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Examination of the Mechanisms Responsible for Tolerance Induction After Intrathymic Inoculation of Allogeneic Bone Marrow

Jon S. Odorico, M.D., Timothy O'Connor, M.D., Luis Campos, M.D., Clyde F. Barker, M.D., Andrew M. Posselt, B.S., and Ali Naji, M.D., Ph.D.

From the Department of Surgery, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania

Objective

This study examined the immunologic mechanism(s) responsible for the induction of transplantation tolerance in rats pretreated with intrathymic inoculation of donor strain bone marrow.

Summary Background Data

Induction of unresponsiveness may involve deletion and/or inactivation of donor-reactive T-cell precursors maturing in a thymus harboring donor alloantigen or generation of regulatory/suppressor cells. It was reasoned that, if unresponsiveness is caused by deletion of alloreactive clones, the presence of additional thymic tissue devoid of donor alloantigen permits normal maturation of T-cells and, thus, prevents induction of tolerance. However, if unresponsiveness were primarily mediated by regulatory/suppressor cells, the presence of noninoculated thymic tissue should not affect the induction of tolerance.

Methods

Three strategies were used to define the cellular basis of cardiac and islet allograft survival in WF recipients of intrathymic LEW donor bone marrow as follows: (1) inoculation of bone marrow either into the native thymus and/or into an ectopic thymus, (2) limiting dilution analyses of the frequency of precursor cytotoxic T-lymphocytes (CTLp), and (3) adoptive transfer to syngeneic secondary hosts.

Results

Inoculation of bone marrow into only one lobe of the native thymus and/or into an ectopic thymus did not promote consistent survival of subsequent LEW cardiac allografts. Tolerant hosts displayed significant reductions in CTLp frequencies against donor alloantigens. Adoptive transfer of spleen cells from tolerant WF hosts harboring long-standing cardiac allografts led to permanent survival of LEW cardiac allografts in all secondary recipients. However, transfer of spleen cells from WF animals that received intrathymic LEW bone marrow (but no cardiac allograft) did not promote survival of LEW cardiac allografts in naive secondary hosts.

Conclusions

These results indicate that the unresponsive state after intrathymic inoculation of bone marrow cells is primarily mediated by deletion and/or inactivation of donor-specific T-cell precursors maturing in a chimeric thymus. The demonstration by adoptive transfer studies of putative regulatory/suppressor cells suggested an important role for the persistence of donor alloantigen (supplied by a vascularized allograft) in the maintenance of the unresponsive state.

Permanent donor-specific tolerance to cellular and vascularized organ allografts has been achieved by intrathymic inoculation of donor alloantigen(s) into transiently immunosuppressed adult rodents.[1-6] In these studies, it was obligatory to inoculate the donor alloantigen into the thymus for successful induction of unresponsiveness; tolerance could not be achieved by intravenous administration of the donor alloantigen. Several possible mechanisms can contribute to the induction of unresponsiveness in this model. These include (1) intrathymic deletion and/or inactivation of donor-reactive T-cell precursors and (2) the generation of regulatory/suppressor cells.

The current study was designed to evaluate the cellular basis of induction of tolerance after intrathymic inoculation of donor bone marrow. We reasoned that, if tolerance is caused by deletion and/or inactivation of alloreactive clones, the presence of additional thymic tissue devoid of donor alloantigen would permit normal maturation of alloreactive T-cell populations and prevent induction of unresponsiveness. However, if unresponsiveness were primarily mediated by suppressor cells, inoculation of only a portion of an animal's thymic tissue should permit generation of the regulatory cell populations without affecting the induction of tolerance. We therefore examined the survival of cardiac allografts in rats that had been pretreated with donor bone marrow in the following manner: (1) inoculation of both native lobes of the thymus, (2) inoculation of only one native thymic lobe after surgical separation of the lobes, (3) inoculation of either the native thymus or of a syngeneic ectopic thymus graft, and (4) inoculation of syngeneic ectopic thymus grafts in animals subjected to native thymectomy. In addition, the potential role of suppressor/regulatory cells in the development of tolerance after intrathymic injection of bone marrow was assessed by the ability of lymphoid cells from putatively tolerant hosts to promote cardiac allograft survival in naive secondary recipients.

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Address reprint requests to Ali Naji, M.D., Ph.D., Department of Surgery, Hospital of the University of Pennsylvania, 3400 Spruce Street, 4 Silverstein, Philadelphia, PA 19104.

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MATERIALS AND METHODS

Construction of Auxiliary Thymus Grafts

Where noted, prospective adult WF ($RT1^u$) rat recipients (weight range, 150 to 175 g) were grafted with either one whole WF neonatal (< 48 hours old) thymus beneath the left renal capsule or one whole neonatal WF thymus beneath each renal capsule 6 weeks before intrathymic inoculation of donor bone marrow. In animals that harbored single syngeneic renal subcapsular thymus grafts, either the native thymus or the ectopic thymus was injected with 25×10^6 Lewis (LEW, RT1*l*) bone marrow cells. Other animals underwent native thymectomy and simultaneous bilateral renal subcapsular thymus grafting and were inoculated 6 weeks later with LEW bone marrow into a single ectopic thymus graft. Only animals in which histologic examination revealed the presence of healthy renal subcapsular thymus grafts and did not demonstrate thymic remnants in the mediastinum were included in this study. A separate group of animals underwent surgical separation of the native thymic lobes with care being given to preserve the vascular and lymphatic channels of each thymic lobe. In these animals, either the left or right lobe of the thymus was inoculated with 25×10^6 LEW bone marrow.

Recipient Pretreatment

Bone marrow cells were prepared from the long bones of adult male LEW rats and were inoculated intrathymically into adult male WF rats as previously described.[2] Prospective WF recipients received inocula of $20–25 \times 10^6$ LEW bone marrow per thymus, followed immediately by intraperitoneal administration of 1 mL of rabbit antirat lymphocyte serum (ALS, Accurate Chemical, Westbury, NY). No further immunosuppression was given throughout the duration of the experiment. Control animals received intrathymic injections of saline. All animals were housed in the pathogen-free laboratory animal facilities of the University of Pennsylvania Medical Center.

Heart Transplantation

Heterotopic heart transplantation was performed using the modified technique of Ono and Lindsey.[7] Cardiac allograft survival was monitored by daily palpation of the graft; rejection was considered complete with cessation of myocardial contraction and was confirmed by histologic examination.

Pancreatic Islet Transplantation

Islets were prepared from LEW donors by collagenase digestion and Ficoll density centrifugation as previously described.[8] Freshly isolated pancreatic islets were transplanted beneath the renal capsule of WF recipients rendered diabetic with intravenous streptozotocin (65 mg/kg). Graft survival was monitored by daily blood glucose measurements, and rejection was defined as two consecutive days of recurrent hyperglycemia (glucose, > 200 mg/dL).

In Vivo Adoptive Transfer

Sublethally irradiated (450 rad) naive WF animals received an intravenous injection of syngeneic spleen cells (200 to 250 $\times$ 10^6), and 24 hours later, they underwent transplantation with LEW cardiac allografts. No immunosuppression was used. Syngeneic spleen cells were obtained from (1) naive WF donors, (2) long-term WF recipients of LEW cardiac allografts rendered tolerant by intrathymic inoculation of LEW bone marrow in conjunction with administration of ALS 100 days earlier, or (3) WF rats pretreated with intrathymic LEW bone marrow and ALS but not subsequently transplanted with a LEW cardiac allograft.

Limiting Dilution Analysis

Limiting dilution analysis was performed as previously described.[1] Briefly, limiting numbers of cervical lymph node cells were cultured in replicates of 24 wells with irradiated (2000 rad) donor strain LEW or "third-party" DA ($RT1^a$) lymph node stimulator cells in the presence of 10% α-methyl mannoside-treated supernatants from concanavalin A (Con A)-stimulated rat spleens. After 7 days of culture, cytotoxic activity was assessed in a 4.5-hour ^{51}Cr release assay against DA and LEW Con A spleen cell blast targets. Wells were considered positive if the value of ^{51}Cr release was more than three SD above the mean ^{51}Cr release of control wells not containing effector cells. Precursor cytotoxic T-lymphocyte (CTLp) frequencies were derived by linear regression analysis.

Statistics

Statistical significance between experimental groups were analyzed using the Wilcoxon rank-sum test for nonparametric data.

RESULTS

Cardiac Allograft Survival After Intrathymic Inoculation of Donor Bone Marrow

In accordance with our previous reports, LEW cardiac allografts had permanent survival (median survival time [MST], > 200 days) in WF recipients pretreated 2 weeks earlier with 20 to 25 $\times$ 10^6 LEW bone marrow intrathymically in conjunction with a single intraperitoneal dose of ALS (Table 1).

We hypothesized that the unresponsive state resulted from the deletion and/or inactivation of RT1*l*-reactive T-cell precursors in a WF thymus bearing LEW alloantigen(s). It follows that, if deletion or functional inactivation of alloreactive clones were responsible for cardiac allograft survival, the presence of an uninjected thymus lobe would allow normal reconstitution of the T-cell repertoire, including the donor specific alloreactive clones. Thus, induction of unresponsiveness should be prevented unless all thymic tissues contained alloantigen. To test this hypothesis, prospective WF recipients underwent surgical separation of the two lobes of the native cervical thymus and received LEW bone marrow cells into one lobe of the bisected native thymus. All animals received a single intraperitoneal dose (1 mL) of ALS at the time of thymic inoculation. Two weeks later, WF recipients were transplanted with heterotopic LEW cardiac allografts. Five of six LEW cardiac allografts were rejected promptly by WF recipients in which the conditioning inoculum of LEW bone marrow was confined to a single thymic lobe (MST, 33 days, Table 1).

We next assessed the impact of a syngeneic (WF) auxiliary thymus, previously transplanted ectopically to the kidney, on the ability of donor LEW bone marrow cells inoculated into the native thymus to promote unresponsiveness. In this model, the ectopic thymus was not inoculated with donor bone marrow. In animals harboring ectopic syngeneic (WF) thymus grafts, inoculation of both lobes of the native thymus with LEW bone marrow did not promote consistent long-term survival of LEW cardiac allografts; five of eight animals promptly rejected their cardiac allografts (MST, 25 days). Similar results were obtained in animals in which the syngeneic ectopic thymus graft was inoculated with LEW bone marrow but the native thymus was left uninjected. In these hosts,

Table 1. SURVIVAL OF LEW CARDIAC ALLOGRAFTS IN WF RECIPIENTS

Group	Status of Native Thymus	Ectopic WF Thymus		Site of BMC Inoculum*†	Individual Cardiac Allograft Survival (d)	MST (d)
		R RSC	L RSC			
I	Intact	—	—	None	14, 17, 20, 23, 25, 38, 50, 62	24
II	Intact	—	—	Both lobes of native thymus	14, >200 × 8	>200
III	Bisection	—	—	One lobe of native thymus	9, 16, 18, 47, 58, >100	33
IV	Intact	—	+	Both lobes of native thymus	12, 13, 17, 19, 30, >150 × 3	25
V	Intact	—	+	Single ectopic thymus	9, 16, 18 × 2, 40, 57 > 150 × 2	29
VI	Thymectomy	+	+	Single ectopic thymus	7, 8, 9, 27, >35, >60	18

RSC: renal subcapsule; MST: median survival time.
* 20–25 × 10^6 LEW bone marrow cells (BMC)/thymus.
† All animals received 1 ml of ALS IP at time of BMC inoculation.
Group I *vs.* II, II *vs.* IV, II *vs.* V, II *vs.* VI, p = 0.001; Group II *vs.* III, p = 0.004; Group I *vs.* III, p = 0.045; Group I *vs.* IV, p = 0.02; Group I *vs.* V, p = 0.347.

six of eight LEW cardiac allografts were rejected with the same time frame as in the control animals (MST, 29 *vs.* 24 days), although two heart grafts did survive permanently (Table 1, group V).

In a series of ongoing experiments, prospective WF recipients harboring bilateral syngeneic ectopic thymus grafts were subjected to native cervical thymectomy. Inoculation of only one of the two ectopic renal subcapsular thymus grafts with LEW bone marrow so far has not been found to promote consistent acceptance of LEW cardiac allografts; four of six animals have already rejected their allografts (Table 1, group IV).

Limiting Dilution Analysis of CTLp

The effect of intrathymic inoculation of LEW bone marrow on the donor-reactive frequencies of CTLp was examined *in vitro* by limiting dilution analysis. CTLp frequencies of lymph node cells from WF animals given bilateral intrathymic inocula of LEW bone marrow in conjunction with ALS and tolerant of LEW islet allografts (MST, > 120 days) were compared with those of naive WF animals. In three separate analyses, tolerant animals displayed a significant reduction (50% to 75%) of CTLp frequency to donor strain LEW alloantigens compared with untreated controls (Fig. 1). In contrast, CTLp frequencies to third-party DA alloantigens were not significantly different between the two groups.

Adoptive Transfer of Spleen Cells

An *in vivo* adoptive transfer protocol was used to delineate the role of suppressor/regulatory cells in the development of tolerance after intrathymic inoculation of donor bone marrow. Survival of LEW cardiac allografts was examined in secondary WF hosts (subjected to 450 rad of sublethal irradiation) after intravenous transfer of syngeneic spleen cells from the following groups of animals: (1) naive WF, (2) WF treated by intrathymic inoculation of LEW bone marrow and bearing established cardiac allograft (> 100 days after cardiac transplantation), and (3) WF rats 60 days after their intrathymic inoculation of LEW bone marrow cells (but not bearing cardiac allografts). Transfer of spleen cells from WF hosts harboring LEW cardiac allografts led to permanent survival of LEW cardiac allografts in all secondary WF recipients (MST, > 100 days; n = 6; Table 2). By contrast, spleen cells from naive WF animals or WF recipients of intrathymic LEW bone marrow (but not harboring LEW heart allografts) did not transfer tolerance to secondary WF hosts as evidenced by rejection of LEW cardiac allo-

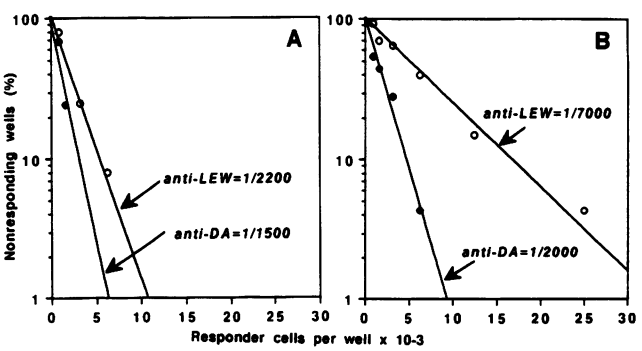

Figure 1. Effect of intrathymic bone marrow transplantation on CTLp frequencies. A representative experiment of three performed is shown. (A) Anti-LEW (○, f = 1/2200) and anti-DA (●, f = 1/1500) CTLp frequencies of naive WF. (B) Anti-LEW (○, f = 1/7000) and anti-DA (●, f = 1/2000) CTLp frequencies of a WF animal rendered tolerant to LEW renal subcapsular pancreatic islets (normoglycemic > 120 days) by pretransplant administration of intrathymic LEW bone marrow and ALS.

Table 2. SURVIVAL OF LEW CARDIAC ALLOGRAFTS IN SECONDARY WF RECIPIENTS AFTER ADOPTIVE TRANSFER OF PUTATIVE SUPPRESSOR CELLS*

Group	Spleen Cell Donor	Individual Cardiac Allograft Survival (d)	MST (d)
I	Naive WF	11, 14, 18, 30	16
II	WF tolerant to LEW cardiac allograft	>100 × 6	>100
III	WF inoculated with IT LEW BMC (no cardiac allograft)	18, 19, 32, 90	25

MST: median survival time.
* Secondary hosts were irradiated with 450R before transfer of 200–250 × 10^6 syngeneic spleen cells. Group I *vs.* II, II *vs.* III, $p = 0.005$; Group I *vs.* III, $p = 0.057$.

grafts in all recipients. (MST, 16 days; n = 4 and MST, 25 days; n = 4; Table 2). In an *in vitro* coculture assay, neither spleen cells from WF hosts tolerant of LEW cardiac allografts nor spleen cells from untransplanted WF recipients of intrathymic LEW bone marrow were able to exert donor-specific suppression of the allogeneic mixed lymphocyte culture response (data not shown).

DISCUSSION

The landmark report concerning "actively acquired tolerance" of cellular and tissue allografts has been the impetus for many subsequent studies on the role of the thymus in acquisition of tolerance to self and foreign major histocompatibility antigens.[9] In these studies, donor strain lymphohematopoietic cells were inoculated intravenously into newborn rodents, a procedure that was found to result in long-lasting and specific unresponsiveness to donor alloantigens. Despite the spectacular success of this experiment in newborn rodents, attempts during the last 40 years to induce transplantation tolerance by the same strategy in adults have been unsuccessful, unless accompanied by extensive myeloablative conditioning of the recipients by drugs or radiation to prevent rejection of the bone marrow graft.

Related to ongoing attempts by many investigators to find a method of achieving classic tolerance in adult animals was our recent report that pancreatic islets transplanted into the thymus of adult allogeneic hosts are protected from rejection. Our initial interpretation of this finding was simply that the thymus was a previously unrecognized immunologically privileged transplant site.[1] However, an even more interesting implication of these studies, possibly promising in the quest for adult tolerance, was our concomitant finding that residence of foreign islets in the thymic microenvironment also rendered the recipients unresponsive to allografts of the donor strain even when they were transplanted extrathymically, under the kidney capsule or to the liver. Subsequently, we have also observed that animals conditioned by intrathymic inoculation of various cell types (bone marrow, spleen, or liver cells) would also permanently accept transplants of the donor strain whether they were of islets or vascularized heart or liver, demonstrating the generality of this approach in promoting specific unresponsiveness in adult hosts.[1–3,8] In searching for an explanation of these findings, we hypothesized that the intrathymic residence of donor alloantigen, that is, thymic microchimerism, acts by promoting deletion and/or functional inactivation of donor-reactive clones before their migration from the thymus to form the peripheral immune repertoire.[1] The current experiments were designed to define the cellular basis of the prolonged survival of cardiac and islet allografts in allogeneic hosts conditioned by intrathymic inoculation of donor bone marrow. The basic model used was inoculation of donor bone marrow, either into the entire thymus, or alternatively, into only a part of the native thymus, and/or into an ectopic thymus (grafted beneath the kidney capsule). We first confirmed that, as in our previous experiments when allogeneic bone marrow was inoculated into both lobes of the thymus of rats treated with a single dose of ALS, tolerance of the donor strain tissue was uniformly achieved. It should be noted that the intrathymic inocula appear to diffuse throughout the parenchyma of the lobe in which an injection is made, a finding readily confirmed by immunohistologic examination. In the next series of experiments, donor bone marrow was inoculated into a single lobe of a surgically bisected native thymus. This variation from the technique of inoculating the entire thymus did not promote unresponsiveness to donor cardiac allografts with any consistency. This suggested to us that, even though alloreactive clones may undergo deletion in the inoculated thymic lobe, the induction of tolerance would be negated by expansion of alloreactive clones in the noninoculated portion of the thymus. Most of the hosts that had been constructed to harbor two thymuses and received foreign bone marrow into only one or the other rejected their cardiac allografts, demonstrating that all thymic tissue must harbor donor alloantigen if tolerance induction is to be accomplished by intrathymic bone marrow inoculation.

Somewhat surprisingly, however, a few of the animals that received inocula of donor marrow into either the native or the ectopic thymus (but not both) maintained

their cardiac allografts and were thus considered operationally tolerant. Several possibilities may account for these inconsistencies. It was conceivable that, after inoculation, some donor bone marrow cells may exit from the inoculated thymus and, eventually, home to the second noninoculated thymus. In this case, the presence of donor bone marrow at the site of thymic inoculation in one case and the site of recirculation to the noninoculated thymus in the other case would sometimes be sufficient to cause unresponsiveness to a subsequent allograft—either by deletion or some other mechanism. The transient period of immune deficiency that follows treatment with ALS might further promote this effect by preventing the alloimmune destruction of circulating bone marrow cells before their entry into the second thymus. In addition, in the case of a surgically manipulated native thymus or in the case of renal subcapsular grafts, the usual anatomic features (such as lymphatic pathways) might also be altered, a perturbation which might result in greater "leakage" of inoculated cells from the thymus and their subsequent recirculation.

Limiting dilution analysis of the CTLp frequencies were performed in long-term WF hosts rendered tolerant of LEW islet allografts by intrathymic inoculation of LEW bone marrow concomitant with a single intraperitoneal injection of ALS. These *in vitro* studies indicated a significant reduction in the major histocompatibility complex class I-restricted RT1*l* reactive T-cells in tolerant hosts compared with naive ones. It seems unlikely that the observed reduction of CTLp frequencies in tolerant hosts could be attributed to the effect of ALS administered to these animals at the time of intrathymic donor bone marrow inoculation. Tolerant hosts received a single dose of ALS 200 days before limiting dilution analysis of their lymph node cells for determination of CTLp frequencies. Moreover, the fact that reduction of CTLp frequency was donor specific and no differences were seen in the CTLp frequency against third-party donor alloantigens further argues against the role of ALS in limiting dilution assays. Nevertheless, CTLp frequencies to donor alloantigen, although significantly reduced, were not completely absent in tolerant hosts. In fact, limiting dilution analysis revealed a 50% to 75% reduction of the CTLp frequency specific for RT1*l* alloantigens rather than complete elimination of alloreactive cells. Thus, it could be argued that the remaining population of donor-reactive cells should easily be sufficient to initiate and complete the destruction of the allografts. A possible explanation is that the nondeleted populations of CTLps were rendered anergic by the pretreatment of recipients with intrathymic donor bone marrow. If such remaining alloreactive cells are indeed incapable of allograft recognition, they might exert regulatory/suppressor cell functions that mediate unresponsiveness. Evidence in support of the immunomodulatory activities of tolerant cells have been established in published studies, suggesting a cell–cell interaction as a mechanism of transplantation tolerance.[10]

In several models of donor-specific unresponsiveness, the tolerant state has been shown to correlate with the presence of lymphoid cells with suppressor/regulatory functions.[10,11] In our study, the adoptive transfer of spleen cells from animals that received intrathymic donor bone marrow (but no cardiac allograft) did not promote permanent cardiac allograft survival in secondary hosts, although slight prolongation was seen (MST, 25 days *vs.* 16 days in the controls, $p = 0.057$). This marginal effect is contrasted with that seen in adoptive transfer experiments in which spleen cells from animals pretreated with intrathymic bone marrow and also harboring long-standing donor strain cardiac allografts proved to be transferring long-term tolerance to cardiac allografts in secondary hosts. These findings indicate that, in this model, optimal generation of suppressor/regulatory cells requires the combined effect of both intrathymic conditioning inoculum and the presence of a long-standing vascularized allograft. The cardiac allograft may contribute to the maintenance of unresponsiveness by shedding the donor alloantigen, which may undergo reprocessing by the host thymic antigen-presenting cells. Additional migration of graft-derived dendritic and/or antigen-presenting cells to the host thymus may contribute to the persistence of thymic microchimerism, thus promoting deletion and/or functional inactivation of donor-reactive cells.

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Discussion

DR. PETER J. MORRIS (Oxford, United Kingdom): It's a pleasure to be asked to open the discussion of this paper. Since the first description in *Science* by Andrew Posselt, Clyde Barker, and Ali Naji in 1990 of the induction of tolerance to islet allografts by injection of islets into the thymus together with antilymphocyte globulin treatment, this work has continued to expand and has attracted enormous interest in the transplantation world. Indeed, I think it's fair to say that it has almost created an industry in the study of intrathymic injection to induce tolerance.

The studies presented today address the question of the mechanism by which the tolerance induced after allogeneic bone marrow injection into the thymus is produced. I think the experimental design is truly elegant and truly simple, which I guess is what makes it so elegant. Indeed, it's reminiscent of those beautifully simple experiments in classical cellular immunology performed by our recorder when he was a very young man with Rupert Billingham many, many years ago.

The authors have shown that if the thymus is not uniformly in contact with the injected bone marrow alloantigen, then tolerance does not occur. For example, if they separate the lobes of the thymus and inject allogeneic bone marrow into one lobe only, or if they implant two thymic grafts from the recipient strain beneath the capsule of each kidney (*i.e.*, syngeneic grafts) in a thymectomized animal and inject only one such thymus, then again tolerance does not occur.

However, I would have liked to have seen one more control group here; namely, the demonstration that injection of an ectopic thymus beneath the kidney capsule in a thymectomized animal would indeed induce tolerance. Perhaps the authors have that information and they might share it with us.

Next, they demonstrate that adoptive transfer of spleen cells from an animal bearing a long-surviving cardiac allograft will transfer tolerance to an irradiated syngeneic recipient (the so-called suppressor phenomenon). However, they are not able to transfer suppression with spleen cells from animals given intrathymic injection and antilymphocyte globulin (ALG) 2 weeks before testing for suppressive activity (at the time at which a cardiac allograft would be implanted and not rejected). On this basis, the authors conclude that suppressor cells are responsible for the maintenance of tolerance but not its induction in this particular model.

I'm not sure that this conclusion is correct and would remind them of the work from Kathryn Woods' laboratory in my own department a few years ago performed by Robert Quigley, who was a Ph.D. student at the time. They showed in a model of allograft tolerance in the rat, where this was produced by the intravenous injection of donor alloantigen in the form of blood 1 week before renal transplant, that if you examine the spleen for suppressor cells at 7 days after injection (the time you would implant a kidney that would not be rejected), you could not demonstrate suppressor cells in the spleen. But they were present 1 or 2 days after injection, and at 7 days these cells had moved out of the spleen and could be found in the thoracic duct compartment.

So it's just possible that in this model, too, at the time the spleen was examined (2 weeks after the intrathymic injection and ALG treatment), the suppressor cells, whatever suppressor cells are, were not present in the spleen but had migrated and perhaps might be demonstrated in another compartment.

Finally, I'd like the authors to comment on the phenomenon demonstrated in their model in general. For example, their model does seem to represent a relatively weak one in that cardiac allografts have a mean survival of 25 days in untreated animals. Perhaps they could tell us something about the application of this phenomenon in stronger rodent models or in the mouse.

Also, could they say something about the need to use antilymphocyte globulin in this model? Does this allow the innoculated cells to survive in the thymus till the mechanism of tolerance induction has taken place? Or, is there some other more subtle explanation that they might provide? And indeed, can you produce this effect with other T cell depleting agents such as anti-CD4 or anti-CD8 antibodies?

Again in this model they've used bone marrow as the alloantigen that has been injected into the thymus. I'd like to know whether this is the optimal form in which alloantigen is presented to the thymus in this model and, furthermore, do they have any evidence of chimerism in their model?

In conclusion, I would like to congratulate the authors on another beautiful study. I'm sure it's one of many more to come and we look forward to hearing much more about this very exciting phenomenon over the coming years. Thank you.

DR. PAUL S. RUSSELL (Boston, Massachusetts): As Peter Morris has already told us, this subject generates a lot of interest among those of us in transplantation. It's directed toward the objective we've all wanted and worked for for many years: the induction of specific unresponsiveness.

As we've progressed, it has become clear that specific unresponsiveness can come about by more than one mechanism. It isn't a single Holy Grail, it's a whole cluster of different possible mechanisms. Three have been mentioned here for cellular responsiveness: the deletion of reactive cells, the inactivation of reactive cells, and the suppression of their reactivity.

Specific T cell deletion is what I would accept as the only definition of tolerance. Cell inactivation seems to occur more in the periphery and not in the thymus. So deletion may be the more likely mechanism for what's being shown here by limiting dilution analysis.

I share Peter Morris' interest in whether or not one can in-