of karyotype analysis in our case. RFLP analysis using minisatellite DNA probes to variable tandem repeat segments is a more powerful method with the capability of detecting minor populations representing 1% to 5% of the total DNA.29,30 The case reported here, to our knowledge, is the first in which multiple hypervariable DNA probes unequivocally identified the presence of third-party cells repopulating the marrow of a patient who had received a T-cell–depleted marrow graft. The presence of these cells with a distinctive banding pattern in both marrow and peripheral blood on two separate occasions provided unequivocal evidence for engraftment by third-party cells and excluded the possibility of simple peripheral-blood contamination from a prior transfusion. This case also represents the first use of oligonucleotide-based HLA typing using PCR amplification for testing engraftment. This permitted the unequivocal assignment of an HLA type to the third-party cells. It also revealed a difference between the recipient and donor that was not detectable by serology. In the future, PCR of variable tandem repeat segments or HLA loci will likely become a powerful complimentary method in such engraftment studies.

Our report has potential implications for irradiation of blood products before transfusion into immunosuppressed patients. While we cannot unequivocally exclude human error, review of the irradiation records indicated that all blood products were dosed at a cesium irradiator setting of 2,000 rad. The dosing rate is also adjusted on a yearly basis to allow for decay of the cesium source and to maintain accurate dosimetry. Blood-product irradiation is designed to inactivate immunocompetent lymphocytes to prevent transfusion-associated GVHD.31 This effect of irradiation on lymphocyte proliferation and function has been studied by several investigators. Valerius et al12 measured thymidine incorporation into lymphocytes at 96 hours after stimulation with PHA and pokeweed mitogen and found only a 90% reduction in activity after 1,500 rad, a 97% reduction after 5,000 rad, and complete ablation after 10,000 rad. MLC reactivity was completely abolished by 500 rad. Similarly, Button et al13 irradiated lymphocytes with 500 to 8,000 rad, stimulated them with PHA or concanavalin A, and measured DNA synthesis after 4 days of culture. At 7,500 rad there was no thymidine uptake, at 5,000 rad 1.5% of the cells incorporated thymidine, and at 1,500 rad 15% of the cells incorporated the isotope.

Several investigators have argued that the MLC system is a more valid indicator than mitogen response with which to judge the potential for transfused irradiated cells to initiate a GVH response.32,33 Data on the abrogation of MLC reactivity by irradiation are not uniform. While two reports note MLC responses are abolished at 500 rad,14 another notes reactivity after 1,500 rad in selected donors.34 Furthermore, the development of GVHD in allogeneic BMT patients with nonreactive MLCs does occur. Thus, the radiation-induced abrogation of MLC reactivity may not strictly correlate with an inability to develop transfusion-associated GVHD.

The clinical expression of third-party alloreactivity is not limited to transfusion-associated host-directed responses (GVHD). Transfused immune cells also can react against the donor graft, and their inactivation by irradiation is equally critical in preventing graft rejection. Whether identical third-party immune-effector cell populations are involved in mediating both GVHD and graft rejection is not known. Experimental evidence, however, suggests that in some cases 1,500 rad may be an inadequate dose of irradiation to inactivate cell populations potentially capable of causing graft rejection. Banisadre et al35 showed that mitogen-activated T cells were able to suppress hematopoietic progenitors after 15 days
of culture. This effect was reduced by irradiation with 1,000 or 2,000 rad but could not be abrogated until 3,000 rad was administered. Browall et al have proposed that the radiation sensitivity of NK activity is controlled by X-linked codominant genes and that approximately 10% of the population may bear the radioresistance-conferring heterozygous or hemizygous phenotypes. Heterozygous partially resistant women exhibited only a 50% decline in NK activity after irradiation with 3,000 rad. This phenomenon theoretically could influence the ability of irradiated NK cells to mediate graft rejection. Finally, Lowenthal et al showed that mitogen activation of lymphocytes before irradiation increased their radioresistance. One could speculate that in the case reported here, presensitization of the blood-product donor through either prior transfusion or pregnancy, and/or lymphocyte activation via nonspecific immune stimulation, might have yielded more radioresistant T/NK cell subpopulations, which could mediate alloreactivity against the donor graft. Alternatively, the expansion of third-party cells might derive from a special immunogenetic relationship of DR7 to DR4, DRw6 favoring cell proliferation.

Remlinger et al reported the clinical finding of both graft rejection and GVHD in a marrow transplant patient, presumed due to an unirradiated granulocyte transfusion, but suggested that irradiation of blood products with 1,500 rad might be an inadequate dose to inactivate transfused lymphocytes. To address this question, we used a limiting dilution assay system to assess quantitatively the precursor frequency of irradiated lymphocytes able to respond to mitogen in a polyclonal fashion. Our data revealed a steep dose–response curve beyond 500 rad but documented the persistence of lymphocytes that retained their replicative potential after 1,500 to 2,000 rad. This system measures the proliferative capacity of lymphocytes over a 12- to 14-day period, independent of their need to produce essential growth factors such as interleukin-2. Antigen-specific lymphocytes, which undergo clonal proliferation and directly or indirectly mediate GVHD, were not specifically quantitated in our system. However, prior work in our lab and by others indicates that the frequency of antigen-specific (clonal) lymphocytes is 1 to 3 logs lower than mitogen-responsive (polyclonal) lymphocytes, depending on the degree of antigen disparity. In a patient receiving blood products that are not matched at HLA loci, the degree of alloreactivity and frequency of precursor T lymphocytes should be high, given the polymorphic nature of the HLA system. The lymphocyte content in red-cell and single-unit platelet packs averages between 4 × 10⁵ and 5 × 10⁶ lymphocytes per unit. Thus, only a small number of potential effector cells would survive irradiation in a previously nonactivated lymphocyte population. If our frequency estimates are valid, only a small number of lymphocytes may be necessary to mediate graft rejection or GVHD.

Several factors may affect the efficacy with which immunocompetent lymphocytes are inactivated by ex vivo irradiation. First of all, it is important to note that there can be up to 30% variation in the actual dose of radiation delivered to portions of a blood bag using a Gammacell 1000 Irradiator. At an irradiator setting of 1,500 rad, dosimetric calculations indicate that a blood product would receive 1,200 to 1,650 rad; at 2,000 rad there would be a variance of 1,600 to 2,200 rad. Based on our limiting dilution analysis curve (Fig 5), this variance might alter the frequency of surviving T cells. Irradiation-mediated lymphocyte inactivation is also affected by environmental conditions such as temperature and oxygenation. Hypoxia is known to decrease the radiation cell kill as compared with the fully oxygenated state at equivalent radiation doses. This effect occurs almost entirely between oxygen tensions of 0 and 30 mm Hg. The oxygen tension in red-cell or platelet collection bags (CLX; Cutter, Elkhart, IN) employed at our center, however, does not drop below 30 mm Hg at any time during storage (personal communication, J. Grote, Baxter Labs, Deerfield, IL, September 1988) and should not have affected lymphocyte inactivation. The issue of temperature is more complex. In murine thymocytes, lowering the temperature from 37 to 25°C decreases radiation-induced cell kill. In other cell systems, however, the results have been contradictory. At our center, red cells are stored at 1 to 6°C, while platelets are maintained at 20 to 24°C. We are unaware of any reports that have examined this effect at temperatures lower than 25°C in in vitro irradiated lymphocyte systems. The contribution of this effect to lymphocyte inactivation remains difficult to assess.

The proliferative capacity of third-party lymphocytes in MLC reactions or mitogen-driven assays may not be the sole measure with which to assess their potential for mediating graft rejection or GVHD. Nichols et al have shown that lymphocytes primed in one-way MLC can mediate cytotoxic responses against human-donor lymphocytes in standard cell-mediated lympholysis (CML) assays even after being irradiated in excess of 5,000 rad and despite losing their replicative potential. Inouye et al have also shown that lymphocytes irradiated at 1,000 to 2,000 rad in vitro can produce lymphokines such as interleukin-2. Consequently, even if the proliferative potential of a lymphocyte population has been abrogated, other immune functional capabilities may still be operative. Furthermore, recent evidence suggests that lymphokine production may stimulate other effector cells to produce the tissue damage seen in GVHD. Specifically, lymphokines and secondary immune effector cells have been shown to be proximate mediators of acute GVHD in murine models. These immune functions may be less radiosensitive, especially in activated or presensitized lymphocyte populations.

The number of immune-competent lymphocytes necessary to mediate graft rejection or GVHD in man is not known. Instances of transfusion-associated GVHD after fresh plasma infusions have suggested that small numbers of lymphocytes may be able to mediate this event and that the degree of immunosuppression of the recipient may be equally critical. T-cell depletion of the donor marrow may decrease the ability of the graft to withstand attack from alloreactive third-party immune-effector cells. The present case shows that graft rejection and GVHD, secondary to blood-product transfusion, can occur after T-cell–depleted marrow transplantation. Its occurrence may be more fre-
quent than previously thought, as many cases of GVHD are causally ascribed to the donor, and most cases of graft rejection are attributed to host-derived mechanisms, even without definitive documentation. Since normal blood elements (platelets, granulocytes, erythrocytes) are minimally affected by radiation doses less than 5,000 rad, our data would suggest that irradiation of blood products to doses of 3,000 to 4,000 rad may be prudent to obviate the risk of third-party-mediated graft rejection and GVHD. Determination of the frequency of this phenomenon in T-cell-depleted marrow transplants will require a high degree of clinical suspicion and use of sensitive molecular techniques with which to examine marrow engraftment.

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Third-party-mediated graft rejection and graft-versus-host disease after T-cell-depleted bone marrow transplantation, as demonstrated by hypervariable DNA probes and HLA-DR polymorphism

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