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BONE MARROW TRANSPLANTATION IN MICE

The ability to engraft mice with a hematopoietic system derived from another mouse provides the opportunity to study a variety of cell functions. Genetic disparities between donor and host owing to mutation, gene extinction, overexpression or selected insertions have provided models to unravel pathways of differentiation, function and even pathology. Prerequisite to success, however, is the application of specialized husbandry technologies. These preferably include HEPA-filtered air and individually ventilated microisolator cages, sterilized food, water and bedding, strict barrier procedures and a pathogen-free environment certified through a sentinel monitoring program. Prospective pathogen free recipient mice should be kept in these conditions from at least 3 days prior to irradiation. All animal transfers and any access to the cages during the study should be done in a BSC or LAF Transfer Station with full garbing and handling standards for barrier maintenance of pathogen free mice. Additionally, recipient mice may be maintained from the three days prior to irradiation on sterile water acidified with approximately 1 ml of 1 N HCl in a 16 oz. water bottle. This is done to reduce bacterial growth in water bottles from regurgitated food. Intestinal ulceration from supralethal whole body irradiation can result in peritonitis and septicemia. This reduces the risk.

Irradiation

Recipient mice are given 850-1100 r whole body lethal irradiation from an x-ray or gamma irradiation source. Commonly, irradiators produce or may be set to deliver about 85 - 165 cGy/min. The RAD SOURCE Technolgies RS2000 is an excellent device to accomplish this. The irradiation procedure, itself, is painless.

Generally more than 850 cGy are necessary to achieve total ablation of host hematopoietic tissues, necessary for a complete lymphohematopoietic engraftment. Mixed chimerism will result if less than 100% of the host hematopoietic stem cells (HSC) are eliminated. This is a greater dose than that which will result in 100% mortality. Lower whole body doses may result in sufficient HSC ablation to prevent survival of the mice, but not be sufficient to ablate all the HSC. This distinction is critical since lethal doses will be less than the supralethal doses required for extinction of all host HSC.

Since radiosensitivity decreases with age, it is best to use recipients that are >12 weeks old. Also, some strains of mice are more susceptible to doses of irradiation than others. BALB/c mice are particularly sensitive. Similarly, some mutations, especially those that affect DNA repair mechanisms, will exhibit increased radiosensitivity. Sufficient supportive care (strict barrier husbandry procedures) and adequate HSC replenishment are necessary to bridge the gap between ablation and repopulation.

Donor cell preparation

The donor HSC population is normally obtained from the long bones of isogeneic mice, preferably 4-6 weeks old. The younger animals tend to have higher concentrations of stem cells within their marrow cavities. Normal 18-22 g mice yield approximately 18-22 x 10^6 nucleated cells/femoral marrow. One tibia would yield approximately 8-12 x 10^6 cells and 1 humerus will generally hold 2-6 x 10^6 cells. Therefore one could anticipate obtaining about 50-75 x 10^6 cells/6 long bones from a single donor animal. Other, less common sources of HSC include the pelvic bones, spleen and fetal liver. It is generally considered sufficient to administer 1-2 x 10^7 marrow derived cells/recipient for a successful engraftment. As few as 5 x 10^6 cells may be successful but some recipients may die. Conditions that affect HSC in the donor population will necessarily impact the requisite number of cells.

Donor animals are preferably euthanatized by cervical dislocation. Harvest of bone marrow cells for transplant needs to be done rapidly after death without intervention of chemical means that could complicate the functional status of stem cells. Published results indicate CO_2 euthanasia can affect several parameters potentially of significance to hematopoietic cell populations (see Selected References). Time to death with CO_2 is reported to be between 45 and 60 seconds. During this time mice can be excited and irritated. Skillful use of cervical dislocation for euthanasia results in a more rapid death, usually within 15 seconds. This allows for a prompt stem cell harvest.

Following euthanasia, the abdomen is swabbed with alcohol or other appropriate disinfectant that has the added benefit of wetting the fur. The abdominal skin is cut and the skin removed from the animal in a rapid motion that pulls it off the torso and down onto the extremities. The long bones of the legs are cleared of adherent muscle tissue with the scissors and a scalpel. Whole bones are removed by cuts at the head of the femur and at the knee joint. The tibia and femur from each side are removed and scraped clean using a scalpel on a sterile field. The termini are removed at the epiphyses with the blade of the scalpel. The shaft of the bone is held by a forceps and sterile balanced saline solution used to flush the contents of the marrow into a sterile conical centrifuge tube. The cell suspensions should be collected and maintained on ice during the entire procedure and cold isotonic fluids should be used. A 22 gauge needle, fitted on an appropriately sized syringe, is adequate. The volume of fluid used to flush the marrow should be no less than 3 ml and generally not more than 10 ml. The suspending fluid can be RPMI 1640, Hank's or Dulbecco’s BSS or any number of other isotonic salt solutions. It is not necessary that the media be nutrient but only isotonic to maintain the cells viable until injection. For multiple flushes a sufficient volume of fluid may be withdrawn from the tube after larger marrow clumps from prior flushes have settled, reducing the reflushing of cells. [Note: It is best to use an 18 gauge needle to refill the syringe so that suspended cells are less disrupted by shear forces when drawn back into the syringe. The shear forces are quite considerable within the barrel of the syringe and the shaft of the needle.] This procedure is repeated for each of the bones, pooling their contents.

Once the marrows are flushed, a sterile Pasteur pipette or plastic transfer pipette is used to aspirate the cell suspension. A minimum of 35 aspirations will insure adequate dispersal of the cells. This can be verified visually with hemocytometer. The bone marrow disperses quite readily. An aliquot of the suspension is taken for viable cell count in an hemocytometer. Viability, not just enumeration electronically, is necessary. If greater than 10% of the cell population is non-viable, something is wrong with the procedure and it should be checked. Generally greater than 95% viability is seen. By using the estimates of # cells/long bone, and knowing the number of cells required and # of injections to be done, it is possible to calculate the number of cells per ml and the total number of cells necessary for the transplantations.

Cell transfer

Once the number of viable cells is determined in the suspension and the appropriate dilutions are made, aliquots are injected into a lateral tail vein of the mouse. The procedure is challenging and survival of the mouse is wholly dependent on success. It sounds quite simple, but is not. One of the 1ateral tail veins is used to access the circulatory system; use of a 26 or 27 gauge need1e is preferable, fitted onto a 1 cc syringe with the volume markings up. The injection volume should be somewhere between 2/10 and 5/10 of an m1 and dilutions made so the final number of injected cells will be delivered in this volume. Cell suspensions are drawn up, a mouse placed in an appropriate holder and tail swabbed with alcohol and then dried immediately with a gauze or "Kim Wipe". The needle is inserted into the tail vein, bevel up, and the appropriate amount of cell suspension delivered. A successful injection is readily known because the vein blanches out ahead of the injection and the fluid volume is freely delivered, i.e., no

resistance. No anesthetic is used for this procedure. Animals are then returned to their cages and cards marked. There is no need to change needles between injections. However, with 26 – 30 ga needles, the thickness of the tail skin will dull them after a few (3 – 6) injections. When replacing the needle, realize that the amount of air in the hub of a fresh needle is sufficient to cause a fatal air embolism.

Assessing results

In a successful graft, one can normally expect complete hematopoietic repopulation within 14-20 days and immunological reconstitution with 21-35 days. After about 30 days, mice are effectively reconstituted and routine husbandry practices may be applied. In the absence of complications from infection, animals do not evidence any painful reactions to the procedure.

Classic signs of discomfort and unthriftiness need to be monitored in all mice. These include, but are not limited to: inappetance, inability to obtain feed and water, difficulty breathing, diarrhea, infection, circling, convulsions, flaccid or spastic paralysis, >20% loss of body weight, moribund or premoribund state. In general fewer than 10% of recipient mice die from marrow transplants done correctly. If they do, several things may be considered:

- the radiation dose may have been too high (inappropriately calculated);
- the HSC content of the infusion population may have been too small;
- supportive husbandry was inadequate;
- genetic or strain susceptibilities were not considered;
- recipients were too young;
- histocompatiblity was not considered.

Incompatible bone marrow transfusions generally do not lead to acute Graft Versus Host Disease (GVHD). Normal spleen cell populations will. However, one will find secondary disease, i.e. delayed GVHD in animals from 21 days after engraftment. This presents as splenomegaly, unthriftiness, kyphosis and finally death.

Determination of mixed chimerism in Bone Marrow engraftment

One can determine the % donor engraftment by any number of strategies. In some studies it may not be necessary. However, as a general rule it is advisable to establish this. HSC from donor animals with a CD45.1 background may be used to rescue recipients with a CD45.2. A gender difference is also possible to use, male donors and female recipients (but not the reciprocal). Percent engraftment may then be determined by PCR for X and Y chromosome-specific genes. Either procedure should be done after a minimum of three weeks. Peripheral blood may assessed by flow cytometry for the percentage of CD45.1 (donor) and CD45.2 (recipient) leukocytes using standard techniques. PCR requires isolation of nucleated WBC and may be done best with marrow cells harvested after study termination.

Suggested Background References:

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