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Posttransplant Administration of Donor Leukocytes Induces Long-Term Acceptance of Kidney or Liver Transplants by an Activation-Associated Immune Mechanism

Yiqun Yan,* Suma Shastry,* Craig Richards,* Chuanmin Wang, † David G. Bowen,* Alexandra F. Sharland,* Dorothy M. Painter,‡ Geoffrey W. McCaughan,* and G. Alex Bishop2*

Donor leukocytes play a dual role in rejection and acceptance of transplanted organs. They provide the major stimulus for rejection, and their removal from the transplanted organ prolongs its survival. Paradoxically, administration of donor leukocytes also prolongs allograft survival provided that they are administered 1 wk or more before transplantation. Here we show that administration of donor leukocytes immediately after transplantation induced long-term acceptance of completely MHC-mismatched rat kidney or liver transplants. The majority of long-term recipients of kidney transplants were tolerant of donor-strain skin grafts. Acceptance was associated with early activation of recipient T cells in the spleen, demonstrated by a rapid increase in IL-2 and IFN-γ at that site followed by an early diffuse infiltrate of activated T cells and apoptosis within the tolerant grafts. In contrast, IL-2 and IFN-γ mRNA were not increased in the spleens of rejecting animals, and the diffuse infiltrate of activated T cells appeared later but resulted in rapid graft destruction. These results define a mechanism of allograft acceptance induced by donor leukocytes that is associated with activation-induced cell death of recipient T cells. They demonstrate for the first time that posttransplant administration of donor leukocytes leads to organ allograft tolerance across a complete MHC class I plus class II barrier, a finding with direct clinical application. The Journal of Immunology, 2001, 166: 5258–5264.

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Transplantation
Orthotopic liver or kidney grafts were performed, and in the case of kidney grafts, the contralateral kidney was removed 3 days after transplantation. Techniques for liver, kidney, and skin grafting have been reported previously (13). For some experiments, donors were given 10 Gy of whole body irradiation 1 wk before liver transplantation as described (9).

Immunohistochemical staining
Localization of leukocyte populations was by immunostaining of frozen sections by an indirect immunoperoxidase technique (18). Primary Abs were obtained from Dr. J. Sedgwick (DNAX Research Institute, Palo Alto, CA). They were OX2, reactive with MHC class II; OX27, reactive with donor (PVG) MHC class I; OX39, reactive with CD25; R73, reactive with αβ TCR; OX35 plus W3/25 1:1 mixture, reactive with CD4; OX42, reactive with CD11b/c expressed by monocytes/macrophages and dendritic cells (19); and MOPC 21 (Sigma, St. Louis, MO), negative control. Apoptotic cells were identified in frozen sections by TUNEL staining as described previously (20) with reagents obtained from Boehringer Mannheim (Mannheim, Germany).

Flow cytometry
Tissues were disrupted by passing the organs through a 100-mesh sieve. Leukocytes were purified by centrifugation of the washed cell suspension on isotonic Percoll (Pharmacia Biotech, Uppsala, Sweden) 1.05 g/ml for 15 min at 840 × g. The leukocyte-containing pellet was washed and analyzed by four-color flow cytometry as described (17). Abs to CD4 and PVG MHC class I were detected by anti-mouse Ab conjugated to fluorescein (F226; Sigma). R73-PE and OX39-biotin were obtained from Serotec (Oxford, U.K.); streptavidin-allophycocyanin was obtained from Molecular Probes (Eugene, OR). Dead cells were identified with propidium iodide at 1 μg/ml and excluded from analysis.

Measurement of cytokine mRNA expression
Cytokine mRNA in spleen was measured by quantitative RT-PCR as we have described previously (17). Total RNA was isolated and reverse-transcribed, and then aliquots of cDNA were measured for cytokine mRNA expression in a quantitative PCR. Quantification was by a noncompetitive method with external standards of known numbers of cytokine cDNA molecules. In some experiments, the number of cytokine mRNA molecules was adjusted to account for sample-to-sample variation with a GAPDH-positive internal control. In some experiments, quantitative PCR was performed by using a Model 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA) and a dye-labeled probe. For IL-2, the probe sequence was 6FAM-TTG CCC AAG CAG GCC ACA GAA TTG- TAMRA, and the amplification primers were CCC CAT GAT GCT CAC TTC GTG GTT TA (forward) and ATT TTC CAG GCA CTG AAG ATG TTT (reverse). For IFN-γ, the probe sequence was 6FAM-CCT TTT GCC AGT TCC TCC AGA TAT CCA AGA-TAMRA, and the amplification primers were AGT CGT CAA GAG AAC TAT TTT AAC TCA AGT AGC AT (forward) and CTG CTT CTC AAG TAT TTT CGT GTT AC (reverse). Dye-labeled primer and probe sets were designed by using Primer Express software (PE Applied Biosystems). Amplification primers were used at 300 nM concentration and some experiments were performed at 200 nM in Taqman universal PCR master mix (PE Applied Biosystems).

Preparation of spleen leukocytes and leukocyte subsets
Spleen leukocytes were aseptically prepared by mashing the spleen and then lysing the RBC in NH4Cl buffer as described (17). Cells were washed and injected at a dose of either 2 × 107 or 6 × 107 i.v. in 1 ml of isotonic saline. This cell population consisted of a mixture of T cells, B cells, myeloid cells, and small numbers of NK cells, and RBC and platelets were not detectable. In some experiments, spleen cells were fixed by incubation in 2% paraformaldehyde (BDH, Poole, U.K.) in PBS for 20 min on ice, washed thoroughly with PBS containing FBS, and injected i.v. For separation of leukocyte subsets from recipient spleen, aliquots of 1.5 × 107 spleen leukocytes were incubated with either OX27, R73, OX35 plus W3/25, or MOPC 21. After incubation for 30 min on ice and washing in PBS, the cells were incubated with 6 × 105 magnetic beads (Dynal, Oslo, Norway), washed, and separated on a magnetic column as described previously (13) before RNA extraction.

Statistical analyses
Comparison of survival data was by log-rank analysis of the product-limit estimate of Kaplan and Meier as described previously (9). Cytokine mRNA molecule numbers were analyzed by Mann-Whitney U test, and infiltration and apoptosis in tissue sections were analyzed by unpaired t test. Error bars for cytokine mRNA expression and infiltration and apoptosis show the mean ± SD of three separate animals.

Results
Posttransplant administration of donor leukocytes induces allograft acceptance
Rat kidney allografts in the strain combination of PVG donor to DA recipient (PVG→DA) mismatched at all MHC class I and II loci were rejected with a median survival time of 7 days (Table I). Treatment of the recipients with donor leukocytes at completion of transplantation led to long-term acceptance of the transplanted kidneys. All recipients of 6 × 107 spleen leukocytes survived for >300 days, and treatment with 2 × 107 leukocytes resulted in long-term acceptance of the majority of kidneys (Table I). Long-term surviving recipients of PVG kidney allografts plus PVG leukocytes were tested 250 days after transplant with a PVG donor strain skin graft. In three of four animals so tested, the skin graft survived for >100 days, showing that donor leukocyte treatment led to tolerance. Donor leukocytes failed to prolong renal allograft survival when their viability was impaired by fixation (Table I).

Unmodified liver transplant survivors were accepted in the PVG→DA strain combination without a requirement for conventional immunosuppression or donor leukocyte treatment (Table I). Irradiation of the liver donors abrogated tolerance, and administration of donor leukocytes at the time of transplantation of these irradiated livers resulted in restoration of graft survival (Table I), as we and others have shown previously (9, 13, 21). Liver allografts from PVG donors were rejected in the high responder Lewis rat strain,

Table I. Donor leukocytes induce long-term acceptance of kidney and liver transplants

<table>
<thead>
<tr>
<th>Organ Transplanted</th>
<th>Strain Combination8</th>
<th>Treatment6</th>
<th>Survival (days)6</th>
<th>MST (days)6</th>
<th>p (vs untreated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney PVG→DA</td>
<td></td>
<td></td>
<td>6,6,6,7,9,10,13</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Kidney PVG→DA</td>
<td>2 × 107 PVG spleen cells</td>
<td>＞ 168, 253, ＞ 268, ＞ 272</td>
<td>261</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Kidney PVG→DA</td>
<td>6 × 107 PVG spleen cells</td>
<td>＞ 300×4</td>
<td>＞ 300</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Kidney PVG→DA</td>
<td>6 × 107 fixed PVG spleen cells</td>
<td>6,8,8,9</td>
<td>8</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Liver PVG→DA</td>
<td>＞ 100×6</td>
<td>＞ 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver PVG→DA</td>
<td>Donor irradiation (10 Gy)</td>
<td>12,12,12,14,15,16,56,＞ 100×2</td>
<td>15</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Liver PVG→DA</td>
<td>Donor irradiation (10 Gy)</td>
<td>35,97,＞ 100×7</td>
<td>＞ 100</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Liver PVG→Lewis</td>
<td>＞ 14,14,15,17</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver PVG→Lewis</td>
<td>6 × 107 PVG spleen cells</td>
<td>＞ 100×5</td>
<td>＞ 100</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

8 Strains used were PVG (RT1b), DA (RT1a), and Lewis (RT1b) and were mismatched at all MHC class I and class II loci.
6 Treatment involved injection of cells into the recipient or irradiation of the donor or a combination of both.
7 Pathology analysis showed hydronephrosis, probably due to ureteric obstruction.
although rejection could be prevented by administration of donor leukocytes to the recipient at the time of transplantation (Table I). Histological examination of the long-term accepted grafts showed little or no evidence of chronic rejection but did reveal occasional small perivascular aggregates of leukocytes that had not damaged the graft or blood vessels within it. The above results showed that large numbers of PVG donor leukocytes administered at the time of transplantation converted rejection of both kidney and liver allografts to long-term acceptance.

Rapid migration of donor leukocytes to lymphoid tissues

PVG leukocytes administered i.v. to DA recipients of PVG renal allografts at the time of transplantation migrated rapidly to recipient lymphoid tissues. Flow cytometric analysis showed that donor cells comprised 4.7 ± 0.5% (n = 3) of recipient spleen leukocytes 1 day after renal transplantation supplemented with 6 × 10⁷ leukocytes. This declined rapidly to 1.6 ± 0.6% on day 3. Immunohistochemical staining demonstrated the presence of large numbers of donor cells (849 ± 97 cells/mm²) in the periarteriolar lymphoid sheaths of recipient spleens on day 1. Many also migrated to the paraaortic lymph nodes draining the graft (288 ± 132 cells/mm²). By contrast, very few donor cells had migrated to the spleen and lymph nodes of recipients of kidney allografts transplanted without additional donor leukocytes, with only 15 ± 8 cells/mm² and 10 ± 6 cells/mm², respectively, being detected on day 1.

Tolerance-associated immune activation

The levels of IL-2 and IFN-γ mRNA expression were compared in the spleen of recipients of allografts that were undergoing either rejection or tolerance. Marked activation, as measured by these parameters, was observed early after transplantation in the spleens from recipients of tolerant but not rejecting grafts (Fig. 1). There was a significant (p = 0.05) increase of IL-2 mRNA 1 day after transplantation in the tolerant leukocyte-treated kidney or liver recipients compared with untreated kidney recipients undergoing rejection or normal nontransplanted animals (Fig. 1A). The increase in IL-2 mRNA in kidney-tolerant animals was rapid and transient, characterized by a peak on day 1 that had disappeared by day 3, similar to the transient increase in IL-2 mRNA observed in recipient lymphoid tissues of animals during liver allograft tolerance (16). No detectable increase in IL-2 expression was observed in the spleen of recipients of untreated kidney allografts undergoing rejection or in syngeneic controls. IFN-γ mRNA showed a similar pattern of increase in tolerant kidney or liver allograft recipients that significantly exceeded that observed in untreated recipients or syngeneic controls (p = 0.05; Fig. 1B).

Cellular source of the IL-2 and IFN-γ mRNA in tolerant allograft recipients

The spleens of recipients of PVG→DA liver allografts were separated into subpopulations of leukocytes on day 1 after transplantation. The expression of IL-2 mRNA in these subpopulations is shown in Fig. 2A. Recipient cells expressed significantly (p = 0.05) more IL-2 than did donor cells. The activation-associated increase in IL-2 mRNA, compared with cells from a normal spleen, was confined to recipient cells from tolerant animals. Donor cells, analyzed by flow cytometry, comprised 1.4 ± 0.1% (n = 3) of the spleen leukocytes, and produced 3.0 ± 1.3% of the amount of IL-2 mRNA produced by recipient cells. T cells, which comprised 37.4 ± 2.7% of the spleen population, produced 97.6 ± 35% of the IL-2, whereas non-T cells only yielded 2.4 ± 0.9% of
pressed 71.6 liver-tolerant animals were followed by an extensive, diffuse infiltrate of T cells and IL-2R-expressing (IL-2R⁺) cells in the tolerant kidney grafts with a high ratio of IL-2R⁺ cells to T cells on day 3 after transplantation (Fig. 3; Table II). However, by day 5 after transplantation comparable numbers of T cells were present in the interstitial areas of both rejecting and tolerant kidney grafts. At the same time, the proportion of IL-2R⁺ cells in these areas had decreased in tolerant vs rejecting grafts (Fig. 3). Thus, the kinetics of the activated T cell response in tolerance and rejection differed markedly, with infiltration of large numbers of T cells and activated cells being seen mainly in the interstitial areas of tolerant grafts at least 2 days before their appearance in rejecting grafts. By contrast, the infiltrate in rejecting grafts was limited mainly to perivascular sites until day 5 after transplantation, when a marked increase in interstitial infiltrate occurred (Table II). Thus, it appears that tolerance is associated with altered patterns of T cell infiltration into the graft.

Graft-infiltrating leukocytes were stained for markers of T cells, IL-2R, and donor MHC class I to confirm that the infiltrate in day 3 tolerant kidneys was composed of recipient activated T cells and not injected donor cells that had localized to the transplanted kidney. The majority of T cells in the infiltrate was of recipient origin (Fig. 4A), donor cells comprising only 4.8 ± 2.6% of the T cell infiltrate. Many of the T cells in the infiltrate were activated as shown by the observation that 32 ± 5% of T cells expressed IL-2R α-chain. On further analysis of T cells, the majority (61.7 ± 0.3%) proved to be CD4⁺, a considerable proportion of which were IL-2R⁺ (43.7 ± 4.0%) (Fig. 4B).

The monocyte/macrophage and dendritic cell infiltrate was identified with the OX42 Ab reactive with CD11b/c expressed on these cells. Their pattern of infiltration was similar to T cells in that on day 3 there were significantly more (p = 0.004) monocytes/macrophages in the interstitial areas of tolerant than rejecting kidneys (Table II). By day 5 there were similar numbers of these cells in the interstitial areas of both tolerant and rejecting kidneys.

Early apoptosis in the lymphoid tissues and graft is associated with tolerance

To examine whether activation of T cells in tolerant animals led to their subsequent death by apoptosis, sections of kidney allografts and of recipient spleen were stained by TUNEL to identify apoptotic cells. TUNEL staining of tolerant kidney transplants on day 3 identified large numbers of apoptotic cells in the same location as the interstitial infiltrate of T cells and IL-2R⁺ cells (Fig. 5A). In contrast, there were few detectable apoptotic cells in the rejecting kidneys at the same time (Fig. 5B). Examination of the spleen of kidney allograft recipients showed a corresponding large increase in apoptotic cells in the periarteriolar sheaths of tolerant (Fig. 5C) compared with rejecting animals on day 3 (Fig. 5D).

Table II. Comparison of graft infiltrates during kidney allograft acceptance and rejection

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<tr>
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<td></td>
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</tr>
<tr>
<td>Normal PVG kidney</td>
<td>14 ± 6²</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>Day 3 KTx</td>
<td>865 ± 243²⁶</td>
<td>71 ± 23²</td>
</tr>
<tr>
<td>Day 3 KTx + cells</td>
<td>275 ± 124</td>
<td>206 ± 21</td>
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<tr>
<td>Day 5 KTx</td>
<td>2476 ± 758</td>
<td>604 ± 153</td>
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<td>1735 ± 269</td>
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* Monocytes/macrophages and dendritic cells were identified by expression of CD11b/c.

弯曲数/mm² of leukocyte subsets was identified by immunohistochemical staining of kidney tissue (mean ± SD of three replicate animals).

⁻ P<0.05; **, p<0.005; *** p<0.001.

FIGURE 3. Analysis of the graft interstitial infiltrate in PVG→DA kidney allografts. Immunohistochemical staining was used to identify the infiltrate of IL-2R⁺ cells and T cells in grafts of untreated recipients (rejection) or in recipients that were treated with 6 × 10⁷ donor spleen cells to become tolerant (tolerance). Results show the ratio of IL-2R⁺ cells to T cells in the interstitial areas of kidney allografts. * There was a significantly (p = 0.02) greater ratio of IL-2R⁺ cells in tolerance on day 3 (n = 3 per group).

the level of IL-2 mRNA expressed by the T cell population. The CD4 population, which comprised 31.0 ± 3.6% of spleen leukocytes, was mainly responsible for the increased IL-2 mRNA in spleen. Non-CD4 cells, which consisted of CD8 T cells, B cells, myeloid cells, and small numbers of NK cells, only expressed 3.9 ± 1.7% of the total amount of IL-2. These results show that recipient CD4 T cells produced >90% of the IL-2 mRNA in the spleen of tolerant animals.

IFN-γ mRNA, shown in Fig. 2B, was expressed predominantly by recipient compared with donor cells (p = 0.05), with only 4.4 ± 3% of the total IFN-γ being produced by donor cells. In contrast to IL-2, IFN-γ was expressed by both T cells, which expressed 71.6 ± 41.5% of the total, and non-T cells, which expressed 28.4 ± 16.3% of the total. CD4 and non-CD4 cells produced approximately equivalent amounts of IFN-γ mRNA. Thus, in quantitative terms, the rapid increase in cytokine mRNA in spleens of tolerant animals was largely attributable to activation of recipient CD4 T cells in the case of IL-2 and of recipient T cells and non-T cells in the case of IFN-γ.

T cell activation and macrophage infiltration in tolerant kidney allografts

The early cytokine increases found in the spleens of kidney or liver-tolerant animals were followed by an extensive, diffuse infiltrate of T cells and IL-2R-expressing (IL-2R⁺) cells in the tolerant kidney grafts with a high ratio of IL-2R⁺ cells to T cells on day 3 after transplantation (Fig. 3; Table II). However, by day 5 after transplantation comparable numbers of T cells were present in the interstitial areas of both rejecting and tolerant kidney grafts. At the same time, the proportion of IL-2R⁺ cells in these areas had decreased in tolerant vs rejecting grafts (Fig. 3). Thus, the kinetics of the activated T cell response in tolerance and rejection differed markedly, with infiltration of large numbers of T cells and activated cells being seen mainly in the interstitial areas of tolerant grafts at least 2 days before their appearance in rejecting grafts. By contrast, the infiltrate in rejecting grafts was limited mainly to perivascular sites until day 5 after transplantation, when a marked increase in interstitial infiltrate occurred (Table II). Thus, it appears that tolerance is associated with altered patterns of T cell infiltration into the graft.

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Counting of TUNEL<sup>+</sup> cells showed that there were significantly more of these cells in the spleen (p = 0.0003) and transplanted kidney (0.006) of tolerant compared with rejecting animals on day 3 (Fig. 6). By day 5 this difference had disappeared and there were similar numbers of apoptotic cells in both tolerance and rejection (Fig. 6). It is possible that the increase in TUNEL<sup>+</sup> cells in the rejecting kidney on day 5 might have been attributable to apoptosis of kidney parenchymal cells, reflecting damage to these cells as a result of rejection, which was usually complete by day 7. These results examining apoptosis in the transplanted kidney and recipient spleen indicate that early cell death was associated with tolerance rather than rejection.

Discussion

According to published evidence, donor leukocytes most effectively prevent rejection when given at least 1 wk before transplantation (5, 6). Such treatment is of little practical value in clinical transplantation programs, which depend on cadaver donors as the major source of organs. There is also the risk of patient presensitization by donor leukocyte pretreatment. The results presented here provide the first demonstration of markedly prolonged survival of either liver or kidney allografts when the recipients were given donor leukocytes at the time of transplantation, despite the use of completely MHC-mismatched strain combinations normally associated with rapid rejection. Recipients of kidney allografts treated in this way ultimately developed tolerance to subsequent skin grafts of the same strain as the kidney donor. Presumably, the explanation for the discrepancy between our studies and those of previous investigators is related to the choice of strain combination or organ transplanted. Previous studies have examined heart or skin allografts in mouse or rat models that are likely to present a greater barrier to induction of tolerance than kidney or liver allografts (5, 6, 13). Nevertheless, the transplant models examined here do reflect the strength of the barriers encountered in human transplantation, especially considering that human renal transplants, where possible, are matched at the MHC.

Our results show that donor leukocytes rapidly migrated to recipient lymphoid tissues, which are the site of initiation of the immune response to transplanted organs (22, 23). Within 24 h of their migration to the recipient spleen, rapid immune activation occurred, accompanied in the case of tolerance, but not rejection, with increased expression of IL-2 and IFN-γ mRNA. These results for renal allograft tolerance induced by administration of donor leukocytes at the time of transplantation closely parallel previous findings in spontaneous tolerance of liver allografts that show a rapid increase in splenic IL-2 and IFN-γ mRNA. This increase was not observed during rejection of liver (16), kidney (17), or skin (24) allografts.

Subsequent to immune activation in the recipient lymphoid tissues, an extensive diffuse infiltrate of activated recipient T cells, monocytes and macrophages appeared in the tolerant kidney grafts. This diffuse infiltrate differed markedly from the localized perivascular infiltrate that was initially observed in the untreated rejecting kidneys. At the same time, programmed cell death was
observed in the tolerant kidneys and in the splenic periarteriolar lymphoid sheaths of the recipients of these kidneys, but not in those undergoing rejection. Thus, there was a close concordance between kidney allograft tolerance induced by donor leukocytes and spontaneous acceptance of liver allografts, which show an early infiltrate of activated T cells accompanied by activation-induced cell death (AICD) within the tolerant graft (17, 25) and in the recipient lymphoid tissues (17).

Analysis of the cell subsets producing IL-2 and IFN-γ in the spleen and of the activated cells in the graft showed that recipient and not donor cells were activated during induction of tolerance, suggesting exhaustion of the rejection response. The immune activation and subsequent exhaustive differentiation of recipient T cells culminating in AICD described here for leukocyte-induced transplant tolerance might also be important in other models of allograft tolerance. Once such model involves treatment of cardiac allograft recipients by blockade of the costimulatory interactions of B7 with CD28 and of CD40 with CD40 ligand. In this case there was an absolute requirement for IL-2 and IFN-γ for tolerance of cardiac allografts (26, 27) consistent with a central role of immune activation in acceptance of these grafts. AICD also has been proposed as an explanation for the finding that inhibition of apoptosis prevents induction of transplantation tolerance (28), whereas promotion of apoptosis with the immunosuppressive drug rapamycin promotes acceptance (29).

This mechanism suggests novel means to promote tolerance in this model that may be relevant to clinical transplantation. As immune activation and apoptosis is associated with this form of tolerance, drugs such as rapamycin (29), methotrexate (30), or bisindolylmaleimide VIII (31), which promote apoptosis, might synergise with donor leukocyte administration. Some immunosuppressive drugs might potentially inhibit this form of transplant tolerance because of their inhibition of immune activation. For example, liver transplant tolerance is inhibited by corticosteroids (16), whereas cardiac allograft tolerance induced by costimulatory blockade is reversed by cyclosporine (32). Moreover, in a primates

FIGURE 6. Time course of apoptosis in periarteriolar lymphoid sheaths of recipient spleens (A) or in transplanted kidneys (B) of PVG→DA kidney transplants. Apoptotic cells were identified by TUNEL staining of sections of untreated recipients (rejection) or of those that were induced to accept the kidney grafts by treatment with $6 \times 10^7$ donor spleen cells (tolerance). Significantly more apoptotic cells were observed in tolerance compared with rejection on day 3 ($*, p = 0.006$; $**, p = 0.0003$; $n = 3$ per group).

References


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