Immune effector mechanisms in heart transplant rejection

Ofer Binah

It is well established that immunological mechanisms are responsible for the pathophysiology of several heart diseases, some contributing significantly to morbidity and mortality worldwide. Of the multiple cellular and humoral components participating in the overall immune response in heart diseases, this article mainly deals with cellular immunity mediated by cytotoxic T lymphocytes (CTL). Three important heart diseases have been associated with CTL induced myocardial damage: (1) heart transplant rejection; (2) myocarditis and dilated cardiomyopathy; (3) Chagas heart disease.

Heart transplant rejection

"Rejection is the body’s normal protective response to a perceived threat. The body’s unusual response to a foreign substance (antigen) is to attack and destroy that antigen. Thus, when the immune system recognizes the transplanted organ as a foreign (non-self) it seeks to destroy it. The body’s ability to distinguish self from non-self is the basis for the rejection process."\(^1\)

Myocarditis and dilated cardiomyopathy

The aetiology and pathogenesis of these two related diseases are still highly controversial and clearly beyond the scope of this article.\(^2,3\) It is commonly thought that myocarditis, initiated by a viral infection, often coxsackievirus B3, may progress in a significant number of cases to dilated cardiomyopathy. While the precise pathological mechanisms of dilated cardiomyopathy are far from clear (and agreed upon), there is a rather wide agreement that CTL mediated autoimmune response contributes to the ongoing cardiac damage, often leading to terminal heart failure.\(^4,5\)

Chagas heart disease

This is the most frequent cause of congestive heart failure and sudden death in endemic areas of Trypanosoma cruzi infection of central and south America. It is estimated that 16 to 18 million people are infected, and 100 million are at risk of infection.\(^6\) T cruzi is an obligatory intracellular protozoan transmitted to humans by haematophagous insects, known as triatomites, as well as by blood transfusions. Following the acute infection by T cruzi, there is in most cases a long (up to 20 years or more) asymptomatic phase, after which 15-20% of infected individuals develop clinical manifestations of usually fatal cardiomyopathy.\(^7,8\)

Of these three heart diseases in which immune responses, and specifically cellular immunity, play an important role, I shall concentrate on the immunological rejection of the transplanted heart; at the same time, in most cases the discussion of the effector mechanisms of CTL induced damage, is also relevant to myocarditis, dilated cardiomyopathy, and Chagas heart disease.

Heart transplant rejection

Cardiac transplantation is now accepted treatment for end stage myocardial failure.\(^9,10\) Following the pioneering work of Shumway and Lower in perfecting the surgical technique, advances in rejection monitoring and improved immunosuppression brought about a progressive increase in survival rates, which has resulted in increased number of transplant procedures. Heart transplantation is performed in over 200 centres worldwide, and around 2000 transplantations have been done in the USA alone.\(^11\) Immediate mortality resulting from heart transplantation is decreasing, while survival time is constantly increasing: about 90% of the patients live over one year after surgery, while 70% live up to five years.\(^12,13\) However, although the surgical procedure of heart transplantation has been satisfactorily mastered and in spite of improved immunosuppressive therapy, immunological rejection of the transplanted organ and the related graft atherosclerosis are the major obstacles to higher survival rates.

Types of transplants

Transplanted organs are divided into four categories (not all applicable to heart transplantation), based on their origin and the "genetic" relation to the recipient. (1) Autogeneic graft: This is performed within the same individual (or animal) and is obviously not rejected. An organ or tissue (skin, blood vessel, bone) is removed from one site and grafted at a different location. (2) Syngeneic graft: A graft is transferred within genetically identical individuals, such as in identical twins, or in an inbred animal colony, and is not rejected. (3) Allogeneic graft: A graft is transferred from one individual to another, both belonging to the same species, but differing in their genetic repertoire. This is the most common mode
Allograft is recognized as non-self, rejection occurs by means of the immune system to destroy foreign tissues. The cellular reaction is initiated mostly by helper T (Th, CD4+) lymphocytes, which were initially sensitized by the graft antigens (mainly MHC class II alloantigens, see discussion below) presented to them by antigen presenting cells and by the donor endothelium. In graft rejection, antigen presenting cells may originate from two different sources: those of the recipient (that is, indirect presentation, as in "ordinary" immune response), or those derived from the donor graft (that is, "direct presentation").

Once sensitised, Th lymphocytes trigger the production of allograft specific cytotoxic (killer) T lymphocytes (CTL), which then destroy the non-self tissues. At the same time, Th lymphocytes recruit macrophages into the rejection site by releasing various cytokines. In turn, macrophages release interleukin 1 (IL-1), which further stimulates the system by promoting IL-2 production by T lymphocytes. The allospecific CTL then attack alloantigens, mainly those belonging to the class I major histocompatibility complex (MHC) antigens. Consequently, the cytolytic machinery is activated and the CTL stage a lethal attack to destroy donor cells displaying non-self antigens. Humoral rejection, on the other hand, is the destruction of the graft by specifically directed antibodies, originating from preformed antibodies (previous blood perfusions or pregnancy), or from allospecific antibodies formed after transplantation.

The molecular basis of allorecognition

Since in most cases the donor heart is immunologically incompatible with the recipient's tissues, an immunological reaction is initiated against the transplanted heart, which is directed against specific surface antigens located on myocardial and endothelial cells.

The major histocompatibility complex

The T cells involved in transplant rejection recognize donor specific antigens (peptides) in association with MHC antigens expressed on the graft. The structure of the T cell receptor is such that T cells can only see peptide antigens associated with MHC molecules. Indeed, the strongest immunological reaction against a transplanted graft is initiated as a result of an interaction between the recipient's immune system and the donor's polymorphic set of membrane proteins (human lymphocyte antigens, HLA) encoded by the major histocompatibility gene complex, MHC. MHC antigens are divided into two groups: class I and class II. Class I MHC antigens are present on all nucleated cells, but their concentration varies among different cell types and depends on the "functional" state of the cell. Class II MHC antigens are more selective in their distribution, and are expressed only on a few cell types, such as dendritic cells, macrophages, B lymphocytes, and endothelial cells. Most importantly, the expression of both classes of MHC antigens on myocytes and endothelial cells is not constant, and is modulated by various stimuli, including graft rejection.

Why does histoincompatibility occur? It occurs mostly because the MHC antigens display a remarkable polymorphism, which enables the organism to respond to a large variety of foreign antigens, but at the same time, it is also the major obstacle to a successful transplantation. The presence of many alleles encoded by each locus, combined with at least six loci of the human MHC (located on the short arm of chromosome 6), makes it extremely unlikely that two unrelated individuals will carry identical MHC antigens. How do MHC antigens work? In simple terms, each MHC molecule (class I and class II) has a deep groove into which a protein fragment or a short peptide can bind. Because this peptide is not a part of the MHC molecule itself, it can vary from one molecule to another...
to the other. Thus, on self or healthy cells, these peptides originate from self-proteins and therefore do not evoke an immune response. However, in infected or transformed cells, or in histoincompatible transplanted tissue, the peptides presented in association with the MHC molecules are foreign (non-self), and therefore initiate an immune response. In addition to the MHC antigens, other antigens contribute to a different extent to the rejection process. Among these are the blood group antigens (antigen A and B), which are expressed on red blood cells and vascular endothelium.31-34

Accessory molecules involved in allore cognition and lymphocyte activation
In addition to the T cell receptor and MHC molecules, a large number of cell surface molecules on lymphocytes and antigen presenting cells participate in cell-cell interaction during antigen presentation (fig 234). The accessory molecules on the lymphocyte contribute to lymphocyte recognition and activation by: (1) functioning as adhesion molecules, thereby strengthening the interaction between T cells and antigen presenting cells (that is, LFA-1 and LFA-2); (2) modifying the transmembrane signal initiated by the T cell receptor (that is, CD4 or CD8); (3) Initiating transmembrane signalling (for example, protein kinase activity), distinct from that triggered by the T cell receptor. The most likely candidates for the T cell receptor associated antigen presenting cells participate in cell-cell interaction during antigen presentation (fig 2).34-36 The accessory molecules on the lymphocyte contribute to lymphocyte recognition and activation by: (1) functioning as adhesion molecules, thereby strengthening the interaction between T cells and antigen presenting cells (that is, LFA-1 and LFA-2); (2) modifying the transmembrane signal initiated by the T cell receptor (that is, CD4 or CD8); (3) Initiating transmembrane signalling (for example, protein kinase activity), distinct from that triggered by the T cell receptor. The most likely candidates for the T cell receptor associated tyrosine kinase are the related c-src family proto-oncogenes p59fyn and p56lck.34-36 The interaction between the T cell receptor and target cell antigens also causes intracellular polymerisation of skeletal organelles and proteins (for example, talin), and of the microtubule organising centre, both of which are involved in directing streaming of intracellular vesicle towards the sarcolemma.40-42

Types of heart transplant rejection
Heart transplant rejection is commonly divided into three main categories: (1) hyperacute rejection; (2) acute rejection; (3) chronic rejection.

Hyperacute rejection – Hyperacute rejection of vascularised organs, which occurs within seconds to several hours after transplantation, is mainly caused by pre-existing antibodies, which include: (1) antibodies against blood A and B antigens found on the donor red blood cells and organ; (2) anti-MHC antibodies in recipients previously exposed to allogeneic tissues; and (3) natural antibodies reacting with endothelial antigens of other species. These antibodies initiate the hyperacute rejection process by binding to antigens on the vascular endothelium and triggering a three step process.45-47 First, stimulated endothelial cells activate the complement cascade, causing cell destruction. Second, complement components also trigger the production of kalikrein, bradykinin, and other vasoactive substances. Third, activated endothelial cells secrete humoral products which attract phagocytes and stimulate platelet agglutination. Phagocytes and platelets enhance blood coagulation, which leads to vascular thrombosis. Finally, the activation of the endothelial cells causes alterations in the expression of endothelial adhesion molecules, which attract to the graft a variety of immune system components, including inflammatory cells such as neutrophils and platelets.48-50 These reactions lead to graft oedema, haemorrhage, and vascular thrombosis causing ischaemia and graft rejection.49 Once initiated, the process of hyperacute rejection cannot be blocked. Nowadays, hyperacute rejection is very rare and can be prevented by screening the potential recipient's blood group and HLA antigens before transplantation.

Chronic rejection – Chronic rejection begins at a variable time after transplantation, as early as one week, and may progress for several years. While the initial immunological response in chronic rejection is relatively weak (in contrast to hyperacute rejection), it intensifies over time, resulting in gradual graft deterioration. Although other possibilities may exist, it is suggested that chronic rejection mainly results from humoral immune responses against the donor (non-self) tissues. Antibodies produced by sensitised B cells activate the complement system and cause platelet aggregation at the rejection site. This in turn causes fibrin accumulation on the endothelium, eventually leading to stenosis and coronary occlusion (that is, graft atherosclerosis). Lack of blood supply to the myocardium leads to ischaemia and failure of the transplanted heart.50 After it has been initiated, the chronic rejection cannot be stopped, and retransplantation is the only therapeutic alternative.

Acute rejection – Most frequently, acute rejection occurs within weeks to months after transplantation, and becomes progressively uncommon with time. Acute rejection is initiated by the recognition of non-self alloantigens (MHC class I and II) by macrophages and precursor T8 lymphocytes. This interaction results in secretion of cytokines, and differentiation and proliferation of T8 cells, which then release IL-2, inducing proliferation of donor specific CTL. As a result of IL-2 mediated "positive feedback", more IL-2 receptors are expressed on CTL membranes, causing exponential proliferation of CTL capable of destroying target cells. Hence, the accumulation of graft specific CTL is a major cause of acute rejection. Additionally, CTL also secrete y interferon (IFN-y), which further activates macrophages, increases the expression of class II MHC antigens on endothelial cells, and accelerates rejection. In addition, T8 lymphocytes, cause differentiation of B cells, which produce and secrete allospecific antibodies.51-53

Cytotoxic T lymphocytes and heart transplant rejection

Cytotoxic mechanisms of T lymphocytes
Before discussing specific CTL-myocardial interactions, I shall briefly summarise potential mechanisms whereby CTL can destroy target cells. Studies carried out during the 1970s44-46 have identified and characterised three stages in CTL-target cell interaction: (1) recognition and conjugate formation; (2) programming for lysis and delivery of the "lethal hit"; and (3) target cell lysis, detachment of the CTL.
from the target, and recycling to initiate another cytotoxic interaction. Of the discrete stages (the first was partially discussed), I shall now focus on the delivery of the lethal hit and mechanisms of cytotoxicity.

Several theories attempt to explain the nature of the lethal hit delivered by killer lymphocytes. However, thus far no single mechanism can account satisfactorily for the entire process of CTL mediated cytosis. This apparent controversy is explained by the existence of multiple mechanisms for target cell destruction; some may act directly on the target cell by means of pore formation, while others which require specific receptor for their initiation, trigger programmed cell death ("apoptosis"), causing target cell disintegration.

**Killing by pore formation**

The theory of target cell destruction by pore formation culminating in colloid osmotic collapse is largely based on observations that certain CTL and natural killer cells have within their cytoplasm lytic granules containing pore forming material(s). Following conjugate formation, the secretory machinery is activated, and lytic granules fuse with the CTL membrane and release their content into the lymphocyte-target cell intercellular space. Among the granule constituents are the pore forming protein perforin, lysozymes, and serine esterases termed granzymes. The major cytolytic component of lytic granules is perforin, a 70 kDa protein which is structurally related to the C9 component of the complement system. According to the perforin hypothesis, in the presence of calcium ions perforin polymerises onto the target cell membrane and forms transmembrane pores with an internal diameter of 16-20 nm. These highly non-selective pores enable large ion fluxes of $10^{-10}$ mol-s$^{-1}$, which result in disruption of the electrochemical gradients, eventually leading to colloid osmotic collapse of the affected cell. Interestingly, perforin is found in a variety of cell types, most commonly in natural killer cells, activated CTL (CT8$^+$), and in most cytotoxic CD4$^+$ cells. Perforin synthesis can be readily induced by in vitro cultivation of various lymphocyte cell lines in the presence of IL-2. In vivo expression of perforin has been demonstrated in T lymphocytes (CD8$^+$), in autoimmune diseases, and in lymphocytes infiltrating rejecting cardiac transplants. These disease related immunological reactions are associated with increased IL-2 secretion, which may be the cause for induction of perforin synthesis in these lymphocytes. That perforin actively participates in immune responses in vivo was recently shown by Kagi and coworkers, who studied cytotoxicity mediated by T cells and natural killer cells in perforin deficient mice. They found that these mice, which were viable and fertile, had normal numbers of CD8$^+$ T cells and natural killer cells, which did not lyse virus infected or allogeneic fibroblasts, or natural killer target cells in vitro. The mice also failed to clear lymphocytic choriomeningitis virus, and eliminated fibrosarcoma tumour cells with reduced efficacy. As some lytic activity and immune responses persisted in the perforin deficient mice, this study also supports the notion of perforin independent lymphocytotoxicity. The experimental evidence, either supportive of or inconsistent with the perforin hypothesis, has been reviewed in depth.

While the presence of perforin in certain cytotoxic lymphocytes is unquestionable, it is clear that the perforin theory cannot account for all CTL mediated cytosis. An important alternative to the perforin theory, capable of explaining killing by CTL devoid of lytic granules and perforin, is "programmed cell death" or apoptosis (which are not always interchangeable). The term programmed cell death was originally coined by Lockshin and Williams to describe the developmentally regulated death of specific larval muscles following the emergence of the adult moth. In the immunological perspective, programmed cell death refers to any cell death that involves a gene mediated "program" to kill the cell), which is independent of the nature of the triggering stimulus. The term "apoptosis" was proposed by Kerr et al to describe a common series of morphological changes that accompanied the death of cells from a wide variety of tissue sources. During apoptosis, the surface membrane "boils", resulting in blebs formation, referred to as "apoptotic bodies". Thus, with the exception that killing of target cells by CTL is not associated with new gene expression (in the target), CTL-induced apoptosis of target cells is similar to that seen in negatively selected T cells, irradiated lymphocytes, metabolizing tadpole tails, and regressing tumours.

According to the apoptosis theory, killing of target cells by CTL is initiated by CTL-target cell conjugation, mediated by the APO-1/Fas receptor. The APO-1/Fas receptor is a 35 kDa membrane protein which belongs to the nerve growth factor superfamily and is expressed on both lymphoid and non-lymphoid tissues. Upon APO-1/Fas mediated CTL-target cell interaction, a signal (of unknown nature) is transmitted to the cell interior and activates a series of endonucleases and proteases. This in turn cause DNA fragmentation into 180-200 base pair bands, eventually resulting in cell fragmentation and destruction.

**Involvement of lymphocytes in heart transplant rejection**

In heart transplantation, the diagnosis of allograft rejection is primarily made by histological examination of endomyocardial biopsies for infiltrating lymphocytes. In fact, the degree of "mononuclear infiltration" in endomyocardial biopsies (allowing sequential histological examination of the post-transplantation myocardium) is the basis for the commonly used Billingham criteria for grading acute cardiac rejection. Phrased in more clinical terms, "the sequence of histologic changes in unmodified classic cell mediated acute cardiac rejection consists of infiltration of the graft by mononuclear cells, initially in a perivascular location. Gradually the mononuclear cells, predominantly lymphocytes and immunoblasts, make their way into the interstitium, eventually causing the destruction of myocytes". Interestingly, a correlation exists between the degree of cellular infiltration and the frequency of in vitro growth of graft infiltrating lymphocytes in culture. Furthermore, it was also found that graft infiltrating cytotoxic T cells with high affinity receptors for the donor antigens are present in the transplanted heart before the histological signs of rejection have developed, and might therefore be used as a prognostic factor. There is also evidence correlating the severity of rejection with lymphocyte infiltration; studies show good correlation between the level of lymphocyte infiltration to the graft, and the decline in the haemodynamic performance and contractile function of the heart.

Numerous studies in experimental animals and in human patients have clearly shown that CTL (both CD4$^+$ and CD8$^+$) infiltrate the rejecting transplanted heart. For example, Carlquist and his coworkers studied human cardiac infiltrating lymphocytes by in vitro expansion in IL-2. Of 28 graft infiltrating lymphocyte cultures from 17 recipients, 17 were comprised predominantly of CD4$^+$ T cells and 10 predominantly of CD8$^+$ T cells; one culture had equal numbers of CD4$^+$ and CD8$^+$ cells. The mean percentage of T cell
subsets for all cultures were as follows: CD4+ 49 (SEM 29)%; CD8+ 42 (31)%. Usually (but not always), T lymphocytes (CD4+) react with endothelial cells expressing class II MHC antigens, while cytotoxic lymphocytes (CD8+) react with myocardial cells expressing class I MHC.84-100 Thus, while the importance of T cells in graft rejection has been convincingly demonstrated in numerous studies, uncertainty still exists as to the relative contribution and importance of CD8+ and CD4+ in meditated heart transplant rejection.101 102 And although this review mainly deals with CTL and heart transplant rejection, it is worthwhile mentioning that, at least in coxsackievirus B3 induced myocarditis in mice, the first wave of cells infiltrating the diseased heart is mainly composed of lymphocytes, consistent with the morphology of natural killers which express perforin mRNA.6 It should be stressed that while traditionally, the donor’s “pure” myocardial cells were considered to be the major target for the recipient’s lymphocytes, the role of the endothelium as an active participant in allograft rejection becomes increasingly apparent. This was largely due to the discovery of a variety of adhesion molecules (ICAM-1, ELAM-1, VCAM-1) and the determination of their role in leukocyte adhesion and interaction with endothelial cells in heart rejection and in other disease states.103-111 That adhesion molecules participate in heart transplant rejection was recently shown by Isobe and co-workers.112 They report an indefinite survival of cardiac allografts between fully incompatible mice strains when monoclonal antibodies (mAb) to intercellular adhesion molecule-1 (ICAM-1) and leucocyte function associated antigen-1 (LFA-1) were simultaneously given for six days after transplantation. Similarly, daily in vivo administration of MK-2, an mAb to the endothelial adhesion molecule VCAM-1, to murine histo incompatible cardiac allograft recipients resulted in prolongation of graft survival.113

Perforin and granzymes in graft infiltrating lymphocytes – As perforin and granzymes (especially granzyme A) have been strongly implicated in cell mediated cytotoxicity, attempts have been made to determine whether they are expressed in graft infiltrating lymphocytes. However, a major problem in interpreting the data from experiments performed in vitro on lymphocytes isolated from the graft is that both perforin and granzyme mRNA are rapidly induced by IL-2, which is required by most T cell lines for maintenance in culture.114 115 And although the presence of perforin and granzymes does not prove active participation in lymphocytotoxicity, it is important to determine their expression in vivo. Indeed, in situ hybridisation to mRNA has been used to detect single cells expressing perforin and granzymes in vivo.116 117 In a study carried out by Griffiths and coworkers,118 granzyme A and perforin mRNAs (using in situ hybridisation) were determined in graft infiltrating lymphocytes and were used as markers for rejection after cardiac transplantation. Twenty nine different biopsies from 17 patients who had undergone cardiac transplantation were examined; 12 biopsies showing evidence of rejection contained lymphocytes expressing granzyme A and perforin. In seven biopsies showing no signs of clinical rejection, granzyme A and perforin were not expressed in infiltrating lymphocytes. Furthermore, it was also found that granzyme A and perforin are not only expressed in lymphocytes infiltrating to an actively rejecting site, but can also serve as early predictive markers of transplantation rejection.71 117

**Figure 3** Tracings illustrating the effect of cytotoxic T lymphocytes (CTL) on myocyte motion. (A) Lymphocyte:myocyte ratio 1:5:1; time interval after addition of lymphocytes to myocytes is indicated above each strip. In this example, cytotoxicity was manifested within 30 min by decreased amplitude of motion and an increase in the rate and irregularity of beating. (B) Higher concentration of lymphocytes was used (12:1). Cytotoxicity, manifested by a decreased amplitude of motion, was apparent by 15 min of exposure to CTL, and was reversible after removal of CTL by washing the myocytes with Ca2+-, Mg2+-free Hanks’ solution containing 1 mmol litre-1 EGTA and restoration of normal culture medium. Adapted with permission from reference 118.

**Figure 4** Changes in the action potential in a concanavalin A (Con A) treated ventricular myocyte conjugated with peritoneal exudate cytotoxic T lymphocyte (PEL). (A) Action potential recordings are shown in Tyrode solution containing 10 μmol litre-1 Con A (upper trace), addition of PEL in the absence of Con A (middle trace), and in Con A treated myocyte conjugated with PEL (bottom trace) (arrows indicate delayed afterdepolarisations, DAD). (B) DAD in a Con A treated ventricular myocyte conjugated with PEL. Recordings were obtained 20, 40, 45, and 47 min after conjugate formation. Temperature 25.0°C, cycle length 5 s. Adapted with permission from reference 123.
Immune effector mechanisms in heart transplant rejection

immensely, there is only sparse information on the actual mechanisms employed by CTL to damage and destroy their respective cardiac target cells. Among the first attempts to investigate these mechanisms in vitro is the study by Barry's group. The in vitro model consisted of splenic lymphoid cells obtained from mice 8-10 days after placement of a vascularised abdominal cardiac allotransplant which were restimulated in vitro with irradiated donor-type splenocytes for five days. The effects of these allogeneically stimulated lymphocytes on syngeneic and donor strain fetal cultured myocytes were determined by recording myocyte motion ("functional" assay), and by a $^{51}$Cr release assay (a measure of cytotoxicity). Figure 3 depicts representative tracings illustrating the effect of CTL on myocyte motion at two CTL:myocyte ratios: (A) 1.5:1; (B) 12:1. "Cytotoxicity", manifested by a decrease in motion amplitude and an increase in rate and irregular beating, was apparent by 15 minutes of exposure to CTL, and was reversible after removal of CTL. $^{51}$Cr release which followed the contractile abnormalities was calcium dependent and was prevented by pretreatment of sensitised 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 were involved. In a study that was perhaps more closely related to CTL attack on virus infected myocytes, Hassin and coworkers examined functional changes in mengovirus infected neonatal rat myocytes, induced by mengovirus sensitised CTL. On the basis of their experiments showing CTL induced attenuation of contraction (although a direct interaction was not demonstrated), oscillations in the plateau phase of the action potential, and increased total myocyte interchangeable calcium (verapamil preventable), the authors concluded that the cytotoxic effect induced by CTL probably results from altered calcium handling causing marked increase in intracellular calcium concentration ($[Ca^{2+}]_i$).

To investigate mechanisms whereby CTL destroy ventricular myocytes, for the past several years we have been studying: (1) the direct CTL-myocyte interaction; and (2) effects of CTL derived lytic constituents on isolated ventricular myocytes. To study the in vitro CTL-myocyte interaction we used the patch clamp technique to record membrane currents and action potentials from concanavalin A (Con A) treated guinea pig ventricular myocytes, conjugated to mouse peritoneal exudate CTL (PEL) (fig 4A). While Con A alone or the addition of PEL in the absence of Con A had no effect on action potential configuration (for as long as 60 minutes), in a conjugated myocyte (bottom trace), action potential duration was markedly shortened, action potential amplitude and resting potential ($V_{rest}$) were reduced, and most importantly, delayed afterdepolarisations were generated (at 30 and 60 minutes). Figure 4B shows the progressive increase in amplitude of delayed afterdepolarisations in a conjugated myocyte undergoing cytocidal interaction with PEL. These oscillatory potentials, which are remarkably similar to delayed afterdepolarisations caused by cardiac glycosides, are indicative of increased $[Ca^{2+}]_i$, and may be relevant to the mechanism of CTL mediated cytotoxicity. PEL-induced electrophysiological alterations were associated with myocyte death as demonstrated by an increase in $V_{rest}$, a decrease in action potential amplitude, and a marked prolongation of action potential duration.

Figure 5  Morphological changes in Con A treated ventricular myocytes conjugated with PEL. Photographs were obtained immediately upon conjugate formation (A, $t = 0$) and at 15 (B), 30 (C), and 45 (D) min after conjugate formation. Conjugates were formed by addition of PEL to the recording bath containing myocytes bathed in Tyrode solution containing 10 μg ml$^{-1}$ Con A. Temperature 25.0°C. Adapted with permission from reference 123.
shortening [fig 5, within 28.9(SEM 2.8) min] followed by complete cell destruction [within 43.5(4.3) min], supporting the notion of CTL induced [Ca$$^{2+}$$] overload. That the release of calcium from intracellular stores and [Ca$$^{2+}$$] overload are involved in CTL induced damage is further suggested by (1) the ability of ryanodine to delay and caffeine to inhibit CTL induced damage, and (2) the ability to modulate the time course of PEL induced changes by altering the calcium buffering capacity of the recording pipette solution. These results therefore suggest that damage inflicted by sensitised CTL to ventricular myocytes, probably mediated by [Ca$$^{2+}$$], overload, can contribute to the overall decline in cardiac function during heart transplant rejection. Similar mechanisms can also contribute to destruction of endothelial cells by CTL, culminating in functional derangement of the graft.

Since under certain circumstances, perforin appears to be an important mediator of cellular immunity, we investigated how CTL derived lytic granules and purified perforin affect guinea pig ventricular myocytes. Exposure of myocytes to lytic granules (or perforin) resulted in specific morphological and electrophysiological changes indicative of [Ca$$^{2+}$$], overload, invariably followed by ineffectibility and myocyte destruction (fig 6). The involvement of [Ca$$^{2+}$$], overload in these changes was shown by fura-2 imaging of [Ca$$^{2+}$$], before and after exposure to granules (fig 7). The common explanation for perforin toxic action is based on its ability to induce large pores (channels) in the target cell membrane; indeed, as was shown in other experimental settings,126 we found that in ventricular myocytes, perforin induced single channels with open times of up to several seconds, a mean conductance of 860 pS, and a reversal potential of -8.2 mV (fig 8). These highly non-selective channels can mediate massive [Ca$$^{2+}$$], accumulation, and can therefore account for most of the observed effects. As CTL participate in the immunological rejection of the transplanted heart, it is conceivable – but remains to be shown – that part of this damage is inflicted by perforin containing lytic granules.

**Damage to ventricular myocytes by immune cytokines** – The appreciation that conditions such as idiopathic dilated congestive cardiomyopathy associated with lymphocytic

---

**Figure 6** Effect of perforin on action potential in a guinea pig ventricular myocyte. Action potential is shown in control (Tyrode solution containing 1.8 mmol-litre$$^{-1}$$ Ca$$^{2+}$$), and at various intervals after application of perforin (8 μl into a 0.5 ml recording bath). Temperature 23.0°C, cycle length 5 s. Adapted with permission from reference 122.

**Figure 7** Effect of CTL derived lytic granules on intracellular Ca$$^{2+}$$ concentration ([Ca$$^{2+}$$]) in a guinea pig ventricular myocyte. [Ca$$^{2+}$$], was monitored by fura 2 imaging. A myocyte is shown in the control state, and at various intervals after application of lytic granules to the bath. Initial [Ca$$^{2+}$$], and cell length were 62 nmol-litre$$^{-1}$$ and 100 μm, respectively. Adapted with permission from reference 120.

**Figure 8** Single channels induced by purified perforin in guinea pig ventricular myocytes. (A) Recordings of whole cell membrane currents in a myocyte held at different holding potentials. Note reversal of current polarity at positive potentials. (B) Current-voltage relations of channels induced by perforin in a guinea pig ventricular myocyte. Each point represents the mean (n = 3-15) of the channel current amplitudes measured at a different holding potential; bars = SEM. The conductance and reversal potential (E$$_{rev}$$) calculated by means of linear regression analysis are 860 pS and -8.2 mV, respectively (p < 0.05). Temperature 24.0-25.0°C. Adapted with permission from reference 122.
myocarditis and cardiac allograft rejection are accompanied by reversible cardiac impairment has led Lange and co-workers to search for an alternative mechanism to CTL mediated irreversible cytotoxicity. Indeed, in some cases of mild and moderate acute rejection, extensive myocardial necrosis is absent and the symptoms of cardiac dysfunction are reversible upon augmentation of the immunosuppressive therapy. These investigators therefore suggested that certain forms of leucocyte mediated cardiac diseases may be caused by non-lethal effects of immune cells or their respective cytokines. Using cultures of spontaneously beating neonatal rat ventricular myocytes, Lange’s group found that activated immune cells produce cytokines which reversibly inhibit contractile and cyclic AMP responses of cardiac myocytes to β adrenergic stimulation. These cytokines, most probably IL-1 and tumour necrosis factor released by macrophages, reversibly modulate cAMP metabolism in myocytes, which appears to be mediated by alterations in G, with uncoupling of the β receptor from adenylate cyclase. While these important studies suggest that soluble immune cytokines are likely contributors to the reversible cardiac dysfunction observed in heart diseases such as heart transplant rejection and idiopathic cardiomyopathy, additional work is required to determine whether cytokine mediated immunomodulation of cardiac function can be used as a target for immunosuppression.

Concluding remarks

The involvement and significant contribution of immunological mechanisms to certain heart diseases is well established. Specifically, in heart transplant rejection, a wide spectrum of mechanisms is employed by the recipient’s immune system to reject the donor’s (non-self) tissues, causing graft failure. As replacement of the terminally diseased heart is the only possible treatment for thousands of patients each year, tremendous efforts have been devoted to perfect the surgical procedure of transplantation and to improve immunosuppression. However, in spite of these remarkable achievements and better tissue typing and matching, immunological rejection (and graft atherosclerosis) still constitutes the major obstacle to long term successful transplantation.

One area in which understanding is rather limited is that of the mechanisms employed by CTL to damage and destroy myocytes and endothelial cells of the donor heart. Not only are the in situ cytotoxic mechanisms still an enigma, but the intracellular events in the target cell leading to its destruction are unaccounted for. Thus the elucidation of the cytotoxic mechanisms operating to destroy the transplanted heart may contribute to the development of improved treatments for combating heart transplant rejection.

Supported by grants from the Israel Academy of Sciences-Basic Research, The Israeli Ministry of Health, the US-Israel Binaional Science Foundation, and the Rappaport Family Institute for Research in the Medical Sciences. Also supported by grants to William Chasin (HL-32093-ID).

Key terms: heart transplant rejection; myocarditis and cardiomyopathy; cytotoxic T lymphocytes; ventricular myocytes; lytic granules; perforin.

Received 19 May 1994; accepted 4 July 1994. Time for primary review 25 days.


20 LaRosa FG, Talmage DW. Synergism between minor and major histocompatibility antigens in the rejection of cultured allografts. Transplantation 1985;39:480-5.


32 Oriol R. Tissular expression of ABH and Lewis antigens in humans and animals: expected value of different animal models


Immune effector mechanisms in heart transplant rejection


Downloaded from https://academic.oup.com/cardiovascres/article-abstract/28/12/1748/327773 by guest on 18 February 2019