Editorial

Immunoregulatory role of chimerism in clinical organ transplantation

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Introduction and brief history

Infusions of stem cells and other bone marrow-derived cells are increasingly being used for the possible induction of specific tolerance in solid organ transplantation, besides their conventional use for conferring new immune and myelopoietic systems to immuno-ablated individuals. Even though a wealth of knowledge is available from experimental animal models, this brief review will dwell exclusively on what we know of, or can speculate about, the immunobiology of chimeric cells of donor phenotype residing in human organ transplant recipients.

The classic observations of Billingham *et al*¹ in 1953, that H-2 disparate donor bone marrow-derived cells could bring about specific acquired tolerance to skin allografts in fetal or new-born murine recipients (before self vs non-self recognition occurred in immune ontogeny) laid the foundations for the goal of establishing donor-specific tolerance in human organ transplantation. However, it was not until the late 1970s that it was first observed that multiple non-specific and subsequently donor-specific blood transfusions in humans often led to an improved allograft acceptance in kidney transplant recipients.^{2,3} Subsequently, it was demonstrated that post- or peri-transplant infusion of donor bone marrow cells together with T cell depletion prolonged and sometimes brought about indefinite allograft survival in adult murine, canine and primate recipients in the absence of chronic immunosuppression.^{4–7} These observations have for the most part been associated with the development of chimerism, but a cause and effect relationship has been the subject of controversy.⁸ The first clinical attempt to use donor bone marrow cells in human solid organ transplantation was that of Monaco *et al*⁹ in kidney transplants performed in Boston with concomitant polyclonal anti-lymphocyte globulin induction therapy. Subsequently, Barber *et al*¹⁰ in Alabama reported initial encouraging results in kidney transplant recipients who also received iliac crest bone marrow from human cadaver donors. Cyclosporine, azathioprine, and steroid-based maintenance therapy together with an induction course of polyclonal anti-lymphocyte globulin was used. There was some improvement in short-term graft survival with fewer rejection episodes.

However, the differences with the control group apparently were not as marked in a later report.¹¹ More recently, Fontes *et al*¹² at Pittsburgh, using tacrolimus- and steroid-based maintenance therapy (no antibody induction) have initiated clinical trials in recipients of several types of organ allografts using vertebral body donor bone marrow cells. These studies were derived from the previous observations of this group documenting long-term microchimerism of bone marrow-derived cells in liver and even kidney transplant recipients, some of whom had stopped immunosuppression for several years.¹³ Our own clinical experience with this procedure began in July 1994. Since that time, over 350 liver (or liver/intestinal), 111 kidney, 25 kidney/pancreas, and five kidney/islet transplants have been performed in our center accompanied by donor bone marrow cell infusions.^{14–19}

Chimerism and controversy

The term chimerism was popularized in transplantation biology by Medawar (see Ref. 20) based on the observations of Owen²¹ in freemartin calf dizygotic twins describing a mixture of blood cells due to cross-circulation in the common placenta *in utero*. This type of chimerism, established during the fetal or newborn stages, has always been synonymous with a state of lifelong unresponsiveness to donor alloantigens. Similarly, mixed allogeneic chimeras of Sachs and co-workers prepared by the inoculation of both syngeneic and allogeneic bone marrow to lethally and subsequently sublethally irradiated adult hosts produced long-term donor specific tolerance.^{22,23} However, there is vigorous controversy with regard to the role and the extent of chimerism needed, especially in the adult human to achieve organ transplant acceptance. It remained unclear whether chimerism induces allograft acceptance or whether it is only an epiphenomenon resulting from the transplantation of a vascularized organ.^{8,24} Even to the contrary, chimerism (more recently designated as microchimerism) mediated by means of blood transfusions, organ transplantation, or pregnancy has been associated with allo-sensitization and even to allograft rejection.^{25–27} Recently we have obtained direct evidence linking increased chimerism in the bone marrow compartment with the absence of graft loss and *in vitro* evidence of donor-specific unresponsiveness in human organ transplant recipients (Refs 15–19, 28, also see below).

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Detection of chimerism

Differences between the donor and the recipient in polymorphic genetic markers or their products have been used for the detection of chimerism by employing a variety of fluorescent and molecular methods in a multiplicity of studies.²⁹ Major polymorphic differences that are exploited in clinical transplantation include HLA polymorphism, gender (XX-XY chromosome) differences, variable number of tandem repeat sequences (VNTR) and various cytogenetic markers. Polymerase chain reaction (PCR)-based detection of MHC gene and Y chromosome sequence differences with a sensitivity of 0.001–0.0001% is the most widely used method of the determination of chimerism.^{24–27} Under this umbrella of PCR applications, sequence-specific oligo probe hybridization (SSOPH), sequence-specific priming (SSP) and single strand confirmation polymorphism (SSCP) are notable methodologies. The disadvantages of these studies are the absence of cyto-identification of the chimeric cells. Immunophenotyping by flow cytometric analysis is less sensitive (<0.001%), but can be more informative as to the subset composition of chimeric cells.³⁰ Detection of macrochimerism (>1%) can be done by Southern blotting for MHC polymorphic genes and VNTR sequences.³¹ Similarly, fluorescent *in situ* sequence hybridization (FISH) can be applied especially when gender differences between the donor and the recipient are involved.^{32,33} Garcia-Morales *et al*³⁴ have described a powerful method, PCR-flow combining the sensitivity of PCR technology and the resolving power of flow cytometry. It has been used extensively at this center for the detection and characterization of microchimerism in a number of clinical organ transplant models.^{15,16,19}

Distribution and phenotypic nature of chimeric cells

The infused donor bone marrow cells or passenger leukocytes that migrate out of the transplanted organ in the non-immunosuppressed murine recipients first circulate through the blood stream and rapidly disappear.³⁵ In the immunosuppressed patients given donor bone marrow infusions, their numbers in the peripheral circulation are highest during first 3 months after transplantation and then gradually decrease until they approach minimal detection levels of most cellular assay systems by 1 year post-transplantation.^{13,24} They can for the most part still be detected by molecular methodologies. It is presumed that the chimeric donor cells home into various tissues where they take up residence and/or multiply depending on the cell type and the microenvironment.^{17,36}

The chimeric cells that have been identified in human organ transplant recipients so far belong to a number of lineages including the stem cells, dendritic cells, myeloid precursors and various lymphoid subpopulations.^{15–17,28,36} Starzl *et al*¹³ described the presence of cells of dendritic morphology in the lymph nodes, skin and blood of organ transplant recipients. Subsequently, the same group confirmed the presence of donor dendritic cells in recipient PBL propagated in GM-CSF and IL-4 enriched medium.³⁷ The latter study further demonstrated that chimeric donor

cells included lymphoid (T, B and NK) cells and macrophages. The PCR-flow analyses of Garcia-Morales *et al*^{15,16} have shown that the chimeric cells of donor genotype present in the peripheral blood and bone marrow of kidney, kidney–pancreas and liver transplant recipients who had been augmented with donor bone marrow cells included CD34⁺, CD3⁺ and CD19⁺ cells (unpublished). These studies have indicated that the number of donor cells gradually increases in the bone marrow with a concomitant decrease in the peripheral blood.¹⁹ This gradual increase in the bone marrow may be due to the homing of donor marrow cells from the general circulation and/or due to the propagation of cells which had already taken residence in their natural protective marrow environment.¹⁸ When purified using anti-HLA antibodies to donor mismatched antigens and magnetic microbeads, a substantial percentage of these recipient derived donor (RdD) chimeric cells were found to be CD3⁺, TcR- $\alpha\beta$ ⁺ and CD28⁺ T cells.²⁸ However, these RdD cells lacked CD40 L (CD154) indicating that they might not be activated T cells even though they were presumably in an environment in which they were constantly stimulated by the recipient alloantigens. They were also deficient of CD80 and CD86 receptors normally observed on antigen presenting cells. The RdD cells also did not contain significant percentages of NKB-1⁺, CD14⁺, CD34⁺ or CD33⁺ cells. A significant proportion of these immuno-isolated RdD cells remained undetermined (ie lineage negative) as they did not have any of the conventional lymphoid, myeloid or erythroid cell markers. The yield of RdD cells by this method of purification ranged from 0.1% to 0.9% of the starting material in the recipient bone marrow compartment.

A number of studies including our own have shown that bone marrow cells freshly obtained from human iliac crest or vertebral bodies have immunoregulatory properties.^{38,39} Our more recent studies have shown that a number of purified subpopulations cells from cadaver donor vertebral bone marrow, including pluripotent CD34⁺ stem cells and their CD34 negative early progeny of both lymphoid and myeloid lineages, inhibited MLR and CML responses of laboratory volunteers reacting to irradiated spleen cells from the same cadaver donor.⁴⁰ These included bone marrow cells positively selected for CD38⁺, CD2⁺, CD5⁺ and CD1⁺ lymphoid cells (all were depleted of CD3⁺ cells), as well as CD33⁺ (but CD15 negative) myeloid precursors. Furthermore, we had also shown that purified CD34⁺ stem cells from bone marrow differentiated into CD3⁺ T cells when cultured *in vitro* with repeated allogeneic stimulation in growth factor supplemented medium.⁴¹ In contrast, positively selected CD15⁺ myeloid cells did not show any inhibitory activity and positively selected CD19⁺ B cells showed variable regulatory effect.⁴⁰

Regulatory functions of RdD chimeric cells

The rationale for the infusion of donor bone marrow cells in organ transplant recipients is to enhance donor chimerism, which in turn is expected to induce tolerance. This hypothesis was based on a number of animal studies^{4–7} and on the observations of Starzl *et al*^{13,36} previously mentioned.

In 1995, Burlingham *et al*⁴² described a case report in which a patient was found to be functionally tolerant to a maternal kidney allograft as evidenced by good graft function 5 years after cessation of all immunosuppressive drug therapy, and with the absence of donor-specific CTL activity in either bulk or limiting dilution analysis (LDA) cultures. Even though removal of the donor chimeric cells in primary cultures failed to reverse the unresponsiveness, restimulation of primary cultures with donor cells plus exogenous IL-2 completely reversed the CTL unresponsiveness. More importantly, addition of fresh patient PBL, as well as donor cell-enriched fractions to tertiary MLR cultures, inhibited the generation of anti-donor CTL. Recently, we have extended these observations.²⁸ Besides phenotypically characterizing the recipient derived donor (RdD) chimeric cells (see above), we assessed them functionally as modulators in MLR and CML assays. Since fresh PBL and iliac crest marrow from the living related donors (LRD) was also obtained after 1 year and yearly subsequently in post-operative follow-up, it was possible to directly compare these freshly obtained cells from the donor concomitantly with the donor chimeric cells from renal transplant recipients who had received perioperative donor iliac crest marrow cells.

At 1 year post transplant almost 50% of these LRD-kidney/DBMC recipients tested so far exhibited donor-specific unresponsiveness in MLR and CML reactions²⁸ and this trend was further increased at 2–3 years (unpublished). This was in contrast to maintenance of reactivity of the recipients to third-party alloantigens and to mitogens. Additionally, this donor-specific MLR and CML reactivity has not returned in the non-reactors at the later times. Clinically, however, it is too early to observe any statistically significant differences in rejection episodes and graft survival between the LRD-kidney/BM recipients and the control LRD-kidney recipients without donor bone marrow infusions, but with identical immunosuppressive treatment (both groups with graft survival of >90% at 3 years). On the other hand, among our cadaver-kidney recipients, those who had received perioperative vertebral body donor bone marrow cell infusions have had less chronic rejection and better graft survival than the control cadaver kidney recipients.¹⁹ It may be speculated that donor cell-derived chimerism may help to induce donor-specific hyporesponsiveness *in vivo* at least in the presence of continuing basal immunosuppression.

Post-operatively, in the LRD-recipients with residual positive anti-donor immune responses, the RdD cells inhibited recipient anti-donor MLR and CML responses significantly more strongly than freshly isolated and similarly treated iliac crest bone marrow cells from the donor (Figure 1). At higher concentrations purified RdD cells from the recipient bone marrow (and PBL) also inhibited the MLR of the recipient to third-party allogeneic stimulator cells.²⁸ However, with decreasing numbers of RdD cells, this non-specific inhibition disappeared, while the donor-specific inhibition remained statistically significant. Taken together these results clearly support the notion that the infused donor cells play a positive role in the induction and/or maintenance of transplant hyporesponsiveness post-operatively.

Figure 1 Inhibition of recipient immune responses to donor alloantigens by RdD cells. Mixed lymphocyte reaction: 1×10^5 PBL from renal transplant recipients were stimulated with 1×10^5 irradiated PBL from the living related donors in presence of the indicated donor modulator cells and standard ^3H -thymidine incorporation assays were performed on day 8 ($n = 7$). Cell-mediated lymphocytotoxicity: 3×10^6 PBL from renal transplant recipients were stimulated with 3×10^6 irradiated PBL from the living related donors in presence of the indicated donor modulator cells and standard 4-h ^{51}Cr release assays were performed on day 8 ($n = 4$). The data were calculated as percentage inhibition from the values of the control cultures where the modulators were additional irradiated donor PBL.

Concluding remarks

It might be speculated that donor bone marrow cell infusions in organ transplant recipients initiate a two-way MLR, ie host-versus-graft (HVG) with the recipient mounting anti-donor immune responses on the one hand, and on the other, graft-versus-host (GVH) by the infused bone marrow cells and passenger leukocytes.¹³ The greater immunoreactive capabilities of the (mature) recipient T cells to donor alloantigens are nullified by the combined suppressive effects of the donor marrow cells and immunosuppressive therapy. Since the infused bone marrow cells are less susceptible to immunosuppressive therapy,⁴¹ a functional immune equilibrium or immunological 'truce' postulated by Starzl *et al*¹³ may be created even with the proportionately lower number of infused donor bone marrow cells (when compared to total immune cells of the recipient). Absence of clinical GVH disease^{15–17} and decreased incidence of chronic rejections¹⁹ in donor bone marrow infused (immunosuppressed) kidney transplant recipients are possible manifestations of this functional immune equilibrium. The infused donor cells exist in the general circulation initially, but the immunomodulatory subsets gradually home into various tissues and remain there, where they act as a continued source of donor regulatory cells. In the short term, the regulation by donor chimeric cells may involve the induction of anergy⁴² possibly by incomplete antigen presentation in the absence of costimulatory molecules⁴³ or carrying tolerogenic allopeptides⁴⁴ or even by the transduction of an as yet undefined negative signal. Additionally, the chimeric cells even in their low numbers may cause infectious tolerance^{45,46} by the development of suppressor T cells in the recipient possibly by a mechanism similar to the induction low zone tol-

erance described nearly 30 years ago.^{47,48} However, prolonged and sustained anergy may perhaps lead to the eventual deletion of donor reactive cells, thus bringing about classical immunologic tolerance.

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