Mechanisms of Immune-Mediated Myocyte Injury

William H. Barry, MD

Immune-mediated myocyte injury can occur during rejection of the transplanted heart, as a consequence of viral myocarditis, and possibly contributes to cardiac dysfunction in patients with dilated cardiomyopathy. Injury may be reversible and manifest as a transient depression of ventricular function and/or electrophysiological stability, or it may be irreversible and lead ultimately to myocyte necrosis and progressive chronic congestive heart failure and/or death. The injury process may be primarily directed at vascular cells, in which case myocyte injury may be secondary to impairment of delivery of oxygen and substrates, or the process may directly involve the cardiac myocyte, in which case the injury is primary. Cellular and humoral components of the immune system may participate in both primary and secondary as well as reversible and irreversible injury.

In this discussion, we will review work that has contributed to our current understanding of the mechanisms by which myocyte injury occurs in different clinically significant conditions. We will focus primarily on the types of immune effector cells involved and the mechanisms by which they appear to damage myocytes because there is more recent information available on these topics. However, humoral processes also will be considered. From a clinical standpoint, prevention of initiation of an immune response against the heart is undoubtedly the most desirable means of reducing immune-mediated myocyte injury; however, this is not always feasible or possible. An understanding of the injury processes involved may result in better interventions to modify and/or reduce cardiac damage.

Functional Changes in the Intact Heart During Immune-Mediated Injury

Changes in systolic and diastolic function in the intact heart have been documented to occur during cardiac transplant rejection and myocarditis. Studies in both experimental animals and humans\(^1\)\(^-\)\(^11\) have shown that hemodynamic deterioration can occur coincident with allograft rejection and is correlated with the degree of lymphocyte infiltration and the extent of myocyte necrosis. Abnormalities in systolic function during rejection are frequently present. For example, Hansen et al\(^1\) found that the peak left ventricular filling rate was not changed during rejection, but that end-systolic volume was increased. The systolic dysfunction that occurs during rejection may also be reversed by treatment of the rejection process. For example, Stevenson et al\(^8\) reported a transplant recipient with acute left ventricular dysfunction and cardiogenic shock that was associated with only mild histological changes. Left ventricular function in this case improved rapidly after treatment with intravenous steroids and gradually returned to normal. de Marchena et al\(^9\) described a patient with severe, reversible right ventricular systolic dysfunction during acute rejection. Myles et al\(^6\) have demonstrated that the ultrastructural changes associated with rejection and indicative of myocyte injury were also reversible with treatment.

Abnormalities in diastolic function may also occur during rejection. Davies et al\(^2\) found elevated diastolic filling pressures early after transplant in a number of patients in whom the ejection fraction, measured with radionuclide techniques, remained in the normal range. They suggested that the hemodynamic changes observed were more compatible with "restriction" of ventricular filling and that an abnormality of diastolic compliance might be involved. Czerska et al\(^7\) found that rejection in patients was associated with a decreased normalized peak rate of left ventricular posterior wall thinning and a decreased diastolic mitral valve slope, whereas systolic function, as defined by fractional shortening, was not altered. They also concluded that the rejection process resulted in impaired ventricular diastolic compliance without changes in systolic function. Amende et al\(^10\) found no changes in ejection fraction or end-systolic volume during acute rejection; end-diastolic volume decreased, as did peak diastolic filling rate. However, Valantine et al\(^11\) found that although a restrictive-constrictive physiology (measured by Doppler echocardiography) occurred in about 15% of allograft recipients, it was consistently associated with impaired systolic performance.

Echocardiographic measures of wall thickness and ventricular mass show significant increases during rejection, and it has been postulated that decreased compliance during rejection is due to vascular inflammation with capillary leakage and interstitial edema.\(^12\)\(^-\)\(^16\) Indeed, Valantine et al\(^17\) have shown a strong correlation between immunohistochemical markers of microvascular inflammation and indices of diastolic dysfunction. Several reports, however, have documented decreases in the peak rate of chamber expansion during the rapid filling phase in early diastole, suggesting slowed myo-
cardial relaxation. Thus, it remains somewhat unclear whether abnormal diastolic function during rejection is the result of slowed relaxation, decreased chamber compliance, or both. However, it is clear that both altered systolic and diastolic ventricular function can contribute to hemodynamic alterations during rejection. Furthermore, depression of systolic function can be reversible.

Although much more data are available regarding the alterations in ventricular function that occur during transplant rejection, it is likely that depression of ventricular function can also result from immune mechanisms present during myocarditis. For example, Mason et al20 and Parrillo et al21 have reported that treatment with immunosuppressive drugs can be associated with recovery of ventricular systolic function in some patients with myocarditis. However, it should be emphasized that the immune mechanisms involved in myocarditis may differ from those in allograft rejection; a benefit of immunosuppression on recovery of ventricular function in patients with myocarditis is not established.

Immune Mechanisms Potentially Important in Cardiac Injury

A complete detailed review of the immune system is beyond the scope of this article. The interested reader is referred to recent texts that cover this topic in depth.22,23 However, a brief consideration of some of the fundamental processes involved may be helpful for orientation and to familiarize the reader with some of the terminology used. The immune response consists of three phases: the cognitive phase, in which a lymphocyte recognizes a foreign antigen; the activation phase, in which lymphocytes recognizing specific antigens proliferate (clonal expansion) and then differentiate; and finally, an effector phase, in which activated lymphocytes carry out their appropriate function to eliminate the foreign antigen.22 B lymphocytes express antibody molecules on their surfaces that bind foreign antigens, causing them to differentiate into producers of specific antibodies that are responsible for humoral immunity. T lymphocytes express receptors that recognize peptide sequences presented to them by antigen-presenting cells within the major histocompatibility complex (MHC) surface molecule of the antigen-presenting cells.

As reviewed by Springer24 and shown in Fig 1, there are two classes of MHC molecules. Class I molecules bind endogenously synthesized peptide antigens and are primarily recognized by cytotoxic T lymphocytes (CTL). Helper T lymphocytes (HTL) recognize endocytosed peptides that are bound to class II MHC molecules on the antigen-presenting cells. These are CD8 molecules on the surface of the CTL and CD4 molecules on the surface of the HTL. These molecules, in conjunction with the T-cell receptor–CD3 complex, form receptors for class I and class II MHC molecules and their bound peptides (depicted as small circles at the end of the MHC molecules in Fig 1). Adhesion of the T cell to the antigen-presenting cells or target cell is facilitated by specific adhesion molecules such as lymphocyte function-related antigen (LFA)-3 and CD2, and intracellular adhesion molecule-1 (ICAM-1) and LFA-1, which will be discussed later. Antigen-presenting cells for HTL include macrophages, B lymphocytes, dendritic cells, and endothelial cells.

Interaction of the peptide-MHC complex with the HTL receptor–CD3 complex results in a rise in T-cell cytosolic [Ca2+] and activation of protein kinase C. Both this rise in [Ca2+], which appears to be due to release from intracellular stores and to Ca2+ influx, and activation of protein kinase C are involved in T-cell activation.25 The rise in [Ca2+], activates the Ca2+-calmodulin–regulated phosphatase, calcineurin. The substrate for calcineurin is NFATp, a DNA-binding phosphoprotein that is present in unstimulated T cells. It forms a complex with the nuclear regulatory proteins fos and jun, the production of which is stimulated by protein kinase C. D Dephosphorylation of NFATp by calcineurin increases the ability of the NFATp–fos–jun complex to activate translation of lymphokine genes,26 resulting in the production by the HTL of a variety of cytokines, including interleukin-2 (IL-2).

Cytokines are critical components involved in the initiation and stimulation of immune and inflammatory responses.27 Natural killer lymphocytes can be stimulated by IL-2 to become lymphokine-activated killer cells, which can nonselectively lyse target cells. Some natural killer cells have low-affinity receptors that recognize IgG molecules bound to antigens on the surface of a target cell and cause antibody-dependent cell-mediated cytotoxicity.28 Tissue injury mediated by cellular components of the immune system may also occur via a delayed hypersensitivity-type reaction (DHT).29 The DHT reaction is initiated by CD4+ T-lymphocyte recognition of foreign antigen presented by antigen-presenting cells and the resultant secretion of cytokines (see Table 1, References 28 through 33).
IL-2 causes proliferation of antigen-activated T cells and has autocrine effects stimulating the synthesis of cytokines by T cells, including IL-2 itself, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). TNF-α stimulates the capacity of venular endothelial cells to bind and activate leukocytes by upregulating their expression of adhesion molecules. Expression of these adhesion molecules promotes attachment of neutrophils, lymphocytes, and monocytes to the endothelium. Subsequent secretion of IL-8 and monocyte chemoattractant protein-1 causes increased mobility of leukocytes, facilitating their adhesion to and migration through the vascular endothelial layer and extravasation into the tissue. Monocytes that have left the vascular system differentiate into macrophages. Macrophages are activated by INF-γ and by bacterial products such as LPS. Macrophages can kill phagocytosed microorganisms by production of oxygen radicals and can stimulate an acute inflammatory response via the production of such mediators as platelet-aggregating factor, prostaglandins, and leukotrienes. These mediators cause an inflammatory reaction that also contains a number of neutrophils, which can damage surrounding cells via production of proteases and free radicals. In addition, activated macrophages themselves can directly kill certain cells, especially malignant tumor cells, via their production of TNF-α and toxic metabolites such as reactive oxygen and nitrogen intermediates. Tissue destruction by a DHT reaction initiated by HTL has been proposed to be the principal mediator of allogenic rejection.

Much attention has also been focused on the mechanisms of activation and subsequent lysis of a target cell by CTL. Binding of the CTL T-cell receptor–CD3 complex to the target cell class I MHC surface antigen results in the activation of the CTL. This recognition-adhesion stage occurs within minutes in the presence of Ca²⁺ and Mg²⁺. This stage can proceed in the absence of Ca²⁺ if Mg²⁺ is present. In the absence of Ca²⁺, this attachment usually does not result in injury or lysis of the target cell by the CTL. However, in the presence of Ca²⁺ and after contact with the target, the CTL rounds up, the nucleus moves away from the target cell, and lymphocyte granules redistribute adjacent to the target within 10 minutes. CTL cytoplasmic granules then fuse with the cell membrane, and granular material is released into the space between the CTL and the target cell. It appears that the activation of CTL by the receptor-antigen interaction involves a breakdown of polyphosphoinositides caused by phospholipase C activation, resulting in production of diacylglycerol and inositol-1,3,5-triphosphate (IP₃). Diacylglycerol causes activation of protein kinase C, and a rise in CTL [Ca²⁺], occurs. This rise in [Ca²⁺], may be due in part to IP₃-induced intracellular Ca²⁺ release. However, a sustained increase in Ca²⁺ influx mediated by Na⁺-Ca²⁺ exchanger also appears to be required for T-cell activation.

The subsequent delivery of the "lethal hit" requires Ca²⁺ and results in lysis of lymphoblast target cells within 30 minutes to 1 hour. Although Ca²⁺ is required for the delivery of the lethal hit, it is not required for subsequent lysis of the cell; thus, lysis does not appear to be due only to Ca²⁺ overload. Martz et al suggested that Ca²⁺ is required as a stabilizing or activating factor for some essential protein involved in the injury process. This protein may be associated with CTL granules. Granules isolated from activated CTL can be partially purified and, in the presence of Ca²⁺, can induce the formation of functional ion channels of large conductance and low ionic selectivity in membranes of isolated cells as well as in artificial bilayer membranes. The active granule component appears to be a 70- to 75-kDa pore-forming protein, perforin, which polymerizes only in the presence of 50 to 100 μmol/L Ca²⁺. The mechanisms by which pore-forming proteins result in target cell lysis are not completely understood. As reviewed by Henkart, one theory is that pore-forming proteins, when inserted into the plasma-

### Table 1. Inflammatory Cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Principal Cell of Origin</th>
<th>Principal Action</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>IL-2</td>
<td>Activated T cell</td>
<td>Autocrine T-cell growth factor. Stimulates production of IL-2, TNF-α. Activates natural killer cells.</td>
<td>28</td>
</tr>
<tr>
<td>IL-1</td>
<td>Activated macrophages, endothelial cells</td>
<td>Stimulates T-cell activation. Induction of inflammatory metabolites. Activates endothelial cells and stimulates cytokine production.</td>
<td>29</td>
</tr>
<tr>
<td>IL-6</td>
<td>Monocytes, macrophages, T cells, endothelial cells</td>
<td>Stimulates differentiation of B cells. Stimulates production of plasma proteins by hepatocytes.</td>
<td>30</td>
</tr>
<tr>
<td>INF-γ</td>
<td>Activated T cells</td>
<td>Activates monocytes. Increases production of oxygen radicals by macrophages. Increases expression of MHC class I and II antigens.</td>
<td>31</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Activated macrophages</td>
<td>Activates endothelial cells. Stimulates production of cytokines. Can induce direct lysis of some cell types.</td>
<td>32</td>
</tr>
<tr>
<td>IL-8</td>
<td>Activated macrophages, lymphocytes, endothelial cells</td>
<td>Chemo-attractant for neutrophils and causes neutrophil stimulation.</td>
<td>33</td>
</tr>
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IL indicates interleukin; INF-γ, interferon-γ; TNF-α, tumor necrosis factor-α; and MHC, major histocompatibility complex.
lemna, cause "colloid osmotic lysis" of the cell due to a selective increase in membrane permeability of the target cell to small ions, with dissipation of transplasmalemmal ionic concentration gradients. The macromolecules inside the cell then exert an unbalanced osmotic pressure across the membrane, causing the cell to rupture and release cytoplasmic contents. Although this mechanism seems to account for lysis of some target cells, this may not be the primary mechanism for all target cells.

For example, Ostergaard and Clark have reported that some CTL can kill one target cell in the absence of Ca\(^{2+}\) and yet require Ca\(^{2+}\) for lysis of another. More recent work from this group has shown that 4,4’-diisothiocyanato-2,2’ disulfonic acid (DIDS), which inhibits degranulation, does not inhibit Ca\(^{2+}\)-dependent lytic activity in some CTL. Takayama and Sitkovsky have also suggested that T-cell receptor-triggered exocytosis of cytolytic granules may not be required for target cell lysis by CTL, and Young et al. have suggested that there may be multiple mediators that are acting in concert or independently in CTL-induced cell killing. Sanderson used time-lapse photography to study target cells during attack by cytotoxic T cells. He described "zeiosis," or target cell membrane blebbing, which occurred at a variable time before cell lysis. He proposed that zeiosis may result from massive changes in the cytoskeletal system. Zeiosis is not seen in time-lapse studies of killing of antibody-coated tumor cells by complement-mediated lysis, which is also believed to occur by colloid osmotic lysis induced by pore formation. Liu et al. have shown evidence that murine CTL contain a cytolytic factor other than perforin. The factor is antigenically related to TNF and lymphotoxin and requires several hours to induce maximal lytic activity, which may be due to DNA fragmentation. The presence of target cell membrane blebbing, cytoskeletal alterations, and DNA fragmentation is consistent with CTL-induced apoptosis, or "programmed cell death."

This process is characterized by activation of a nonlysosomal Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease, which gives rise to a characteristic "ladder" pattern on DNA electrophoresis. However, it appears that genome digestion is not absolutely required for CTL-mediated target cell death by an apoptosis mechanism.

Thus, CTL as well as cellular components of a DHT reaction can certainly cause target cell lysis, although the mechanisms of killing may vary with the target cell type and the nature of the CTL. We will now consider whether these cellular components of the immune system contribute to indirect (due to vascular effects) and direct myocyte damage.

**Cell-Mediated Vascular Injury**

Vascular alterations caused by immune processes are an important cause of indirect myocyte injury. The recognition of endothelium as foreign plays an important part in the initiation of the immune response to the transplanted allograft. Allogeneic class II human leukocyte antigens (HLA) encoded by the MHC complex are expressed at a low level by endothelial cells in the grafted heart. Interaction of circulating HTL with these antigens results in activation of the T cells, and subsequent production of cytokines such as INF-\(\gamma\), TNF-\(\alpha\), and IL-1. INF-\(\gamma\) increases the expression of class II antigens on endothelial cells, and TNF-\(\alpha\) and IL-1 stimulate the expression of adhesion molecules such as ICAM-1 and selectins on the surface of endothelial cells (see Table 1 and Fig 1). These adhesion molecules interact with receptor molecules on the surface of circulating monocytes and neutrophils. For ICAM-1 the receptor is LFA-1 (also called CD11a/CD18 and \(\alpha_i/\beta_2\) integrins). The specific receptors on the lymphocyte for P-selectin (and E-selectin, also known as endothelial-leukocyte adhesion molecule-1) remain unidentified (see Fig 1).

The expression of these molecules on lymphocytes promotes their activation and adherence to, followed by migration through, the endothelium. The endothelium can be injured by locally high concentrations of cytokines and by natural killer lymphocytes, CTL, and possibly by HTL. This ultimately results in the development of an "inflammatory" endothelium, with leakage of albumin and other macromolecules into the interstitium documented to occur early in the rejection process. It is also noteworthy that increased expression of endothelial-cell human class II MHC antigen (HLA-DR) has been noted to occur in some patients with dilated cardiomypathy and myocarditis. It is likely that interstitial edema resulting from endothelial injury increases wall thickness and perhaps accounts for the decrease in ECG voltage frequently noted during rejection episodes. It may also be the cause of decreased ventricular chamber compliance, resulting in an increase in filling pressures. It is also apparent that chronic vascular injury can occur after cardiac transplantation. This can result in functional changes such as impaired endothelial cell-dependent vasodilatation and eventual small-vessel obstruction and large epicardial coronary artery athroclerosis. The resulting obstructive coronary artery lesions can cause myocyte dysfunction and necrosis as a result of impaired delivery of oxygen and substrates (ischemia). Although immune-mediated vascular injury is of enormous clinical importance, particularly in allograft rejection, the major focus of this discussion is the causes of direct myocyte injury.

**Cell-Mediated Direct Myocyte Injury**

We shall now consider the evidence that CTL, cytokines, and components of the DHT response can cause direct myocyte injury and/or dysfunction.

**Cytotoxic T Lymphocytes**

Traditionally, it has been believed that CD8+ CTL-mediated lysis of parenchymal (and possibly vascular) cells is a very important mechanism of tissue injury during allograft rejection. As pointed out by Abbas et al, CD8+ cells comprise a significant fraction of the cellular infiltrate in grafts undergoing rejection, and cloned lines of alloreactive CD8+ cells have been demonstrated in some experiments to adoptively transfer cellular graft rejection. Also, parenchymal cells such as cardiac myocytes express class I MHC molecules and can therefore presumably be lysed by CD8+ cells. However, there is considerable question as to whether the degranulation–secreted toxin mechanism is the normal mode of killing used by CTL generated in primary allograft reactions in vivo. Skepticism in this regard has also developed in part
from the fact that perforin initially could not be demonstrated in primary CTL. However, Nagler-Anderson and associates have demonstrated that small quantities of perforin can be detected in primary CD8+ CTL, although the quantity is considerably smaller than in cloned CTL. Furthermore, this group has been able to demonstrate message for perforin in primary peritoneal-exudate CTL and Young et al. have detected perforin (using polyclonal antibodies and immunoelectron microscopic techniques) in CTL obtained from mice infected with lymphocytic choriomeningitis virus. These results strengthen the hypothesis that primary CTL could be inducing injury of ventricular myocytes in vivo by a pore-forming protein mechanism.

Most of the studies of CTL-mediated cytotoxicity have been carried out using lymphoblast cells as a target; however, it is quite likely that the nature of the injury produced may depend on the type of target cell studied and that parenchymal cells may be more resistant to CTL-mediated injury than lymphoblasts. Therefore, it is important to consider data available regarding the effects of lymphocytes on myocyte target cells. Hassan et al. detected physiological changes in Mengo virus–infected cultured rat cardiac myocytes, induced by lymphocytes sensitized to Mengo virus. Prolongation of the duration of the action potential plateau occurred within 50 minutes, with delayed relaxation and slowing of the relaxation half-time. Extended exposure caused irreversible depolarization; however, early washout of CTL or addition of verapamil resulted in reversal of the abnormal contraction and the electrophysiological changes. More recent experiments from this group have shown that lymphocytes from rats sensitized to injected cardiac myocytes by subcutaneous injection in Freund’s adjuvant can induce similar contractile and electrophysiological abnormalities in cultured neonatal rat ventricular myocytes. A soluble substance appears to mediate these effects, as they could be mimicked by supernatant collected from cultures of myocytes and lymphocytes. Gorelik et al. have shown that lymphocytes isolated from mice infected with Trypanosomatidae cruzi for 1 to 4 weeks induced a negative inotropic effect in intact mouse atrium. This effect was blocked by inhibitors of the cyclooxygenase pathway, was mediated by CTL, and was postulated to be due to production of prostaglandin E2. The process was not allospecific because it was observed in syngeneic tissue. Huber et al. have shown CTL-mediated lysis of Coxsackievirus-infected myocytes by allosensitized CTL. These studies all suggest that direct CTL-mediated myocyte injury can occur in myocarditis and autoimmune experimental models. We have also investigated direct myocyte injury produced by CTL in a heart transplant rejection model. Splenic lymphoid cells were obtained from mice 8 to 10 days after a heterotopic (intraabdominal) cardiac allograft. These splenocytes were stimulated in vitro for 5 days by exposure to irradiated donor-strain splenocytes. The resulting mixed lymphocyte reaction contains a large number of CTL and induces injury of cultured donor-strain cultured fetal myocytes, manifest by 51Cr release (see Fig 2). The injury could be prevented by elimination of the CD8+ component of cells from the mixed lymphocyte reaction population and was allospecific in that self (syngeneic) and unrelated third-party strains of cultured myocytes were not injured.

Successful therapy of rejection frequently results in recovery of normal ventricular function. The classic model of an irreversible lethal hit delivered by CTL would suggest that CTL are not responsible for this reversible dysfunction, given that cardiac myocytes do not regenerate after injury leading to cell death. However, Young et al. found that cultured chick embryo skeletal myocytes showed marked membrane depolarization at doses of perforin severalfold lower than those required to cause lysis. Jones et al. reported that cells of an erythroleukemia cell line showed transient increases in intracellular calcium and transient permeability to propidium iodide without cell lysis when exposed to sublethal doses of perforin. They suggested that some nucleated target cells are able to remove perforin pores from the membrane (as has been shown with serum complement pores), and they estimated the half-life of the perforin pores to be 90 seconds. These studies suggest that, at least in the case of perforin-mediated injury, CTL may be able to cause reversible target cell injury without lysis. Results from our laboratory are consistent with this hypothesis. The initial phase of CTL-myocyte interaction is associated with a decrease in the amplitude of myocyte motion, and this depression of contractility is reversible on washout of lymphocytes (Fig 2). Subsequent studies have shown that the
negative inotropic effect on cultured myocytes induced by CTL is due to a decrease in the Ca\(^{2+}\) transient, as well as membrane depolarization (R.D. Ensley, M. Ives, L. Zhao, M. McMillan, J. Shelby, and W.H. Barry, unpublished data, 1993). Both the myocyte lysis and the contractile abnormalities can be inhibited by pretreatment of lymphocytes with the degradation inhibitor DIDS, indicating that a component of the CTL granule is probably responsible for the changes we have observed. This possibility is supported by the findings of Binah et al.\(^{76}\) who have reported that lytic granules derived from CTL can cause an initial membrane depolarization and decrease in the Ca\(^{2+}\) current, followed by lysis of adult rat ventricular myocytes. Thus, results from a variety of laboratories indicate that CTL can directly injure myocytes, that the injury probably involves an effect of the CTL granules (perforin), and that the injury can be reversible. However, while these results indicate that CTL could account for a component of myocyte injury during cardiac allograft rejection (and myocarditis as well), they do not prove that this is occurring in vivo.

A number of studies have investigated the types and functional activities of cells infiltrating rejecting hearts.\(^{79-82}\) As pointed out by Ascher,\(^{83}\) studies of the in vitro function of infiltrating cells accumulating in a rejecting allograft may provide insights into the in vivo activity of the cells. The infiltrating cell population from rejecting rat cardiac allografts is composed of macrophages, T and B lymphocytes, neutrophils, basophils, and eosinophils.\(^{79-81}\) Strom et al.\(^{82}\) have shown that these cells have cytotoxic activity that can be attributed to donor-specific CTL, as well as antibody-dependent lymphocyte-mediated cytotoxicity. However, the degree of cytotoxicity assayed against lymphocyte target cells was relatively low (\(^{95}\)–\(^{96}\)%) and showed only modest allogeneic specificity (1% to 6%). Furthermore, in these studies the effector-infiltrating cell population was centrifuged in a Ficoll-Hypaque gradient that would tend to remove neutrophils. Thus, the possible contribution of this cell type to cell injury may have been underestimated.

Cells infiltrating a rejecting murine heart may be harvested by collagenase treatment.\(^{84}\) We have examined the cytotoxicity of these “heart-infiltrating cells” (HIC) against cultured adult and fetal mouse myocytes. The cells making up the HIC population are shown in Table 2. The population consists primarily of lymphocytes, macrophages, and neutrophils. In preliminary experiments,\(^{85}\) we have found that, although the HIC population contains a similar number of CTL, the injury of myocytes produced by HIC cells differs markedly from that produced by mixed lymphocyte reaction cells. The latter, as previously described,\(^{75}\) appears to be due entirely to the CD8\(^{+}\) (CTL) component. Injury produced by HIC is less intense and does not appear to be allospecific, in that self and third-party strains of myocytes are injured as severely as allospecific target cells. This finding suggests that although CTL are certainly present in the rejecting heart, they appear functionally relatively ineffective in producing cytotoxicity against myocyte target cells, and injury produced by HIC may also result from other nonallospecific mechanisms. These results are consistent with a recent report by Bishop et al.\(^{86}\) in which the effects of in vivo depletion of mice of CD8\(^{+}\) cells on rejection of a heterotopic allograft were examined. These investigators found that although depletion of CD8\(^{+}\) cells virtually eliminated CTL from the infiltrating cell population (estimated by limiting dilution analysis), rejection of the graft occurred in a normal fashion. This finding also raises the possibility that cells other than CD8\(^{+}\) CTL are effecting injury of the rejecting transplanted heart. We will now consider the cell types and mechanisms that could be involved and the evidence favoring their importance.

### Macrophages and Neutrophils

As shown in Table 2 and discussed previously, a variety of cell types besides CTL are present in the HIC population, including HTL, macrophages, and neutrophils. We have previously shown that CD4\(^{+}\) HTL present in a mixed lymphocyte reaction do not induce lysis of cultured fetal mouse myocytes.\(^{75}\) Early work by Christmas and MacPherson\(^{87,88}\) showed that macrophages infiltrating a rejecting rat heart were not able to cause cultured myocyte lysis as detected by \(^{51}\)Cr release, although macrophages were able to inhibit spontaneous contractions of myocytes. The results of Strom et al.\(^{82}\) also suggest that macrophages obtained from rejecting hearts have a relatively small cytolytic effect. Thus, cell types other than HTL or macrophages in the total HIC population must be considered as potential causes of myocyte lysis.

As previously discussed, natural killer cell and antibody-dependent cell-mediated cytotoxicity may be produced during allograft rejection. It is also possible
that neutrophils present in the infiltrating cell population can cause myocyte injury. It is widely recognized that neutrophils are important mediators of tissue injury in inflammation.90 These cells normally are engaged principally in defending the body against invading microorganisms, but if parenchymal cells are identified as foreign or damaged, the destructive mechanisms of the neutrophil can lead to significant cell injury. As summarized by Weiss,90 the plasma membrane of the neutrophil contains an NADPH oxidase enzyme that can generate reactive oxygen radicals. In addition, neutrophils contain granules consisting of toxins and proteolytic enzymes. When the neutrophil is activated, the oxidase generates oxygen radicals, and granules fuse with the plasma membrane and release their toxic components into the interstitial space. It is likely that both these components contribute to target cell injury.

Entman and associates90 have shown that the adhesion of isolated human neutrophils to adult canine myocytes is increased by the induction of expression of the intercellular adhesion molecule ICAM-1 on the myocyte surface by exposure to TNF-α, IL-1, and IL-6. Adhesion results from the interaction of myocyte ICAM-1 with CD11/CD18 integrins expressed on the surface of activated neutrophils, and the resulting neutrophil-myocyte interaction results in neutrophil-induced oxidative injury followed by contracture of the myocyte.91 (Fig 3). Interestingly, the oxidative injury is intracellular and is not prevented by extracellular free radical scavengers, but is inhibited by monoclonal antibodies to ICAM-1, CD11b or CD18, or by intracellular radical scavengers. It is also important to note that low concentrations (<10 U/mL) of cytokines were sufficient in these studies to induce adhesion molecule expression on the surface of adult myocytes.92 The significance of these findings in relation to ischemia-reperfusion injury has been emphasized,91 but they may be also relevant to myocyte injury in immune processes. Isobe et al93 have recently reported that survival of murine cardiac allografts can be markedly prolonged by treatment of recipient animals with monoclonal antibodies against ICAM-1 and LFA-1 (CD11a/CD18). Interestingly, the monoclonal antibody treatment did not completely prevent the initial leukocyte infiltrate but inhibited myocyte necrosis. Monoclonal antibody treatment in this report also induced prolonged tolerance. It is possible that the prevention of myocyte necrosis noted in this model is due to inhibition of the interaction between neutrophils and myocytes as well as to interference with ICAM-1-dependent, T lymphocyte-mediated effects. As free radicals can also cause reversible and irreversible depression of myocyte function,94,95 it seems possible that neutrophil-mediated injury could contribute to functional abnormalities of the ventricle during inflammation associated with myocarditis or rejection as well. However, direct experimental evidence that neutrophils participating in a DHT reaction cause myocyte injury is not available at present.

**Effects of Cytokines**

Although macrophages and HTL infiltrating a rejecting heart do not appear likely to be capable of directly causing myocyte necrosis, they could contribute to the alterations in contraction and relaxation observed during immune-mediated myocyte injury by production of cytokines. A variety of cytokines recently have been shown to have effects on myocytes, but considerable differences have been described regarding whether a direct negative inotropic effect or only a blunting of the effects of catecholamines is produced, and whether the effects are due to stimulation of nitric oxide (NO) production. For example, Gulick and colleagues97 have reported that cultured neonatal myocytes incubated for 72 hours in dilutions of medium conditioned by activated rat splenic macrophages have reduced cyclic AMP and positive inotropic responses to catecholamines. Subsequent studies have shown that IL-1 and TNF-α are the major pro-inflammatory factors mediating this effect.98 Balligand et al99 have reported a similar phenomenon in cultured adult rat ventricular myocytes and showed that the blunting of the catecholamine response could be inhibited by the L-arginine analog N⁰-monomethyl-L-arginine (L-NMMA). Furthermore, this effect of macrophage-conditioned medium was associated with release of NO by myocytes and required at least 12 hours to develop. In these experiments, as in those of Gulick et al,97 there was no direct negative inotropic effect produced but only a blunting of the positive inotropic response to β-adrenergic stimulation (see Fig 4). These observations suggest that cytokines (including IL-1 and TNF-α) produced by activated macrophages can cause an increase in inducible NO synthase activity in cardiac myocytes, and that the resulting increased production of NO can modulate inotropic responsiveness.

Fig 3. Bar graphs showing relation of neutrophil adherence to oxygen radical-dependent myocyte fluorescence. Adult canine myocytes were preincubated with 4 U/mL recombinant interleukin (IL)-1b for 3 hours and then loaded with intracellular oxidation probe 2',7'-dichlorodihydrofluorescein diacetate. Loaded myocytes were suspended with neutrophils at a ratio of illness:1.50 in complete medium. An aliquot of the cell suspension was placed in a cuvette, and zymosan-activated serum (ZADS) was added to stimulate neutrophils at time 0. Samples of the cell suspension were evaluated for neutrophil-myocyte adherence (expressed as mean number of neutrophils per myocyte, bars), and fluorescence was determined using a fluorometer (excitation wavelength, 488 nm; emission wavelength, 521 nm) and plotted as a line. The anti-CD18 monoclonal antibody, R15.7 (50 µg/mL), was present as indicated. Increased neutrophil adherence and intracellular oxidation in myocytes was produced by IL-1 and ZADS, and these effects were inhibited by the monoclonal antibody against CD18. (From Entman et al91 with permission of authors and publisher.)
Other studies have suggested that inflammatory cytokines may cause a direct negative inotropic response. First, depression of myocardial function has been reported after administration of high doses of IL-2 during chemotherapy.100 and IL-2–stimulated cultured human mononuclear cells produce a soluble factor that causes a reversible depression of contractility of isolated perfused rat hearts.101 Finkel et al102 have shown that high concentrations of TNF-α, IL-2, and IL-6 cause a rapid negative inotropic effect in isolated hamster papillary muscles that can be prevented by L-NMMA, suggesting that enhanced NO production by a constitutive NO synthase is involved (Fig 5). Brady et al103 have recently reported that an increase in NO production in cardiac myocytes induced by exposure to endotoxin can cause a direct depression of contractility of isolated adult rat ventricular myocytes that can be reversed by the guanylate-cyclase inhibitor methylene blue, indicating that the negative inotropic effect may be related to increased levels of cyclic GMP. McMorn et al104 have reported a direct negative inotropic effect of acetylcholine in rat ventricular myocytes related to shortening of the action potential duration, with a resulting decrease in the inward Ca^{2+} current. Taken together, these findings suggest that inflammatory cytokines may produce a direct negative inotropic effect by increasing production of NO within cardiac myocytes, which may cause activation of guanylate cyclase. However, recently Yokoyama et al105 have shown that TNF-α induces a direct negative inotropic effect in the isolated adult cat heart and in isolated adult cat ventricular myocytes that is associated with a decrease in the Ca^{2+} transient, without a change in the L-type Ca^{2+} current, and that is not inhibited by blockers of NO production or arachidonic acid metabolism. Thus, the extent to which inflammatory cytokines can cause a direct negative inotropic effect, as opposed to only a blunting of the positive response to catecholamines, and the mecha-

![Figure 4](http://circ.ahajournals.org/)

**Fig. 4.** Bar graphs showing inhibitory effect of LPS-activated macrophage-conditioned medium on contractile response to isoproterenol. Adult rat ventricular myocytes were preincubated in control medium (open bar) or in control medium diluted 50% (vol/vol) with medium conditioned by endotoxin-activated rat alveolar macrophages (LPS+ solid bar) or by macrophages not previously exposed to endotoxin (LPS−; hatched bar). A, After 24 hours, these media were removed, and baseline contractile function was determined in myocytes from each preincubation group, paced at 2 Hz, and superfused with physiological buffer at 37°C. Baseline amplitude of shortening was not altered by macrophage-conditioned medium. B, The amplitude of shortening after exposure to 2 nmol/L isoproterenol relative to the baseline amplitude of shortening was significantly reduced by LPS+ macrophage-conditioned medium (*P < 0.05 for LPS+ compared with LPS− or with control). (From Balligand et al99 with permission of authors and publisher.)

![Figure 5](http://circ.ahajournals.org/)

**Fig. 5.** Graphs showing direct negative inotropic effects of increasing concentrations of tumor necrosis factor-α (TNF-α) (A), interleukin (IL)-6 (B), and IL-2 (C) alone (e). In the presence of Nω-monomethyl-L-arginine (L-NMMA; 10 mmol/L) (c) the negative inotropic effect was inhibited (n=6). Values represent the means±SEM of six different determinations in six different papillary muscle preparations. (From Finkel et al102 with permission of authors and publishers.)

![Figure 6](http://circ.ahajournals.org/)

**Fig. 6.** Bar graph showing survival of self (C3H) and donor (BALB/c) cultured adult mouse ventricular myocytes coincubated for 24 hours with C3H cytotoxic T lymphocytes (CTL) produced in a mixed lymphocyte reaction (MLR) (left) or in 1000 U/mL of interleukin (IL)-2, tumor necrosis factor-α, interferon-γ, and IL-4 (right). Even these high concentrations of cytokines had no effect on survival of myocytes, whereas CTL caused virtually complete lysis of myocytes in an allospecific fashion (means±SEM, n=4).
Humoral Mechanisms of Injury

The major mechanism of cell injury mediated by the humoral or antibody-dependent component of the immune system is via the activation of complement via the classic pathway involving binding of the C1 component of complement by an antigen-antibody (IgG or IgM) complex on the surface of a target cell. A cascade of reactions is initiated that results in the formation of the membrane attack complex, which consists of multiple C9 molecules and a C5-8 complex, arranged into a plasma membrane pore with a diameter of about 110 Å, or 11 nm. The structure of this pore is similar to that formed in the plasma membrane by perforin during CTL-mediated cell lysis mentioned above. It should be noted that antibodies differ in the extent to which they bind C1 and thus initiate the formation of a membrane attack complex, because a C1 molecule must bind to several Fc components of Ig. IgM contains five Fc regions, whereas IgG contains only one, and therefore IgM is a more effective complement-fixing antibody than IgG.

Humoral components of the immune system can contribute to both direct and indirect myocyte injury. The presence of preformed HLA antibodies in the serum of recipients of a cardiac allograft may result in "hyperacute" injury to the vascular endothelial cells of the cardiac allograft, resulting in rejection within hours. Chronic vascular injury during allograft rejection may also occur by humoral mechanisms. Preformed xenoantibodies are present in the serum of a given species and can cause direct injury of myocytes (and other cell types) in a recipient organ from a different species. Antibodies may be involved in antibody-dependent cell-mediated cytotoxicity in allograft rejection and could also affect myocyte survival and function in myocarditis and cardiomyopathy. However, as recently reviewed by Herskowitz et al, the pathogenetic role of autoantibodies in myocardial injury associated with myocarditis and/or cardiomyopathy (and transplant rejection) is not well established. Indeed, it may be that circulating autoantibodies are a marker for, rather than a cause of, myocardial damage. However, circulating autoantibodies to intracellular antigens may cross-react with sarcolemmal proteins of functional importance. For example, Schulteis et al have described autoantibodies to the adenine nucleotide translocator in patients with dilated cardiomyopathy. Morad et al have shown that antibodies against this translocator can interact with the L-type Ca²⁺ channel and increase the Ca²⁺ current in isolated myocytes. This could cause myocyte injury by inducing Ca²⁺ overload. These antibodies may also interfere with energy metabolism. Limas and associates have described the presence of autoantibodies against the β-adrenergic receptor in patients with dilated cardiomyopathy that may alter catecholamine responsiveness. Autoantibodies against other intracellular antigens such as myosin and heat-shock proteins have also been described, but the pathophysiological significance of these antibodies is not supported by experimental evidence at this time.

Summary

Much progress has been made in defining the mechanisms by which altered systolic and diastolic function of the heart may be produced by components of the immune system activated during allograft rejection and myocarditis and in patients with dilated cardiomyopathy. It is clear that injury of the vascular bed can occur via both humoral and cellular mediators and probably accounts for the acute alterations in ventricular compliance that occur during allograft rejection, as well as the accelerated development of graft atherosclerosis. Altered myocyte function and lysis can be produced by CTL in vitro, but the importance of this injury process in vivo remains uncertain. Other cells present in the inflammatory infiltrate can also affect myocyte function and survival. Neutrophils may cause lysis of myocytes, and cytokines produced by infiltrating macrophages and HTL may reach a sufficient concentration in the interstitial microenvironment to decrease myocyte catecholamine responsiveness and/or directly depress myocyte contractility. Humoral antibodies to myocyte cell surface antigens may cause cell damage by an antibody-dependent cytotoxic cell mechanism or by directly binding to and altering sarcolemmal receptor and/or ion channel function. Further elucidation of the extent of involvement of these different mechanisms in specific clinical settings may provide a basis for improved therapy of immune-mediated cardiac injury and dysfunction.

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