Immunomagnetic T-Lymphocyte Depletion (ITLD) of Rat Bone Marrow Using OX-19 Monoclonal Antibody

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Abstract  Graft versus host disease (GVHD) may be abrogated and host survival prolonged by in vitro depletion of T lymphocytes from bone marrow (BM) prior to allotransplantation. Using a mouse anti-rat pan T-lymphocyte monoclonal antibody (OX19) bound to monosized, magnetic, polymer beads, T lymphocytes were removed in vitro from normal bone marrow. The removal of the T lymphocytes was confirmed by flow cytometry. Injection of the T-lymphocyte-depleted bone marrow into fully allogeneic rats prevents the induction of GVHD and prolongs host survival.

Keywords: Immunomagnetic T-lymphocyte depletion, bone marrow, allogeneic transplantation, OX-19 monoclonal antibody, graft-versus-host disease.

Graft-vs-host disease (GVHD) is frequently observed after bone marrow transplantation and, in selected instances, following small intestinal transplantation.1,2 This phenomenon is thought to be due to immunocompetent donor cells, especially T lymphocytes, reacting to cells of a histoincompatible, immunologically comprised host.3 Alloreactive, donor-recipient cell–cell interaction with lymphokine release, perhaps enhanced in an unknown way by bacteria or viruses, results in the pathologic damage seen in epithelial-lined organs.4–6 A variety of techniques have been used to remove the alloreactive donor T lymphocytes and prevent GVHD.7–12 However, these methods are generally complex, time-consuming, expensive, and in some cases nonspecific in their removal of T lymphocytes. They may also be toxic to the host. In this manuscript, we demonstrate

Abbreviations: ITLD, immunomagnetic T-lymphocyte depletion; GVHD, graft-versus-host disease; BM, bone marrow; MoAb, monoclonal antibody; HBSS, Hank’s balanced salt solution.
that binding of the pan anti-T-lymphocyte monoclonal antibody (MoAb) OX-19 to
monosized, magnetic polymer beads successfully removes T lymphocytes from
rat bone marrow. This novel technique, which relies upon magnetic separation of
T lymphocytes from bone marrow, is simple, rapid, relatively inexpensive, and
nontoxic, and can be used in a sterile environment.

Methods and Materials

Animals

Forty-five inbred AC(Rti) and LEW(Rtll) rats were bred and purchased from
Harlan-Sprague Dawley (Indianapolis, IN). The animals were housed in conven­
tional facilities and fed commercial rat chow (Wayne Lab-Blox F-6, Chicago, IL),
and acidified tap water ad libitum.

T-Lymphocyte Depletion of Rat Bone Marrow

Magnetic, monosized polymer beads were coated with covalently bound, af­
finity-purified sheep anti-mouse IgG directed against all mouse IgG subclasses.
(Dynal-Dynabeads M450-11001, Great Neck, NY). The beads were incubated
with 10 \mu L. fetal calf serum mg of beads (Fig 1A). After 24 h of incubation at 4°C,
the beads were washed three times in Hank’s balanced salt solution (HBSS), then
incubated with 1 \mu L of OX-19 (Accurate Chemical and Scientific Corp.) for every
milligram of beads using the same incubation conditions (Fig 1B).

Bone marrow was harvested from the tibias and femurs of ACI rats. Cell
counts and viability were determined using trypan blue exclusion. Viability
always exceeded 90%. The bone marrow was incubated for 30 min at room
temperature with OX-19-coated beads in 5–10 mL of HBSS containing 0.05
mg/mL of gentamicin (Fig 1C), with gentle mixing performed every 5 min. Magnetic
separation within a round-bottomed glass test tube was accomplished using a Mag­
netic Particle Concentrator (Dynal MPC-1) containing cobalt–samarium magnets.
The bead/OX-19/T lymphocyte complexes were attracted to the magnet and
quickly immobilized against the test tube wall (Fig 1D). The supernatant, con­
taining stem cells, progenitor cells, and B cells, was easily removed from the test
tube and the T-lymphocyte-depleted mixture adjusted to a cell concentration of
33.3 \times 10^6 cells/mL for reconstitution. Bead-to-T-lymphocyte ratios of both 20:1
and 60:1 were used for T-cell depletion, based upon an estimated T-cell popula­
tion in rat bone marrow of 5%.

Flow Cytometry

The analysis of cell surface-associated markers by flow cytometry was conducted
by placing 1 \times 10^6 bone marrow cells (±ITLD) in 12 × 75-mm glass tubes in 0.1
mL of staining buffer (PBS, pH 7.4, 0.1% NA Azide, 2% FCS). The test antisera
or normal serum was added (1:10 to 1:100 final dilutions) for 30 min at 4°C. The
cells were washed twice and resuspended in FITC-labeled anti-IgG directed
against the primary antibody (rat anti-mouse IgG, H and L chain specific, Boe-
Immunomagnetic T-Lymphocyte Depletion

**Step 1:**

Monosize Magnetic Bead + Fetal Calf Serum (11110 (101l/mg bead) → 24 hr 4°C

**Step 2:**

OX-19 mouse a rat + MAb directed against all T cells (1 lI11mg bead) → 24 hr 4°C

Figure 1. Preparation of immunomagnetic beads bound to OX-19 monoclonal antibody and the T-lymphocyte depletion technique. **Step 1:** The beads already bound with sheep anti-mouse IgG are incubated for 24 h at 4°C with fetal calf serum to bind unbound, nonspecific sites. **Step 2:** OX-19 is incubated under the same conditions and attaches to the sheep anti-mouse IgG. **Step 3:** The bead/OX-19 complex binds to T lymphocytes present in the ACI bone marrow by virtue of the affinity and avidity of the MAb for T lymphocytes. **Step 4:** A cobalt–samarium magnet attracts the beads that contain 20% magnetite to the side of the test tube. The T lymphocytes remain bound to the OX-19 during this process. The supernatant is T-lymphocyte-depleted ACI bone marrow.

hinger-Mannheim, Indianapolis, IN). After a 30-min incubation at 4°C, the cells were washed twice and analyzed for fluorescence staining on an Ortho Spectrum III (Ortho Diagnostics, Braintree, MA) flow cytometer. Following ITLD, the magnetic bead/lymphocyte mixture was diluted 10:1 and 40:1 with HBSS and stained by a Wright-Geimsa technique. The presence of cell-bead rosettes which consisted of 6–8 beads clustered as a rosette around small, mature lymphocytes was microscopically confirmed.
Lewis rats were allowed to acclimatize for 1–2 weeks in the experimental animal facility and were fed acidified water containing tetracycline hydrochloride (100 mg/L) and neomycin sulfate (10 mg/L). A dose of 1000 rads of total body irradiation using a $^{137}$Cs source at 97 rads/min was administered to the recipient Lewis rat 4–6 h prior to reconstitution. The recipient was anesthetized with 3.6% chloral hydrate by intraperitoneal injection and surgically prepared with Betadine and 70% ethanol. An upper midline celiotomy was performed. The intestines were eviscerated and retracted to the left upper quadrant. A total of $50 \times 10^6$ bone marrow cells from ACI rats (with or without ITLD) suspended in 1.5 mL of HBSS were injected into the exposed infrahepatic vena cava above the renal veins through a 27-gauge needle (Fig 2). Hemostasis was secured and the viscera

**Reconstitution of Rat Bone Marrow**
were restored to their normal intra-abdominal position. The fascia and skin were closed in two layers with 4-0 Dexon.

**Recognition of GVHD**

The diagnosis of GVHD in the irradiated recipients was based upon previously described clinical and histopathologic findings.\textsuperscript{13,14} All 21 recipient animals were assessed for clinical GVHD. An animal was considered to exhibit clinical GVHD if four of the following signs were observed: diffuse erythema, hyperkeratosis of the foot pads, dermatitis, weight loss, generalized unkempt appearance, or diarrhea. Ten necropsy specimens from the non-ITLD group were taken of the ear, tongue, liver, spleen, intestine, and mesenteric lymph nodes on days 18–25 and assessed for pathologic examination. Five ear biopsies from the ITLD group taken on days 30–60 were also examined. Specimens were processed routinely for light microscopy. The important microscopic histopathologic features observed were the same as previously reported.\textsuperscript{15,16}

**Results**

**Nonspecific Cell Loss**

Rat bone marrow contains approximately 6% T lymphocytes.\textsuperscript{17} A 1:1 bead-to-BM cell ratio is approximately equivalent to a 20:1 bead-to-BM T-lymphocyte ratio.
ITLD using a ratio of 20:1 beads to BM T lymphocytes resulted in an overall loss of 8–15% of the bone marrow cells. This suggests mild to moderate nonspecific cell binding by the beads or bead/OX-19 complexes. When a 60:1 ratio was used, nonspecific cell loss was 31%. Use of either of these ratios did not prevent engraftment of ITLD BM cells in an allogeneic host.

**Flow Cytometry**

T-lymphocyte depletion is confirmed by flow cytometric measurements which demonstrated at least a $1.5 \log_{10}$ depletion of T lymphocytes when compared to untreated ACI BM cells (Fig 3). Flow cytometric analysis of untreated ACI BM cells mixed with FITC conjugated OX-19 contains 5.9% T lymphocytes. Depletion of the bone marrow with ITLD reduced the T lymphocytes to a maximum of 1.8%. The percentage of T lymphocytes in this latter group may be artifically elevated due to poor resolution at the lower limits of the flow cytometer capabilities.

**Elimination of GVHD**

Eleven lethally irradiated Lewis rats developed severe clinical and histopathologic manifestations of GVHD after transplantation with ACI BM. Ninety percent
Figure 3. Flow cytometric studies of ACI rat bone marrow before and after ITLD. (A) FITC-conjugated OX-19 is bound to untreated ACI bone marrow (5.9% T-lymphocyte population of 18,772 total cells). (B) FITC-conjugated OX-19 is bound to ITLD ACI bone marrow (1.8% T-lymphocyte population of 9654 total cells). Note the absence of fluorescent cells of the ITLD group (along the x axis) indicating the removal of T lymphocytes by this technique. This trial is representative of at least a 1.5 log depletion of T lymphocytes.

of the recipients were dead by 25 days and all were dead by 48 days. Ten lethally irradiated Lewis rats receiving ITLD ACI BM engrafted and survived for greater than 165 days. No clinical or histopathologic evidence for acute GVHD was observed in this group of animals.

The most consistent and sensitive measure of the presence of GVHD is the appearance of single basal epithelial cell necrosis with lymphocyte infiltration in the tongue and the ear (Fig 4). The livers of affected animals frequently exhibited lymphocytic infiltration of the portal triads with focal cellular injury to the bile duct epithelium. Other histopathologic changes included lymphocytic depletion of lymph node paracortical areas and splenic arteriolar sheaths.
Figure 4. Photomicrographs of the epidermis of the ear of lethally irradiated Lewis rats. (A) Received untreated ACI BM 22 days previously. Single epithelial cell necrosis with an adjacent lymphocyte (satellitosis) is seen. (H & E ×560). (B) Received ITLD ACI BM 34 days previously. Normal epithelium. (H & E ×450).
Discussion

The incidence of GVHD and the resultant morbidity and mortality to the host after bone marrow transplantation is eliminated by T-lymphocyte depletion of the donor BM. Various methodologies have been developed to improve the specificity and efficiency of the T-lymphocyte depletion with variable results. These methods include irradiation, lectin soybean agglutination/sheep red blood cell rosetting, immunoabsorption using a Sepharose column, immunotoxins, complement mediated cytotoxicity, and pharmacologic inhibition.

Irradiation successfully eliminates T lymphocytes but is nonspecific and radioresistant T-lymphocytes can be spared. Physical separation techniques take advantage of the cell surface molecules that phenotypically identify T lymphocytes and their individual subsets. Pan T, helper T, and suppressor T cell populations may be isolated through the use of MoAb in human or animal bone marrow models. T cells may be eliminated from bone marrow by sheep red blood cell rosetting and enhanced by soybean agglutination of the non-rosetting cell population. Immunoabsorption of T lymphocytes using monoclonal antibodies bound to Sepharose is effective and has the advantage of individual subset removal. However, SRBC/soybean agglutination and immunoabsorption procedures are complex and time-consuming. Immunotoxins such as ricin may also be used; ricin linked to specific monoclonal antibodies can selectively kill T lymphocytes. Complement-fixing monoclonal or polyclonal antibodies successfully deplete bone marrow of T cells and inhibit GVHD, but certain complement systems may be toxic to bone marrow precursor cells and many monoclonal antibodies, including those that define rat T-lymphocyte subsets, do not fix complement. In addition, the immunotoxin and complement systems may harm the progenitor cells or the stromal microenvironment. Pharmacologic methods, such as methotrexate and cyclophosphamide, are relatively ineffective at preventing GVHD in humans. GVHD has been reportedly abrogated by cyclosporine and anti-thymocyte globulin, but the results have been inconsistent and toxicity may be a concern.

Monosized, magnetic polymer beads were initially developed to improve resolution in liquid chromatography. The addition of magnetite (20% by weight) to the beads allows them to be used for magnetic separation. The surface of the bead is partly hydrophobic, allowing antibodies to be bound by physical adsorption, and partly hydrophilic due to the presence of hydroxyl groups that can be chemically activated to covalently bind antibodies.

OX-19 is a MoAb derived by fusing spleen cells from a mouse immunized with rat thymocyte glycoproteins. The antibody detects a cell surface-associated glycoprotein that is present on thymocytes and mature peripheral T lymphocytes. OX-19 does not react with cells other than lymphoid cells in the intestine, thymus, spleen, and lymph nodes. When the OX-19/bead complex is incubated with rat bone marrow, T lymphocytes within the marrow bind to the MoAb-bead complex and magnetic separation depletes the marrow of T lymphocytes.

The magnitude of the depletion is difficult to establish due to the small number of mature T lymphocytes in the bone marrow and the potential generation of additional T lymphocytes from the remaining progenitor cells. The percentage of OX-19 lymphocytes in normal ACI marrow is approximately 6% and ITLD re-
roduces the number of OX-19 cells to levels of less than 2%. This procedure has a potential for efficient removal of lymphocytes from the bone marrow; up to a 6 log depletion of Rael Burkett lymphoma cells from human bone marrow has been obtained using 2 cycles of immunomagnetic depletion. Our preliminary evidence that rats do not develop GVHD after receiving ITLD-treated allogeneic bone marrow demonstrates that the T lymphocytes have been reduced below a level functionally capable of inducing GVHD.

ITLD is a novel, simple, rapidly performed in vitro technique that is nontoxic to the recipient. It is relatively inexpensive: $4 \times 10^9$ rat bone marrow cells can be depleted of T lymphocytes using 10 mL of immunomagnetic beads and small amounts of MoAb. The availability of specific monoclonal antibodies to a variety of cell surface molecules makes this immunomagnetic bead a model with a wide range of potential applications.

Summary

A highly efficient technique of T-lymphocyte depletion using rat bone marrow is described. It involves the binding of OX-19, a MoAb directed against all rat thymocytes and mature peripheral T lymphocytes, to monosized, magnetic polymer spheres. Magnetic separation of T lymphocytes after mixing the allogeneic bone marrow with the bead/OX-19 complex provides for a simple, rapid depletion of T lymphocytes from the bone marrow. In vitro studies using flow cytometry and the prevention of GVHD in a fully allogeneic rat bone marrow model have been used to demonstrate the effectiveness of the depletion procedure.

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References

Immunomagnetic T-Lymphocyte Depletion


