Tolerance: Defining, Achieving, and Measuring It: Report from The Tolerance Assay: Where Are We Now? Workshop, Transplant 2001

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Transplantation tolerance remains the goal of clinical transplantation and the focus of a large amount of research. Despite anecdotal reports,[1] a rational scheme for reliably inducing and maintaining clinical transplant tolerance has not been defined. Nonetheless, clinicians and researchers realize the need for designing and testing assays capable of defining the immunologic fingerprint of a tolerant state. At the recent Transplant 2001 meeting, the 2nd joint meeting of the American Society of Transplantation (AST) and American Society of Transplant Surgeons (ASTS), we were charged with moderating a workshop entitled, *The Tolerance Assay: Where Are We Now?* A very lively discussion resulted in the consensus, which we report here.

Prior to the workshop, we both agreed that one of the great challenges in developing a reliable tolerance assay is the lack of a universal definition of "tolerance." The ability to measure tolerance using a laboratory test clearly requires a "gold standard" definition of tolerance itself. The workshop started with a fairly textbook definition of transplantation tolerance:

> The normal function of a transplanted organ, without exogenous immunosuppressive therapy, in the absence of a pathologic donor-specific immune response, but accompanied by an otherwise fully competent immune system.

The 60 to 100 people attending the workshop initially agreed that this was a reasonable definition. However, upon probing a bit further, there were many different opinions on the details of that definition. In an effort to facilitate the discussion, we used a series of questions originally formulated by Dr. Charles Orosz regarding how to best model human tolerance in rodents. It was thought that developing criteria for defining tolerance in rodents, where controlled testing can be performed, would be easier than initially attempting to define tolerance in humans. The questions are printed below, and the resulting consensus was quite surprising to us all.

To model human allograft tolerance, allograft tolerant mice (or rats):

(a) must, (b) should, (c) may, (d) may not, or (e) should never:

1. Display micro-chimerism,
2. Display altered T cell responses to donor antigen,
3. Accept a similar allograft without further therapy,
4. Accept a donor-matched skin graft,
5. Display chronic-rejection like histologic changes,
6. Display persistent inflammation within the graft,
7. Require intermittent immunosuppression,
8. Lose an allograft 200 to 300 days after transplantation,
9. Require preparative irradiation,
10. Display clonal deletion of graft-reactive T cells,
11. Produce donor-reactive IgG,
12. Require preparative injection of donor bone marrow.

Results

1. A few individuals thought micro-chimerism *must* or *should be* present. In contrast, the majority thought that micro-chimerism *may* or *may not* be displayed.
2. All participants thought that donor-reactive T cell responses *should be* altered, either through deletion of the reactive cells or through regulation (suppression, cytokine immune deviation, etc).
3. All participants thought that a 2nd allograft of the same type *must/should be* accepted without further treatment.
4. Most participants thought that donor-matched skin graft *should be* accepted. This is the ideal situation, in which the strongest stimulus is accepted.
5. All participants thought that chronic rejection-like histologic changes *should never be* present.
6. Mononuclear cell infiltration within the graft is acceptable, but tissue damage resulting from that infiltration is never acceptable. One point made was that the presence of an infiltrate may be related to the underlying disease, rather than due to alloantigenicity.
7. Participants thought that intermittent immunosuppression *may be* used, although the absence of intermittent immunosuppression is preferable.
8. Graft loss after 200 to 300 days *may be* acceptable, especially given that the average life span of a mouse is not much more than 365 days.
9. Participants did not think that preparative irradiation must be required, but its use is acceptable as long as the re-

cipients are immuno-competent after transplantation.

10. Clonal deletion of graft-reactive T cells is considered necessary by some participants, but not by the majority. Most participants thought that regulation or blockade of the donor-reactive T cells is sufficient.
11. In general, donor-reactive IgG *should not be* present, according to most participants. However, a significant proportion raised the issue of protective antibodies.
12. Preparative injection of donor bone marrow is not required, but is acceptable.

Overall, the consensus opinion was much more vague than the original proposed definition. An animal model for graft tolerance that best models tolerance in humans will exhibit long-term, but not necessarily indefinite, graft survival and will exhibit no evidence of chronic histopathologic changes *attributable to chronic rejection*. The animals should accept a 2nd allograft of the original donor strain and, ideally, accept a skin graft of the original donor strain. In terms of immune function, donor-reactive T cells and alloantibodies should either be absent or should exhibit a protective or regulatory phenotype.

The participants were then asked an additional question: To be considered a viable model of the human allograft tolerance, does the rodent model need to be operative in one inbred strain, in several inbred strains, or in outbred mice? The consensus was that ideally the model would be operative in outbred mice but that showing the tolerance using at least 2 disparate recipient strains was sufficient (for example, BALB/c and C57BL/6).

The group next considered whether any of the existent rodent models actually meet all of these criteria. The 3 models most discussed were those introduced and initially studied by Megan Sykes (irradiation and preparative bone marrow transplant, reviewed in ref. 2) and by Kathryn Wood (DST and anti-CD4 antibody treatment[3,4]), along with Anita Chong's modification of the protocol originally described by the Larsen/Pearson group at Emory (anti-CD154 antibody and donor bone fragment at the time of transplantation[5]). In the latter system, femur fragments are placed under the kidney capsule on day 0 and antibody is administered on days 0-3, 5, 7, 9, 11, and 13 relative to heart transplantation. A challenge with a 2nd heart is performed on days 60-90. The tolerant animals accept donor, but not 3rd party, grafts. The tolerance is associated with a peripheral loss of donor-reactive IFNγ production by T cells, but central T cell deletion does not occur. Allograft tolerance has been induced by this approach in inbred mice (C57BL/6) and in mice on a mixed background (129 × B/6 × DBA/2). The Chong adaptation at this time appears to meet the criteria of only peri-operative immunosuppression: an absence of alloantibody, no chronic rejection, acceptance of a 2nd graft, as well as donor-matched skin graft, and rejection of 3rd party allografts as a measure of immuno-competence. The other tolerance-inducing protocols discussed have not been fully evaluated in multiple strains, although a variation of the Sykes model has been used successfully in larger animals and nonhuman primates. However, concerns were raised by some participants about immuno-competence of the treated animals following the Sykes model, and/or the presence of chronic histo-pathologic changes in the graft in some situations following the Wood model. Based on this discussion, there is still a significant amount of basic science experimentation required before we have an ideal model of tolerance in rodents. It is only when such a model is established that we can perform a variety of rigorously controlled laboratory tests to determine which one(s) best predict or correlate with the tolerant state.

Despite the above deficiencies, the group next discussed how one would go about measuring tolerance in humans. It is obvious that certain experimental protocols, including placement of 2nd grafts or skin grafts, could not be performed in clinical trials and that other surrogate markers need to be established. It was further noted that long-term graft survival with normal graft function in the presence of minimal immunosuppression might suggest a clinically tolerant state but that more stringent immunologic criteria might help define truly tolerant individuals. Eventual, total discontinuation of immunosuppression with maintenance of normal organ function is the ultimate goal. The proposed tolerance assays would be used to evaluate the alloimmune repertoire in an effort to confirm tolerance and to define the presence of a potentially pathologic immune response prior to graft dysfunction.

A variety of assays capable of measuring immune reactivity were discussed with the caveat that there are no definitive data and only preliminary information about any of the proposed assays. Proliferative hypo-responsiveness to donor cells in an Mixed Lymphocyte Reaction (MLR), the lack of killing in Cytolytic T Lymphocyte (CTL) assays, the absence of donor-reactive recall Enzyme-Linked Immuno-SPOTs (ELISPOTs) for type 1 cytokines (i.e., IFNγ), and the absence of mRNAs for perforin/granzyme in graft biopsies, and the absence of alloantibodies were considered consistent with, but not diagnostic of, a tolerant state. In some situations, the presence of micro-chimerism was also considered by the group to be consistent with tolerance. An additional intriguing assay discussed by the group was the trans vivo Delayed-Type Hypersensitivity (DTH) assay. In this assay, control antigens and solubilized donor antigen are mixed with recipient cells and tested for their ability to mediate DTH in mice by simply measuring skin thickness. Recent studies have shown that Peripheral Blood Leukocytes (PBLs) from some tolerant patients can specifically suppress otherwise potent immune responses as measured by this assay, providing a readout that correlates well with tolerance.[6] Whether or not this approach or any of the proposed assays will be useful for defining or identifying tolerance in the clinic remains to be determined in prospective trials.

The group consensus was that many of the proposed assays need to be evaluated in the context of animal models in which stringent criteria can be used to define tolerant versus nontolerant states. Until this is done, it will be difficult to interpret any results of studies performed in humans. Nonetheless, attempts at human tolerance induction are being performed in the clinical setting, and immune monitoring is therefore desirable. It seems that under these circumstances, the best approach to evaluate the usefulness of any of the proposed assays is to simultaneously study the same tolerant and nontolerant individuals (defined based on clinical criteria) using as many of the available assays as possible. In this way, it might be possible to correlate responsiveness, lack of responsiveness, or the presence of regulatory immunity with a particular clinical outcome. This is indeed the approach being championed by the NIH-funded Immune Tolerance Network, a multicenter program focusing on induction and measurement of tolerance in human disease. It is hoped that this effort, among many others, will provide us with better insight into the best ways to define and measure tolerance in the laboratory—and at the bedside.

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