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BRIEF COMMUNICATIONS

EVIDENCE FOR ENGRAFTMENT OF HUMAN BONE MARROW CELLS IN NON-LETHALLY IRRADIATED BABOONS[1]

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Background. **Prior to organ harvesting, an attempt was made to modulate the donor's immune responses against prospective xenogeneic recipients by infusion of "recipient-type" bone marrow.**

Methods. **For this purpose, baboons conditioned with total lymphoid irradiation were given 6×10^8 unmodified human bone marrow cells/kg body weight with no subsequent treatment.**

Results. **Animals survived until they were euthanized at 18 months. Using primers specific for human chorionic gonadotrophin gene, the presence of human DNA was confirmed by polymerase chain reaction in the blood of one animal for up to 18 months after cell transplantation; in the other animal, xenogeneic chimerism became undetectable in the blood at 6 months after bone marrow infusion. However, tissue samples obtained from both animals at the time they were euthanized had evidence of donor (human) DNA. Additionally, the presence of donor DNA in individually harvested colonies of erythroid and myeloid lineages suggested that infused human bone marrow cells had engrafted across the xenogeneic barrier in both baboons.**

Conclusions. **Bone marrow transplantation from human to baboon leads to establishment of chimerism and modulation of donor-specific immune reactivity, which suggests that this strategy could be reproducibly employed to create "surrogate" tolerogenesis in prospective donors for subsequent organ transplantation across xenogeneic barriers.**

Insight into the probable mechanisms of allograft and, by inference, xenograft acceptance has been provided by the recent discovery of persistent ubiquitous donor cells in long-surviving human organ recipients (*1*, *2*). It has been demonstrated in humans and in experimental models that these donor cells seeded from the transplanted organ are multilineage "passenger leukocytes" of bone marrow (BM*) origin and include stem cells (*3*, *4*). We have postulated that the commingled donor–recipient immunocyte populations progressively become mutually nonreactive (tolerant). Their reciprocal nullification may explain why major histocompatibility complex-disparate organs of all kinds (*1*, *2*) and unaltered donor BM can be safely engrafted in noncytoablated recipients without causing graft-versus-host disease (GVHD) (*5*, *6*).

We report here observations suggesting that the two-way paradigm will be relevant in strategies for xenotransplantation. Unmodified human BM cells were infused into baboons that had been conditioned by total lymphoid irradiation (TLI). Although TLI is cytoreductive, it leaves the recipient arm of the two-way paradigm partially intact, with a consequent low incidence of GVHD. Unequivocal evidence was obtained of xenoengraftment and maintenance of long-term, widespread, multilineage human leukocyte chimerism without GVHD or other adverse effects.

Three healthy juvenile baboons (*Papio anubis*) were obtained from the Southwest Foundation for Biomedical Research (San Antonio, TX) and housed at the Montefiore Hospital, University of Pittsburgh Medical Center. This is an American Association for Accreditation of Laboratory Animal Care approved facility. The animals were quarantined and rigidly screened for viral and microbial infections before and after arrival in Pittsburgh. After induction of anesthesia with ketamine (10 mg/kg i.m.; Parke-Davis, Morris Plains, NJ), maintenance supplementation with isoflurane (FORANE, Omega, Liberty Corner, NJ), and endotracheal intubation, two of the baboons were submitted to 750 cGy of TLI (cobalt-60; Eldorado 78 Teletherapy Unit, AECL, Ottawa, Canada); the lungs, liver, head, and limbs were spared. The third

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* Abbreviations: BM, bone marrow; GVHD, graft-versus-host disease; MLR, mixed leukocyte reaction; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; TLI, total lymphoid irradiation.

animal, which received 750 cGy of total body irradiation, died from BM aplasia after 14 days and will not be discussed further.

Immediately after the TLI, 6×10^8/kg unmodified human BM cells were infused into the baboons through an intravenous catheter placed in the femoral vein, with no change in vital signs. The cryopreserved BM cells had been procured from 32- and 10-year-old cadaveric human donors, as described elsewhere (*5*). The animals were administered prophylactic therapy for *Candida*, Gram-negative bacteria, and herpes virus. The two animals (M10 and M11) treated with TLI appeared healthy throughout the course of the experiments and showed no signs of GVHD. After 18 months, both were euthanized with an overdose of sodium pentobarbital (Abbott Laboratories, Chicago, IL).

Evidence of donor leukocyte chimerism was sought in peripheral venous blood samples 3, 6, and 12 months after reconstitution, and in blood and multiple tissue samples collected at the time the two animals were euthanized at 18 months. Chimerism was looked for in the baboon blood and tissue samples, using immunohistochemical and polymerase chain reaction (PCR) technologies. The immunohistochemical and flow cytometric analysis yielded inconclusive results because monoclonal antibodies directed against either donor HLA class I or human CD45 (pan-leukocyte marker) cell surface antigens exhibited marked cross-reactivity with baboon cells. This observation was confirmed by a microlymphocytotoxicity assay (Biotest AG, Dreieich, Germany) and Terasaki second HLA 72 well tray (lot 11, One Lambda, Inc., Canuga Park, CA).

Consequently, we depended exclusively on PCR for the determination of chimerism, using probes directed against the human chorionic gonadotropin gene. Primers (5′ AACCCCGTGGTCTCCTACG-3′ and 5′ GGGAGGATCGGGGTGTCC-3′) were selected from the middle exon of the β-subunit of human chorionic gonadotropin, and were specific for humans and failed to amplify the corresponding baboon sequence. The specificity of the method was established by amplification of DNA obtained from the peripheral blood mononuclear cells (PBMC) of the normal healthy humans and baboons that served as positive and negative controls, respectively. Fresh or frozen vertebral BM cells and PBMC were examined. In addition to direct examination of these specimens, the PCR assay also was used to detect the presence of human cells in colonies obtained from hematopoietic progenitor cell assays.

All other tissues were fixed in formalin at the time of euthanasia, necessitating formaldehyde extraction before DNA purification. This was achieved by placing a small piece of the tissue into a seamless cellulose dialysis tubing (cut-off, 12 kDa) which was subsequently dialyzed for 12 hr at room temperature against 4 L of buffer (10 mM Tris HCl and 1 mM EDTA [pH 8.0]). After two sets of hot-start PCR amplifications (35 cycles/set), the products were resolved by electrophoresis on an agarose gel and visualized using ethidium bromide staining. To preclude false positive or negative results, comparably aged (in formaldehyde) fixed tissue samples (liver) obtained from normal humans and baboons were used as appropriate controls. Additionally, to rule out contamination, three independent sets of molecular analysis were performed on freshly isolated DNA. The sensitivity of the PCR assay was 10^{-2} (1:100).

Evidence for human cell engraftment was obtained by hematopoietic progenitor cell assays. When the baboons were euthanized, their vertebral columns were resected *en bloc* for subsequent isolation of the BM cells that were used in this assay. A known concentration of cells (2×10^5) was suspended in Metho Cult GF H4434 medium (Stem Cell Technologies, Vancouver, BC) and dispensed in triplicate into 1 ml of tissue culture plates and incubated for 14 days at 37°C in 5% CO_2 in air. At the end of incubation, colonies of burst-forming unit–erythroid (BFU-E), colony-forming unit–granulocyte and macrophage (CFU-GM), and granulocyte colony-forming unit–granulocyte-erythroid, megakaryocyte, and macrophage (CFU-GEMM) were scored. PCR was used to identify handpicked colonies that had donor DNA.

To assess recipient's immunocompetence, a one-way mixed leukocyte reaction (MLR) was performed with the PBMC obtained from the chimeric baboons at the time they were euthanized. A constant dose (5×10^4 cells/well) of freshly isolated baboon PBMC (used as responders) was co-cultured for 6 days at 37°C with equal numbers of gamma-irradiated (20 Gy), cryopreserved, third-party human splenocytes (used as stimulators) in a 96-well, round-bottom microtiter plate (Becton Dickinson, Lincoln Park, NJ) in 5% CO_2 in air. For the final 20 hr, 1 μCi of [^{3}H]-thymidine (NEN, Boston, MA) was added to each well and its degree of incorporation was determined by liquid scintillation counting. As controls, responses of M10 and M11 PBMC were determined against gamma-irradiated autologous and allogeneic (baboon) PBMC. Results were expressed as mean counts per minute (cpm).

Serial blood samples were also obtained to ascertain the

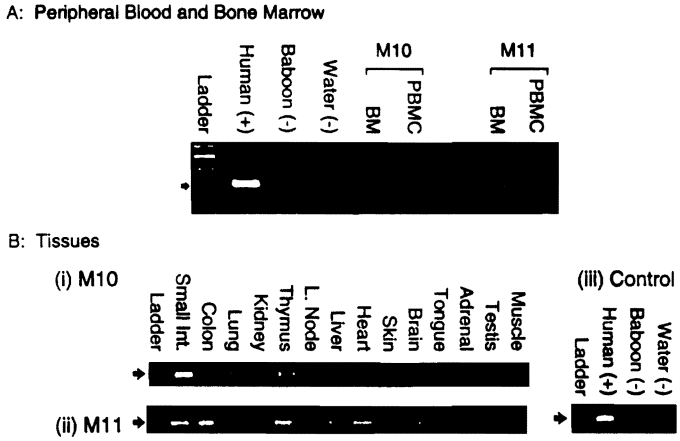

FIGURE 1. (A) PCR analysis of BM and PBMC obtained from animals (M10 and M11) at the time they were euthanized (18 months after reconstitution). Amplification was performed using primers specific for the 203-basepair segment of the human chorionic gonadotropin gene, and the products were resolved by electrophoresis on a 1.5% agarose gel (see text). The bands were visualized using ethidium bromide staining. DNA obtained from the PBMC of normal human and baboon served as positive and negative controls, respectively (arrow: 203-basepair product of interest). (B) Detection of human DNA in formaldehyde-fixed–dialyzed tissues obtained at the time of death (18 months after transfusion) from two baboons (i: M10; ii: M11) who underwent cytoreductive conditioning with TLI before reconstitution with human BM. For controls (iii), same-aged (in formaldehyde), fixed-dialyzed tissue (liver) obtained from normal human and baboon were used as positive and negative controls, respectively (arrow: 203-basepair product of interest).

TABLE 1. Detection of donor (human) DNA in the colonies obtained from hematopoietic progenitor cell assays (HPCA) generated from baboon BM cells harvested at the time of death[a]

Animals	BM cells	HPCA[b]		
		BFU-E	CFU-GM	CFU-GEMM
M10	+	+	+	+
M11	+	+	+	+

[a] Animals were euthanized 18 months after reconstitution.
[b] BFU-E, burst-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte-macrophage; CFU-GEMM, colony-forming unit-granulocyte-erthyroid megakaryocyte-macrophage.

presence of donor cells by PCR. In one baboon (M11), donor (human) DNA was detectable in all peripheral blood specimens, whereas in baboon M10, it was not found in the 12-month blood samples. However, human DNA was found in the blood, BM (Fig. 1A), and all tissue samples (Fig. 1B [i]) obtained at the time baboon M10 was euthanized, with the exception of muscle. This distribution of donor DNA was as impressive as that in baboon M11, in whom all specimens taken at death (except lung) were positive (Fig. 1B [ii]). Furthermore, as presented in Table 1, donor DNA was present in individually harvested colonies of BFU-E, CFU-GM, and CFU-GEMM, thereby confirming stem cell engraftment. It should be noted that human cytokines were used for the generation of these colonies, which allowed for relatively preferential expansion of hematopoietic stem cells of human origin. Additionally, moderate proliferative activity was also evident in cells obtained from naive baboons when cultured under similar conditions, which suggests the partial cross-reactivity of the biological effects of these cytokines. However, despite these observations, no attempt was made to ascertain the proposition of colonies of various origin, thus obviating the determination of the level of human xenochimerism.

These findings indicate that under the appropriate circumstances, chimerism can be readily and safely induced across the human→baboon barrier. Although we were initially surprised, this was consistent with previous observations that isolated xenogeneic cells do not undergo hyperacute rejection even when transplanted from disparate species whose whole organs undergo infarction immediately after revascularization by surgical anastomosis (summarized in [7]). Additionally, using one-way MLR assays, both animals were found to be immunocompetent, exhibiting responses against allogeneic (baboon) and xenogeneic third-party (human) stimulators (Fig. 2). Incidentally, the nonavailability of donor splenocytes and preirradiation PBMCs from the BM recipients precluded any meaningful evaluation of donor-specific immunomodulation in these animals.

With the genetically closer circumstances of baboon→human transplantation, organs are not abruptly rejected (*7*). However, a "slow motion" version of hyperacute rejection occurs, despite complete control of cellular rejection (*7*); in the best-studied cases, there was unmistakable evidence of complement activation (*7*). Consequently, we have concluded that more drastic modification of both the xenografts and their recipients will be necessary for long-term engraftment, even using concordant species (*7*). The studies reported here were designed to explore that possibility.

Most clinically oriented organ xenotransplantation research (*7*) has focused on preventing complement activation directly by removing its precipitating cause (i.e., xenogeneic antibodies) or by making the organ xenograft a less vulnerable target (e.g., the creation of transgenic donors) (*8–10*). Even if successful, these approaches will be only a first step in breaching the genetic barrier of xenotransplantation.

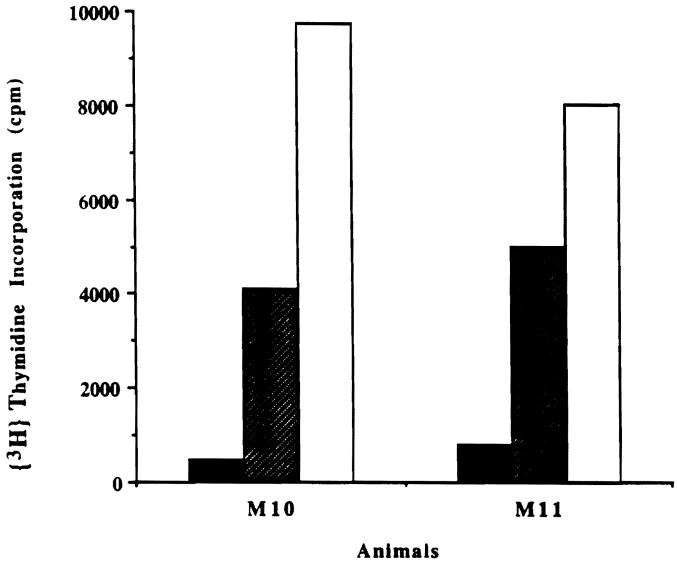

FIGURE 2. A one-way MLR was performed using freshly isolated PBMC (5×10^4 cells/well) obtained from chimeric baboons at the time they were euthanized (18 months after reconstitution) as responders with an equal number of gamma-irradiated cells obtained from various source used as stimulators. Stimulators: (■) autologous PBMC, (▨) third-party human splenocytes, ([□]) allogeneic (baboon) PBMC. The results are expressed as mean of [^{3}H]-thymidine incorporation.

The experiments reported herein could have clinical implications for the next step. The two TLI-conditioned adult baboons given human BM developed persistent human leukocyte chimerism, with evidence suggesting the possibility of stem cell engraftment. Striking evidence for the presence of donor DNA was found in most of the organs commonly transplanted clinically. The ability to induce coexisting xenogeneic donor and recipient leukocyte populations in parenchymal as well as hematolymphopoietic organs may allow various subsequent manipulations (e.g., adoptive transfer of chimeric marrow and/or xenotransplantation of the chimeric organs [*11*]).

It is noteworthy that the achievement of stable transpecies donor leukocyte chimerism is not limited to closely related species. Zanjani et al. (*12*) have successfully produced stable multilineage human donor leukocyte chimerism in sheep by inoculation of fetuses early in gestation with stem cells purified from adult human BM and with nonparenchymal cells from livers of aborted human fetuses. The humanized and ostensibly normal sheep (now 7 years old) have been a prime source of stem cell-rich chimeric BM that has been adoptively transferred to fetuses. Similar observations have also been made in baboon→pig BM chimeras (*11*). Neonatal pigs who received unaltered baboon BM cells had unequivocal evidence for the presence of donor cell chimerism in their peripheral blood and BM for up to 17 months after infusion. Additionally, there was evidence for induction of stable do-

nor-specific immune modulation as determined by in vitro MLR assays (*11*).

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IMPROVED TECHNIQUE OF HETEROTOPIC CERVICAL HEART TRANSPLANTATION IN MICE[1,2]

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Background. **The method for mouse vascularized heart transplantation have been described using suture and cuff techniques. Technical problems have limited its widespread use. Here, we describe our method of modified cervical heterotopic transplantation with the cuff technique.**

Methods. **By using a smaller Teflon cuff (external diameter 0.6 mm, internal diameter 0.4 mm) and superfine-tip forceps, it became possible to directly pull the edge of the carotid artery and evert the proximal end of the artery over the cuff. Similarly, the external jugular vein could be easily everted over a 22-gauge cuff with this direct pulling method.**

Results. **By these modifications, the operation time was reduced. It usually takes 20 min for the donor harvest, 15 min for preparation of the cervical vessels, and 15 min for anastomosis. All procedures from the donor harvest through skin closure of the recipient mice can be completed within 1 hr, and ischemic time is within 25–40 min.**

Conclusions. **This method can be used to investigate cyclophosphamide-induced tolerance and mechanisms of reperfusion injury.**

The technique for heterotopic heart transplantation in mice was first described by Corry et al. in 1973 (*1*). Although this model has been used in some laboratories to study the immunology of graft rejection (*2*, *3*), technical problems associated with the anastomosis of small vessels seem to have limited its widespread use. On the other hand, Matsuura et al. (*4*) reported a method of cervical heterotopic heart transplantation using the cuff technique. Although they stated that their method was easier than other methods, their method still has technical problems. To greater reduce operating time and make this cuff technique easier, we have developed a more simplified method of heterotopic cervical heart transplantation.

Female C3H/He Slc ($H2^k$) mice were bred and maintained at the Institute of Experimental Animals, Kyushu University, Fukuoka, Japan. Female AKR/J Sea ($H2^k$) mice were obtained from the Seiwa Experimental Animal Institute, Oita, Japan. Recipients were used at 12–16 wk of age.

The mice were anesthetized with pentobarbital at 0.5 mg/mg. An operating microscope with 20 times magnification (OME-JA; Olympus Inc., Tokyo, Japan) was used for the procedure. Operations were performed by a single surgeon

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[2] All procedures were recorded and edited on videotape (8 min, VHS or SVHS). A videotape can be sent to the reader upon request to the corresponding author.

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