Myocyte Injury and Contraction Abnormalities Produced by Cytotoxic T Lymphocytes

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Background. The mechanisms by which ventricular function is altered during cardiac transplant rejection are not well understood. Therefore, an in vitro model system has been developed to facilitate investigation of lymphocyte-mediated myocyte injury.

Methods and Results. Splenic lymphoid cells were obtained from mice 8–10 days after placement of a vascularized abdominal cardiac allograft and were restimulated in vitro with irradiated donor-type splenocytes for 5 days. Cytotoxic effects of these allogenically stimulated lymphocytes on syngeneic and donor strain fetal cultured myocytes were determined by a 51Cr release assay at different lymphocyte to myocyte ratios. 51Cr release from donor strain myocytes was detectable within 1 hour of exposure, was maximal by 3–5 hours of coincubation with sensitized lymphocytes, and was allospecific. Cell injury manifest by 51Cr release was calcium dependent and was inhibited by pretreatment of lymphocytes with phorbol ester to deplete protein kinase C. Myocyte injury was also prevented by pretreatment of sensitized lymphocytes with anti-Thy 1.2 or anti-CD8 antibody plus complement but not by treatment with anti-CD4 antibody, indicating that CD8+ cytotoxic T cells are involved. Altered myocyte contractile motion preceded myocyte lysis (51Cr release), was characterized by an initial reversible decrease in amplitude of contraction, and was followed by rapid and irregular beating with eventual complete cessation of contraction. Contractile alterations induced by sensitized lymphocytes were inhibited by elimination of CD8+ cells.

Conclusions. Myocyte injury can be produced by sensitized cytotoxic T lymphocytes in vitro and is calcium and protein kinase C dependent. The contractile abnormalities produced appear to be similar to those observed in cardiac transplant patients undergoing rejection, and thus this model system promises to allow investigation of the mechanisms involved. (Circulation 1991;83:1410–1418)

Heart transplantation has evolved into an effective therapy for the treatment of end-stage heart disease. During the past 5 years, more than 10,000 heart transplant procedures have been performed and reported to the registry of The International Society for Heart Transplantation.1 During this time, the success rate has improved, with a 1-year survival rate now greater than 90% in some series.2 Advances in immunosuppressive therapies have accounted, in part, for this increase in graft survival. Nevertheless, rejection and associated graft dysfunction after cardiac transplantation still remain significant clinical problems.

For example, depressed ventricular contractility occurs during episodes of allograft rejection in heart transplant recipients.3,4 In general, the severity of hemodynamic dysfunction is correlated with the degree of cellular lymphoid infiltration of ventricular myocardium, and dysfunction may precede myocardial cell necrosis and, thus, be reversible. Indeed, Myles et al5 demonstrated that ultrastructural abnormalities in human cardiac allografts (increased glycogen granules, condensation and streaming of myofilaments, clumping of Z band material at the cell periphery, and loss of sarcomeric units) can resolve after successful treatment of episodes of rejection. In

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addition, complete recovery of depressed systolic function detected during rejection by changes in ventricular wall motion and hemodynamics can occur with immunosuppressive therapy. However, the cellular pathophysiological mechanisms involved that account for both reversible and irreversible components of cardiac allograft injury during rejection remain poorly defined.

Studies in experimental animals from a number of laboratories have indicated that T lymphocytes play a central role in cardiac allograft rejection. Helper T cells (CD4+), expected to react primarily with allogeneic vascular endothelium and interstitial dendritic cells expressing class II major histocompatibility complex antigens, may induce graft injury by secretion of lymphokines and recruitment of nonspecific macrophages and monocytes, which can cause graft injury by a delayed type hypersensitivity reaction. On the other hand, cytotoxic T lymphocytes (CD8+), which interact primarily with myocytes expressing major histocompatibility complex class I antigens, can produce cell injury directly, either by a calcium-dependent or a calcium-independent mechanism. However, the relative importance of CD4+ and CD8+ T lymphocytes in cardiac injury during cardiac allograft rejection remains somewhat uncertain.

Studies in the intact heart are complicated by possible damage to the vascular bed by both humoral and cellular mechanisms during rejection, possibly leading to myocyte dysfunction by limitation of oxygen and substrate delivery. Tissue-cultured myocardial cells have proved useful for determination of cellular pathophysiological events that correlate with ventricular dysfunction and injury during conditions simulating ischemia. We have attempted to apply this approach to the study of mechanisms of cardiac allograft rejection by means of a murine heart transplant model. The goals of this work were to determine whether allogeneically sensitized cytotoxic T lymphocytes injure cultured ventricular myocytes in vitro, whether injury is correlated with functional abnormalities, and whether the injury produced by these cells is calcium dependent.

Methods

Animals

C3H/HeN (MTV-) (H-2k) and BALB/c (H-2d) mice were obtained from NCI Frederic Cancer Research Facility, Fredric, Md. They were housed at a density of six animals per 18×28-cm cage with food and water supplied ad libitum. Animal care was in accordance with National Institute of Health guidelines, and all experiments were approved by the University of Utah Animal Care and Use Committee.

Heart Transplantation

Heterotopic vascularized heart transplants in mice were performed with the technique of Shelby and Correy. Mice were anesthetized with a single dose of 0.1 ml 3.6% solution of chloral hydrate/10 g body wt. The abdomen of the recipient animal (C3H) was incised, and the infrarenal abdominal aorta and vena cava were dissected free for a length of about 2 mm. Proximal and distal ties of 4.0 cotton were placed around the vessels. A midline incision was then made in the donor animal (BALB/c) after anesthesia and injection of 1 ml heparin (200 units/ml) into the inferior vena cava. The heart was then removed, and the inferior and superior venae cavae of the donor heart were ligated and divided. The aorta and pulmonary artery were divided, and the pulmonary veins were ligated with one ligature. The donor heart was briefly placed in a cooled, lactated Ringer's solution, then sutured in the abdomen of the recipient by joining the donor ascending aorta to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava in an end-to-side fashion with 10-0 nylon suture material. Normal sinus rhythm usually resumed shortly after reinitiation of perfusion, and contraction of the perfused (but nonworking) heart in the abdominal cavity was palpable after operation. The success rate with this technique was more than 90%. All operations were performed with a Zeiss operating microscope at a ×25 magnification. With the strains used in these studies, rejection of the heart occurred during an 8–12-day period.

Tissue Culture of Mouse Ventricular Myocytes

The cell culture technique that was used was a modification of that described by Ishida et al. Briefly, fetal hearts were obtained from mice at 16–20 days of gestation, minced in Ca2+-Mg2+-free Hanks' solution, and then serially digested with collagenase 75 units/ml (Worthington Biochemical Corp., Freehold, N.J.) at 37°C. Cells that were released by collagenase were placed into a 50% fetal calf serum collagenase-inhibitor solution in an ice bath. After the ventricular fragments were completely dissolved, the released cells were washed and subsequently suspended in culture medium containing 6% fetal calf serum, at a concentration of 0.75×105 cells/ml. Cells were plated in 96-well microtiter tissue culture dishes (1.5×103 cells/well) (Corning Glass Inc., Corning, N.Y.), grown for days, then changed to serum-free medium to inhibit fibroblast proliferation. Spontaneous contractions developed by day 2 of culture, and cells spread into a confluent layer that contracted synchronously by days 4–5.

Removal and Culture of Sensitized Lymphocytes

Mice were killed 8–10 days after transplantation when palpable contractions of the allograft had ceased, and the spleens from the recipient animals were harvested. A single-cell suspension was prepared by gently extruding the lymphoid cells from the splenic capsule. Cell clumps were broken up by vigorous aspiration and expulsion of the cell suspension with a 5-ml pipette. Lymphocytes were separated from red blood cells in the spleen by hypotonic lysis of red blood cells and resuspended in RPMI
1640 medium at 2.0×10⁶ cells/ml as described by Lynch et al.⁰¹⁸ Lymphocytes obtained from the spleen were then cultured for 5 days in the presence of irradiated (3300 R) donor strain lymphocytes at a responder to stimulator ratio of 1:1 in RPMI 1640 medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, and penicillin or streptomycin antibiotics.

Addition of Lymphocytes to Myocytes
To rapidly and consistently expose cultured ventricular myocytes to lymphocytes in the cytotoxicity assay, immediately after addition of lymphocytes to the multiwell plates, we centrifuged the plates at 20g for 3 minutes to precipitate the lymphocytes onto the myocyte layer. The number of lymphocytes required to achieve a specific lymphocyte to myocyte ratio for each well was calculated based on the initial plating density of myocytes. Lymphocytes were thoroughly washed and then resuspended in fresh medium immediately before addition to myocytes.

Detection of Cultured Myocyte Injury
Cultured ventricular myocytes were labeled with ⁵¹Cr at a concentration of 2 μCi/well for 1 hour. Cells were then washed and covered with fresh media before exposure to lymphocytes. At the end of the incubation period, the supernatants were collected, and total released counts were measured on a gamma counter (Micro-Medic Systems, Inc., Huntsville, Ala.). The percent ⁵¹Cr release was calculated by dividing the amount released in each treatment well minus the spontaneous release, by total counts released by cells in wells treated with NP-40 detergent minus spontaneously released counts.¹⁹-²¹ Experiments were performed in triplicate.

Measurement of Cultured Myocyte Motion
Myocyte motion was measured by previously described techniques.²² Cultured myocytes in tissue culture wells, or attached to a glass coverslip, were placed on the stage of an inverted phase contrast microscope (Diaphot, Nikon) and were magnified with a ×40 objective. A videomotion detector was used to monitor changes in position of a plastic microsphere attached to the surface of cultured myocardial cells during contraction and relaxation. The analog output of the motion detector was recorded with a Teac R80 instrumentation tape recorder and a laboratory strip chart recorder.

Antibody-Mediated Lysis of Lymphocytes
Concentrations of complement and antibody required to lyse specific lymphocyte subpopulations were determined. Minimal lymphocyte lysis (<10%) was detected with complement treatment in the absence of added antibody. Lymphocytes were washed and diluted to a concentration of 9×10⁶ cells/ml and were plated (9×10⁶ cells per well) in a 96-well microtiter plate, and antibody-mediated lysis was accomplished in a two-step process. First, antibody was added to the cells in each of the wells, and the plate was incubated on ice for 45 minutes. The different antibodies and concentrations used in this study were as follows: GK1.5 (anti-CD4, 1:20),²³ 53-6.4 (anti-CD8, 1:50),²⁴ and 30-H12 (anti-Thy-1.2, 1:20)²⁴ plus facilitating antibody MAR 18.5. After incubation with the particular antibody, the plate was centrifuged; the supernatant was discarded; and rabbit complement was added at a previously determined sufficient concentration (1:4) to cause cell lysis. The plate was incubated at 37°C for an additional 30 minutes; afterward, the cells were centrifuged; the supernatant again was discarded; and the cells were resuspended in RPMI 1640 before their use in the cell-mediated myocyte injury assay as described above.

Statistical Analysis
The significance of differences between means in paired comparisons were assessed by means of Student’s t test with Bonferroni’s correction for multiple comparisons. Data are presented as mean±SEM.

Results
Lymphocyte-Mediated Myocyte Injury
We first sought to determine the most efficient method to produce cytotoxic lymphocytes. We initially used lymphocytes isolated from the cardiac allografts undergoing rejection. Although some injury of myocytes could be produced by these lymphocytes, the yield was too low for our intended studies. We, therefore, used lymphocytes isolated from spleens of animals undergoing cardiac transplant rejection. As shown in Figure 1, lymphocytes that underwent primary in vivo stimulation by transplant rejection, but that were applied directly to myocytes without undergoing further in vitro stimulation by exposure to irradiated donor strain lymphocytes, produced minimal ⁵¹Cr release relative to control release. Lymphocytes obtained from a naive animal (no heart transplant) that had been stimulated in vitro by exposure to irradiated donor strain splenocytes produced some cytotoxicity. In comparison, allogenically reactive lymphocytes generated by combining primary in vivo stimulation (by transplantation) with secondary in vitro stimulation (by coculture with irradiated allogeneic cells) reproducibly caused maximal cytotoxicity, with 60–80% ⁵¹Cr release obtained at lymphocyte to myocyte ratios as low as 3:1 and 6:1. Statistical comparison of triplicate means from separate experiments in which lymphocytes were sensitized both in vivo and in vitro compared with in vitro sensitization alone at a lymphocyte to myocyte ratio of 12:1 showed a significant difference in percent ⁵¹Cr release (83.2±7.3 versus 39.6±4.1, n=4–5, p<0.002).

To examine the allogeneic specificity of the lymphocyte-mediated cytotoxicity in this model, we mea-
Maximal injury manifest by $^{51}$Cr release was observed when recipient strain C3H lymphocytes were sensitized in vivo by the rejection of a transplanted donor BALB/c heart and in vitro by exposure to irradiated donor BALB/c splenocytes (■). C3H lymphocytes sensitized in vivo by the transplant only showed little cytotoxicity against BALB/c myocytes (□), whereas those sensitized in vitro only were cytotoxic (○). Each point indicates the mean±SEM for $^{51}$Cr release from myocytes from triplicate wells.

Fig. 2. Plot of chromium-51 release from BALB/c myocytes (closed symbols) and from C3H myocytes (open symbols) induced by exposure to C3H lymphocytes sensitized in vivo and in vitro against BALB/c (rectangles) or C3H lymphocytes sensitized in vitro only against BALB/c (circles). Maximal cytotoxicity was produced by both in vivo and in vitro sensitization, and C3H lymphocytes sensitized against BALB/c did not injure syngeneic (C3H) myocytes (see text).

Fig. 3. Plot of time course of release of chromium-51 from BALB/c myocytes induced by C3H lymphocytes sensitized both in vivo and in vitro. Lymphocyte to myocyte ratio of 6:1 was used. $^{51}$Cr release was time dependent and was apparent within 1 hour of exposure of myocytes to sensitized lymphocytes. Each point indicates the mean±SEM for $^{51}$Cr release from triplicate wells.

Kinetics of $^{51}$Cr Release Induced by Cytotoxic Lymphocytes

We next sought to determine the early time course of $^{51}$Cr release from myocytes induced by exposure to cytotoxic T lymphocytes. $^{51}$Cr release from myocytes was measured at 0, 1, 2, 3, and 5 hours after the addition of lymphocytes at a lymphocyte to myocyte ratio of 6:1. Significant levels of $^{51}$Cr release were detected as early as 60 minutes after addition of cytotoxic effector cells, and $^{51}$Cr release increased progressively and reached a plateau by 3–5 hours (Fig. 3). Thus, the above experiments indicated that primary cytotoxic T lymphocytes produced in a mixed lymphocyte culture can induce cultured myocyte injury. We next sought to gain some insight into the mechanisms of injury and lymphocyte types involved.

Lymphocyte Types Mediating Cytotoxicity

The mixed lymphocyte culture prepared as described above contains a variety of cells including helper T cells (CD4$^+$), cytotoxic T cells (CD8$^+$), B
lymphocytes, macrophages, and monocytes. We, therefore, attempted to determine whether removal of the T cell component of the mixed lymphocyte culture population with anti-Thy-1.2 (pan T cell) antibody plus complement could block the cytotoxic effect of these cells. In addition, we further phenotyped the cytotoxic T lymphocytes by negative selection by pretreatment of mixed lymphocyte culture cells with complement and anti-CD8 antibody (53-6.4) or an anti-CD4 antibody (GK1.5). As shown in Figure 4, pretreatment of the mixed lymphocyte culture cells with 30-H12, an antibody against Thy-1.2 antigen expressed on all T cells, completely inhibited the $^{51}$Cr release from myocytes in the subsequent cytotoxicity assay. This indicates that the T-cell component of the mixed lymphocyte culture is involved in the cytotoxic effects observed. In addition, the anti-CD8 antibody 53-6.4 also decreased cytotoxicity, whereas pretreatment with the anti-CD4 antibody GK1.5 had no effect on cytotoxicity. These results indicate that the cells mediating the cytotoxic effects on myocytes are CD4$^+$, and CD8$^+$ T cells.

**Inhibition of $^{51}$Cr Release by EGTA**

As recently reviewed by Young, the binding of the cytotoxic T lymphocytes to the target cell is Mg$^{2+}$ dependent and can result in injury or lysis of the target cell by either Ca$^{2+}$-dependent or Ca$^{2+}$-independent pathways. Activation and subsequent degranulation of the cytotoxic T lymphocytes requires an increase in Ca$^{2+}$, and activation of protein kinase C. Thus, the calcium dependence may result from an effect on the activation of the cytotoxic T lymphocytes or an effect of Ca$^{2+}$ on the subsequent mechanisms involved in injury of the target cell. Therefore, we examined the effects of a reduction in the concentration of extracellular Ca$^{2+}$ on the injury process by adding EGTA to wells of myocyte target cells either immediately, 30 minutes or 1, 2, or 3 hours after addition of the cytotoxic lymphocytes. $^{51}$Cr release from allogeneic myocytes induced by cytotoxic T lymphocytes was almost completely inhibited when 2 mM EGTA was added immediately and was significantly reduced when added within 30 minutes after exposure of myocytes to cytotoxic lymphocytes (Figure 5). This concentration of EGTA lowers Ca$^{2+}$ to approximately 22 nM but only lowers Mg$^{2+}$ to approximately 0.38 mM, which is a level sufficient to support cytotoxic T lymphocyte–target cell adhesions. This result suggested that a calcium-dependent mechanism was involved in the action of these cytotoxic T lymphocytes early during the incubation period but indicated that Ca$^{2+}$ is not required for subsequent lysis of the myocytes.

To verify that the protective effects of adding EGTA early were not due to prevention of lymphocyte–myocyte interaction by the slight lowering of Mg$^{2+}$ by EGTA, we also exposed cytotoxic T lymphocytes to myocytes in the presence of 2 mM

**FIGURE 4.** Bar graph of phenotypic characterization of allo- genically reactive cytotoxic T lymphocytes. Negative selection of mixed lymphocyte culture cells was performed with complement and antibodies against CD4$, CD8$, and Thy-1.2 (pan T cell) antigens. Chromium-51 release induced by exposure of myocytes to selected lymphocyte subsets for 5 hours, at a lymphocyte to myocyte ratio of 6:1, was used to determine cytotoxic activity. All antibodies were used at the concentration stated in “Methods,” in the presence of rabbit serum complement at 1:4. These concentrations of antibody plus complement killed 35–49% of cells in the mixed lymphocyte culture population, as estimated by Trypan blue exclusion (see “Methods”). Complement alone (C$'$) or C$'$+MAR was not toxic to lymphocytes and did not inhibit cytotoxicity. Asterisk indicates significant difference (p<0.05) in the lytic activity of the antibody-selected cells relative to that of the C$'$-treated controls. Bars indicate the mean±SEM for $^{51}$Cr release from triplicate wells.

**FIGURE 5.** Bar graph of effects of lowering [Ca$^{2+}$]$_e$ on myocyte chromium-51 release induced by exposure to sensitized lymphocytes for 5 hours. A lymphocyte to myocyte ratio of 6:1 was used. Stock solution of 20 mM EGTA in culture medium was diluted 1:10 in the co-culture wells at 0 and 30 minutes and 1, 2, and 3 hours after addition of lymphocytes. These assays were performed in complete RPMI medium, at 0.52 mM [Ca$^{2+}$] and 0.48 mM [Mg$^{2+}$]. After addition of 2 mM EGTA, estimated free [Ca$^{2+}$] and [Mg$^{2+}$] levels were 22 nM and 0.38 mM, respectively. Protection against cytotoxic effects of lymphocytes was observed when [Ca$^{2+}$] was reduced within 30 minutes of exposure of myocytes to lymphocytes. Each point indicates the mean±SEM for triplicate individual wells. *p<0.05 vs. control when no EGTA was added.
EGTA supplemented with 2 mM Mg\(^{2+}\). The results of this experiment also indicated that lowering [Ca\(^{2+}\)] with EGTA but maintaining a high [Mg\(^{2+}\)] also completely inhibited cytotoxic T lymphocyte–induced cytotoxicity in this system (Figure 6). Prolonged treatment with 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) causes depletion of protein kinase C within cytotoxic T lymphocytes and thereby blocks subsequent activation and degranulation of cytotoxic T lymphocytes.\(^{28}\) We, therefore, also examined the effects of 18-hour exposure to 200 nM PMA on cytotoxicity produced by the mixed lymphocyte reaction cells against cultured myocytes, and the results are also shown in Figure 6. Treatment with PMA also prevented myocyte \(^{51}\)Cr release induced by sensitized lymphocytes. Thus, the time course of cell injury produced by sensitized lymphocytes, the calcium dependence of this process, and the effects of exposure to PMA\(^{28}\) are consistent with a lytic mechanism involving cytotoxic T lymphocytes induced by interaction with a cell–surface alloantigen (most likely major histocompatibility complex class I antigen) on the myocyte target cell.

**Induction of Physiological Abnormalities in Myocytes by Cytotoxic T Lymphocytes**

We next examined whether changes in myocyte cell motion or rhythm of contraction could be detected during cytotoxicity produced by sensitized lymphocytes in this model. In Figure 7 are shown typical examples of effects of cytotoxic T lymphocytes on contraction of cultured myocytes after varying periods of incubation. The most consistent response was an early decrease in the amplitude of contraction, with the subsequent development of irregular beating followed by complete cessation of contraction. These contractility effects were progressive even when superfusion of the myocyte monolayer was restarted after attachment of the lymphocytes. However, removal of lymphocytes by vigorous rinsing for 2 minutes with 1 mM EGTA in Ca\(^{2+}\)-Mg\(^{2+}\)-free Hanks’ solution, followed by resupply of normal medium, resulted in recovery of the amplitude of contraction when cytotoxic T lymphocyte washout was performed early (Figure 7B). This type of recovery was never noted in cultures in which the cytotoxic T lymphocytes were allowed to remain on myocytes.

Both \(^{51}\)Cr release and the negative inotropic effects probably represent components of allogenically spe-
cific injury produced by cytotoxic T lymphocytes. For example, exposure of sensitized lymphocytes to syngenic or third party myocytes did not produce a negative inotropic effect. In addition, myocytes continued to contract normally when exposed for up to 4 hours (lymphocyte to myocyte ratio, 12:1) to sensitized lymphocytes depleted of CD8+ cells by pre-treatment with antibody plus complement, whereas contraction of myocytes was completely inhibited within 30 minutes by identical non-CD8+-depleted lymphocyte cultures (n=6). However, the negative inotropic effect produced by sensitized lymphocytes against myocytes in this model system has an onset within 15 minutes, before the occurrence of cell lysis as indicated by the kinetics of 51Cr release.

Discussion

The processes involved in graft rejection that may account for reversible and irreversible components of myocyte injury were recently reviewed by Cramer.29 Rejection of cardiac allografts is associated with cellular infiltration of the graft by lymphoid cells, including cytotoxic and helper T lymphocytes30 and a substantial number of macrophages and dendritic cells that probably serve to process antigens and scavenge cellular debris.10 However, it has been difficult to ascertain which of these infiltrating cell types has the potential to produce injury to myocytes in the intact heart, and there have been several in vitro approaches to this problem.

Early work by Christmas and McPherson31 suggested that macrophages present in the graft at the time of rejection may contribute to functional abnormalities, perhaps by secretory products such as proteases or through the production of free radicals. In their experiments, macrophages were able to produce cessation of beating of cultured myocytes within 24 hours, but this effect was nonspecific and was not associated with cell lysis as detected by 51Cr release. Parthenais et al19 isolated lymphocytes from the spleens of rats with a rejecting cardiac allograft and examined cytotoxicity against cultured myocyte and nonmyocyte cardiac cells. Although very high effector to target cell ratios were required (≥25:1), these investigators found evidence for direct cell injury (51Cr release), induced by splenocytes sensitized in vivo against nonmyocytes, but injury of myocytes could be produced by sensitized splenocytes only in the presence of immune serum, suggesting that an antibody-dependent cytotoxic cell mechanism was involved.

There have also been several in vitro studies of myocyte injury produced by lymphocytes in models of myocarditis. Leiros et al32 induced autoimmune myocarditis in mice by subcutaneous injection of homogenized myocardium in Freund’s adjuvant. Lymphocytes isolated from the spleens of immunized animals with myocarditis induced a negative inotropic effect and irregular beating in normal atrial tissue from animals of the same strain. The observed effects were shown to be due to the T-cell component of the lymphocyte population. These toxic effects of sensitized lymphocytes could be prevented by lipoygenase inhibitors, suggesting that leukotrienes were mediating cytotoxicity. Hassin et al33 studied changes in membrane potential and motion in cultured rat cardiac myocytes infected with mengo virus that were incubated with lymphocytes sensitized to mengo virus. These investigators found that prolongation of the duration of the action potential plateau occurred within 50 minutes of exposure to the sensitized lymphocytes. In addition, there was delayed relaxation and slowing of the relaxation half-time. Extended exposure of cultured myocytes to the sensitized lymphocytes caused irreversible depolarization. However, early washout of lymphocytes, or treatment with verapamil, resulted in reversal of both the abnormalities of contraction and the electrophysiological changes. Hassin et al33 suggested that the cytotoxic effects of the lymphocytes in these experiments were due to Ca2+-dependent load induced by an increase in the Ca2+ current mediated by the slow calcium channel. Taken together, these studies suggest that cytotoxic T lymphocytes can directly damage myocytes during cardiac transplant rejection, a hypothesis that is also strongly supported by our findings. However, the mechanisms involved remain uncertain.

There have been many in vitro studies of the mechanisms of cytotoxic T lymphocyte cytotoxicity against nonmyocyte target cells, mostly with cloned cytotoxic T lymphocytes, and both Ca2+-dependent and -independent cytotoxicity have been documented. In Ca2+-dependent cytotoxicity, after contact with the target, [Ca2+], in the cytotoxic T lymphocyte increases,34 and lymphocyte granules redistribute adjacent to the target within a few minutes. Cytotoxic T lymphocyte cytoplasmic granules then fuse with the cell membrane, and the granular material is released into the space between the cytotoxic T lymphocytes and the target cell.35 Subsequent delivery of the “lethal hit” also requires extracellular calcium, may be associated with an increase in [Ca2+], in the target cell,34 and results in the onset of target cell lysis within 30 minutes to 1 hour.26,36 Granules isolated from IL-2-stimulated cytotoxic T lymphocytes contain the pore-forming protein perforin, which in the presence of calcium can induce the formation of functional pores of large conductance and low ionic selectivity in membranes of isolated cells and in artificial membranes.37,38 Insertion of these pores in the plasma membrane of the target cell is probably a major mechanism of Ca2+-dependent cytotoxic lymphocyte-mediated cell injury.26 However, Ca2+-independent mechanisms may also mediate cytotoxicity due to cytotoxic T lymphocytes. Ostergaard and Clark39 reported that some cytotoxic T lymphocytes can kill a target cell in the absence of calcium and yet require calcium for lysis of another, suggesting that several mechanisms cause target cell lysis and that the nature of the target cell is an important determinant of the mechanism in-
volved. The causes of Ca\textsuperscript{2+}-independent injury remain obscure, but Liu et al\textsuperscript{40} provided evidence that murine cytotoxic T lymphocytes contain a cytolytic factor antigenically related to lymphotoxin, which requires several hours to induce lytic activity and which may cause DNA fragmentation.

Despite the considerable progress made in these previous studies of cytotoxicity, the extent to which the mechanisms for killing of various target cells by cloned cytotoxic T lymphocytes can be extrapolated to cytotoxicity occurring during in vivo graft rejection has been questioned.\textsuperscript{41} For example, cytotoxic T lymphocytes in vivo, or cytotoxic T lymphocytes prepared in a mixed lymphocyte culture with a brief period of in vitro stimulation by alloantigen as performed in our experiments, have been thought to lack perforin and cytotoxic granules and, thus, to lyse target cells without inducing pores in the plasma membrane.\textsuperscript{41} However, Nagler-Anderson et al\textsuperscript{42} recently showed that primary cytotoxic T lymphocytes prepared in a mixed lymphocyte culture and separated into CD4\textsuperscript{+} and CD8\textsuperscript{+} cell subsets have cytolytic activity only associated with the CD8\textsuperscript{+} cells. In addition, these cells were found to have perforin-like cytolytic activity, although in smaller quantities than present in cloned cytotoxic T lymphocytes. Recently, messenger RNA for perforin was isolated from primary peritoneal exudate cytotoxic T lymphocytes.\textsuperscript{43} These findings suggest that the primary CD8\textsuperscript{+} cytotoxic T lymphocytes obtained from mixed lymphocyte culture, such as those used in our experiments, may indeed injure target cells by a cytotoxic granule-dependent mechanism. Although our results do not exclude other mechanisms, they are consistent with this hypothesis. First, the time course of myocyte injury in our experiments, manifest both by alterations in amplitude of contraction and by \textsuperscript{51}Cr release, was relatively rapid, which is a result consistent with degranulation-dependent cytotoxic T lymphocyte cytotoxicity.\textsuperscript{38,39} In addition, cytotoxic T lymphocyte cytotoxicity against myocytes could be prevented in our experiments by reduction of extracellular calcium concentration by the addition of EGTA or by depletion of protein kinase C, of which both interventions would be expected to inhibit activation and subsequent degranulation of cytotoxic T lymphocytes.

In many in vitro studies of the interaction of cloned cytotoxic T lymphocytes with target cells, the cytotoxic T lymphocytes have been found to deliver a "lethal hit" in which the target cell is irreversibly "programmed" for lysis.\textsuperscript{11,36} In the case of the heart, it is clear from several clinical studies\textsuperscript{3,4,6} that graft dysfunction during rejection manifest by a decrease in systolic function is reversible. This would imply that when the cardiac myocyte is the target cell, cellular dysfunction can be induced during the rejection process, which is not necessarily associated with ultimate progression to cell lysis and necrosis. Consistent with this hypothesis is the observation of Young et al,\textsuperscript{38} who found that perforin proteins derived from cytotoxic T lymphocyte granules can cause depolarization of membrane potential of cultured chick embryo skeletal myocytes, but the concentration of perforin proteins required to induce depolarization of the myoblasts was several times less than that required to cause lysis. This suggests that the intensity of exposure to cytotoxic T lymphocytes may be an important factor in the degree of reversibility of cellular dysfunction.

Our results provide support for this hypothesis. We find that the negative inotropic effect of cytotoxic T lymphocytes, manifest by a decrease in the amplitude of contraction of cultured myocytes, can be reversible if cytotoxic T lymphocytes are removed early (less than 30 minutes), after exposure of myocytes to the lymphocytes. Thus, these cultured ventricular myocytes also appear to survive a low level or brief exposure to cytotoxic factors produced by cytotoxic T lymphocytes. We do not think it is likely that the negative inotropic effects produced by sensitized lymphocytes are due to soluble mediators because in our experiments the decrease in amplitude of myocyte contraction was progressive once lymphocytes were attached to the monolayer despite continued superfusion of the chamber. Soluble mediators produced by interaction of lymphocyte with myocytes would be expected to be diluted into the large extracellular fluid space and be washed out of the chamber. However, further studies in this model system are needed to identify the cytotoxic factors involved and to elucidate the mechanisms responsible for the initial negative inotropic effects and subsequent myocyte lysis.

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