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September 25, 2008

Nuclear Regulatory Commission  
Rulemaking, Directives, and Editing Branch  
Office of Administration  
Mail Stop T-6D59  
Washington DC, 20555-0001  
Attn: Michael Lesar, Director

Re: CsCI Issue Comments

7/31/08  
73 FR 44780  
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RULES AND DIRECTIVES  
BRANCH  
USNRC

Dear Mr. Lesar:

The University of Cincinnati provides the following comments to NRC-2008-0419, Request for Comments on the Security and Continued Use of Cesium-137 Chloride Sources and Notice of Public Meeting. The University of Cincinnati believes a careful and through review is needed prior to making a decision on the recommendations of the National Academy of Sciences' report. The University of Cincinnati believes discontinuing the use of Cs-137 sources is likely the wrong approach, and instead education and appropriate security measures are a much more worthwhile approach.

Issue No. 1.2: Feasibility of the Use of Isotopes Other Than Cs-137

The University of Cincinnati has three specific concerns with any recommendation to replace Cs-137 with Co-60.

First, many radiation detection/survey instruments are energy dependant. For energy dependant instruments, the University of Cincinnati believes the Cs-137 photon is much more appropriate for the calibration of instruments used in biomedical research and/or medicine.

Secondly, replacing Cs-137 with Co-60 brings up shielding concerns. The half-value layer of lead for Co-60 is 16 mm and the half-value layer of lead for Cs-137 is 8 mm. These values show the amount of lead shielding needed to reduce the dose rate from Co-60 to the same value as from Cs-137 is about double. Therefore, the shielding of a facility designed for Cs-137 may be insufficient if Cs-137 is replaced with Co-60. In addition, self-contained irradiators containing Cs-137 routinely weigh 4000 to 6000 pounds, with the majority of the weight due to shielding. If a Cs-137 self-contained irradiator is replaced with a Co-60 irradiator weighing twice the amount, any needed transportation equipment, including elevators, and/or any operation location other than on a ground floor may be compromised without considerable additional investment in infrastructure.

SUNSI Review Complete  
Template = ADM-013

E-RIDS = ADM-03

Call =  
J. Jankovich (JP52)

Finally, there is a concern resulting from the significant differences in half-life if Cs-137 is replaced with Co-60. Co-60 has a half-life of about 5 years resulting in about 13% of the activity being lost per year. Cs-137 has a half-life of about 30 years resulting in about 2% of the activity being lost per year. Therefore, Co-60 will require source replace and recalibration much more frequently than if Cs-137 is used. Though more frequent recalibration may just be a minor inconvenience, there are significant costs and personnel doses associated with source replacement.

#### Issue No. 2: Use of Alternative Technologies

The recommendations identify the alternate technology as x-ray generators. There are no known x-ray generator devices commercially available for instrument calibration. Though there are x-ray irradiators, the University of Cincinnati has concerns with replacing Cs-137 with x-ray generators.

- Replacement of Cs-137 with an x-ray generator for instrument calibration includes concerns regarding instrument calibration accuracy due to the energy dependence of radiation/survey instruments. Unlike Co-60 and Cs-137 which emit photon radiation with discrete energies, x-ray generators emit photons over a spectrum of energies; therefore, any correction needed for energy response would be difficult.
- Replacement of Cs-137 irradiators with x-ray irradiators for one specific research technique is not only impractical it would have a devastating affect on the research itself. As noted in the attached from Jose Cancelas, MD, PhD, Associate Professor, Director Research Division Hoxworth Blood Center, University of Cincinnati, in addition to reliability problems associated with breakage and overheating of x-ray irradiators, x-ray irradiators cannot be used for myeloablative total body irradiation of mice for stem cell transplantation. In order to achieve the desired dose to the bone marrow, the soft x-rays associated with an x-ray irradiator also result in the mice receiving skin burns.
- Replacement of Cs-137 irradiators with x-ray irradiators for research includes significant concern with the consistency of the dose delivered by x-ray irradiators and the need to routinely calibrate x-ray irradiators. As noted in the attached letter from Aaron Johnson, PhD, Research Assistant Professor, University of Cincinnati, there are hundreds of research studies that use protocols involving irradiation using a Cs-137 irradiator. Also, as noted in the attached research publication by Schwarte and Hoffmann, alternative radiation sources elicit different background effects in experimental subjects, such as altered cytokine productions. Protocol modification to an x-ray irradiator would require permission, time and funding to investigate these effects.

#### Issue No. 3 – Possible Phase-Out of CsCl sources

The University of Cincinnati acknowledges the concerns that have led to consideration of the proposal to discontinue or replace all IAEA category I and II CsCl sources, but is concerned that the scientific and fiscal costs of such a move outweighs the potential benefits.

It is understood that one source of concern is data that indicates CsCl sources can be removed from an irradiator very quickly; however, the University of Cincinnati believes it is more cost effective for physical devices to be used to alert individuals who can respond to an attempted source removal in a timely manner (and hopefully prevent removal) and which increase the time of source removal. The NRC through Orders issued over the past several years has required all

individuals who possess IAEA category I and II Cs-137 sources to significantly increase the security of the Cs-137 sources. Increased security requirements included the installation of physical security devices that alert local law enforcement if a removal or theft of a Cs-137 may be occurring. The University of Cincinnati also understands there are physical security devices that can be added to the source chamber by source vendors which would significantly increase the source removal time.

A major issue associated with the potential requirement for total CsCl source replacement is the logistics of executing the replacement. Currently there is no disposal facility in the United States generally available to all licensees for what would be "Greater than Class C" waste. Currently, IAEA category I and II sources are "disposed" through transfer to the Department of Energy's Offsite Source Recovery Program. Though an excellent program, the program cannot keep up with the limited number of unwanted sources much less handle the tens of thousands of sources that would have to be transferred to the Department of Energy if CsCl source replacement was required.

#### Issue No. 3.1: Potential Rulemaking Issues and Justification for Regulatory Changes

Many of the questions specifically asked under this issue were addressed under Issue 2 above.

#### Issue No. 3.2: Transportation and Storage Issues Associated With Removal of CsCl Sources from Licensee Facilities

One question asked under the issues is "Who should bear the transportation costs?" associated with the removal of CsCl sources. As previously noted the University of Cincinnati does not agree with overall CsCl removal; however, if removal becomes a requirement the University of Cincinnati recommends all costs, including transportation costs, be covered by the Federal Government. Cost will be significant, for example, a recent transfer of an irradiator to the University of Cincinnati indicates the labor costs associated with packaging a source for transport and then transporting the source is over \$30,000.

#### Issue No. 4 – Additional Requirements for Enhanced Security of CsCl Sources

Over the last few years the Federal Government has added security requirements piece-by-piece, and it has not always been clear how all the requirements fit together and maximize effective security. The timetables have often been hard, if not, impossible to achieve and all costs associated with the requirements have been born by the licensee. Therefore, if additional requirements are mandated, the requirements need to have verified enhanced security effectiveness, the timetable for implementation must be readily achievable by licensees, and all costs should be covered by the Federal Government.

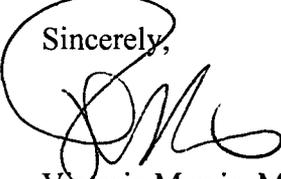
#### Issue No. 5 – Role of Risk Analysis in Potential Future CsCl Requirements.

While not directly related to current uses of CsCl, the University of Cincinnati notes that the NRC's fact sheet on dirty bombs states that the most significant concern from the radioactivity is radiation contamination "creating fear and possibly panic and requiring potentially costly cleanup." Therefore, the University of Cincinnati hopes that any overall strategy for reducing the risks associated with CsCl sources includes support for research on procedures for quick and effective decontamination of CsCl, development of decontamination release guidelines, and education of the public on the true risks of radiation. Minimizing the impact of a dirty bomb both

in economic costs and in societal effect helps minimize the ability of terrorists to use a dirty bomb.

Thank you for the opportunity to provide input. If you have any questions about the comments, do not hesitate to call. Also, I plan on attending the public hearing on September 29 and 30 and could answer questions at that time.

Sincerely,



Victoria Morris, MS, CHP  
University of Cincinnati  
Radiation Safety Officer

C: Radiation Safety Committee  
S. Degen, PhD  
D. Plas, PhD  
J. Cancelas, MD, PhD  
A. Johnson, PhD  
S. Spreitzer

## **X rays vs Gamma rays in myeloablative total-body irradiation schemes in stem cell transplantation in mice.**

Hematopoietic stem cell transplantation is a routine therapy administered to patients with cancer and multiple genetic defects, including immunodeficiencies and stem cell failure syndromes. This technique has been possible thanks to the technological advances of the 20<sup>th</sup> century including basic knowledge of stem cell biology and use of high-energy sources of ionizing radiation. Hematopoietic stem cell biology has largely benefited from the development of successful, homogenous, reproducible myeloablative total body irradiation (TBI) methods (7-15.75 Gy) methods in animal models. These irradiation schemes have allowed complete myeloablation, which can be rescued by hematopoietic stem cell transplantation. These protocols are so accurate that they allow the specific analysis of the hematopoietic reconstitution of one single cell, without major background drawbacks regarding to remaining endogenous hematopoiesis or increased lethality of the recipient mice due to overdose of soft tissues. These protocols largely depend on the use of high-energy sources of gamma rays which allow homogeneity and large penetration in bone tissues, where the bone marrow (the hematopoietic tissue) resides.

Alternatively, X-rays have represented an alternative way to irradiate tissues. Recent technical improvements have allowed a more efficient use of X-rays, minimizing irradiation, reducing time of exposure and decreasing the usual technical problems of X-ray machines (breakage and overheating). In addition, recent issues related to nuclear installation security have increased the expectations of X-rays as a replacement of Gamma ray sources for most applications.

Unfortunately, X-rays dependent energy deposited in tissues highly depends on the mean atomic number of the tissue in which the deposit occurs. Gamma-rays and electrons have the advantage that the energy absorbed by the tissue is much less dependent on the atomic number, so that the drawbacks of very high cortical bone absorption (with its twin problems of dosage inhomogeneity and radionecrosis) are largely avoided.

Multiple laboratories in the world have tried unsuccessfully to develop methods using X-rays to successfully irradiate to complete myeloablation animals (usually, mice) for stem cell transplantation in a homogenous, consistent way that allows consistent rescue with stem cell transplantation. The current perspectives of applications of stem cells in regenerative medicine and the answers to specific questions of relationship between hematopoietic stem cells and the nurturing bone marrow milieu are clearly dependent on the use/administration of ionizing radiations that achieve consistent, reproducible radiation with activity on the hematopoietic bone marrow cavity.

For these reasons, I consider that X-rays administration cannot replace Gamma rays in myeloablative total body irradiation for stem cell transplantation.

Jose A. Cancelas, MD, PhD

Research Division Director and Medical Director for Cell Therapies, Hoxworth Blood Center.  
Assoc. Prof. of Pediatrics, Division of Experimental Hematology, Dept. of Pediatrics, Cincinnati Children's Hospital Medical Center.



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September 3, 2008

Victoria Morris, MS, CHP  
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Dear Victoria,

I have become aware of thoughts to remove CsCl irradiation from the University of Cincinnati. Our research team relies on this radiation source to conduct our science pertaining to defining the immunological factors that contribute to multiple sclerosis. Our laboratory has secured \$1.4 million in federal and private funding for this work, not including indirect monies that we have also received that go straight to helping the university run. We need this radiation source to perform bone marrow transplants and adoptive transfer of blood derived cells to definitive test the role of specific genes. I am unaware of alternative protocols and will be essentially shut down if the gamma irradiator is removed. **Therefore, removal of this source will render us unable to conduct these funded studies. This could ultimately result in our leaving the University of Cincinnati, taking our federal funding with us.** That is how serious this matter is. We simply cannot replace the 500 protocols that require the CsCl irradiator. We also do not have permission or time to use federal and private funding to "explore" and "invent" alternative protocols that currently do not exist.

Sincerely,

A handwritten signature in black ink, appearing to read 'Aaron J. Johnson'.

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## Influence of Radiation Protocols on Graft-vs-Host Disease Incidence After Bone-Marrow Transplantation in Experimental Models

Sebastian Schwarte and Matthias W. Hoffmann

### Summary

Bone-marrow transplantation is an approved curative treatment for many hemato- and oncologic diseases. Nevertheless, the severe acute clinical course of graft-vs-host disease (GVHD) after allogeneic bone-marrow transplantation is frequently fatal, and is to date not curable. Acute GVHD must, therefore, be prevented from the start of the bone-marrow transplantation by immunosuppressive medication, causing sometimes serious side effects. Therefore, new preventive strategies are tested, starting with animal experiments. Often mice are chosen for this kind of trial, and the clinical protocol of bone-marrow transplantation is transferred into the experimental settings.

The first step to induce an acute GVHD is whole-body irradiation of the recipients. Several methods are available for this purpose: the most common is a  $^{60}\text{Co}$  source ( $\gamma$ -irradiation); less common are a  $^{137}\text{Cs}$  source ( $\gamma$ -irradiation) and a linear (particle) accelerator (photons). Differences between these radiation techniques can occur and can unexpectedly interfere with the results of the experiments. In this chapter, the materials and methods for bone-marrow transplantation in mice, with particular emphasis on the different radiation techniques, are explained; furthermore, the advantages and disadvantages in regard to the underlying physical principles will be discussed.

**Key Words:** Radiation; whole body irradiation; bone-marrow transplantation; cobalt; cesium; linear accelerator; particle accelerator; incidence; GVHD; GVHR; mice.

### 1. Introduction

Bone-marrow transplantation is an approved curative treatment for many hemato-oncologic diseases, such as immunodeficiency (SCID, Wiskott-Aldrich syndrome), acute leukemia, aplastic anemia, chronic leukemia, hemoglobinopathies (thalassemia major), myelodysplasia, enzymatic defects (Gaucher's disease), lymphoma, myeloma, and after whole-body irradiation of carcinomas with myelosuppressive side effects. Allogeneic bone-marrow transplantation is the treatment of choice for patients with lymphomas or leukemias. Despite significant improvements since the beginning

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of the modern era of allogeneic bone-marrow transplantation, the side effects of this therapy can be tremendous and life-threatening. In particular, the disease caused by the immunological reaction of immunocompetent donor cells against the immunocompromised host (graft-vs-host disease [GVHD]) is still a major problem. An acute and a chronic form of GVHD can be differentiated. The acute form of GVHD can be very dangerous and life-threatening and manifests mainly at the skin, the liver, and the gastrointestinal tract. The chronic form resembles an autoimmune disease, and severe complications are mainly caused by the immunosuppressive therapy.

The treatment of a patient with a severe acute clinical course of GVHD is frequently not successful. Acute GVHD must, therefore, be prevented by potent immunosuppressive drugs, with their inherent range of risks and side effects. Therefore, a main goal in bone-marrow transplantation is to specifically suppress the GVH reaction without impairing the physiological immune response to pathogens and residual leukemic cells. For this reason, it is of major importance to develop more specific therapeutic strategies in bone-marrow transplantation.

Mice are frequently used as experimental models to test new therapeutic approaches in the treatment of GVHD after allogeneic bone-marrow transplantation. They have the advantage that several genetically defined inbred, transgenic, and knock-out mouse strains are available. These can be used to examine GVHD pathophysiology and GVHD prevention in several informative combinations. In the following, a mouse model is presented that allows the study of GVHD under defined conditions.

The first step to induce acute GVHD is whole-body irradiation of the host. In the clinical setting, this serves three purposes: first, lethal irradiation destroys malignant cells underlying the leukemic disease. Second, irradiation destroys host lymphocytes and in turn prevents or ameliorates the rejection of the allogeneic bone-marrow inoculum. Third, irradiation is thought to create space in the bone marrow by destroying host bone-marrow cells, thereby improving the engraftment of the injected bone-marrow cells. Several methods are available to irradiate animals in an experimental setting: the most commonly used radiation source is  $^{60}\text{Co}$  (γ-irradiation); less frequently used are  $^{137}\text{Cs}$  (γ-irradiation) and the linear (particle) accelerator (photon irradiation).

After irradiation, bone-marrow cells are purified from the donor. In the clinical setting, multiple bone-marrow aspirates are harvested from the donor, whereas in animal models bone-marrow cells are obtained from the donor tibia and femur.

Finally, bone-marrow cells are injected intravenously. The outcome is followed by regularly checking the weight and inspection for signs of GVHD.

## 2. Materials

1. Phosphate buffered saline (PBS): 28.0 mM  $\text{NaH}_2\text{PO}_4$ , 7.2 mM  $\text{Na}_2\text{HPO}_4$ , and 0.14 M NaCl; 9.55 g/L bidistilled water; without calcium and magnesium; may be stored indefinitely without contamination at 4°C.
2. Anesthesia with ketamine (ketamine hydrochloride) and Rompun (xylazine hydrochloride): mixture of 0.5 mL ketamine hydrochloride and 0.5 mL xylazine hydrochloride, dissolved in 9 mL sterile PBS; light-sensitive; stable at room temperature for up to 1 mo. Ketamine (10%): 0.08 mL/100 g i.p. (80 mg/kg); Rompun (2%): 0.01 mL/100 g i.p. (2 mg/kg). Two-hundred fifty microliters per mouse.

3. Irradiation equipment: a  $^{60}\text{Co}$  cobalt source, a linear accelerator, or a  $^{137}\text{Cs}$  cesium source.
4. Mouse cages made of plexiglas for irradiation.
5. Medium 199 (TC 199): Hank's salt solution with L-glutamine without  $\text{NaHCO}_3$ : 10.61 g/L bidist. water; stable at  $4^\circ\text{C}$  for 2 mo if kept sterile.
6. One liter hemolysis buffer: pH 7.3, 8.34 g  $\text{NH}_4\text{Cl}$ , 0.037 g EDTA, and 1.00 g  $\text{NaHCO}_3$ ; stable at  $4^\circ\text{C}$  for up to 2 mo if kept sterile.
7. One hundred milliliters trypan blue: 8 mL of a 2% stock solution, dissolved in a 92-mL NaCl solution; store at  $4^\circ\text{C}$  indefinitely.
8. Surgical instruments (forceps, scissors).
9. Fifteen-milliliter plastic tubes.
10. Polystyrene cell-culture dishes (Petri dishes).
11. Sieve with 0.6-mm mesh size.
12. Sieve with 0.2- $\mu\text{m}$  mesh size.
13. Fifty-milliliter polypropylene tube.
14. Counting chamber (Neubauer's hematocytometer).
15. BL/LB, 0.40  $\times$  12 mm, 27 gage  $\times$  0.5 in. disposable injection needles.
16. BL/LB, 0.90  $\times$  40 mm, 20 gage  $\times$  1.5 in. disposable injection needles.
17. Five-milliliter, 10-mL, and 20-mL disposable syringes.

### 3. Methods

Mice should be kept under clean, preferably "specific pathogen free" conditions. Following lethal irradiation and bone-marrow reconstitution, the mice are temporarily immunodeficient until the bone marrow has produced sufficient immunocompetent cells to protect the mice from infections.

The anesthetized recipients are lethally irradiated 24 h before the injection of donor cells. Donor animals are sacrificed about 6 h before the planned injection to allow for the collection of bone marrow and lymph-node cells (*see Note 1*), and to have sufficient time for cell processing.

Donors and recipients should be age and sex matched in order to avoid undesired immune responses to minor (H-Y) antigens that might affect the GVH reaction under study.

#### 3.1. Irradiation of the Recipients

To prevent rejection of the allogeneic bone-marrow cells; the recipients are lethally irradiated by a single dose of whole-body irradiation. The recipient animals are contained in special cages during the irradiation.

##### 3.1.1. Anesthesia

To achieve a homogenous radiation effect, the animals are kept still by general anesthesia. Therefore, the animals are anesthetized with 250  $\mu\text{L}$  of the ketamine-Rompun mixture, applied intraperitoneally with a 27-gage needle. Anesthesia lasts for about 20 min and is sufficient for the duration of the irradiation procedure.

##### 3.1.2. Cage Design

The size of the radiation cage must be large enough to accommodate the mice in one plane, side by side, to achieve a homogenous irradiation effect. The cage should contain air holes to provide sufficient ventilation. Usually, a cage contains a maxi-

imum of eight mice. A suitable radiation cage can measure 16 cm in length, 10 cm in width, and 2.5 cm in height. The lid of the cage should close directly above the animals with as little space between them and the lid as possible (*see Note 2*). A mouse cage for irradiation can be made of safety glass, plexiglas, or cardboard carton, and should be secured to prevent escape of the animals.

### 3.1.3. Irradiation Equipment

*See Note 3.* Irradiation with a  $^{60}\text{Co}$  source is carried out at a distance of 75 cm between the device and the animals for a radiation dose of 9 Gy. A 0.5-cm thick plexiglas plate should be placed on top of the cage to achieve optimal tissue distribution.

## 3.2. Collection of Donor Cells

For the collection of bone-marrow and lymph-node cells the animals are sacrificed by cervical dislocation, following anesthesia with carbon dioxide. The level of anesthesia is checked by controlling for spontaneous movements, posture of the head, reflexes, spontaneous breathing, and heartbeat of the mouse.

### 3.2.1. Preparation of Lymph-Node Cells

1. Donor animals are fixed on their back and disinfected with 70% EtOH.
2. Then a transverse skin incision is made, starting 1 cm below the xiphoid process.
3. The skin is manually pulled upwards and downwards, exposing the abdomen and thorax up to the neck and front legs.
4. The submandibular, axillar, and brachial lymph nodes are removed with a curved anatomic forceps, taking care not to include surrounding fat.
5. The lower part of the skin is drawn caudally towards the hind legs, and the inguinal lymph nodes are removed.
6. Then a midline incision is made through the abdominal wall to expose the intestines. Lymph nodes are collected from the intestinal mesentery, taking particular care to exclude fat.
7. Lymph nodes are collected in sterile, ice-cold TC 199 medium in 15-mL tubes that are usually kept on ice in a styrofoam container.
8. Under sterile conditions, the lymph nodes are transferred to a sterile sieve (mesh size: 0.6 mm) and ground with a spade. Subsequently, the sieve is rinsed with 10 mL TC 199. The cell suspension is collected in a cell-culture dish (Petri dish) and is flushed through another sieve (mesh size: 0.2  $\mu\text{m}$ ) to exclude cell debris.
9. The resulting single-cell suspension is collected in a 50-mL plastic tube and is centrifuged (10 min, 300g, 4°C). Cells are taken up in 10 mL sterile PBS, and viable cells are counted after trypan blue staining in a microhemocytometer.
10. The optimal number of cells for induction of GVHD depends on the chosen model (*see Note 4*).

### 3.2.2. Preparation of Bone-Marrow Cells

After collecting the lymph-node cells, the bone marrow is harvested.

1. Bone-marrow cells are harvested from the tibia and femur of donor mice: after stripping the skin from both hind legs, the femoral bones are disarticulated at the hip joint. Care is taken not to damage or splinter the bones, as this makes later flushing inefficient. Subsequently, muscle tissue is carefully dissected away from the bones. The bones are collected in 20 mL sterile TC 199 at 4°C in 50-mL plastic tubes.

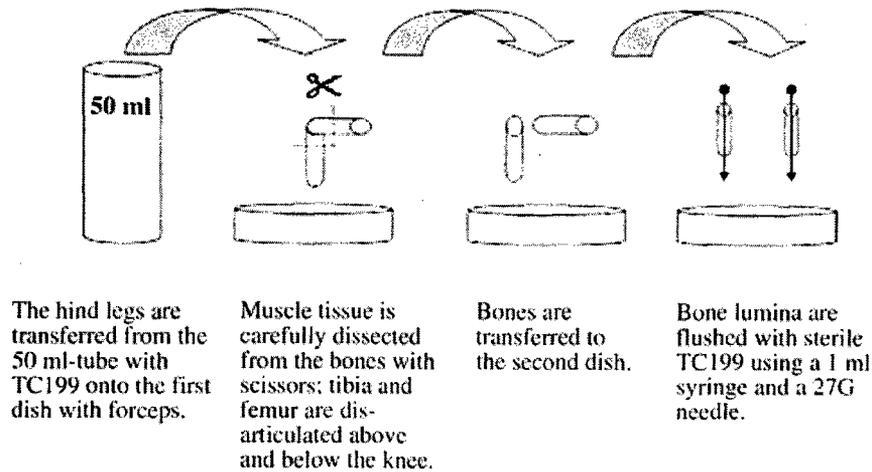


Fig. 1. Harvesting of bone-marrow cells.

2. Under sterile conditions, three cell-culture dishes are prepared, each containing 10 mL sterile TC 199. Scissors and forceps should be disinfected with 70% EtOH regularly during this process. First, the bones are collected in the first dish. Femur and tibia are disarticulated at the knee joint (*see Fig. 1*) and the separated bones are collected in the second dish. There, the distal ends of both bones are cut off with scissors to access the bone-marrow cavity. The bone marrow is harvested by flushing the bone cavities with a 27-gage needle on a 1-mL syringe containing TC199 into the third dish.
3. Bone-marrow cells are then repeatedly resuspended with a 20-gage needle on a 20-mL syringe (TC 199) until the cell suspension is homogenized. Then cells are transferred to a 50-mL plastic tube and centrifuged (10 min, 4°C, 300g).
4. After centrifugation, the bone-marrow cell suspension is incubated with sterile hemolysis buffer at room temperature for 5 min. Then it is filtered through a sieve (mesh size: 0.2  $\mu\text{m}$ ) to remove bone fragments and other particulate material. The cell suspension is collected in a 50-mL plastic tube and is washed twice in sterile PBS. Thereafter, the cells are taken up with 10 mL sterile PBS to count them with trypan blue (vitality control).
5. Similar to the clinical situation, in which  $2 \times 10^8$  bone-marrow cells per kg body weight are administered to the patient, mice should receive on average  $5 \times 10^6$  cells (*see Note 5*).

### 3.3. Injection of Donor Cells

To inject the cells intravenously, the four tail veins of the mouse can be expanded and visualized by patting them with a swab and warm water. Water should not be too hot or boil. It is recommended to inject the tail vein with no more than 200  $\mu\text{L}$  of volume. Only the lateral tail veins are suitable for injections.

For this procedure the mouse must be restrained in a holding device. At best, the mouse is placed in a body-adjusted plastic tube or cylinder to restrain the mouse inside the tube, and to allow the tail to hang out. Care should be taken not to exert unnecessary force against the animals.

The injection is carried out with a 27-gage injection needle on a 1-mL disposable syringe.

### 3.4. Diagnosing GVHD

After bone-marrow reconstitution, the recipients are checked every other d for at least 100 d (*see Note 6*) for weight changes, and are checked daily for survival. In addition, the skin is inspected and the defense reaction is tested. If skin lesions such as alopecia, desquamations, or ulcerous-erosive changes appear, the animals should be sacrificed. Similarly, animals should be sacrificed upon severe weight loss or apathetic behavior. The liver, the gastrointestinal tract, and the skin should be examined by histology. It is recommended to take photos for documentation of skin lesions and a hunched back. *See Note 7* for the clinical course of chronic GVHD.

## 4. Notes

1. In contrast to human beings, in whom acute GVHD is caused by allogeneic bone-marrow cells alone, mature T-cells must be added to the bone-marrow inoculum in most experimental mouse models in order to induce acute GVHD. It is reported that with total body irradiation of 8.5 Gy (<sup>60</sup>cobalt source) and  $3 \times 10^7$  bone-marrow cells in a major histocompatibility complex (MHC)-I disparate mouse model, about 40% of recipients survived the first 3 mo after bone-marrow transplantation (*1*). Many studies confirm that the murine bone marrow contains only a low frequency of mature T-cells (1–3% of all bone-marrow cells) (*2–5*). In marked contrast, the proportion of immunocompetent T-cells in humans amounts to  $8\% \pm 1.6\%$  of the bone-marrow pool (*6*), and is therefore considerably higher than in the mouse. Another factor necessitating the addition of mature T-lymphocytes from secondary lymphoid organs (spleen, lymph nodes) is the high proportion of immunoregulatory cells in murine bone marrow. Thirty to 50% of bone-marrow  $\alpha\beta$ -T-cell antigen receptor (TCR) T-cells are NK1.1<sup>+</sup> cells (*7*); about 30% of CD3<sup>+</sup> cells are CD4<sup>+</sup>CD8<sup>-</sup> double negative T-cells (*8*). So far, experiments in autoimmune disease and GVHD models have proven the immunoregulatory function of both of these cell types (*9–14*). The mechanism of immunoregulation has been attributed to the secretion of interleukin (IL)-4 by these cells (*5*). Therefore, NK1.1CD4<sup>+</sup>CD8<sup>-</sup> T-cells have been termed “natural suppressor cells” (*15*). Both the low frequency of mature immunocompetent T-cells and the high percentage of immunoregulatory T-cells could explain the unsuccessful induction of GVHD in mice reconstituted with bone-marrow cells only. This is why, even with higher cell numbers, mature T-cells cannot induce a GVHD reaction as expected—they are still suppressed by the concomitant increase in the number of transferred suppressor cells. In summary, in contrast to the human system, bone-marrow cells alone are unable to reliably induce a GVH reaction in the mouse. It is necessary to add mature T-cells from secondary lymphoid organs, such as lymph nodes or spleen, to increase the ratio of immunocompetent vs immunoregulatory T-cells in the bone-marrow inoculum (*see Note 4* for further details).
2. Should an alternative design of an irradiation cage be used, it is important to remember that the effect of irradiation is more diminished, the greater the distance between the animals and the lid of the cage. The dose needed to achieve the same irradiation effect increases with the second power of the distance between the irradiation source and the target. Ideally, the lid should close directly on top of the back of the animals in order to achieve perfect irradiation distribution. To achieve optimal radiation results, it is necessary to optimize the radiation conditions. First, it is important to construct an optimal cage. If the lid does not close directly above the back of the recipients, a higher radiation dose might be necessary. In our own experience, the mortality of recipients of allogeneic

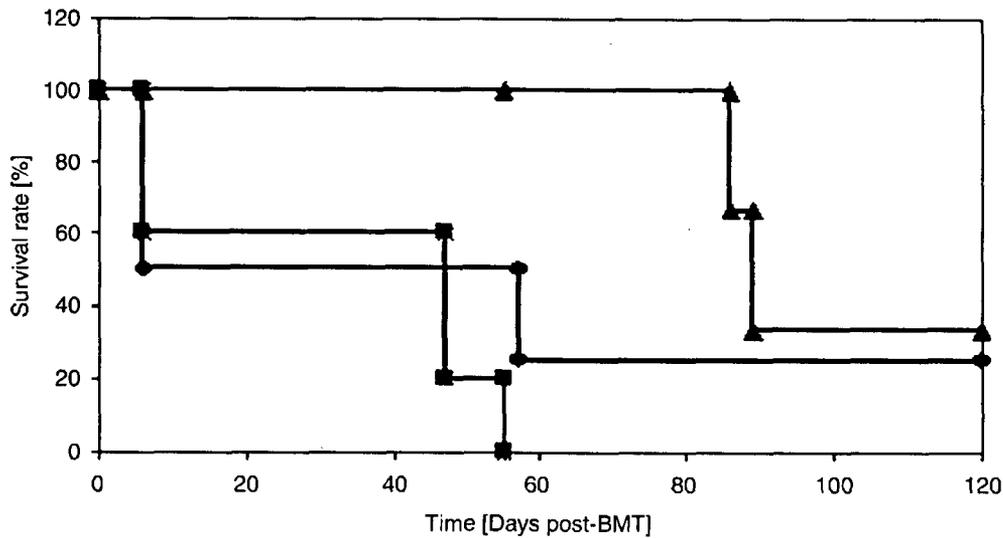


Fig. 2. Lethal graft-vs-host disease after irradiation by a particle accelerator (5 MeV) in cages of different height (2.5 cm, 5 cm).  $K^b$  mice were reconstituted with  $5 \times 10^6$  Des-T-cell antigen receptor (TCR) (anti- $K^b$  TCR) bone-marrow cells. Three experimental groups were analyzed: (1) 12 Gy irradiation, 5 cm height,  $1 \times 10^6$  added lymph-node cells (LNC); (2) 11 Gy, 2.5 cm,  $1 \times 10^6$  added LNC; (3) 11 Gy, 2.5 cm,  $5 \times 10^6$  added LNC.

bone marrow was higher in a group that was irradiated with a smaller dose (11 Gy) of a particle accelerator in a smaller box (2.5 cm high;  $n = 5$ ; survival rate: 0% at 55 d after bone-marrow transplantation) than with a higher dose (12 Gy) of the same particle accelerator in a higher box (5 cm high;  $n = 3$ ; survival rate: 33% after 89 d post bone marrow transplantation) (unpublished, Fig. 2). This can be explained by the build-up effect of radiation beams. With too much space between lid and the irradiated objects, the build-up dose will be lost and has to be “rebuilt” in the object itself. In this scenario, the dose maximum is not optimally focused, which can lead to misleading results. In summary, it is recommended that the irradiation cage should have a maximum height of 2.5 cm.

As mentioned above, another modification to focus the build-up dose and the depth dose, and to optimize the radiation effect, is to place a plate on the cage. Usually, a plexiglas plate is put on top of the cage to distribute the radiation homogenously and to focus the maximum of the radiation optimally on the body of the recipient. The optimum thickness of such a plate can vary from 1 to 4 cm, depending on the radiation device and the employed energy of the radiation source. Alternatively, the use of moulages has been recommended in the literature (16,17) for oncologic radiation patients. Moulages are wax molds (lexan) with tissue-like characteristics adapted to the body forms of the patients. In the experimental situation, the moulages would have to be placed on top of the cage.

3. Different radiation devices are available for GVHD induction in experimental models. The three most commonly used are  $^{60}\text{cobalt}$ ,  $^{137}\text{cesium}$ , and particle (linear) accelerators. The biological effects, for instance with respect to GVHD induction or side effects, differ considerably depending on the radiation source used. Characteristics of these radiation sources are summarized in Table 1.

**Table 1**  
**Characteristics of Different Radiation Sources**

| <sup>60</sup> Cobalt   | Linear accelerator   | <sup>137</sup> Cesium  |
|--|--|--|
| <ul style="list-style-type: none"> <li>• Naturally found radioactive source</li> <li>• Radioactive decay</li> <li>• β- and γ- radiation<sup>a</sup></li> <li>• Energy: 1.25 MV<sup>b</sup></li> <li>• Maximum dose at 0.5 cm depth</li> <li>• Formation of penumbra<sup>c</sup></li> <li>• Half-life: about 5.25 yr</li> </ul> | <ul style="list-style-type: none"> <li>• Electrons are accelerated by electromagnetic high frequency waves</li> <li>• Photon radiation</li> <li>• Used energies (monoenergetic): between 6 MV and 25 MV</li> <li>• Maximum doses with:<br/>23 MV at 3.0 cm depth; 6 MV at 1.5 cm depth</li> <li>• No formation of penumbra<sup>c</sup>; extensive continuous spectrum</li> <li>• No half-life</li> </ul> | <ul style="list-style-type: none"> <li>• Naturally found radioactive source</li> <li>• Radioactive decay</li> <li>• β- and γ- radiation<sup>a</sup></li> <li>• Energy: 662 keV<sup>b</sup> (monoenergetic)</li> <li>• Maximum dose at &lt;0.5 cm depth</li> <li>• Formation of penumbra<sup>c</sup></li> <li>• Half-life: about 30 ys</li> </ul> |

<sup>a</sup> β-radiation is absorbed by the technical device; only the γ-radiation will have an effect.

<sup>b</sup> The <sup>60</sup>cobalt source emits two equally powerful γ-lines of 1.17 and 1.33 MeV. For practical reasons they are calculated as one monochromatic line of 1.25 MeV. In contrast, the <sup>137</sup>cesium source emits only one monoenergetic line.

<sup>c</sup> Within the radiation field, the dose is roughly constant, but decreases towards the margins of the radiation field. In the area within these margins, the dose reduction is geometrically very steep; outside of these margins and the radiation field it converts into a flat runner. The dose value in the main axis of the radiation field is defined as 100%. The distance between two distinct dose values in the area of the radiation field margins (dose reductions) is called "penumbra." The term "penumbra" therefore deals with the flat dose reduction at the margins of the radiation field. Often the distance between the 80% and the 20% value will be given (again, 100% is according to the main field axis). The formation of the penumbra is influenced by the size of the radiation source and its focus, and by the construction of the collimator system. Particle accelerators have a penumbra region of only a few millimeters, which are therefore smaller than those of γ-irradiation devices (17).

**Table 2**  
**Results of Different Irradiation Modalities With the Same Radiation Dose (9 Gy) and Injected Lymph-Node Cell Number <sup>a</sup>**

| Radiation device     | Number of treated mice | Dose (Gy) | Height of cage (cm) | Cell number of injected lymph-node cells | Death rate (Days after BMTx) | Survival rate (%) |
|----------------------|------------------------|-----------|---------------------|--|------------------------------|-------------------|
| Cobalt               | 6                      | 9         | 5                   | 5 × 10 <sup>5</sup>                      | 7, 11 (2x), 14 (2x), 23, 27  | 0                 |
| Particle accelerator | 12                     | 9         | 5                   | 5 × 10 <sup>5</sup>                      | 7, 8, 12, 14, 42, 46, 73     | 42                |
| Cesium               | 5                      | 9         | — <sup>b</sup>      | 5 × 10 <sup>5</sup>                      | 20, 82, 113                  | 40                |

<sup>a</sup> For legend, see Table 1.

<sup>b</sup> For technical reasons, the mice were placed inside a special metal cylinder (18 cm high, base area 58 cm<sup>2</sup>). The metal cylinder with the mice inside rotated around its vertical axis close to the cesium source for equivalent dose distribution.

Experiments were performed to compare the different radiation sources after allogeneic bone-marrow transplantation (Schwarte and Hoffmann, unpublished). Using 9 Gy irradiation with  $^{60}\text{Co}$  and  $5 \times 10^5$  lymph-node cells plus  $5 \times 10^6$  bone-marrow cells, all recipients died from acute GVHD within 30 d. With the same treatment but with the other radiation devices, recipient mice could live longer than 120 d (**Table 2**).

It can be observed that with these other two radiation techniques, the mice developed skin lesions, such as alopecia or ulcerative-erosive skin desquamations, especially after radiation with the  $^{137}\text{Cs}$  source. Acute GVHD could not be induced by these two radiation modalities. In contrast, it was confirmed that the dose of 9 Gy with the latter two radiation sources was lethal in the absence of bone-marrow reconstitution. All irradiated mice died within 14 d, irrespective of the presence or absence of an acute GVHD reaction.

The high lethality in the radiation control groups (without treatment) showed that the radiation was sufficient to induce a lethal tissue injury, particularly of the bone marrow, but was not able to set the recipient into a GVHD-inducible state.

The different reactions to the radiation techniques and their effects on GVHD results can be explained by the underlying physical principles and some immunological concepts.

The main reason for these findings and different GVHD incidence, dependent on the different radiation devices, is the principle of the build-up dose and the depth dose maximum.

One particularly important quality of these radiation devices is the dose build-up effect with increasing tissue depth. After hitting the body surface, the radiation dose changes with increasing depth along the central axis of the beam, which is caused by various physical effects (*18*). The graphic description of these dose distributions in the radiated object is termed *isodose curves*.

The explanation for the phenomenon of the build-up dose is as follows: at first, as the radiation decreases, the dose is reduced. The relative depth dose of the  $^{60}\text{Co}$  source at the surface amounts to about 90%, of the particle accelerator with 5 MeV to 70%, and with 16 MeV to 40% (*17*).

At the surface of the object, only electrons from the radiation source, the surrounding area (mainly air), and backscattered electrons from the object have an effect; in the irradiated object itself, significantly more electrons are removed and transmitted, proportional to the total amount of available photons (so-called photonfluence).

As a consequence of the limited range of liberated electrons, the total sum of electrons increases with increasing depth until they have lost their motion energy. At the end of their course and, therefore, at the end of their defined depth range, a maximum of liberated electrons can be observed. At the same time, the effect of each liberated, secondary electron adds up to the primary photon beam, which is absorbed by the object and is therefore further diminished. On the whole, an increase of dose is achieved up to a defined depth in the tissue, though the primary beam is decreased.

A depth dose maximum of 100% is achieved with the  $^{60}\text{Co}$  source at 0.5 cm, with the particle accelerator at 1.5 cm (6 MeV) to 3.0 cm (23 MeV), and with the  $^{137}\text{Cs}$  source at less than 0.5 cm. Beyond this depth, the dose decreases, mainly due to the effect of photoabsorption (*17,19*).

With the  $^{60}\text{Co}$  equipment, an acute GVHD can be induced in murine recipients. The depth dose maximum is at 0.5 cm. Beyond this point, the dose decreases steeply. Nonetheless, this is sufficient to induce an acute GVHD. The only technical disadvantage is that due to its short half-life (approx 5 yr) a  $^{60}\text{Co}$  source needs to be checked and replaced at regular intervals.

The  $^{137}\text{cesium}$  source has a lower energy (662 KeV) than  $^{60}\text{cobalt}$  (1.25 MeV) and the linear accelerators. Therefore, the dose depth maximum is  $<0.5$  cm, which is quite close to the surface, even if the dose is built up appropriately under optimized conditions. Below this point, the dose decreases even more steeply than with a  $^{60}\text{cobalt}$  source. This is why the irradiation is most effective at the surface, but significantly decreases in effectiveness with increasing tissue depth. Due to these considerations,  $^{137}\text{cesium}$  is no longer used as a telegamma radiation device in the clinical routine, but only for brachytherapy of tumors (20). Furthermore, the surface effects can explain why in experimental models so many recipient mice develop skin lesions compared to other radiation devices ( $^{60}\text{cobalt}$ , linear accelerator): the maximum radiation effect at the surface leads to a heavy injury of the skin, resulting in a high incidence of skin lesions, but without the effects in the depth. In turn, the recipient organism is not sufficiently immunosuppressed to allow for the induction of acute GVHD; only a mild, chronic GVHD can be induced. Other studies prove the interdependences between radiation source and the induction of GVHD (21).

It has previously been observed that organisms vary in their biological response to the different radiation techniques. With  $^{137}\text{cesium}$ , a higher secretion of IL-1 and IL-6 could be detected, whereas with a conventional X-ray device, a higher secretion of interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  was identified (21).

In contrast, linear accelerators use more energy than the  $^{60}\text{cobalt}$  source. The maximum depth dose is at 1.5 cm (6 MeV) or 3.0 cm (23 MeV). Considering the small size of an animal such as a mouse for irradiation, problems with the use of higher energies (e.g., 23 MeV) may occur. The dose lethally damages the organism. However, irrespective of the death of the irradiated mouse, the suboptimal distribution and depth dose, the same effect as with the  $^{137}\text{cesium}$  source will be obtained. If lower energies are used, irradiation will have to be optimized, i.e., the cage design and the plate for the build-up dosing must be adjusted (2.0 cm for a 6 MeV linear accelerator and 4.5 cm for a 25 MeV linear accelerator).

It was determined that the mouse strains respond differently to irradiation (22). BALB/c, C3H/OuJ, C3H/HeJ, and C57BL/6 mice were exposed to  $^{60}\text{cobalt}$  and  $^{137}\text{cesium}$  sources. The lethality rate decreased in the following sequence: BALB/c  $>$  C3H/OuJ = C3H/HeJ  $>$  C57BL/6. The C57BL/6 mouse strain was, therefore, the most radiation resistant. The reason for this difference is still unclear. It is assumed that probably DNA-repair systems in some strains do not work as efficiently as in other strains, or that the pool of radiosensitive stem cells differs between the mouse strains.

4. Only the addition of mature T-cells from secondary lymphatic organs, such as lymph nodes or spleen, to the bone-marrow inoculum can induce acute GVHD in the mouse (see Note 2 for further details). With that in mind, the number of added cells determines the form and clinical course of the GVH-reaction (23). In a murine GVHD-model with CBA-mice as donors and BALB/c mice as recipients, which had been irradiated 5 to 6 h before bone-marrow transplantation with 750 cGy ( $\gamma$ -irradiation), the recipients were given  $5 \times 10^6$  CBA bone-marrow cells with varying numbers of spleen cells. Less than  $1 \times 10^5$  spleen cells did not lead to any GVH reaction at all, or at least only to a mild course of GVHD;  $2.5$  to  $4 \times 10^5$  spleen cells led to a moderate to severe GVHD;  $1 \times 10^6$  or more spleen cells were associated with a hyperacute form of GVHD in which most of the BALB/c recipients died within 8 d after bone-marrow transplantation (4). The various mouse strains differ in their extent of immune responses. That is why a defined number of cells to induce an acute GVHD cannot be given in general. Nevertheless, in all mouse strains, mature T-cells must be added to induce lethal acute GVHD.

5. In the literature, varying numbers of transferred cells can be found. Therefore, many alternatives to this mentioned cell count are possible. The amount of  $5 \times 10^6$  bone-marrow cells is related to the clinical setting, in which the patient is given  $2 \times 10^8$  bone-marrow cells per kg body weight, calculated with an average mouse weight of 25 g.
6. Alternatives for monitoring for a GVHD course: every d for mortality and once a wk for weight changes. It is dependent on the question of interest and of data survey for statistical analysis. The definition of the acute form of GVHD is that it appears during the first 100 d after bone-marrow transplantation; the chronic form of GVHD by definition starts after 100 d. That is why bone-marrow transplant recipients should be controlled for at least 100 d if the acute form is the objective for experiments.
7. Mice developing a chronic GVHD typically show the following disease course: In the first wk after cell transfer, the mice get visibly and evidently ill: they lose weight and show bristled fur. Then the status of a few mice can worsen, resulting in their death within the next 2 wk. In most cases, however, the recipients recover for a certain period of time. These mice exhibit only marginal body weight loss, the skin lesions improve at the end of the second wk after bone-marrow transplantation, and the mice show regular, almost normal physical activity. After 4 wk, the mice develop a severe and progressive body weight loss with diarrhea and a hunched back. Most of them die within the next 1 to 2 wk; the body weight loss can then be severe, sometimes up to more than 40% (24). The described course of chronic GVHD in mice, also in the study cited above (24), is frequently observed. In that study, the chronic GVHD was caused by injection of purified CD8<sup>+</sup> T-cells, without CD4<sup>+</sup> T-cells. That study is seen as evidence that CD8<sup>+</sup> T-cells more likely induce a chronic GVHD, rather than an acute form. Even in standard textbooks this study is cited for this interrelation (25). It is striking, though, that in publications with observations of a chronic GVHD by CD8<sup>+</sup> T-cells, the recipient mice were lethally irradiated by a <sup>137</sup>cesium source (23–29). Therefore, the general statement that CD8<sup>+</sup> T-cells lead to chronic GVHD cannot be maintained. The reaction of the recipient is also influenced by the radiation source during the conditioning for the bone-marrow transplantation. Since the immunological reactions following irradiation and bone-marrow transplantation do not necessarily lead to an acute GVH reaction, but rather to a mild and chronic GVHD, the donor cells somehow react in another way than expected. In another study, three possible concepts were proposed as explanations for this observation (30): the activation-induced cell death, tolerance by internalization of the T-cell receptor, and anergy of the donor T-cells. The immune response of the recipient and the induction of the GVHD is dependent on various factors, e.g., the combination of the mouse strains and the different radiation modalities. In summary, based on the physical-technical characteristics, it is easiest and most reliable to work with a <sup>60</sup>cobalt source for the induction of an acute GVHD, at least in the experimental setting with mice. In contrast, linear accelerators and <sup>137</sup>cesium sources are rather unreliable for this purpose.

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