

## Peripheral Tolerance to Alloantigen Results from Altered Regulation of the Interleukin 2 Pathway

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### Summary

Tolerance to alloantigen may be induced in rats by administration of blood followed by transplantation of a renal allograft. The mechanism of this tolerance was investigated by directly analyzing the functional activity of graft-infiltrating cells. We have previously shown cytotoxic T lymphocyte infiltration of, and major histocompatibility complex induction on, grafts of tolerant animals. We now report that cells isolated from the grafts of tolerant rats show a reduced expression of the p55 interleukin 2 receptor (IL-2R) chain on the cell surface compared with that seen on the cells of untreated animals. Scatchard analysis further reveals low expression of high affinity IL-2R. This is due to reduced transcription of both IL-2R  $\alpha$  and  $\beta$  chain mRNAs and results in a reduced ability of cells to proliferate in response to IL-2. Cells isolated from tolerant animals are unable to make biologically active IL-2 in culture, whereas cells from untreated animals make high levels. This is not reflected at the mRNA level as the IL-2 gene is induced in both tolerant and untreated animals to similar levels. The induction of tolerance is abrogated by administration of recombinant IL-2 to animals at the time of transplantation. Thus, we conclude that an altered regulation of the IL-2 pathway results in tolerance in these alloantigen-treated and transplanted animals.

Interleukin 2 plays a central role in amplification of immune responses. Further, it has been suggested that an absence of IL-2 and/or other cytokines after antigenic stimulation may be critical in the induction of T and B cell anergy (1, 2). Tolerance to antigens that are expressed within the thymus usually results from deletion of autoreactive cells (3-5), although recent evidence suggests that self-reactive cells may escape deletion and become anergic (6, 7). Tolerance to antigens expressed extrathymically does not seem to involve deletion but may result from the induction of anergy in the autoreactive population (8-10). Tolerance to alloantigens may be induced in adult animals by a single intravenous injection of alloantigen in the form of whole blood (11). In the DA-RT1<sup>a</sup> (DA)→PVG-RT1<sup>c</sup> (PVG) rat strain combination, intravenous administration of 1 ml whole DA blood to PVG animals 7 d before transplantation of a DA kidney leads to indefinite graft survival which is blood donor specific (12). Indeed, these pretreated and transplanted animals may be retransplanted with a cardiac allograft of the same donor strain, 100 d after the primary graft, and the secondary graft also survives indefinitely (our unpublished results). Thus, these animals are tolerant of the original alloantigens. We and others have investigated this transplantation tolerance by analyzing immune events within the transplanted graft and have demon-

strated that the tolerance exists despite the presence of cells reactive with donor alloantigen. Animals contain, in their grafts and peripheral lymphoid organs, fully differentiated, donor-specific, effector cytotoxic cells (12, 13) and can produce at least some cytokines as reflected by an induced level of both MHC class I and II antigens on the transplanted tissue (13, 14). Clearly then, this tolerance is not maintained by a complete deletion of alloantigen-specific T cells. We have now examined the status of the IL-2 pathway in these rats by a direct analysis of intragraft events. Here we present evidence that transplantation tolerance in this model is due to an altered regulation of the recipient's IL-2 pathway that is induced following rechallenge with alloantigen in the form of a transplant. Graft infiltrating cells isolated from tolerant animals do not make biologically active IL-2, although expression of the IL-2 gene is induced, and do not respond normally to IL-2. This poor response to IL-2 is due to decreased expression of both  $\alpha$  and  $\beta$  chains of the IL-2R. Further, this tolerant state can be reversed completely by administering IL-2 at the time of transplantation. These data suggest that, in this model, a state of anergy has developed in the T cells responsible for IL-2 production resulting in transplantation tolerance.

## Materials and Methods

**Animals.** Inbred Lewis-RT1<sup>l</sup> (LEW), blood group D Agouti-RT1<sup>a</sup> (DA), and PVG-RT1<sup>c</sup> (PVG) male rats were obtained from Harlan Olac UK Ltd. (Bicester, UK) or bred in the Biomedical Services Unit at the John Radcliffe Hospital (Oxford, UK).

**Transfusion and Transplantation.** DA rats were given 1.0 ml PVG (donor-specific) blood, intravenously, 7 d before transplantation of a PVG or DA kidney. Control animals were either given 0.5 ml LEW (third party) blood intravenously or nothing. Kidneys were transplanted into the left orthotopic site as previously described (15).

**Preparation of Cell Suspensions.** Kidneys were removed 3 or 5 d after transplantation and digested with collagenase as previously described (12) to release infiltrating leukocytes. LN were removed and lymphocytes teased into a single-cell suspension through a wire mesh.

**Flow Cytometry.** Single-cell suspensions of graft-infiltrating cells were stained at 4°C for 60 min with the following antibodies in the form of tissue culture supernatant: MRC OX1 and MRC OX30 (leukocyte-common antigen); NDS 61, NDS66, and MRC OX 39 (IL-2R p55,  $\alpha$  chain); or MRC OX 21 (human Factor I). After washing, cells were incubated with FITC-conjugated goat anti-mouse Ig (FO257; Sigma Chemical Co., Poole, England). Samples were analyzed on a flow cytometer (Ortho Diagnostic Systems, Inc., Westwood, MA). Antibodies designated NDS were produced in this laboratory (16); hybridomas producing antibodies designated MRC OX and W3/25 were kind gifts of Dr. A.F. Williams (Medical Research Council Cellular Immunology Unit, Oxford, England).

**Scatchard Analysis.** IL-2 was labeled with [<sup>125</sup>I]sodium iodide using Enzymobeads (170-6001; Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's methods to a specific activity of 2.04–2.16  $\times 10^7$  cpm/ $\mu$ g. Radiolabeled IL-2 binding was performed according to the slightly modified (17) method of Robb et al. (18). Briefly, graft-infiltrating cells were resuspended in IL-2-free RPMI (Gibco Ltd., Uxbridge, UK) for 2 h at 37°C. Serial dilutions of radiolabeled IL-2 (in the range of 50 pM to 50 nM) were added to 1.6  $\times 10^6$  cells in a total volume of 500  $\mu$ l RPMI containing 0.1% BSA and 0.1% NaN<sub>3</sub> and incubated at 20°C for 40 min. Free and cell-bound <sup>125</sup>I-IL-2 was separated by centrifugation over a 20% olive oil/80% dibutylphthalate mix. Cell pellets were counted. Nonsaturable binding was determined by incubating cells with a 100–1,000-fold excess of unlabeled IL-2. Specific binding was determined by subtracting nonsaturable binding. Scatchard analysis was used to determine the presence of low and high affinity IL-2 binding sites.

**IL-2 Proliferation Assays.** Cells were incubated at 5  $\times 10^5$ /ml in RPMI containing 10% FCS, 2 mM glutamine, 2.5  $\times 10^{-5}$  M 2-ME and penicillin (45  $\mu$ g/ml), streptomycin (45  $\mu$ g/ml), and kanomycin (90  $\mu$ g/ml) (R10) in a total volume of 200  $\mu$ l in round-well plates for 24 h at 37°C. 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added to each well for the last 4 h of culture. Human rIL-2 (a generous gift of the Cetus Corp., Emeryville, CA) was added to the cultures at the concentrations indicated.

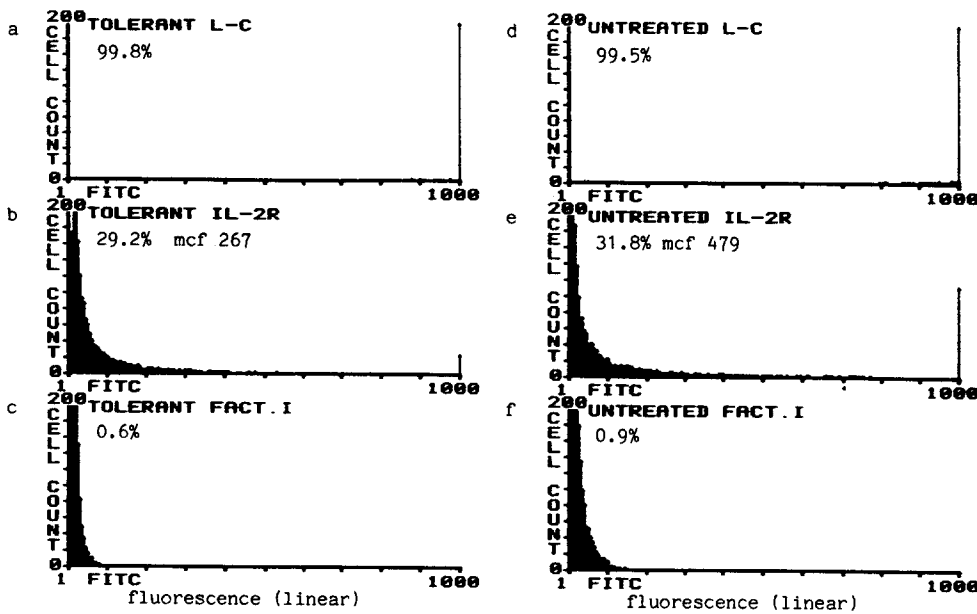
**IL-2 Production Assays.** 7.5  $\times 10^5$  graft-infiltrating leukocytes were incubated with 7.5  $\times 10^5$  irradiated (2,500 rad) DA rat LN cells in a total volume of 1 ml in 24-well plates for 3 d at 37°C. Supernatants of such cultures were then harvested and incubated at various dilutions in 96-flat well plates for 48 h with an IL-2-dependent rat cell line (developed in this laboratory) at 8  $\times 10^5$  cells/ml in R10. During the final 4 h of culture, 1  $\mu$ Ci [<sup>3</sup>H]thymidine was added per well. rIL-2 was used as a positive control.

**Preparation of RNA and Northern Blotting.** Kidneys were removed

and immediately homogenized in guanidine thiocyanate solution (4 M guanidine thiocyanate, 25 mM Na<sub>3</sub> citrate, 0.2% sarkosyl, 200 mM 2-ME). Total RNA from individual rats was pelleted through a cesium chloride gradient (5.7 M CsCl, 100 mM EDTA). 10  $\mu$ g total RNA was denatured by glyoxal treatment (50°C, 60 min in 10 mM sodium phosphate, pH 7.0, 50% DMSO, 17% deionized glyoxal; 26107; BDH Chemicals Ltd., Atherstone, England) and size fractionated on a 0.6% agarose gel. RNA was transferred to nylon membranes (Hybond N RPN 303N; Amersham International, Amersham, UK), fixed under UV illumination and hybridized to [ $\alpha$ -<sup>32</sup>P]dCTP-labeled cDNA probes in 50% formamide, 6  $\times$  SSPE, 0.1% SDS, 0.25% Cadbury's Marvel. cDNA probes for the IL-2 R  $\alpha$  and  $\beta$  chains were prepared in this laboratory (Page, T.H., and M.J. Dallman, manuscript submitted for publication).

**Polymerase Chain Reaction (PCR).** PCR was performed essentially as previously described (19). Briefly, 1  $\mu$ g total RNA was incubated for 5 min at 60°C with 4  $\mu$ g oligo(dT) (27 7858; Pharmacia, Milton Keynes, UK), chilled on ice for 3 min, and further incubated for 40 min at 37°C with 1  $\mu$ l RNAsin (799-017; BCL, UK), 1 mM dNTPs, 400 U reverse transcriptase (murine Molony leukemia virus, 510-8025; Gibco Ltd., Uxbridge, England) and 8  $\mu$ l RT buffer (Gibco Ltd.). After 40 min, a further 400 U reverse transcriptase was added and incubated for 40 min at 37°C. Reactions were then heat inactivated at 70°C for 10 min and 60  $\mu$ l water was added per sample. PCRs were set up using 5  $\mu$ l of this cDNA. To this was added (final concentrations in parentheses) KCl (0.15 M), Tris HCl, pH 8.3 (10 mM), MgCl<sub>2</sub> (1.5 mM), gelatin (0.01%), dNTPs (0.2 mM each), primer A and primer B (1  $\mu$ M each), and 2.5 U Taq polymerase (N801-0046; Perkin-Elmer Corp., Hayward, CA) in a total volume of 100  $\mu$ l. This was overlaid with 50  $\mu$ l mineral oil (M3516; Sigma Chemical Co.). Primer sequences were derived from the previously published mouse IL-2 sequence (20). We have found that these primers amplify a 0.4-kb product from rat IL-2-producing cells whose sequence is identical to that of the recently published rat IL-2 sequence (21). Primers A and B have the sequences 5' AACAGCGCACCCACTTCAA3' and 5' TTGATGATGCTTTGACA3' respectively. Samples were incubated at 93°C for 1 min, 60°C for 2 min, and at 72°C for 1 min. This cycle was repeated 20–60 times using a DNA thermal cycler (Perkin-Elmer Ltd., Beaconsfield, England). Control primers were derived from the rat cytoplasmic  $\beta$ -actin sequence and have the sequences 5' ATGGATGACGATATCGCTG3' and 5' ATGAGGTAGTCTGTGTCAGGT3'. PCR using these primers was as described for the IL-2 primers except that the reactions contained 2.5 mM MgCl<sub>2</sub> and the annealing cycle was performed at 55°C. Samples were analyzed either by gel electrophoresis, using 15- $\mu$ l samples of the amplified DNA or by dot blot. For dot blot analysis 15  $\mu$ l of the PCR mix was removed after varying numbers of cycles, dot-blotted on to nitrocellulose membrane (BA85; Schleicher & Schull, Anderman and Co., Ltd., Kingston-Upon-Thames, England) and hybridized to  $\gamma$ -[<sup>32</sup>P]ATP, end-labeled oligonucleotides whose positions in the cDNA are internal to that of the original 2 primers and whose sequences are 5' CACTGAAGATGTTTC3' and 5' AGCAAGAGAGGTATCCT3' for IL-2 and actin, respectively.

**Treatment of Transplanted Animals with rIL-2.** Animals were transfused and transplanted as described above. On the day of transplantation and for the subsequent 5 d, animals received an intraperitoneal injection of rIL-2 diluted in RPMI containing 10% FCS and antibiotics. 7 d after transplantation, the animals' second kidney was removed so that survival of the animal depended on adequate function of the transplanted kidney.

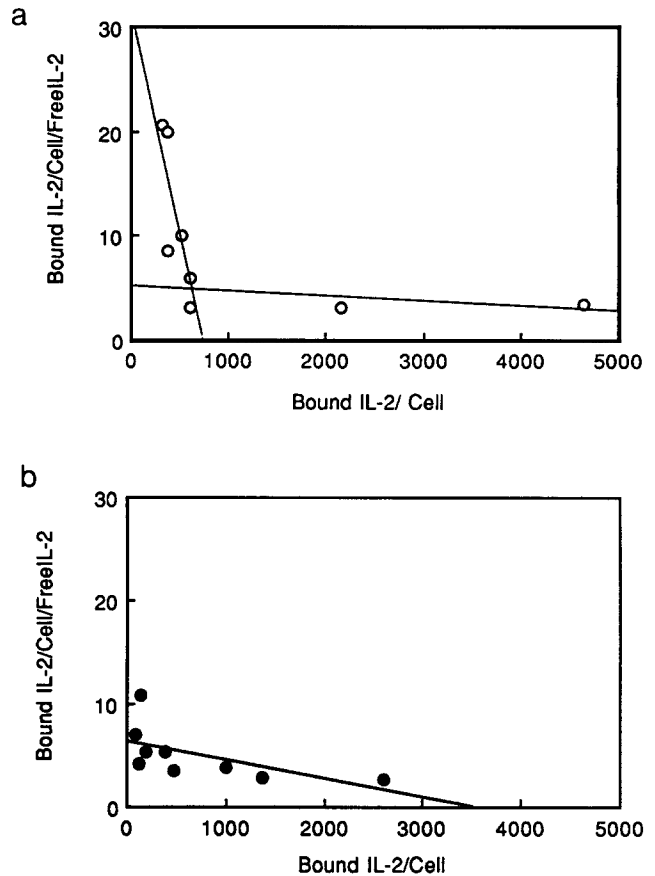


**Figure 1.** Graft-infiltrating cells from tolerant rats express low levels of the IL-2R  $\alpha$  chain. Cells were obtained from the transplants of one tolerant (a-c) and one nontolerant (d-f) animal 5 d after grafting, by collagenase digestion of the grafts, and examined for surface expression of the IL-2R by flow cytometry. Percentage of cells stained with antibodies against leukocyte common antigen, L-C (a and d; MRC OX1+MRC OX30); IL-2R (b and e; NDS61+NDS66+MRC OX39); or human Factor I (c and f; MRC OX21, negative control) is shown with mean channel fluorescence (as an indication of level of expression) of IL-2R-positive cells (mcf). These data are examples from single animals; mean results from groups of animals are given in the text.

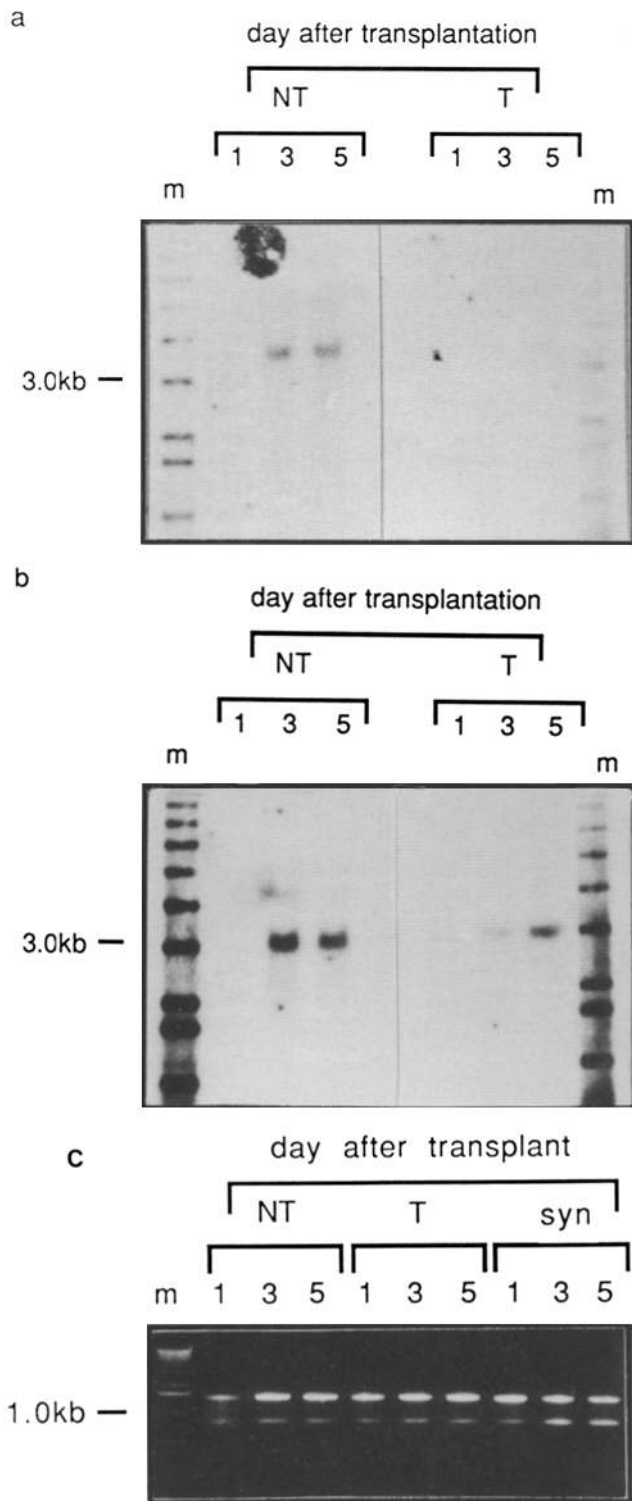
## Results

*The Cell Surface Expression of IL-2R Is Reduced on Graft-infiltrating Cells from Tolerant Animals.* We have previously described that the leukocyte infiltration is similar in grafts from tolerant and untreated (nontolerant) animals (14). The expression of IL-2 receptors on graft-infiltrating cells was examined phenotypically by flow cytometry and Fig. 1 shows an analysis of the cells from one tolerant (a-c) and one nontolerant (d-f) animal. Cells in both types of graft express the IL-2R. However, although the percentage of leukocytes expressing the IL-2R was similar in tolerant and nontolerant animals (means of 34.1 and 37% for six and for four animals, respectively), the level of expression of the receptor, as judged by the mean channel fluorescence, was consistently reduced on cells from tolerant animals (means of 400 and 271 on the cells of untreated [four rats] and tolerant [six rats] animals, respectively; Fig. 1, b and e). Since the antibodies used for this phenotypic analysis only react with the p55,  $\alpha$  chain, of the rat IL-2R, Scatchard analysis was used to examine the expression of high affinity IL-2R, which require the presence of both  $\alpha$  and  $\beta$  chains. This analysis revealed a lack of expression of high affinity IL-2 binding to the cells from tolerant rats (Fig. 2, a and b).

*IL-2R  $\alpha$  and  $\beta$ -Chain Genes Are Weakly Expressed in Tolerant Animals.* Northern analysis using total RNA isolated from the grafts of tolerant rats shows that IL-2R  $\alpha$ -chain gene is barely expressed, whereas strong expression is seen in grafts of nontolerant animals (Fig. 3 a). This result suggests that the low surface expression of the IL-2R  $\alpha$  chain by leukocytes from the tolerant animals was due to a weak induction of the gene. The IL-2R  $\beta$  chain gene is constitutively expressed by rat T cells although its expression is upregulated upon activation (Page, T.H., and M.J. Dallman, manuscript submitted for publication). Fig. 3 b shows that although IL-2R  $\beta$ -chain mRNA is seen in the grafts of both tolerant and nontolerant animals it is expressed at a higher level in those

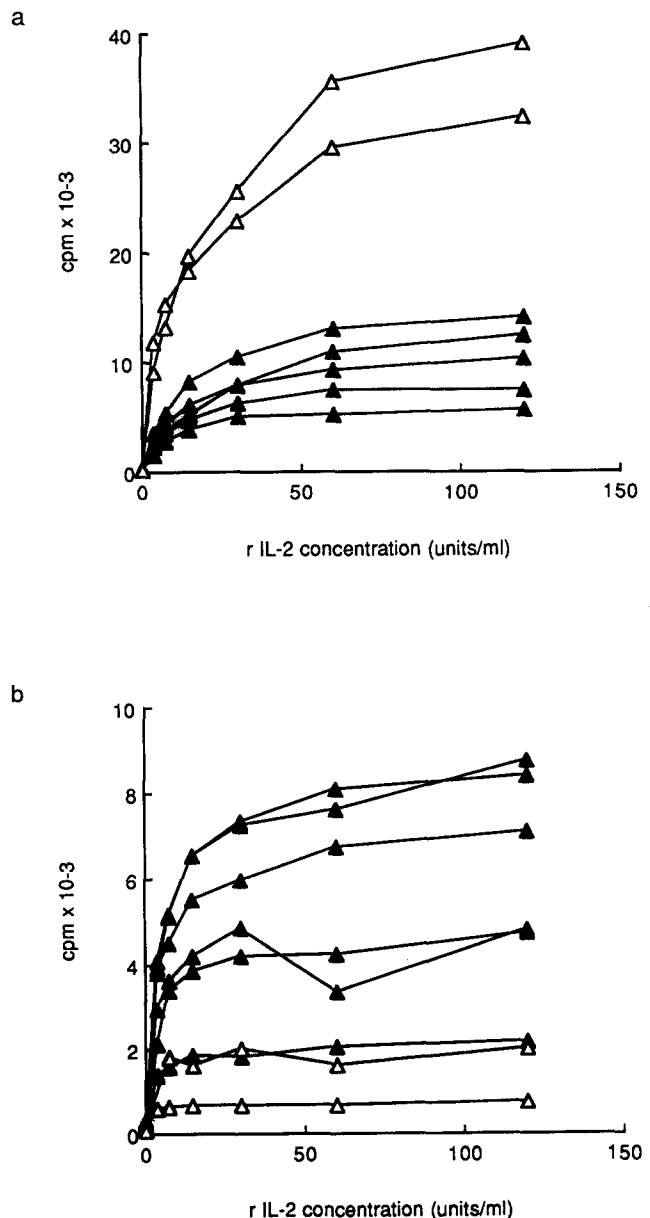


**Figure 2.** The presence of high and low affinity IL-2 binding sites on graft-infiltrating cells from nontolerant (a;  $\circ$ ) and tolerant (b;  $\bullet$ ) rats was investigated using  $^{125}\text{I}$ -labeled IL-2. Graft-infiltrating cells were recovered, 5 d after grafting, by collagenase digestion of the transplants, and incubated with serial dilutions of radiolabeled IL-2. Free and cell-bound IL-2 were separated by centrifugation through oil, and cell pellets were counted. Scatchard analysis was used to determine the presence of low and high affinity IL-2 binding sites. The experiment was repeated with similar results.



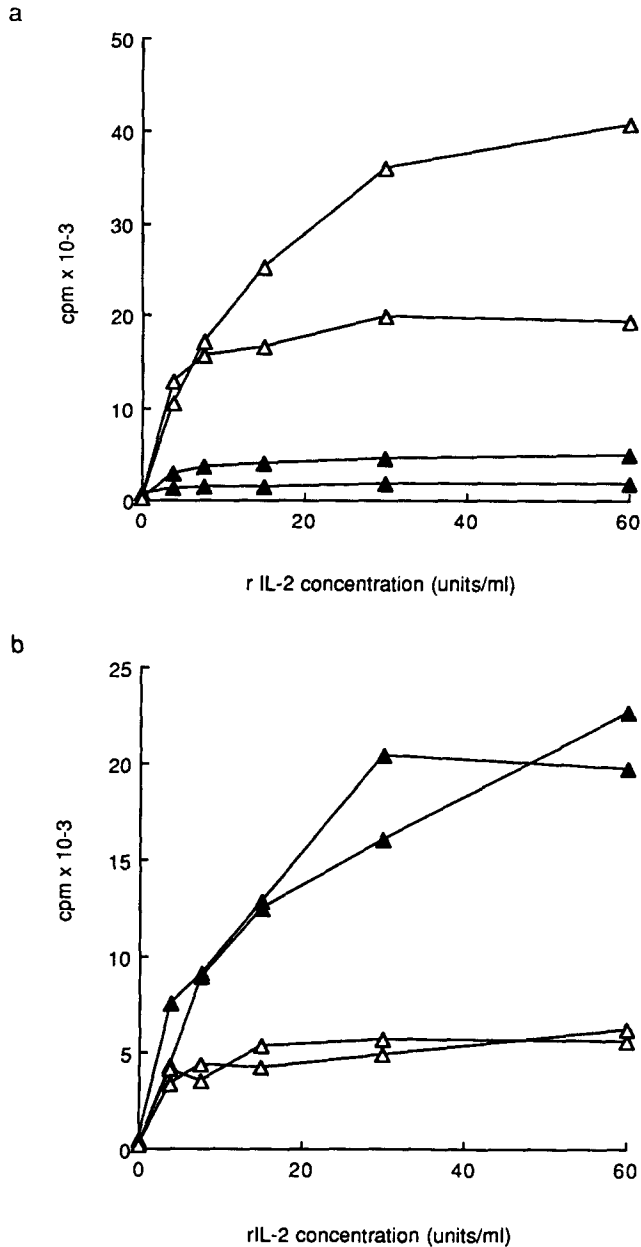
**Figure 3.** IL-2R  $\alpha$  and  $\beta$  chain genes are weakly expressed in tolerant animals. Total RNA was isolated from grafts in tolerant (*T*) or nontolerant (*NT*) animals 1, 3, or 5 d after renal transplantation. 10  $\mu$ g total RNA was subjected to Northern analysis and probed with cDNA probes for the rat IL-2R  $\alpha$  (*a*) or  $\beta$  (*b*) chain. *c* shows the RNA stained with ethidium bromide to show equal loading of all samples. These RNA samples were also used for experiment shown in Fig. 8. This experiment was repeated with essentially the same result.

of nontolerant animals. NK cells have been reported to express the IL-2R  $\beta$  chain (22). The higher level of expression of the  $\beta$ -chain gene by untreated rats is, however, unlikely to be due to increased infiltration by NK cells since NK activity is similar in the grafts of tolerant and nontolerant animals (reference 13, and our unpublished observations). These data indicate, therefore, that low expression of not only the IL-2R  $\alpha$  chain gene but also of the  $\beta$  chain gene contributes to the reduced level of high affinity IL-2 binding sites on the graft-infiltrating cells of tolerant animals.

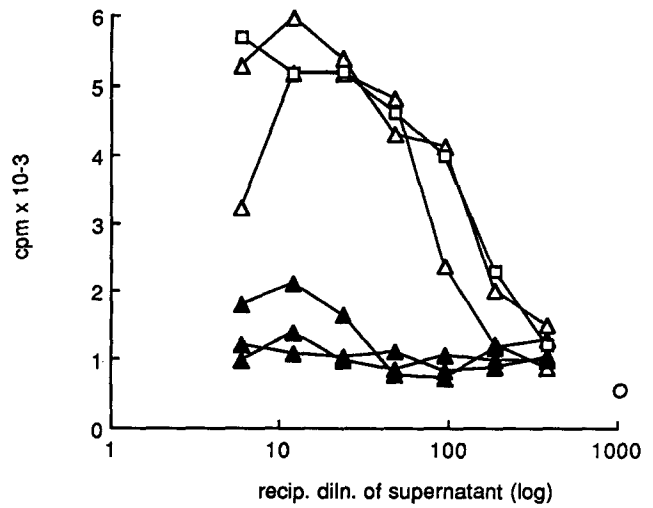


**Figure 4.** Graft-infiltrating cells from tolerant rats do not respond normally to IL-2. Cells isolated from renal transplants were tested for their ability to proliferate in response to IL-2 in a [ $^3$ H]TdR uptake assay. Proliferation ( $\text{cpm} \times 10^{-3}$  incorporated) of cells from grafts of untreated rats ( $\Delta$ ) and alloantigen-pretreated rats ( $\blacktriangle$ ) 5 d (*a*) or 3 d (*b*) after transplant. Each titration represents the results from an individual animal. The experiment was repeated three times with similar results.

*Cells from Tolerant Animals Do Not Develop a Normal Response to IL-2.* Since the cells from tolerant animals express low levels of the high affinity IL-2R, it seemed likely that they would not be capable of proliferating in response to IL-2. Fig. 4 a shows that the cells isolated from the grafts of tolerant animals, 5 d after transplantation, responded very

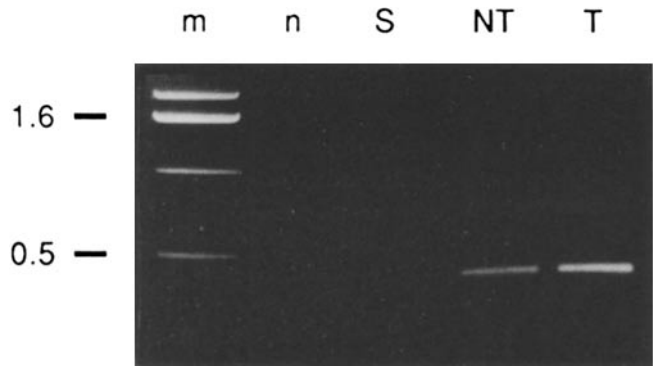


**Figure 5.** Rats pretreated with third-party blood develop a normal response to IL-2. Cells isolated from renal transplants were tested for their ability to proliferate in response to IL-2 in a [<sup>3</sup>H]TdR uptake assay. Proliferation (cpm × 10<sup>-3</sup> incorporated) of cells from grafts of third-party blood-treated animals (Δ) and donor-specific blood-treated rats (▲) either 5 d (a) or 3 d (b) after transplant. Each titration represents the results from an individual animal. The experiment was repeated with essentially the same result.

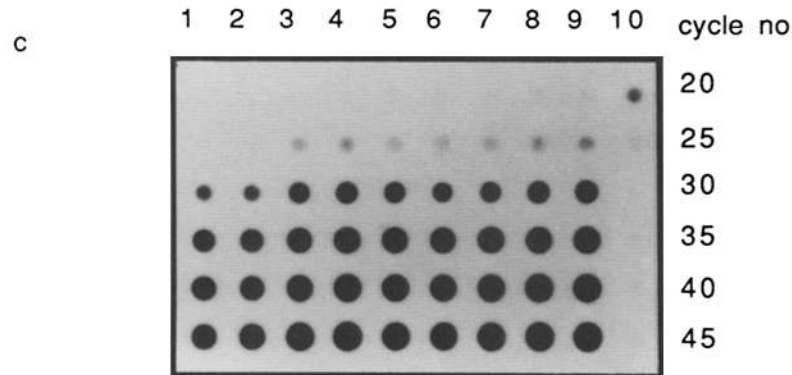
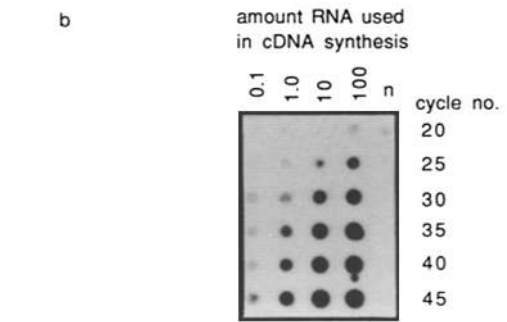
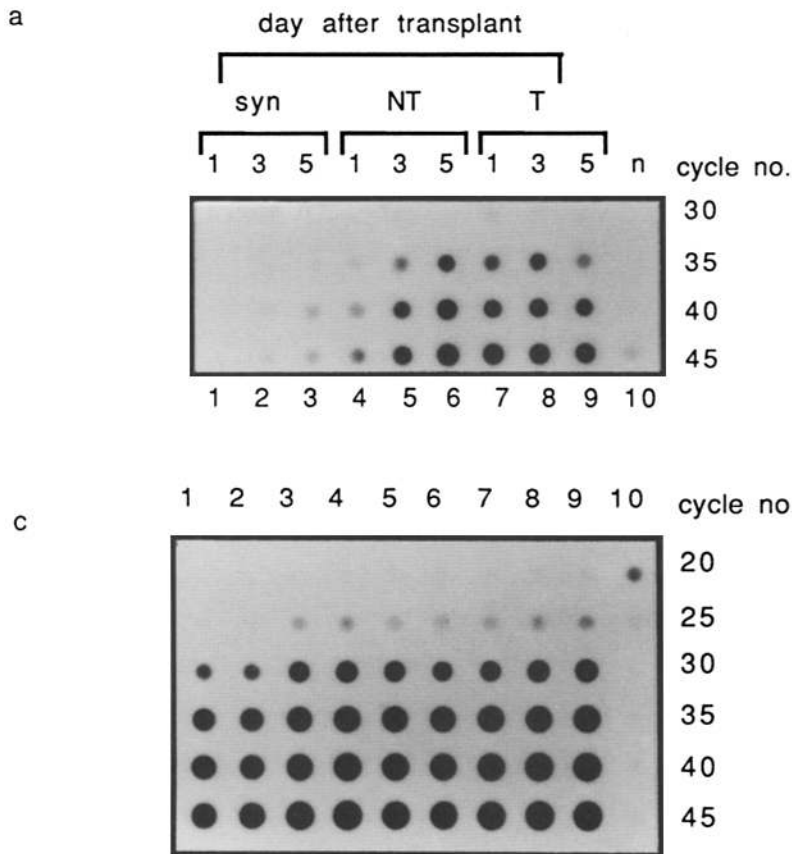


**Figure 6.** Graft-infiltrating cells from tolerant rats are unable to make biologically active IL-2. Leukocytes isolated from allografts of tolerant (▲) and nontolerant (Δ) animals, 3 d after transplantation, were restimulated with alloantigen in vitro. The supernatants from such cultures were assayed for IL-2 activity using an IL-2-dependent rat cell line. rIL-2 at a starting concentration of 6,000 IU/ml was used as a positive control (□); background (○). Each titration represents the supernatant of a culture of cells obtained from an individual rat. The experiment was repeated with similar results.

poorly to rIL-2 compared with cells from grafts that were undergoing rejection in naive recipients. Interestingly, however, cells isolated from tolerant animals showed an early very low response to IL-2 at a time at which cells from untreated rats were unable to respond to IL-2 (Fig. 4 b). As a specificity control for the observed effects on the IL-2R expression, graft-infiltrating cells were isolated from rats that had been preoperatively transfused with third-party blood and therefore will reject their grafts acutely (14), and tested for their ability to



**Figure 7.** Tolerant rats express the IL-2 gene. Expression of the IL-2 gene was assessed in RNA isolated from the grafts of tolerant (T), nontolerant (NT), and syngeneically transplanted (S) rats, 5 d after grafting, using the PCR. m, marker track (kb ladder); n, no cDNA control. 15 μl samples of the amplified cDNA (60 cycles) were electrophoresed on ethidium bromide containing 1.0% agarose gels and visualized under UV illumination. The experiment was repeated on several occasions using RNA isolated from two individual groups of animals with essentially the same results.



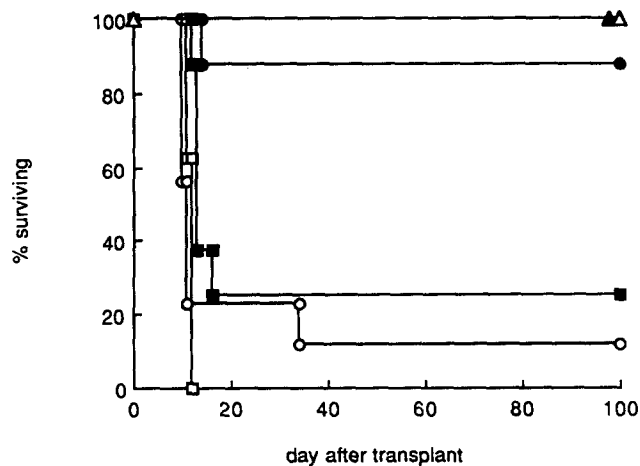
**Figure 8.** Semiquantitative analysis of the level of IL-2 expression. The level of IL-2 mRNA expression was assessed semiquantitatively using PCR followed by dot blot analysis. (a) RNA was isolated from grafts 1 (columns 1, 4, and 7), 3 (columns 2, 5, and 8), or 5 (columns 3, 6, and 9) d after transplantation from syngeneic (*syn*), nontolerant (*NT*), or tolerant (*T*) rats and amplified using IL-2 primers. Dot blots were hybridized with a third IL-2 primer which lies internal to the original two primers in the rat IL-2 cDNA sequence. (b) As a control for quantitation, cDNA synthesis was performed using 0.1, 1, 10, or 100  $\mu\text{g}$  RNA from nontolerant animals and amplified and dot-blotted as in *a* using IL-2 primers. Under these experimental conditions 10-fold differences in target sequence are easily distinguishable. (c) Control PCR using primers and internal oligonucleotide for hybridization to

the dot blot derived from the rat cytoplasmic  $\beta$ -actin sequence. Samples are presented in the same order as in *a*; n, no cDNA negative control. Samples of RNA used here are shown in Fig. 3 C. These experiments have been repeated on five different occasions using RNA isolated from two groups of animals with the same result.

respond to IL-2. Fig. 5 shows that cells isolated from such animals, 3 d after transplantation, respond poorly to IL-2 (Fig. 5 *b*), but develop a good IL-2 response by day 5 (Fig. 5 *a*). Thus the reduced IL-2R expression is confined to the cells of tolerant rats.

**Tolerant Animals Are Unable to Make Biologically Active IL-2.** It is known that the presence of IL-2 upregulates the expression of its receptor (23). It was therefore possible that the low IL-2R expression and poor response to IL-2 of cells from tolerant animals was due to an inability of such rats to make IL-2. Graft-infiltrating cells from tolerant animals were restimulated with alloantigen in culture and the supernatants of such cultures tested for the presence of biologically active IL-2. We were unable to detect any IL-2 activity in the cultures of cells from tolerant rats (Fig. 6).

**Tolerant Animals Express IL-2 mRNA.** We next examined whether the defect in IL-2 production was reflected by the level of messenger RNA induction. Amplification using RNA in the PCR from both tolerant and nontolerant animals resulted in a product of the predicted size for IL-2 (Fig. 7). Further, this product was positive when a Southern blot of the amplified DNA was probed with a third oligonucleotide whose position in the cDNA sequence lay between that of the original two primers. As a control, we used RNA from a syngeneic transplant which was negative in the PCR (Fig.



**Figure 9.** Administration of IL-2 at the time of transplantation prevents the induction of tolerance. Animals were treated and transplanted as usual. On the day of transplantation and for the subsequent 5 d, animals received intraperitoneal injections of rIL-2 diluted in RPMI containing 10% FCS and antibiotics. Animals were given  $60 \times 10^4$  (○),  $30 \times 10^4$  (□),  $15 \times 10^4$  (●) and  $7.5 \times 10^4$  (■) U IL-2 per day (9, 8, 8, 8 animals per group, respectively). Rats given medium alone or medium with IL-2 excipient control buffer survived indefinitely (six animals) (▲). DA rats transplanted with DA kidneys and treated with  $60 \times 10^4$  U IL-2 per day survived indefinitely (five animals) (△). The results are pooled data from 10 different experiments.

7). Since we used the PCR for these analyses, it is difficult to exclude absolutely the possibility that there may be quantitative differences in the levels of IL-2 mRNA in tolerant and nontolerant animals. However, our data (Fig. 8 *a*) repeating the analyses under limiting conditions suggests that this is not the case since we always find similar levels of product even when using few cycles of amplification with low levels of starting RNA. As may be seen from Fig. 8 *b*, these conditions allow easy discrimination of samples containing 10-fold differences in target sequence. Amplification of identical cDNA samples from rejected and nonrejected transplants using primers for cytoplasmic  $\beta$  actin as a control shows equal levels of product in all cases (Fig. 8 *c*).

*Tolerance Induction Is Abrogated by the Systemic Administration of IL-2.* We hypothesized that if the tolerant animals were unable to reject their grafts simply because they were unable to produce IL-2, it should be possible to abrogate the induction of tolerance by injection of IL-2 into animals at the time of transplantation. This approach is not unprecedented since Malkovsky et al. (24) were able to diminish neonatally induced allograft tolerance in vivo by injection of IL-2. Fig. 9 shows that even relatively low doses of systemically administered IL-2 completely abrogated the induction of tolerance and the animals acutely rejected their transplants. This effect was not due to nonspecific toxicity of the IL-2, since animals transplanted with a syngeneic graft and treated with IL-2 at the highest dose survived indefinitely with normal renal function. It seems unlikely that this treatment induced any additional or alternative routes of graft rejection, e.g., LAK cells, since the doses of IL-2 used are much lower than that normally required to induce this activity.

## Discussion

Thus we have demonstrated that a mixed state of unresponsiveness exists in these alloantigen-pretreated, graft-tolerant animals. These animals possess donor-specific cytotoxic cells and make some cytokines in response to alloantigen indicating that, in this model, deletion of specific alloreactive cells, if it occurs at all, cannot be complete. However, we have shown that the IL-2 pathway is abnormally regulated in these animals. IL-2R expression is decreased because of low expression of both the IL-2R  $\alpha$  and  $\beta$  chain genes and this is reflected in a decreased ability of cells to respond to IL-2. This appears to be due to a lack of production of biologically active IL-2 although the IL-2 gene is induced. Indeed, the induction of tolerance may be reversed by the administration of IL-2 to rats at the time of grafting. These data imply that the defect in alloantigen-pretreated animals lies in their inability to produce biologically active IL-2 which in turn results in abnormal IL-2R expression.

Interestingly, it appears in our model that both the initial injection of blood and the transplant are required for tolerance induction since animals given a third party transfusion before transplantation do not show a similar inhibition of the IL-2 response. It seems likely that a primary signal for activation of T cells occurs in this model since there is apparently normal IL-2 gene and low IL-2R gene induction. It is

possible that a secondary signal required for full and sustained T cell activation is, however, missing.

At what level regulation of IL-2 production occurs is not quite clear. Expression of the IL-2 gene clearly occurs, since the PCR analysis does not detect IL-2 gene expression in resting lymphocytes (Dallman, M.J., unpublished results) or, as shown above, in syngeneic transplants. These data therefore give rise to the intriguing possibility that there may be abnormal translational control of IL-2 production in the tolerant animals, although no such regulation has previously been described for the IL-2 gene. It is also possible that the mRNA we observe is not in a mature form. Since the structure of the rat IL-2 gene is unknown, this cannot be formally excluded. However, the PCR primers used span 3 introns of both the mouse and human IL-2 genes (25, 26) and therefore it seems unlikely, given the size of the IL-2 product found in these experiments, that the product is derived from unspliced RNA. Lastly, it is possible that the tolerant animals make functional IL-2 protein but that they simultaneously make an IL-2 antagonist resulting in our inability to observe functional IL-2 activity in the in vitro system. Such an example of a cytokine antagonist has recently been defined for IL-1 and its receptor (27, 28). We are currently investigating all of these possibilities.

Inhibition of the IL-2 pathway may be involved in tolerance induction where T cell anergy has been implicated; this has previously been alluded to by other workers, both when the antigen is presented neonatally (in neonatal tolerance or transgenic models; references 1, 6, 10, 29, and 30) and to adult animals (9). However, none of these studies examine IL-2R  $\alpha$  and  $\beta$  chain expression and the functional consequences of such expression together with an analysis of IL-2 at both the gene and protein levels. Further, only the work of Malkovsky et al. (1) has shown any effect of IL-2 administered to mice in the reversal of neonatal tolerance, but the data were in contrast to those of Tempelis et al. (31) in another model of neonatal tolerance.

The regulation of the IL-2 pathway demonstrated in this animal model of tolerance induction does bear a striking resemblance to the T cell anergy that has been induced in cell culture systems (32–35). In these systems, it has been suggested that T cell anergy results after antigenic stimulation in the absence of a second signal. The nature of this second signal is unclear but appears to require cell–cell contact and acts via nonconventional signal transduction mechanisms (35). A good candidate for such a signaling mechanism is the CD28 molecule which does not appear to signal either through calcium flux or activation of protein kinase C and requires a primary signaling event through either the CD3/TCR or CD2 proteins (36, 37). Activation through this molecule increases both the rate of transcription and mRNA stability of a number of cytokine genes, including IL-2 (38). The role of CD28 signaling in our model of tolerance is currently under investigation.

Recently, the inhibition of cytokine synthesis by Th1 cells has been reported to occur through the elaboration of cytokine synthesis inhibitory factor by Th2 cells (39). This protein does not inhibit production of cytokines by Th2 cells. Thus production of IFN- $\gamma$  is reduced but of IL-4 and IL-5 is

unaffected. However, secretion of lymphokines produced early during the response of Th1 cells, such as IL-2 and TNF- $\beta$ , is not, or is only minimally, affected. Further, this cytokine appears to act by preventing cytokine gene transcription. It thus seems unlikely that the elaboration of cytokine synthesis inhibitory factor could be responsible for the IL-2 regulation observed in our model of tolerance. However, we are currently developing PCR for the analysis of rat IL-4 and TNF- $\beta$  to further investigate this possibility.

It is important to note that, in clinical practice, preoperative, donor-specific blood transfusion has also been shown to improve survival of renal allografts between 1 and 2 HLA

haplotype-disparate, living, related donors and recipients (40). Whether or not similar regulation of the IL-2 pathway is involved in this clinical setting remains to be seen. With respect to this issue, immunosuppressive strategies that directly interfere with the IL-2 pathway, such as the use of cyclosporin A (41) and mAbs that react with the IL-2R (42), have been effective immunosuppressive agents in clinical transplantation. It is apparent then that specific regulation of the immune response at the level of the IL-2 pathway has profound implications for the induction of peripheral tolerance to alloantigens.

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