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Apoptosis Within Spontaneously Accepted Mouse Liver Allografts

Evidence for Deletion of Cytotoxic T Cells and Implications for Tolerance Induction[1]

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MHC-mismatched liver grafts are accepted spontaneously between many mouse strains. The underlying mechanism(s) is unclear. In the B10 ($H2^b$) to C3H ($H2^k$) strain combination used in this study, donor T cells within the liver were rapidly replaced within 2 to 4 days of transplantation with those of the recipient. Freshly isolated liver graft-infiltrating cells harvested on days 4 and 7 exhibited strong CTL responses against donor alloantigens. CTL activity was reduced substantially, however, by day 14, although levels of CTL precursors in the spleen and liver remained high. Examination of the liver allografts by in situ terminal deoxynucleotidyltransferase-catalyzed dUTP-digoxigenin nick end labeling on days 4, 7, and 14 after transplantation revealed prominent apoptotic cells dispersed throughout the nonparenchymal cell population. When acute liver allograft rejection was induced by administration of IL-2 from days 0 to 4 post-transplant (median survival time, 5 days), apoptotic activity (day 4) was reduced substantially, whereas CTL activity was enhanced. Nonparenchymal cells isolated from allografts of unmodified recipients 4, 7, and 14 days after transplantation exhibited significantly higher DNA fragmentation after 18-h culture than cells from liver isografts. Moreover, the level was 4 to 5 times higher than that of cells from IL-2-treated mice (on day 4). These observations suggest that T cell deletion, not regulation, may be responsible for spontaneous liver allograft acceptance. The molecular recognition events that cause apoptosis of infiltrating T cells and why this occurs within liver grafts, but not heart or skin grafts, remain to be elucidated. *The Journal of Immunology,* **1997, 158: 4654–4661.**

Livers transplanted between members of MHC-disparate mouse strains survive indefinitely without a requirement for immunosuppressive therapy. For example, in the C57BL/10 (B10; $H2^b$) to C3H ($H2^k$) strain combination, orthotopic liver grafts are accepted permanently (median survival time (MST),[3] >100 days), while skin or heart grafts are rejected acutely (MST, 10 days) (1). Common to these different types of transplant is a marked T lymphocyte infiltrate that results, except in the case of liver, in graft destruction (2). Acceptance of the liver confers donor-specific unresponsiveness in vivo, e.g., to a challenge skin graft (1, 3). Paradoxically, however, vigorous MLR and CTL responses to donor alloantigens are generated in vitro by graft nonparenchymal cells (NPC) and spleen cells of liver allograft recipients. This phenomenon, described as split tolerance (2), is also observed after rat liver transplantation (4) and occurs in a number of other experimental models of alloreactivity (5–7). The persistence of ex vivo responsiveness, including the capacity to generate donor-specific CTL from the spleen, indicates that clonal deletion of CTL precursors (CTLp) does not occur. Alternative mechanisms of tolerance induction appear to be operative. Thus, a tolerogenic effect of donor liver-derived soluble MHC class I Ags has been suggested (8), but hepatic allografts from class I-deficient donors are also accepted indefinitely (9). More recent observations have implicated donor leukocyte microchimerism (10–12) and, in particular, a role for donor-derived dendritic cell progenitors (13) in liver transplant tolerance. The precise mechanism(s) underlying this phenomenon, however, remains to be elucidated.

Two recent studies in mice have focussed attention on the possibility that apoptotic death of graft-infiltrating cells mediated through activation of the Fas/Apo-1 (CD95) receptor system might contribute to the immune privilege of tissues such as the testis (14) and the anterior chamber of the eye (15). Although apoptosis has been described as a mode of hepatocyte death in allograft rejection (16), and other liver diseases (17), little attention appears to have been paid to programmed cell death in the inflammatory cell infiltrate in liver transplantation.

In the present study, we hypothesized that spontaneous mouse liver allograft acceptance might be due to a high level of in situ death of graft-infiltrating cells, in particular CTL, while CTLp within recipient lymphoid tissue might persist. We first examined the kinetics of cytotoxic activity exhibited by freshly isolated liver allograft NPC. High antidonor CTL activity early (4 days) after transplantation was followed by a marked decline thereafter. Following in vitro stimulation, however, high levels of CTL were still generated by both liver NPC and spleen cells. Evidence was then

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[3] Abbreviations used in this paper: MST, median survival time; NPC, nonparenchymal cells; CTLp, cytotoxic T lymphocyte precursors; TUNEL, terminal deoxynucleotidyltransferase-catalyzed deoxyuridine triphosphate-digoxigenin nick-end labeling.

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obtained for the apoptosis of graft-infiltrating cells, associated with the progressive decline in expression of CTL function. Apoptotic activity was reduced, and CTL activity of freshly isolated cells was increased however, in NPC obtained from mice in which acute liver rejection was induced by the systemic injection of IL-2, a T cell growth factor known to inhibit programmed cell death of CTL (18).

Materials and Methods

Mice

Male C57BL/10J (B10; $H2^b$), C3H/HeJ (C3H;$H2^k$), and BALB/c ByJ ($H2^d$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center (Pittsburgh, PA), provided with Purina rodent chow (Ralston Purina, St. Louis, MO) and tap water ad libitum, and used at 10 to 12 wk of age.

Orthotopic liver transplantation

The B10 to C3H strain combination that permits spontaneous acceptance of liver allografts was used. Operations were performed from B10 donors to C3H recipients under inhalation anesthesia, using methoxyflurane (Pitman-Moore, Atlanta, GA). Livers were transplanted orthotopically, using the technique of Qian et al. as previously described (19). No hepatic artery revascularization was attempted, and no immunosuppressive therapy was used. Isografts (C3H→C3H) were also performed for control purposes. Tissues were obtained from the animals on days 2, 4, 7, 14, 60, and 150 after liver transplantation (day 0). There were at least three animals in each experimental group.

Liver rejection model

As a model of acute primary liver allograft rejection, recombinant mouse IL-2 (Cetus Co., Emeryville, CA; 4×10^5 U/day i.p.) was administered to graft recipients for 5 days, starting on the day of transplant.

Isolation of liver NPC

Livers were perfused in situ with collagenase solution and further digested in vitro, and the NPC were isolated by centrifugation, using self-generating Percoll solution (Sigma Chemical Co., St. Louis, MO) as described previously in detail (20).

Flow cytometric analysis of donor and recipient T cells

Both normal liver NPC and those isolated from liver allografts were analyzed for the relative frequency of pan T cells and T cell subsets expressing donor (B10) or recipient (C3H) MHC class I Ags. Two-color immunofluorescence staining was performed by first incubating the cells (5×10^5/tube) for 60 min at 4°C with 100 μl of phycoerythrin-conjugated anti-CD3 (hamster IgG) or anti-CD4 or anti-CD8 (both rat IgG2a; all mAbs from PharMingen, San Diego, CA). The cells were washed twice, then 100 μl of biotin-conjugated mouse IgG2a anti-mouse H-$2K^b$ (donor) or H-$2K^k$ (recipient) mAbs (PharMingen) was added. After 60-min incubation at 4°C, the cells were washed and then further incubated for 30 min at 4°C with streptavidin-conjugated FITC (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After additional washes, the cells were resuspended in 1% paraformaldehyde and analyzed in a FACScan flow cytometer (Becton Dickinson Co., Mountain View, CA). Appropriate fluorochrome-conjugated, isotype-matched, irrelevant mAbs were used as appropriate negative controls.

Cell-mediated lymphocytotoxicity assay

Freshly isolated liver NPC or spleen cells obtained from C3H recipients of B10 livers were used as effectors. The EL4 ($H2^b$) lymphoma cell line (TIB39; American Type Culture Collection, Rockville, MD) was used as a source of specific target cells. The P815 ($H2^d$) mouse mastocytoma cell line (TIB64, American Type Culture Collection) and the R1.1 ($H2^k$) mouse lymphoma cell line (TIB42; American Type Culture Collection) were used as third party and syngeneic targets, respectively. The target cells were labeled with 100 μCi of [^{51}Cr]Na_2CrO_4 (New England Nuclear, Boston, MA), washed, and plated at a concentration of 5×10^3 cells/well in 96-well round-bottom culture plates (Corning, Corning, NY). Serial, twofold dilutions of effector cells were added to give E:T ratios of 100:1, 50:1, and 25:1 in a total volume of 200 μl/well. The percentage of specific ^{51}Cr release was determined after incubating the plates for 4 h at 37°C in 5% CO_2 in air. An aliquot (100 μl) of supernatant was recovered from each well after centrifugation at 500 × g for 1 min. Maximum ^{51}Cr release was determined by osmotic lysis of the cells. The percent specific cytotoxicity was calculated using the following formula: % cytotoxicity = 100 × [(experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm)]. The results are expressed as the mean ± 1 SD of the percent specific ^{51}Cr release in triplicate cultures. For the in vitro generation of CTL, 2×10^6 C3H splenic or liver T cells from normal mice or B10 allograft recipients were cultured with irradiated syngeneic (C3H) or allogeneic (B10) stimulator spleen cells at a low stimulator:responder ratio (1:4) for 96 h in 24-well plates. The stimulated T cells were then harvested, dead cells were removed by Ficoll purification, and CTL activity was determined as described above.

In situ detection of apoptotic cells in tissue sections

Liver grafts were harvested, and diced tissue samples were placed in embedding medium (Tissue-Tek OCT Compound, Miles, Inc., Elkhart, IN). The tissue was snap-frozen in liquid nitrogen and stored at −80°C until sectioned. Cryosections were cut at 4 μm, mounted on precleaned slides, air-dried overnight at room temperature, then fixed for 10 min at room temperature in 10% neutral buffered formaldehyde (pH 7.4), followed by two washes (5 min each) in PBS. DNA strand breaks were identified by in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) (21). Sections were immersed in 2% H_2O_2 in PBS for 5 min at room temperature to quench endogenous peroxidase activity, then incubated with 20 μg/ml proteinase K (Sigma Chemical Co.) for 15 min at room temperature, washed in PBS, and immersed in reaction buffer supplemented with terminal deoxynucleotidyl transferase and biotinylated dUTP. Sections were incubated in a humid atmosphere at 37°C for 60 min. Each experiment was performed with a negative control (without biotinylated dUTP) and a positive control (10-min pretreatment of sections with 1 mg/ml DNase dissolved in reaction buffer). Irradiated (500 rad) thymocytes incubated for 18 h, then cytocentrifuged onto glass slides (>90% apoptotic cells), also served as positive controls. After washing in stop/wash buffer (30 min at 37°C), anti-digoxigenin peroxidase (Oncor, Gaithersburg, MD) was added (30 min at room temperature). The sections were washed in PBS, and the reaction was developed with 3-amino-5-ethycarbizol. Slides were counterstained with Harris' hematoxylin and mounted with Crystal mount (Biomeda Corp., Foster City, CA). The average number of labeled cells per high power field was determined, and the results are expressed as the mean ± 1 SD.

DNA fragmentation assay

The protocol used was a modification of that described previously (22, 23). NPC (5×10^6) isolated from the grafted livers were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) for 18 h in 24-well plates. The cells were washed three times in PBS and transferred to 1-ml microcentrifuge tubes. After centrifugation, the supernatant was removed, and the cells were lysed by incubation in 400 μl of buffer containing 5 mM Tris, pH 8.0; 1 mM EDTA; and 0.5% Triton X-100 at 4°C for 30 min. The lysates were then spun at 16,250 × g for 30 min, and the supernatants were transferred to separate tubes. A second aliquot of lysis buffer was laid gently onto the pellet, and the tubes were spun again. The supernatant from each tube was removed and added to the previous supernatant. Each pellet was then resuspended in a volume of lysis buffer equal to the total supernatant volume. The supernatant and pellet fractions were sonicated, and the samples were plated in triplicate 100-μl serial dilutions in Dynatech Microfluor 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA). Then, 100 μl of a solution containing 0.6 μg/ml of the fluorescent DNA tag DAPI (4,6-diamidino-2-phenylindole; Sigma Chemical Co.) in a buffer containing 10 mM Tris, pH 8.0, and 100 mM NaCl was added to each sample. The amount of DNA was calculated from the emission at 465 nm, measured on a Dynatech Microfluor plate reader (350 nm excitation). After mathematical conversion of the data, fragmentation was calculated as: % DNA fragmentation = 100 × (DNA in supernatant/DNA in supernatant + pellet).

Results

Quantitation of donor and recipient T cells within liver allografts

Groups of unmodified C3H recipients of B10 livers were killed 2, 4, 7, 14, and 150 days after transplantation. The incidence of T cells and both $CD4^+$ and $CD8^+$ subsets within the freshly isolated NPC populations that were either donor or recipient derived was quantitated by two-color flow cytometric analysis. As shown in Figure 1, the liver transplants were infiltrated rapidly (within 2 days) by recipient MHC class I^+ ($H2K^{k+}$) T cells. By day 4, 95%

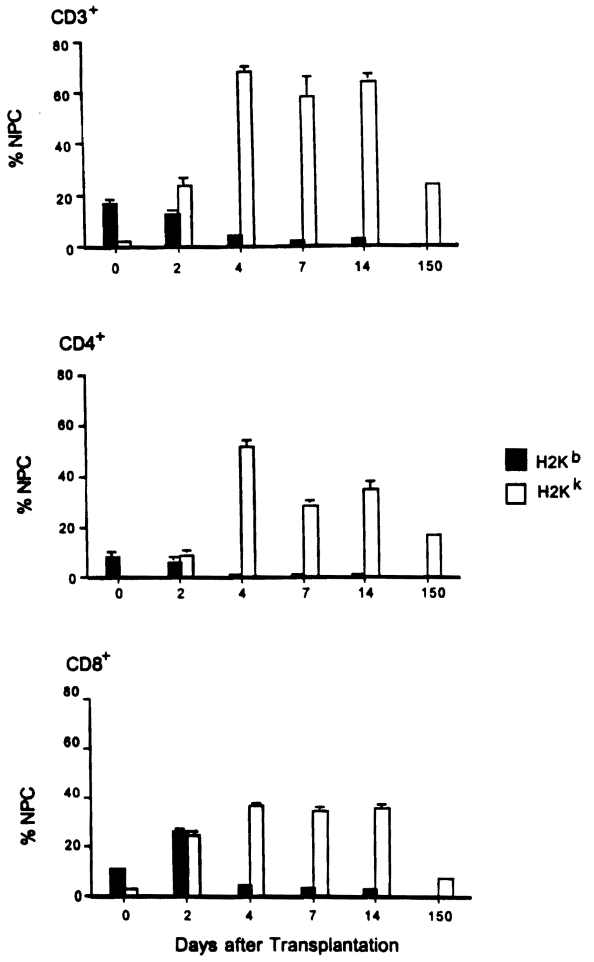

FIGURE 1. Flow cytometric analysis (MHC class I staining) of donor ($H2K^{b+}$) and recipient ($H2K^{k+}$) T cells and subsets in freshly isolated NPC populations from liver allografts (B10→C3H) at various times after transplantation. The results are representative of three separate experiments.

of the $CD3^+$ cells within the liver were of recipient origin. Of these, approximately two-thirds were $CD4^+$ cells. $CD8^+$ cells however, represented a higher proportion of the T cell infiltrate on days 7 and 14. By day 150, the number of (recipient) T cells within liver allografts was similar to that in normal liver.

Kinetics of cytotoxic activity of liver NPC and spleen cells of liver graft recipients

We next analyzed the CTL activity of graft-infiltrating cells isolated from mice killed 4, 7, 14, and 150 days after transplantation. By day 4, freshly isolated liver allograft NPC showed substantial levels of CTL activity (>40% specific ^{51}Cr release at a 100:1 E:T ratio) against target cells expressing donor alloantigens (EL4; $H2^b$), but not toward third-party (P815; $H2^d$) or syngeneic (R1.1; $H2^k$) targets (Fig. 2*A*). After day 4, CTL activity declined to 37% (day 7), 12% (day 14), and subsequently to normal control values (by day 150; Fig. 2*A*). Compared with NPC, freshly isolated recipient spleen cells (Fig. 2*B*) showed much lower levels of cytolytic activity against donor targets (12% specific lysis at a 100:1 E:T ratio) on day 4. There was a decline to 7% on day 7, and normal control values were reached by day 14. These results indicated that donor-specific, cell-mediated immunity, manifested by massive lymphocytic infiltration and high levels of donor-specific CTL within the graft, was induced within a few days of liver transplantation. It was also clear, however, that by day 14, the in situ donor-specific immune response had subsided due to a marked reduction in CTL activity within the graft. In liver-allografted mice in which acute graft rejection was induced by the systemic administration of mouse rIL-2, there was a significant increase in the CTL activity of freshly isolated NPC determined 4 days post-transplant (Table I).

Generation of CTL from the spleens of liver allograft recipients

We then investigated the in vitro generation of CTL from the spleens of liver allograft recipients harvested 4, 7, 14, and 150 days after transplantation. Normal C3H or graft recipient spleen cells were cultured with γ-irradiated B10 splenocytes at a low stimulator:responder ratio for 4 days. The cytotoxic activity of the stimulated C3H cells was then determined against donor-specific, third-party, and syngeneic targets. Compared with normal spleen cells that gave 7.2 ± 0.8% donor-specific cell killing (E:T ratio = 100:1), elevated levels of CTL activity were observed at all time points, with a peak of specific ^{51}Cr release (69.8 ± 3.4% at an E:T ratio of 100:1) 4 days post-transplant (Fig. 3). CTL exhibiting between 43 and 54% specific target cell lysis were generated from spleen cells obtained 7 to 14 days post-transplant. Thus, appreciable levels of donor-specific CTL could still be generated from host lymphoid tissue at least 14 days after transplantation despite liver allograft acceptance. Even by day 150, it was still possible to generate low, but significant, donor-specific CTL activity above that in normal spleen cells (15.4 ± 1.2% specific donor cell lysis). Similar results were obtained for the in vitro generation of CTL from liver NPC of liver allograft recipients (data not shown).

In situ visualization of programmed cell death

We speculated that the diminution in CTL function of the freshly isolated liver graft-infiltrating cells might be due to a high incidence of in situ apoptotic death of activated T cells. The fate of infiltrating mononuclear cells within the graft was therefore evaluated by investigation of their in situ apoptotic activity using the TUNEL assay. Compared with liver isografts (C3H→C3H), which showed only a modest inflammatory cell infiltrate on days 4, 7, and 14, there was heavy mononuclear cell infiltration of the B10→C3H liver allografts. Despite the significant ex vivo donor-specific CTL activity, these graft-infiltrating cells appeared innocuous in vivo, as there was little morphologic evidence of hepatocellular necrosis. Nick end-labeled apoptotic cells within the infiltrates were readily evident by day 4 after transplant (Fig. 4*B*) and increased in number by day 7 (Figs. 4*C* and 5). Thereafter, TUNEL-positive cells became progressively less numerous, although small numbers were evident 60 days post-transplant. In comparison, the number of positively staining (apoptotic) parenchymal cells was lower at all points tested. On day 7, it represented less than one-third of that in the TUNEL positive inflammatory cells. In liver isografts, the incidences of both apoptotic parenchymal cells and NPC were consistently very low (Figs. 4*A* and 5).

The next question addressed was whether apoptosis of graft-infiltrating cells represented a primary mechanism by which livers were accepted spontaneously or constituted an epiphenomenon of the alloimmune response. To further determine the relationship between apoptotic NPC and liver graft acceptance, the incidence of apoptotic graft-infiltrating cells in nonrejecting allogeneic livers was compared with that in rejecting hepatic allografts. Acute liver rejection was induced by the systemic administration of mouse rIL-2. As shown in Figure 5, IL-2 treatment (which resulted in enhanced specific CTL activity within the graft) was accompanied by a lower incidence (compared with that in tolerant animals at the

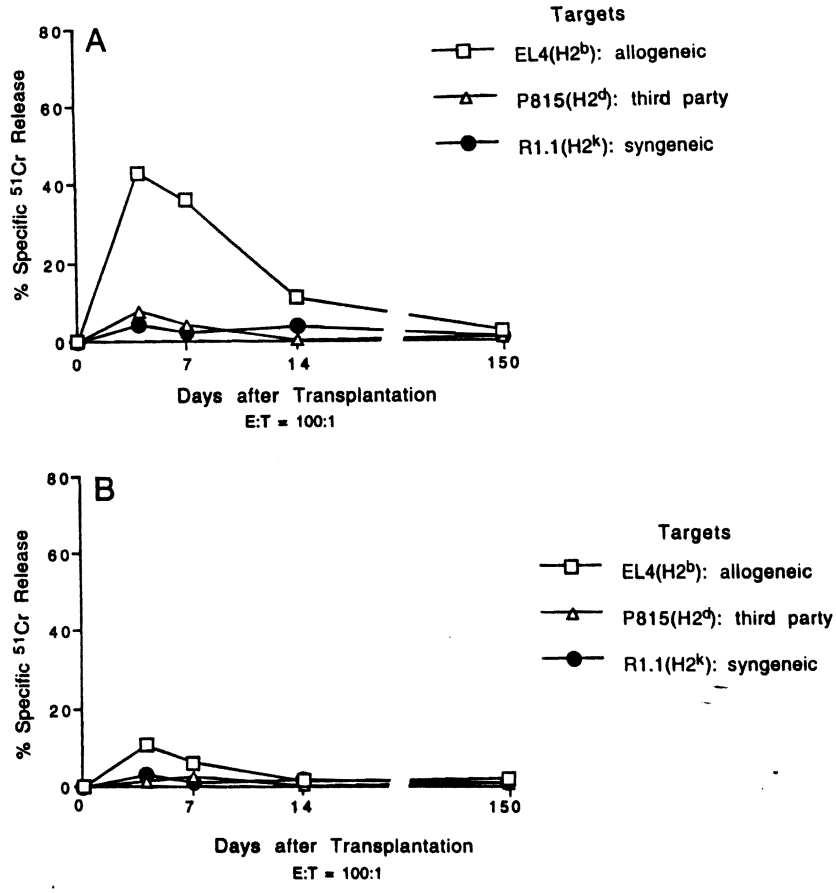

FIGURE 2. Cytotoxic activity of freshly isolated liver NPC (*A*) and spleen cells (*B*) from C3H recipients of B10 hepatic allografts. A 4-h ^{51}Cr release assay was used to determine cytotoxicity against target cells expressing donor ($H2^b$) alloantigen 4, 7, 14, and 150 days after transplantation. Third-party ($H2^d$) and syngeneic ($H2^k$) target cells were also used. Cells were pooled from groups of three transplanted animals, and the experiment was performed three times.

Table I. *Influence of systemic IL-2 on the cytotoxic activity of freshly-isolated NPC from C3H ($H2^k$) recipients of B10 ($H2^b$) liver allografts*[a]

	Targets: % Specific ^{51}Cr Release[b]								
	EL4 ($H2^b$) (allogeneic)			P815 ($H2^d$) (third party)			R1.1 ($H2^k$) (syngeneic)		
Treatment	100:1[c]	50:1	25:1	100:1	50:1	25:1	100:1	50:1	25:1
None	43.1 ± 1.9	33.8 ± 1.6	31.5 ± 2.9	7.8 ± 1.3	5.6 ± 1.3	5.4 ± 0.7	4.0 ± 0.7	2.0 ± 0.5	2.2 ± 0.8
Murine rIL-2 (d 0-3)	64.5 ± 2.7[d]	46.9 ± 1.7[d]	36.0 ± 1.3[d]	12.5 ± 0.1[d]	11.5 ± 1.2[d]	5.4 ± 1.2	2.3 ± 0.5	1.9 ± 0.6	2.1 ± 0.9

[a] Cells were isolated from liver grafts 4 days after transplantation.
[b] Cultures were maintained for 4 h.
[c] E:T cell ratio.
[d] Significantly higher than untreated controls ($p < 0.01$).

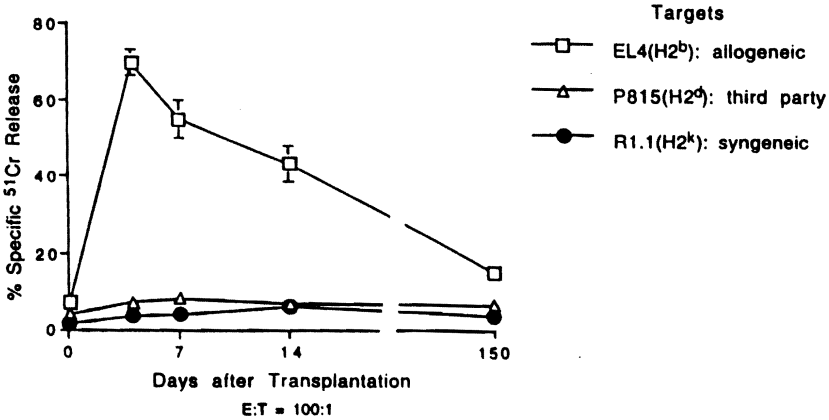

FIGURE 3. Generation of CTL from the spleens of C3H ($H2^k$) mice that received an orthotopic B10 ($H2^b$) liver transplant 4, 7, 14, or 150 days previously. Splenic T cells were incubated for 96 h with γ-irradiated (20 Gy) B10 spleen cell stimulators at a low stimulator:responder ratio (1:4) then used as effectors. The effector cells were cultured with target cells expressing the haplotype of donor (EL4; $H2^b$; □) or with third-party (P815; $H2^d$; △) or syngeneic (R1.1; $H2^k$; ●) targets. Cells were pooled from groups of three transplanted animals, and the experiment was performed four times. Results are the mean ± 1 SD.

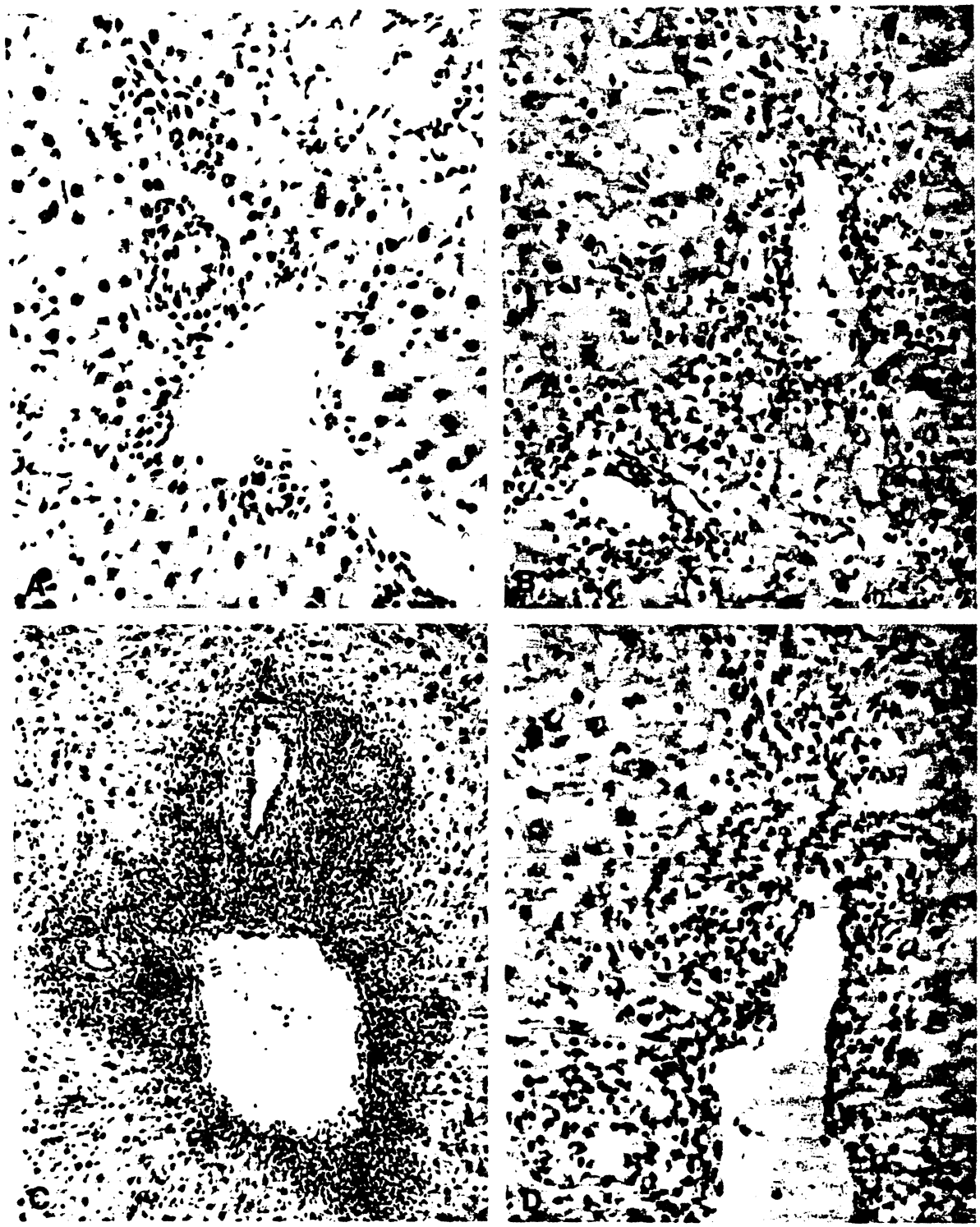

FIGURE 4. Apoptotic cells identified in portal areas of liver grafts by TUNEL staining, as described in *Materials and Methods*. *A*, Isograft (C3H→C3H), day 7; *B*, allograft (B10→C3H), day 4; *C*, allograft, day 7; *D*, allograft plus IL-2 administration, day 4. The cells were counterstained with hematoxylin. Magnification: *A*, *B*, and *D*, ×400; *C*, ×200.

same point (day 4)) of apoptotic cells within the inflammatory infiltrate (see also Fig. 4*D*); by contrast, there was a significant increase in hepatocyte death (TUNEL-positive cells) with IL-2 treatment (Fig. 5). These findings suggest that liver allograft survival may be the result of a relatively high level of apoptosis within the graft-infiltrating cell population that subverts the effector arm of the host immune response.

DNA fragmentation in cultured graft-infiltrating cells

Apoptosis was quantitated in NPC isolated from liver grafts (isografts, allografts, and allografts with IL-2 treatment) 4, 7, and 14 days after transplant (Fig. 6). The cells were cultured overnight (18 h) without stimulation. DNA fragmentation, an indicator of apoptotic cell death, was then quantitated. Cells from accepted allografts showed much higher DNA fragmentation on day 4

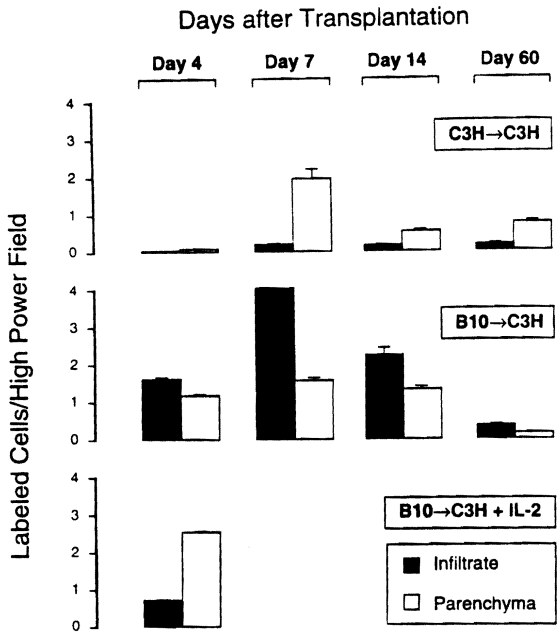

FIGURE 5. Incidence of apoptotic cells determined, as described in *Materials and Methods*, by in situ nick end labeling (TUNEL) within the NPC (infiltrate) and parenchymal cell populations of hepatic transplants (isografts, allografts, and allografts plus IL-2) at various times after transplantation.

(34.8 ± 10.2%), day 7 (40.1 ± 1.2%), and day 14 (25.7 ± 1.9%) compared with cells from isografts (12.6 ± 2.6%, 14.5 ± 5.7%, and 17.5 ± 2.4%, respectively; all $p < 0.01$). Interestingly, NPC from rejecting allografts (IL-2 treatment) exhibited significantly lower DNA fragmentation (7.2 ± 2.3% on day 4 post-transplantation; $p < 0.01$ compared with isografts; Fig. 6) than those from tolerant or isografted mice. These data indicate that acute liver allograft rejection (induced by exogenous administration of mouse rIL-2) is associated with inhibition of graft-infiltrating cell apoptosis.

Discussion

Liver transplantation in mice across MHC and non-MHC histocompatibility barriers results in spontaneous graft acceptance and the induction of donor-specific tolerance. Paradoxically, however, vigorous MLR and CTL responses are generated ex vivo against donor alloantigens by liver graft-infiltrating cells and spleen cells. This phenomenon has been described as split tolerance (2). Similar findings have been reported in rat liver transplantation models (4, 24). The mechanisms underlying this in vivo/ex vivo functional disparity have been investigated extensively, but are still poorly understood. All allotransplanted livers initially undergo a brisk episode of histologic rejection. This is manifested by heavy graft infiltration, but there is little hepatocyte necrosis. The inflammatory response decreases spontaneously within a few weeks (2, 25). In this study, we speculated that programmed cell death of liver-infiltrating cells might underlie functional tolerance. Examination of donor-specific cytotoxic activity of graft-infiltrating cells revealed high early (days 4–7) but rapidly diminishing CTL function against donor. This indicated that donor-specific immunity was induced initially, consistent with the massive lymphocytic infiltrate and the coincident high donor-specific CTL activity observed shortly after liver transplantation. It appears from our analysis of apoptotic cell death that the rapid waning of the in situ immune response may be due to the elimination of donor-specific CTL within the graft. Formal proof of this hypothesis, however, requires the demonstration of selective deletion of graft-infiltrating CTL expressing TCR specific for donor MHC in mice bearing a high frequency of these cells. Thus, Bertolino et al. (26) have recently described the peripheral deletion of autoreactive $CD8^+$ T cells in transgenic mice expressing allogeneic MHC class I in the liver. Notably, in experimental models of autoimmune disease, there is evidence of apoptotic elimination of target Ag-specific T lymphocytes within the affected organ. Moreover, it has been suggested that this mechanism may contribute to the subsidence of inflammation and the development of tolerance (27, 28).

In vitro studies have demonstrated that triggering of primary activated T cells via the TCR may lead to their death by apoptosis, but that resting T cells, such as CTLp, are not susceptible (29–32). We hypothesized that apoptosis of CTL within the liver might provide a mechanistic basis of allograft acceptance. Host recognition of donor alloantigens induces activation and subsequent proliferation of T lymphocytes bearing Ag-specific receptors. In the case of mouse heart and skin grafts, this results invariably in CTL-mediated graft rejection. With liver grafts, however, the initial extensive expansion of Ag-reactive T cell clones and graft infiltration is followed by attenuation of the CTL effector response. Rather than being rejected, the graft survives.

Clonal down-regulation during immune responses is a poorly characterized phenomenon. It seems likely, however, that some form of activation-induced cell death is involved. In vitro studies suggest that the susceptibility of activated CTL to apoptosis may relate to the interaction of CD95 ligand with its cell death-transducing receptor CD95 (Fas/Apo-1) (32). CTLp that lack CD95 (33), however, are less sensitive to apoptosis. As apparent from the findings of the present study, CTLp present in spleen may continue to provide the basis for renewal of the potential effector CTL population after liver transplantation. There are recent reports of high levels of expression of CD95L on parenchymal cells within immunologically privileged sites (34), in particular the mouse testis (14) and the anterior chamber of the eye (15). This has been linked to the death of specific activated T cells and to the maintenance of immune privilege within these sites. Others, however, have challenged the interpretation of these findings on the basis that no data for T cell functions were reported (35). Normal rodent and human livers do not appear to express CD95L mRNA (34, 36, 37), but CD95L mRNA is found within hepatocytes in inflammatory liver disease (36). Thus, CD95-CD95L interactions may provide a mechanism underlying apoptosis of liver allograft-infiltrating T cells. Even though CD95/CD95L interactions may not be essential mediators of T cell-induced allograft damage (38), they may play a regulatory role in both the clonal expression and the subsequent contraction of the T cell response. Others, however, have shown similar CD95L transcripts in mouse heart allograft rejection and tolerance models (39). Interactions between other cell surface-expressed receptor-ligand pairs (e.g., intracellular adhesion molecule-1/LFA-1, and 4-1BB/4-1BBL (40) and the roles of other members of the TNF family, including TNF, CD30 ligand, and CD40 ligand) that may enhance or inhibit CD95-mediated activation-induced cell death are also currently being tested. In addition, specific cytokines produced by liver cells, in particular TGF-β (41), which can induce T cell apoptosis (42), may be involved. One possibility, based on other models of alloreactivity, is that deletion of high affinity T cells may occur in vivo as a result of exhaustive differentiation (7, 26) in the presence of large amounts of donor Ag in the liver. The survival of low affinity cells (that function poorly in vivo) may provide a basis for the strong in vitro response to alloantigens.

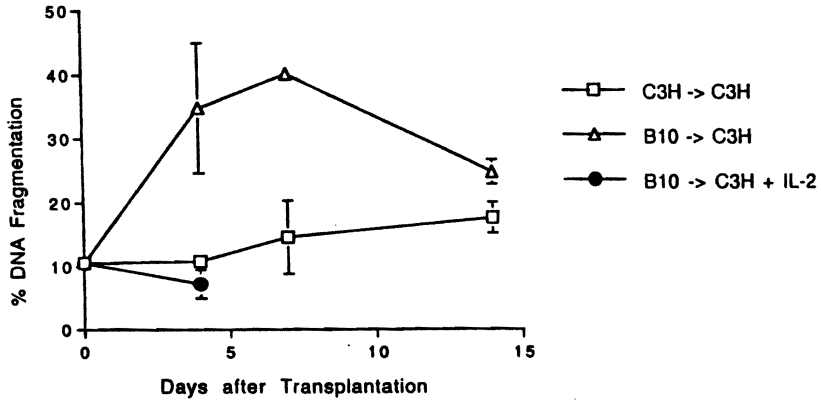

FIGURE 6. DNA fragmentation in NPC isolated from liver allografts 4, 7, and 14 days after transplantation. The cells were cultured overnight (18 h) without stimulation. DNA fragmentation was then measured as described in *Materials and Methods*. Cells isolated from liver isografts and from liver allografts of mice treated with systemic mouse rIL-2 (MST 5 days) were also examined. Cells were pooled from groups of three transplanted animals, and the experiment was performed twice. Results are the mean ± 1 SD.

Taken together, our observations suggest that a mechanism underlying the acceptance of MHC-disparate liver allografts in nonimmunosuppressed mice may be the apoptotic death of alloreactive CTL that occurs predominantly within the graft and is triggered by donor alloantigens. The death of donor-reactive T cells may also be attributable at least in part to interaction with donor liver-derived leukocytes, in particular populations of potential migratory dendritic cells (20, 43) that may express CD95L and have the ability to trigger apoptosis in activated allogeneic T cells (44, 45). Conceivably, apoptosis mediated by factors including specific cytokines or nitric oxide (46, 47) may also occur in donor leukocytes (dendritic cells and macrophages) present within the graft NPC population. This, too, could contribute to the regulation/subversion of what is, essentially, a two-way alloimmune response.

TCR triggering of activated T cells may lead to their death by apoptosis unless the T cells are rescued by growth-promoting signals such as IL-2 and IL-4 (18, 48, 49). The implication of the present findings is that IL-2, either alone or together with additional signals, may protect the CTL from apoptosis, thus predisposing to liver graft rejection.

Liver transplant tolerance is evident in all MHC-disparate mouse strain combinations that have been tested (1). Moreover, Ags introduced into the liver (via the portal venous or oral route) can induce Ag-specific tolerance (50). Several distinctive features of the liver microenvironment may contribute to its immunologic privilege and its inherent tolerogenicity. These include its regenerative capacity and a comparatively heavy endowment of ("passenger") leukocytes (51), including stem cells (52, 53), compared with that of kidney or heart. In rodent liver transplant models, these cells and their cytokine products may be essential for graft survival (11, 12) and may include donor-derived tolerogenic or deletional APC (13), such as may be represented by costimulatory molecule-deficient dendritic cell progenitors derived from the liver (13, 54). Further investigation of the role of programmed cell death in regulating immune responses within liver and other organ allografts (including challenge grafts that are tolerated in liver recipients) may provide new insight into their comparative immune privilege and tolerogenicity.

We have shown for the first time that high relative levels of T lymphocyte apoptosis within the graft-infiltrating cell population are correlated with liver allograft survival in nonimmunosuppressed recipients. Although this finding sheds light on why livers may be accepted in the face of ex vivo alloimmune reactivity, the mechanism of systemic tolerance induced by liver transplantation remains to be elucidated. As shown previously, however, in both mouse and rat models, systemic effects of liver transplantation include the capacity of liver leukocytes (including progenitor and stem cells) (12) to migrate extensively to recipient lymphoid tissues, where they may persist indefinitely (1, 51). These chimeric cells may provide not only a continuing source of allostimulation, but may also have the capacity to modulate the survival and function of alloactivated T cells within the recipient lymphoid system (55).

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