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Fas LIGAND, TUMOR NECROSIS FACTOR-α EXPRESSION, AND APOPTOSIS DURING ALLOGRAFT REJECTION AND TOLERANCE

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Background. **Cytotoxic T cells can induce target cell lysis and apoptosis by different pathways. The interactions of CD95 antigen (Fas) with its ligand (CD95L) and of tumor necrosis factor (TNF)-α with its receptor (TNF-R1) lead to apoptotic cell death. Recently, conflicting studies have been published concerning the expression and the role of CD95L in allograft rejection and tolerance.**

Methods. **In this study, the intragraft expression of CD95/CD95L and TNF-α and the frequency and distribution of apoptotic cells were compared in a model of heterotopic cardiac allograft in the rat in which recipients were either not treated (acute rejection) or pretreated with donor-specific blood transfusion (tolerant).**

Results. **In the acutely rejected allografts, a peak in the expression of CD95L and TNF-α and in the number of apoptotic cells was observed during the first week after transplantation; apoptotic cells were confined to graft-infiltrating cells. In the tolerated allografts, however, levels of graft-infiltrating cell apoptosis and CD95L and TNF-α expression during the same period of time were dramatically lower. The expression of Fas was constitutive and was not modulated during acute rejection or tolerance.**

Conclusion. **This down-regulation of CD95L and TNF-α in allografts rendered tolerant by donor-specific transfusion suggests a role for apoptosis-inducing pathways in acute allograft rejection.**

The role of anti-donor cytotoxic T lymphocytes (CTLs*) in allograft rejection and tolerance remains a subject of controversy (*1*). While some authors have demonstrated a correlation between the occurrence of high CTL frequency in circulating blood and the incidence of allograft rejection (*2*), specific anti-donor CTLs have also been found both in humans and rodents in the blood and allografts of "tolerant" recipients (*2–4*). CTLs can lyse their target cells via the Ca^{++}-dependent granule exocytosis pathway (perforin/granzyme system) and/or via the Ca^{++}-independent CD95 (Fas)/CD95 ligand (FasL) pathway (*5–7*). In addition, it has recently been shown that tumor necrosis factor (TNF)-α is released by $CD8^+$ CTLs (*8*) and may also contribute to CTL-mediated cytotoxicity under certain conditions, as suggested in perforin-FasL–deficient mice (*9*). The final mechanism used by CTLs to induce target cell lysis is apoptosis (*5*), which can be mediated by different molecules including granzyme/perforin, CD95/CD95L, and TNF-α (*1, 6, 7*). The CD95 antigen and the p55 TNF receptor (TNF-R1) are related molecules that contain a death domain in their intracytoplasmic tail and can signal apoptosis (*6, 7, 10, 11*). It has been suggested that apoptosis has a role to play in liver allograft rejection (*12*), but its role in other models of allograft rejection has remained controversial (*13, 14*). Several studies have found increased expression of CD95L and TNF-α during acute rejection of allografts in rodents (*15, 16*) and in humans (*17, 18*). Larsen and colleagues (*15*) have shown that despite the up-regulation of CD95L during acute allograft rejection, the absence of an intact CD95/CD95L pathway did not alter the tempo of rejection, suggesting that CD95/CD95L interactions were not essential mediators of T-induced allograft damage. They suggested, instead, a role for FasL in the regulation of the immune response (*15*). By contrast, using the same strain combination (Fas-deficient *lpr* or FasL-deficient *gld* mice as donor or recipient), Seino et al. (*16*) reported results that pointed to CD95L as making a substantial contribution to cardiac allograft rejection. Interestingly, this effect was confined to the expression of CD95/CD95L by recipient cells and was independent of CD95 expression by grafts.

In a previous study, using a model of heart allograft tolerance induced by transfusion of donor strain blood, we showed that heart allografts were tolerated despite the strong anti-donor cytotoxicity that graft-infiltrating cells displayed in vitro and the high levels of perforin and granzyme A mRNA expressed in vivo (*3*). In this study, using the same model, we investigated intragraft expression of CD95/CD95L and TNF-α, two molecules also involved in CTL target lysis, and measured the proportion of apoptotic cells. During the first week after transplantation, a strong and transient up-regulation of CD95L and TNF-α was observed in the rejected allografts from untreated recipients. Expression of both correlated with the number of apoptotic cells seen in these animals. In contrast, in heart allografts from donor-specific transfusion (DST)-treated recipients, CD95L and TNF-α

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* *Abbreviations*: CTL, cytotoxic T lymphocyte; DST, donor-specific blood transfusion; HPRT, hypoxanthinguanine phosphoribosyltransferase; GIC, graft-infiltrating cells; IL, interleukin; PCR, polymerase chain reaction; RT, reverse transcriptase; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

TABLE 1. List of oligonucleotides used

Primers	Sequences (5′-3′)
CD95L*	atggaactgctttgatctctggagtgataagaaaggcggcc-ttgtg
CD95L-5′	atggaactgctttgatctctgg
CD95L-3′	agattcctcaaaattgatcagag
CD95L-RO	tggcttaggggctggctgtt
Soluble CD95*	ttcatgtgaacctaccaggctgacttgacagcaaaatgg-gcccac
Membranous CD95*	taacttttcgttcaccaggctgacttgacagcaaaatggg-cccac
CD95-5′	gatatgctgtggatcatggc
Soluble CD95-3′	ttcatgtgaacctaccaggc
Membranous CD95-3′	taacttttcgttcaccaggc
CD95-RO	ggactgatagcatctttgagg
TNFα*	ccttacggaacccctatatttataacctgtgactattta-tttat
TNFα-5′	ctactgcttcagctccacag
TNFα-3′	gacccgtagggcgattacag
TNFα-RO	gcttcccaacgctgggtcc

were down-regulated and the peak of expression seen in rejecting animals was absent. In addition, the level of apoptotic cells in these grafts was significantly lower than in rejected grafts.

MATERIALS AND METHODS

Animals, transfusions, and transplantations. Eight- to 12-week-old male LEW.1W (RT1.^{u}) and LEW.1A (RT1.^{a}) rats served as blood and heart donors and as blood and allograft recipients, respectively. These congenic strains were purchased from the Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Blood was collected by cardiac puncture in a heparinized syringe (final concentration: 20 IU/ml). To induce tolerance, allograft recipients were transfused with 1 ml of donor blood 14 and 7 days before transplantation. Heterotopic heart grafting was performed according to Ono and Lindsey's technique (*19*). Graft function was evaluated daily by palpation through the abdominal wall; rejection was defined as termination of palpable cardiac contractility. LEW.1A recipients rejected LEW.1W heart allografts in 6.4±1.7 days, whereas allografts in DST-treated recipients survived indefinitely (*20*).

Immunohistology. At the indicated time point, heart allografts were removed, immediately snap-frozen in liquid nitrogen after they had been embedded in optimal cutting temperature compound (OCT compound, Tissue Tek Miles Laboratories, Elkhardt, IN), and stored at −70°C until use. To detect CD95 ligand, 5-μm cryostat sections were air-dried, fixed in acetone, and incubated with a rabbit polyclonal antibody specific for the amino acids 250–269 mapping at the carboxy-terminus of rat CD95L (Santa Cruz Biotechnology, Santa Cruz, CA), followed by a peroxidase-conjugated chicken anti-rabbit IgG incubation for 60 min at 37°C. Peroxidase was developed with VIP (Vector, Burlingame, CA) and sections were counterstained with hematoxylin. Normal rat testes were used as a positive control. The area of each immunoperoxidase-labeled tissue section was determined by morphometric analysis using the point-counting technique (*21*). With a 121 intersection-squared grid in the eyepiece, sections were examined at a magnification of ×400 by two observers. For each of 15 adjacent high-power fields, the number of positively stained cells superimposed by an intersection was counted and the percentage area of each section occupied by such cells was calculated as follows: ([*number of positive cells under grid intersections*]/[*total number of grid intersections*]) × 100. Results for each time point were expressed as mean values ± SD of the results from four animals.

To detect cell apoptosis by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique, a commercial in situ histochemical assay (Apop Tag, Oncor, Gaithersburg, MD) was used according to the manufacturer's instructions. Briefly, tissues were sectioned (5 μm) and fixed in 10% neutral-buffered formalin (10 min at room temperature) and then in ethanol/acetic acid. The tissue was then treated with the terminal deoxynucleotidyl transferase enzyme and incubated in a humidified chamber at 37°C for 1 hr. After washing, the tissue was treated with peroxidase-labeled antidigoxygenin antibody, incubated for 45 min, and washed. Diaminobenzidene substrate was used for peroxidase development, and the tissue was counterstained with hematoxylin. Negative controls were prepared using the same conditions, with the omission of the terminal deoxynucleotidyl transferase enzyme. Normal rat mammary glands were used as a positive control.

Quantitative-competitive reverse transcriptase (RT) polymerase chain reaction (PCR). Messenger RNA transcripts were measured using a quantitative-competitive RT-PCR assay as previously described (*20, 22*). Briefly, the mRNA, reverse-transcribed into cDNA, is quantified by co-amplification with known amounts of an internal standard DNA differing from the wild type by a four-base pair deletion. After a run-off reaction initiated by one additional fluores-

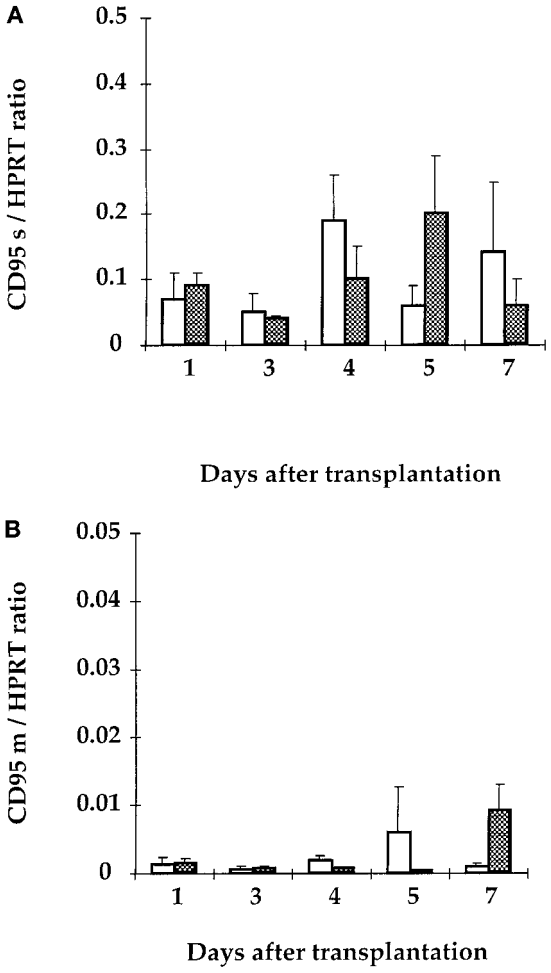


FIGURE 1. Kinetics analysis of the mRNA expression of membrane (A) and soluble (B) forms of CD95 in allografts. Expression of CD95 mRNA, membrane (A) and soluble form (B), was assessed by quantitative-competitive RT-PCR, as described in the *Materials and Methods*. Results for each time point are expressed as the mean ± SD in four animals of the ratio between the number of transcripts of CD95 (membrane [A] and soluble form [B]) and of HPRT in allografts from untreated (□) and DST-treated (▨) recipients.

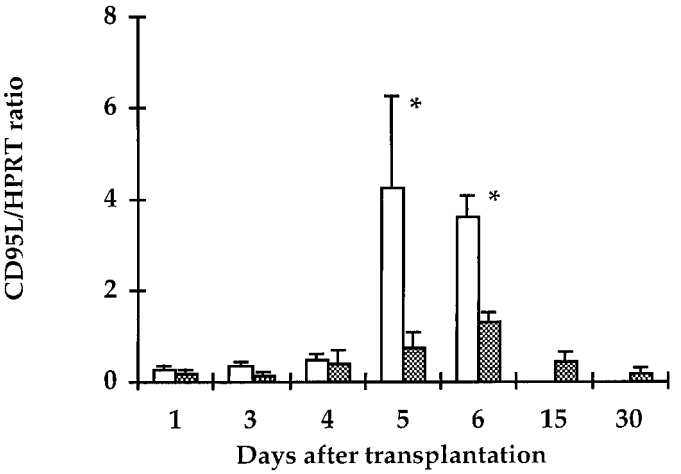


FIGURE 2. Kinetic analysis of CD95L mRNA expression in allografts. Expression of CD95L was assessed in allografts at different time points after transplantation by quantitative-competitive RT-PCR, as described in the *Materials and Methods*. Results for each time point are expressed as the mean ± SD in four to six animals of the ratio between the number of cytokine and HPRT transcripts in allografts from untreated (□) and DST-treated (▨) recipients. *$P<0.01$.

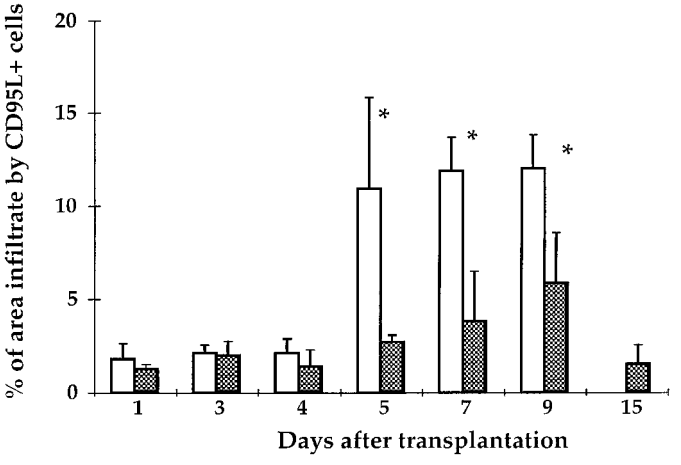


FIGURE 3. Semiquantitative analysis of the expression of CD95L protein by GICs in allografts. At the indicated time point, heart allografts were removed and frozen. Five-μm cryostat sections were incubated with a rabbit polyclonal antibody corresponding to amino acids 250–269 mapping at the carboxy-terminus of CD95L of rat origin, detected by means of peroxidase-conjugated chicken anti-rabbit IgG followed by VIP as a substrate, and slightly counterstained with hematoxylin. The incidence of CD95L protein staining (see Fig. 4, A and B) in heart allograft sections was scored blindly by morphometric analysis using the point-counting technique. Results are expressed as mean values ± SD of the results of four animals per time point. *$P<0.05$

cent nested oligonucleotide, these two species can be visualized on an electrophoresis gel using an automated DNA sequencer. The size difference introduced into the standard DNA makes it possible to distinguish between the two amplified species, i.e., the cDNA and the standard DNA. The Immunoscope software was specially devised to measure, for each detected DNA peak, both its length (with a precision of less than 0.2 nucleotides) and its area (with a precision of less than 5%) (*22*).

Table 1 shows the oligonucleotides used. Standard DNA constructions and mRNA extractions were performed as previously described (*20, 22*). For the cDNA preparation, 10 μg of total RNA from heart allografts were reverse-transcribed using a cDNA synthesis kit (Gibco Life Technologies, Grand Island, NY).

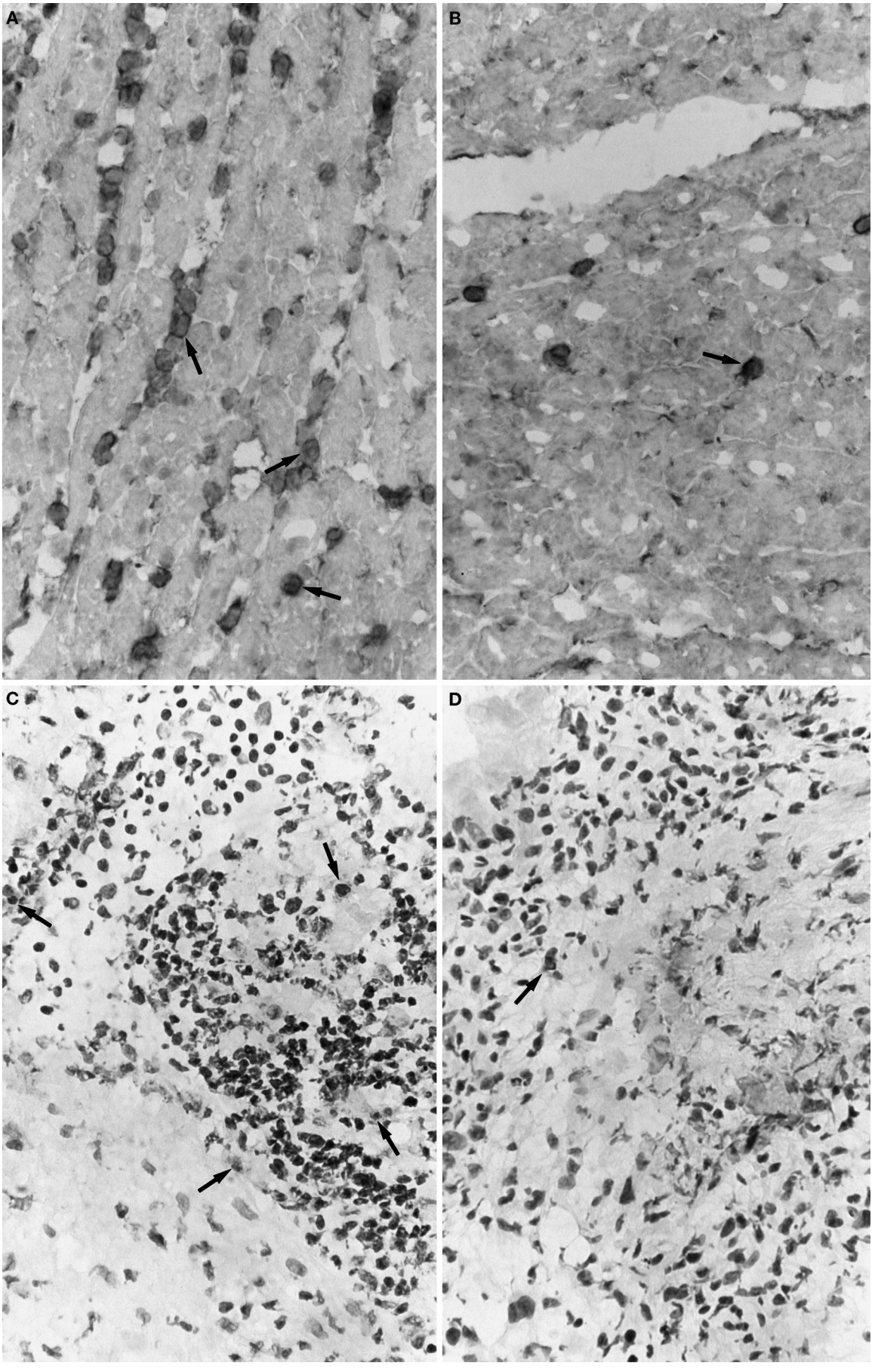
The levels of each mRNA were standardized by measuring in a similar manner the number of transcripts encoding the house keeping gene hypoxanthinguanine phosphoribosyltransferase (HPRT), and results are expressed as the ratio between the number of copies of the studied transcript and the number of copies of HPRT for the same sample.

Statistical analysis. Statistical analysis was performed using a Student's *t* test.

RESULTS

CD95 is constitutive and is expressed equally in allografts from untreated and DST-treated recipients. We and others (*3, 4*) have reported that graft-infiltrating cells (GICs) from DST-treated recipients compared with GICs from untreated recipients exhibit similar levels of in vitro anti-donor cytotoxicity and in vivo granzyme A and perforin mRNA expression (*3*). The usual donor target cells for in vitro cytotoxic assays are stimulated donor splenocytes which, ideally, express activation molecules and also CD95. In this study, we sought to explore the hypothesis that DST-induced allograft tolerance might be associated with a down-regulation in the graft (target cell) of molecules involved in cytotoxic mechanisms, such as CD95. In order to analyze the expression of CD95 in allografts in vivo, we performed a quantitative-competitive RT-PCR analysis using specific primers for the membrane and soluble forms of CD95. Figure 1, A and B, shows the kinetics of expression of the two forms of CD95 in allografts from untreated and DST-treated recipients. During the first week after grafting, CD95s mRNA was constitutive and no significant differences were observed between DST-treated and untreated recipients. CD95 m mRNA levels were found at very low levels (10 times less than CD95s) and no significant difference was seen in the two groups studied.

Differential CD95L and TNF-α expression in allografts from DST-treated and untreated recipients. To measure CD95L expression in allografts, RT-PCR was performed using the method outlined above with primers specific for CD95L. As shown in Figure 2, on day 1 after transplantation, CD95L transcripts in both situations were at low levels, similar to those observed in naive control hearts (day 0, not shown). During the following days (days 3–4), levels of CD95L transcripts in grafts from untreated recipients rose slowly, and then on days 5–6, they underwent a dramatic 10-fold increase. In grafts from DST-treated recipients, the expression of CD95L mRNA also increased over this period but at a much less pronounced rate and with markedly different kinetics. Levels of CD95L transcripts rose only slowly during the first week after grafting and then fell back to baseline levels on day 30. The expression of CD95L at the protein level was also analyzed using immunohistology. Figure 3 shows the kinetics of the semiquantitative analysis of CD95L protein expression in both situations during the first 15 days after transplantation. An example of anti-CD95L staining in allografts from untreated and DST-treated recipients on day 5 after transplantation is shown in Figure 4 (A and B). Two major features were evident from these results.


A
B
C
D

First, the kinetics of CD95L expression as determined by immunohistology were consistent with those determined by RT-PCR analysis of mRNA: a peak on days 5–9 in allografts from untreated recipients and, in contrast, significantly lower expression of CD95L in tolerated allografts. Second, anti-CD95L antibody staining was almost completely confined to GICs.

The kinetics of TNF-α mRNA expression in allografts was also assessed by quantitative RT-PCR. As shown in Figure 5, on days 1 and 3 after transplantation, levels of TNF-α transcripts were low, as in syngeneic grafts (data not shown) in both situations. During the following days (days 4–7), grafts from untreated recipients showed a marked increase in the level of TNF-α transcripts, which peaked on day 4 and decreased thereafter. In contrast, allografts from DST-treated recipients showed only a very low increase in levels of TNF-α gene expression on days 4 to 7.

Apoptotic cells are confined to GICs and decreased in allografts from DST-treated recipients. In order to determine whether apoptosis was a mechanism involved in allograft rejection and tolerance, we analyzed allografts from untreated and DST-treated recipients at different time points, using the in situ TUNEL method to detect DNA fragmentation. Figure 6 shows a semiquantitative analysis of apoptotic cells in allografts using this method. Apoptotic cells were present in both sets of allografts, but in numbers whose mean was significantly higher ($P<0.05$) on days 5 and 6 in those from untreated rats than in those from DST-treated rats. As shown in Figure 4 (C and D), the apoptotic cells were observed mostly among GICs, and surprisingly, none of the sections examined showed detectable evidence of apoptotic myocytes, even within regions of myocyte damage.

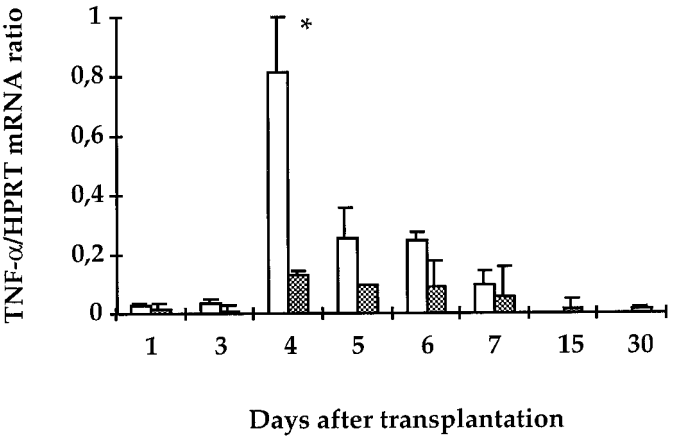


FIGURE 5. Kinetic analysis of TNF-α mRNA expression in allografts. Expression of TNF-α was assessed in allografts at different time points after transplantation by quantitative-competitive RT-PCR, as described in the *Materials and Methods*. Results for each time point are expressed as the mean ± SD in four to six animals of the ratio between the number of cytokine and HPRT transcripts in allografts for untreated (□) and DST-treated (▨) recipients. *$P<0.02$

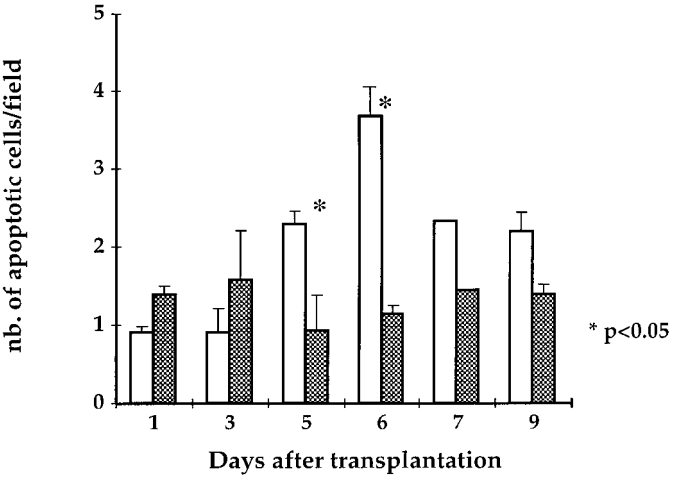


FIGURE 6. Semiquantitative analysis of apoptotic cells by the TUNEL method in allografts. Cell apoptosis was detected by the TUNEL technique; a commercial in situ histochemical assay was used according to the manufacturer's instructions (see *Materials and Methods*). Results for each time point are expressed as mean score ± SD of the number of apoptotic cells/field counted by three different investigators in four allografts from untreated (□) and four from DST-treated (▨) recipients. *$P<0.05$

DISCUSSION

Cytotoxic T cells were originally defined as lymphocytes present during allograft rejection in vivo that were able to destroy in vitro target cells syngeneic with the allograft (*23*). Nevertheless, the role of CTLs in allograft rejection and damage is still a matter for discussion. We have previously shown (*3*) that GICs from DST-treated and untreated recipients express similar anti-donor cytotoxicity when measured in vitro. This phenomenon, described as "split tolerance," has also been demonstrated in other experimental models of alloreactivity (*24, 25*). Furthermore, similar levels of perforin and granzyme A mRNA are expressed in grafts from both groups of recipients (*3*). The perforin/granzyme effector pathway has been described as the main pathway of cytotoxicity for $CD8^+$ T cells (*7*), and our results showing that this pathway was not modified in tolerant animals (*3*) suggested that it does not play an essential role in the effector phase of allograft rejection. Here, we show that the expression of CD95L and of TNF-α is both down-regulated in tolerated allografts compared with allografts from rejecting animals during the first week after transplantation and correlated with a significantly lower incidence of intragraft apoptosis.

The expression of CD95 transcripts was measured in allografts from DST-treated and untreated recipients using a

FIGURE 4. Examples of staining for CD95L and apoptosis in allografts. Expression of CD95L protein in allografts: Heart allograft cryostat sections from (A) control untreated and (B) DST-treated recipients 5 days after transplantation were immunostained with an anti-CD95L antibody (arrows: typical CD95L-positive cells; magnification ×400). Cell apoptosis detected by the TUNEL technique in allografts: A commercial in situ histochemical assay was used according to the manufacturer's instructions (see *Materials and Methods*). Myocardial sections from (C) untreated and (D) DST-treated recipients on day 5 after transplantation are shown. Apoptosis was observed mostly in GICs, usually in groups of cells; the severity varied in different regions. Some apoptotic cells are indicated with arrows (magnification ×400).

quantitative RT-PCR assay. Fas was expressed constitutively in both situations with no significant difference in the level of its expression between the two groups over a 7-day period after grafting. Our results in untreated recipients agree with those of Larsen et al. (*15*), who showed that CD95 transcripts were constitutively expressed in syngeneic and allogeneic grafts. In addition, the fact that tolerated allografts also expressed CD95 transcripts shows that neither a lack nor a down-modulation of CD95 in the target organ can explain the absence of allograft destruction despite the presence of alloreactive CTLs (*3*).

Interestingly, the expression of CD95L (FasL) in allografts from untreated recipients was highly up-regulated (10 times its baseline level) after transplantation, reaching a peak on days 5–6 (Fig. 2). This peak of expression was dramatically down-regulated in allografts from DST-treated recipients. We have previously shown, using this same allograft model (*20*), that during the first week after transplantation, allografts from untreated and DST-treated recipients are heavily infiltrated by recipient mononuclear cells. The profile of cytokine mRNA expression in rejecting allografts is characterized by a strong induction (on days 4–6) of interleukin (IL)-2, IL-10, and interferon-γ. This induction is absent in tolerated allografts, which suggests suppression or anergy of the T-cell response in those animals (*20*). A similar perturbation of the T-cell response is reflected in the decreased expression of the IL-2R in tolerant rats observed in another DST model (*26*). The expression of CD95L reported in the present study has shown that in allograft rejection, FasL was induced with kinetics similar to those of other molecules involved in cell activation and that its expression was down-regulated in tolerated allografts. Since the CD95/CD95L interaction has been shown to contribute to cell activation (*27*), the demonstration of strong expression of CD95L early in the course of the rejection process also suggests that a strategy involving the inhibition of CD95L (such as the use of a CD95-Ig fusion molecule) could have some effect on the rejection process.

Seino et al. (*16*), using a mouse model of allograft rejection, have demonstrated an increased expression of CD95L transcripts in heart allografts. Their study, using CD95-deficient *lpr* or CD95L-deficient *gld* mice as donors or recipients, suggested a role for the CD95/CD95L pathway in allograft rejection that was restricted to the recipient side. The authors hypothesized that CD95L expressed by recipient alloreactive T cells may act on recipient-derived macrophages in the graft to initiate inflammatory tissue damage. Multiple effector mechanisms have been implicated in allograft rejection, but the precise role of monocytes/macrophages in this process is still unknown, despite the fact that they represent 60–70% of the GICs (*20, 28–31*). TNF-α is a cytokine produced by a variety of cells, including lymphocytes, natural killer cells, and monocytes (*32, 33*), and has been shown to play a role in allograft rejection (*34–38*). Nitric oxide and TNF-α are powerful weapons in macrophage-mediated cytotoxicity. In support of a role for monocytes/macrophages in allograft rejection, we have shown (Josien et al., unpublished results) that the expression of inducible nitric oxide synthase (which regulates the production of nitric oxide [*39, 40*]) and TNF-α (this report) was also increased during allograft rejection in this model, correlating with the expression of CD95L. Furthermore, both factors were down-regulated in allografts rendered tolerant by DST, suggesting that macrophage activation was suppressed in these allografts.

It has been suggested that apoptosis may be a mechanism of cell death in liver allograft rejection (*12*); it has also been implicated in acute tubular necrosis and acute renal allograft rejection in humans (*41*). Bergese et al. (*14*) evaluated the frequency and distribution of apoptotic cells in heart allografts in acute rejection and in tolerance. Their results showed that rejecting allografts contain only rare apoptotic cells while, in contrast, tolerated allografts analyzed 60 days after transplantation contain large numbers of them. They concluded that apoptosis does not appear to be indicative of acute rejection and is not related to parenchymal damage, and suggested that apoptosis in tolerated allografts may be related to a mechanism of immunoregulation either in allograft acceptance or chronic vascular remodeling. Our results show an increased frequency of apoptotic cells during the first week after transplantation in rejecting allografts, which was significantly lower in allografts rendered tolerant by DST. However, the fact that the apoptotic cells belong mostly to GICs and that we could not detect apoptotic myocardial cells supports the hypothesis that T-cell anti-donor cytotoxicity with subsequent graft damage does not play a fundamental role in allograft rejection. However, we cannot exclude the presence of apoptotic myocytes among apoptotic GICs. Jollow et al. (*13*) investigated the incidence of apoptosis in myocytes and mononuclear cell infiltrates using endomyocardial biopsy specimens from cardiac transplant patients. They reported that the incidence of apoptotic cells increased with the grade of acute rejection and that apoptosis was again confined to the mononuclear cell infiltrate, suggesting that it could reflect the state of activation of GICs. The discrepancies with Bergese's results in tolerated allografts could be due to the timing of the analysis. Whereas we analyzed tolerated allografts over the phase of induction of tolerance (first week), they performed their analysis 60 days after transplantation.

Our results suggest that in acute allograft rejection, CD95L, TNF-α, and apoptosis play a role that is suppressed in DST-induced tolerance and, given the decrease of apoptosis of GICs, offer a basis for understanding the apparent paradox of the increased numbers of GICs in tolerant animals during the first week after transplantation that have been reported by various authors (*20, 31*). We have also provided some indirect evidence for the possibility that in the effector mechanisms of allograft rejection, monocytes/macrophages play a role that is suppressed in allografts rendered tolerant by DST.

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