Transplantation Tolerance: Of Mice and Men

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Graft 2002; 5; 27

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Most strategies that successfully induce transplantation tolerance in murine models have failed upon translation into clinical therapies, and transplantation tolerance remains an elusive clinical goal. The disparity in outcome between mouse and human studies has led some investigators to cynically question whether data on tolerance in mice have any relevance to humans. The authors believe that this cynicism is overly negative and that principles of immune tolerance in humans can be revealed with mouse models. In this review, the authors discuss why the lessons of transplantation tolerance learned from mice have not directly translated to large animals, and how to overcome this problem.

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Introduction

The fundamentally important issue of a consensus definition of successful induction of tolerance was unresolved until surprisingly recently, when the Immune Tolerance Network (ITN) decided on 4 minimal requirements for clinically relevant tolerance induction. First, tolerance must be specific, preventing deleterious responses to the graft while preserving responsiveness to pathogens. Second, the graft must be protected during the period of establishment of tolerance, with prevention of damage by any abortive immune response that may occur. Third, the prevention of graft injury must be durable, with a stable effect lasting indefinitely despite cessation of active therapy. Fourth, the tolerant state must also be unperturbed during proinflammatory responses to other stimuli such as infections or vaccinations. We discuss 4 criteria, based on those established by the ITN, that we believe should be attained before transplantation tolerance can be declared to have been achieved in mice, and suggest that data from models based on these criteria can be extrapolated to clinical situations.

Criteria of Tolerance in Mice

1. As proof of principle, *1 cohort of grafts should be monitored, preferably for the life of the mouse, or for at least ≥ 150 days after cessation of therapy*. Histology of the transplanted grafts must be examined to demonstrate an absence of deleterious responses to the graft at ≥ 150 days. The extended period of investigation and the histological examination are critical for discriminating between strategies that simply result in delayed chronic rejection and those that can induce clinically relevant tolerance.[1-3] We believe that "normal" histology should include only a minimal cellular infiltration and antibody deposition. Although the observation of otherwise normal tissue histology with dense cellular infiltrate or antibody deposition does not necessarily preclude a tolerant state, caution must be exercised when leukocytic infiltration and antibody deposition criteria are observed. Under such circumstances, we propose that additional histological examinations be conducted at later time points to determine whether chronic tissue damage will eventually manifest.

2. *Tolerant recipients must accept a 2nd donor graft but reject a 3rd-party graft at a normal tempo.* There

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is ample evidence that certain allografts, such as kidney and liver, can be accepted spontaneously in mice despite major and minor histocompatibility barriers.[4-6] These observations lead to the suggestion that long-term graft survival may be due to the inherently tolerigenic properties of certain types of vascularized grafts that prevail after the initial immune responses are inhibited. Thus, the transplantation of a 2nd graft tests whether the long-term survival of the 1st graft is the result of immune tolerance or immune suppression solely within the allograft milieu.

Because of technical limitations, many groups have performed a 2nd graft challenge with a donor-specific skin graft, a tissue that is often different from the 1st graft.[7-9] There is a general consensus that the skin graft is the gold standard because it is the most sensitive to rejection. A recent study by Jones et al.[10] determined that the number of T cells required to elicit skin graft rejection is 6000 times as small as that required for heart rejection. These observations are consistent with the observation that skin graft is the most sensitive marker of immune reactivity. However, we, along with many other investigators, have noted the rejection of skin grafts without rejection of the cardiac allograft. This has led some to raise the possibility of tissue-specific immune responses or of microenvironments that can differentially stimulate or suppress immune response.[5,6,10,11] In light of these considerations, we believe that acceptance of a skin graft may be too rigorous a criterion, and that an adequate and appropriate test of tolerance is the ability to accept the same type of graft as the original graft. The tolerant recipient must, of course, retain the ability to reject the same tissue or organ from a 3rd-party mouse strain.

3. *A robust allograft transplant, with the maximum numbers of antigenic mismatches, is necessary.* Successful strategies for the induction of allograft survival in mouse experimental systems with only low immunological barriers cannot be expected to succeed in nonhuman primates or in clinical trials, where the barriers are much higher. Even in mice, it is apparent that the results from transplant models with only a single minor, or even a single major, histocompatibility mismatch may not apply to models in which there are multiple antigenic mismatches. For instance, early studies of infectious tolerance described the ability of anti-CD4 and anti-CD8 antibodies to control rejection in multiple minor histocompatibility-mismatched and major histocompatibility complex class I plus minor mismatched combinations. However, neither anti-CD4 nor anti-CD8 antibodies were effective when the genetic disparity involved whole major histocompatibility complex plus minor antigens.[12]

In a recent review, Li et al.[13] presented a hypothesis to explain why therapeutic intervention to induce tolerance is tightly associated with the size of the antigen-reactive T-cell pool. Under this hypothesis, apoptosis of antigen-specific T cells is required for the induction of tolerance when the alloreactive T-cell precursor frequency is high, as in major and minor mismatched combinations. However, T-cell apoptosis is not required when the frequency of reactive cells is low.[13] Thus, approaches that inhibit apoptosis may be successful at inducing tolerance across minor antigen barriers but would not be successful across major histocompatibility complex barriers. The hypothesis of Li et al. provides a strong rationale for developing model systems with high degrees of mismatch, comparable to those in the clinical situation.

4. Many factors, including heterogeneous genetic backgrounds, the presence of preformed donor-reactive IgM antibodies, and concurrent inflammatory events resulting from ischemia or infections, can prevent the development of the tolerant state. Thus, *experimental models that incorporate as many of these factors as possible, beyond the simple elegance of basic scientific models of immunity, will most likely provide data relevant to the clinical situation*. For instance, it is known that the genetic predisposition to producing inflammatory cytokines is associated with increased incidence of acute rejection in the human population, whereas individuals who have the genetic marker for high TGF-β production are more susceptible to chronic rejection.[14-16] It is well documented that some of the inbred mouse strains commonly used for experimental purposes represent extreme immunological phenotypes (e.g., in their tendency to generate Th1 as opposed to Th2 responses).[17] The efficacy of any tolerance-inducing regimen, when tested in recipients with different genetic and immunological phenotypes, can provide critical insights into the factors that affect the development of tolerance.[18]

In humans, up to 50% of recipients manifest pretransplant donor-reactive IgM. Counterintuitively, the presence of such antibodies is not a contraindication to transplant, and may even be beneficial in reducing the occurrence of rejection episodes.[19] However, the effect of the presence of such antibodies on tolerance induction has not been adequately investigated. Our preliminary data, comparing recipient mice with pretransplant donor-reactive IgM to those without, suggests that it may be more difficult to induce tolerance in the former. Thus, development of a tolerance protocol that is effective in the face of preformed donor antibodies would be more likely to extrapolate to many human transplants.

In summary, we believe that mice can pave the way to transplantation tolerance in humans if the above factors are given due consideration. We will next discuss the best data for transplantation tolerance in mice and the limitations in the translation for human clinical transplantation.

Central Deletional Tolerance

The most robust and stable tolerance reported in mice is through mixed hematopoietic chimerism.[20,21] In a stable chimeric state, recipients accept highly immunogenic skin and small-bowel grafts across extensive major and minor histocompatibility antigen barriers.[22,23] The tolerant state is stable because donor-reactive cells are permanently deleted when encountering donor hematopoietic cells in the bone marrow and thymus. Although central deletional tolerance through stable long-term chimerism is conceptually elegant and extremely robust experimentally, there are significant problems that prevent its routine clinical application. First, the host conditioning traditionally used to achieve consistent and high levels of allogeneic marrow engraftment is quite toxic. Second, the problem of lethal graft-versus-host disease (GVHD) is formidable and can occur even when only partial HLA barriers are involved. GVHD can be ameliorated by donor marrow T-cell depletion, but this is accompanied by an increased incidence in the failure of bone marrow cells to engraft.

When protocols to induce hematopoietic chimerism in nonhuman primates and humans were modified to clinically acceptable levels of toxicity, inferior hematopoietic chimerism was observed. It was frequently only transient, even when allografts survived long term.[24,25] Because central tolerance in the mouse is absolutely dependent on a stable and high level of chimerism,[26] the observation of even transient chimerism in nonhuman primates with long-term surviving allografts raised the possibility that mechanisms other than clonal deletion may be operative in these models. This possibility is consistent with observations that the dose of bone marrow cells and the level of resulting chimerism in mice can determine whether a central deletional or peripheral tolerance ensues.[27]

Research over the past several years has focused on understanding the minimal requirements for achieving stable chimerism. This work has yielded an exciting non-toxic-based and non-radiation-based regimen of transient costimulation blockade and very large hematopoietic cell doses.[28,29] Four caveats temper enthusiasm for this protocol. First, these studies were performed in mice and thus require confirmation in large animals. Second, the doses of bone marrow cells (10 to 20 donors for 1 recipient) cannot be achieved with cadaveric donors. Third, up to 43% of the recipients failed to achieve permanent chimerism or donor-specific skin graft acceptance.[28] Fourth, large numbers of alloreactive T cells already present in the peripheral lymphoid tissues in the recipient will not be affected by central deletion mechanisms. Thus, peripheral mechanisms of deletion or anergy must also be effective if an approach using costimulation blockade and bone marrow cells is to succeed.[26]

In summary, the concept of central tolerance remains attractive, but its translation into the clinic has been slow because the conditions necessary for achieving mixed hematopoietic chimerism have not yet been achieved using protocols with clinically acceptable toxicities. In addition, protocols that initially were aimed at inducing central deletion tolerance have, by necessity, evolved, so that the separation between central deletional and peripheral tolerance is becoming increasingly blurred.

Peripheral Tolerance

A 2nd approach for the induction of tolerance is through peripheral mechanisms of activation-induced cell death, anergy, immune deviation, and/or the

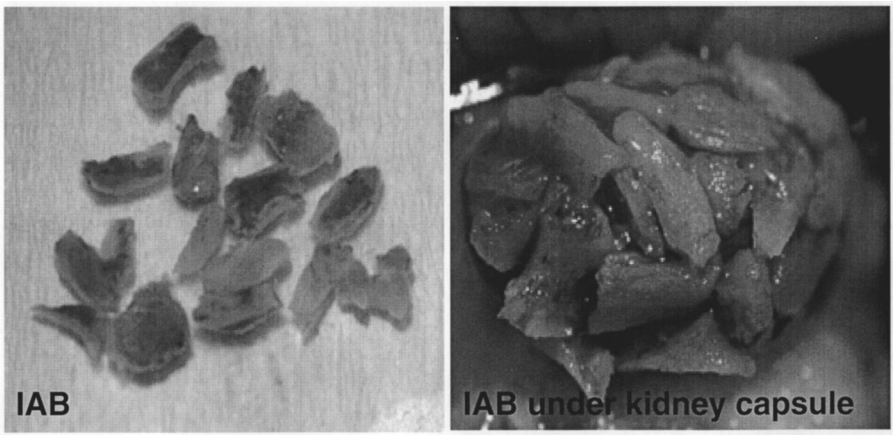

Figure 1. Intact active bone (IAB) fragments from the heads of femurs of donor mice (C3H; H-2^k) placed under the kidney capsule of recipient mice (C57BL/6 ´ DBA2 ´ 129; $H2^{bxd}$).

induction of regulatory T cells.[13,30-33] There are varying degrees of support for each of these mechanisms in the induction of peripheral tolerance, and it is possible that some or all of these events can contribute to the induction or maintenance of tolerance.

A large number of approaches have been reported to induce long-term allograft survival, but the most successful have involved the partial inhibition of T-cell function by blocking signal 1 (anti-CD4 and anti-CD8 mAbs) or signal 2 (anti-CD40L or CTLA4Ig). These approaches are based on the basic principle that inappropriate activation of T cells is not a neutral event, but actually induces prolonged hyporesponsiveness or anergy.[34,35] Over the past decade, in vivo experiments in mice have confirmed this principle and demonstrated that the inhibition of signal 2 (otherwise referred to as costimulation blockade with anti-CD40L and/or CTLA4Ig) results in very effective suppression of the immune response.[1,36] When these approaches were applied to nonhuman primates, especially blockade of CD28-B7 and CD40-CD40L interactions, long-term survival was observed, but in no case was indefinite graft survival achieved.[37-39]

If the data are carefully examined, and attention given to the criteria of tolerance discussed here, the outcomes of the regimens attempted with nonhuman primates could in fact be predicted from rodent data. Costimulation blockade alone does not induce permanent mouse allograft acceptance in many mouse strain combinations (Chong A, unpublished data).[2,18,40] Many of the grafts treated with transient costimulation blockade ultimately succumbed to delayed acute or chronic rejection if the experimental time was sufficient to allow the immunosuppressants to be completely eliminated from circulation and for tissue damage and repair to fully develop. Costimulation blockade did not permanently suppress the alloantibody response, and alloantibody titers increased gradually following cessation of therapy (Chong A, unpublished data). Thus, in general, the observations in mice coincide with the nonhuman primate data that show that the costimulation blockade cannot completely tolerate alloreactivity.

The increasing evidence that costimulation blockade alone cannot elicit allograft tolerance points to a need for adjunct therapies capable of synergizing with costimulation blockade. One of the best-described adjunct therapies involves the administration of donor-specific leukocytes with costimulation blockade.[41,42] A combined therapy of donor-specific leukocytes and costimulation blockade with anti-CD40L allows long-term cardiac allograft survival in euthymic mice and skin allograft survival in thymectomized recipients. Li et al.[40] reported that rapamycin with anti-CD40L and CTLA4Ig allows long-term acceptance of skin

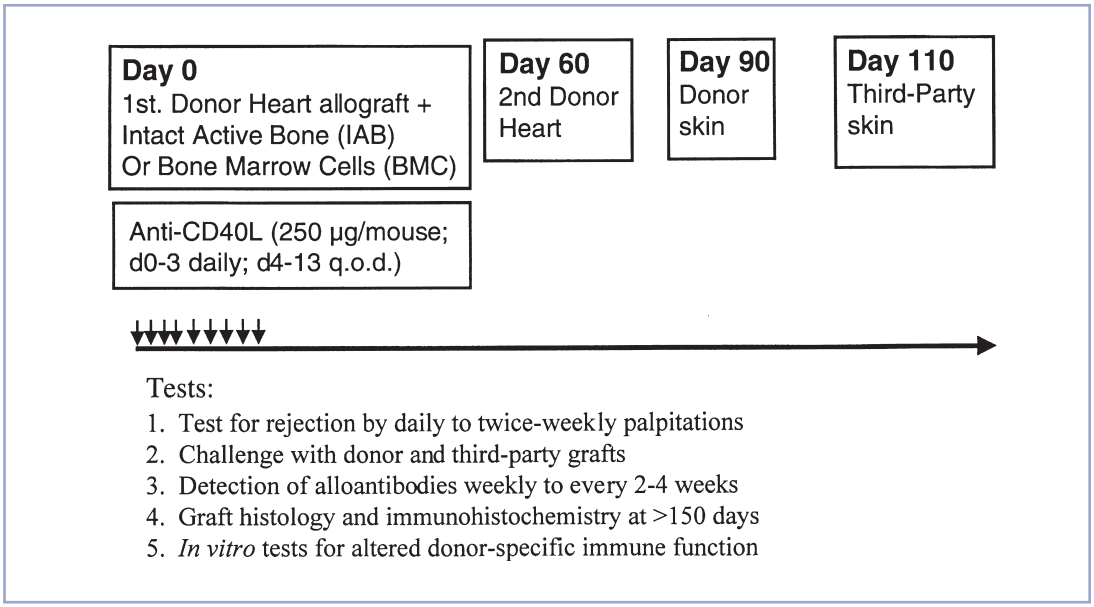

Figure 2. Tests of tolerance induced by intact bone fragments and transient anti-CD40L treatment.

allografts in euthymic mice in a completely major and minor mismatched model. Although the mechanisms of donor-specific leukocytes and costimulation blockade are not completely understood, it appears that powerful peripheral regulatory mechanisms and/or deletion of alloreactive CD8+ T cells are involved.[31,43]

We used fragments of donor-specific intact active bone (IAB) (Fig. 1) and anti-CD40L to induce allograft tolerance. The IAB is placed under the kidney capsule as a sustainable source of donor cells. We reasoned that IAB may have advantages over donor-specific transfusions and bone marrow cell infusions because it can provide a sustained source of donor cells without the need for preconditioning regimens. Cotransplantation of donor IAB, with cardiac allograft and transient anti-CD40L mAb therapy, induced a long-term cardiac allograft survival in which the histology was normal as late as 250 days posttransplantation.[44] These results are remarkable because long-term allograft survival was achieved in a completely major and minor histocompatibility-mismatched system where the recipients had a mixed genetic (129′ C57BL/6′ DBA/2) background. In a formal test of tolerance, these recipients accepted a challenge with 2nd donor-specific heart and skin grafts but were able to reject 3rd-party skin grafts at a normal rate (Fig. 2). In addition, the alloantibody response was significantly suppressed. The extended period of graft acceptance without evidence of chronic rejection allowed us to determine the mechanism of tolerance to be nondeletional peripheral tolerance. In vitro donor-reactive T-cell responses, including mixed lymphocyte reaction and IL-2 and IL-4 production, were comparable to those of rejecting animals with 1 exception: a significantly reduced primed interferon-γ response. Microchimerism was observed in tolerant animals but not in nontolerant animals. As with other studies, whether microchimerism is necessary for tolerance or is the result of tolerance is not known. These results provide the important proof of principle that clinically relevant long-term allograft survival can be attained with nondeletional peripheral tolerance mechanisms.

In summary, methods that can induce transplantation tolerance in mouse models and that are then used to justify investigations in nonhuman primates and humans must fulfill the highest standards of tolerance induction. The criteria we propose here can be achieved in mouse models with sufficient rigor to justify extrapolation to human

transplantation. The definition of an ideal model system for studying tolerance is relevant also for studies on mechanisms and for the identification of surrogate markers of transplantation tolerance.

Acknowledgment

This work was supported in part by a grant (R01 43631) from the National Institutes of Health.

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