Induction of Cytotoxic Gene Expression During Rat Cardiac Allograft Rejection and the Effects of Combination Low Dose Cyclosporine A/Methotrexate Immunosuppressive Therapy in Prolonging Graft Survival

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INDUCTION OF CYTOTOXIC GENE EXPRESSION DURING RAT CARDIAC ALLOGRAFT REJECTION AND THE EFFECTS OF COMBINATION LOW DOSE CYCLOSPORINE A/METHOTREXATE IMMUNOSUPPRESSIVE THERAPY IN PROLONGING GRAFT SURVIVAL

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF CELL BIOLOGY, NEUROBIOLOGY, AND ANATOMY

BY THERESA TORRES PIZARRO

CHICAGO, ILLINOIS MAY 1994
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DEDICATION

My deepest appreciation goes to Linda A. Piccinini, Ph.D. and Elizabeth J. Kovacs, Ph.D. for all their help and support; no better two advisors could be asked for! Also, to all the life-long friendships I have made at Loyola through the "graduate school experience"; I couldn't have made it without you guys. Finally, to my family: Dad, Mom, James, Mamel, Bobby, and Christian, you have always been there for me and words can not explain what this means to me. And to Fabio, your patience and love has been immense throught the writing of this dissertation ..... and finally, it's done!
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<td>BN</td>
<td>Brown Norway</td>
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<tr>
<td>CSA</td>
<td>cyclosporine A</td>
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<td>ECG</td>
<td>electrocardiographic</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>HF</td>
<td>Hanukkah Factor</td>
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<td>IL-2</td>
<td>interleukin-2</td>
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<td>IFNγ</td>
<td>interferon gamma</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LT</td>
<td>lymphotoxin</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MTX</td>
<td>methotrexate</td>
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<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol myristate acetate</td>
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<tr>
<td>post-tx</td>
<td>post-transplantation</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>SMNCs</td>
<td>splenic mononuclear cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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CHAPTER 1
INTRODUCTION

Despite the availability of powerful immunosuppressive agents, rejection of the allografted heart remains one of the major problems associated with cardiac transplantation. The inflammatory process in response to alloantigen is in part due to the production by different cell types of soluble endogenous mediators, including both cytokines and granzymes. The studies contained within this dissertation were designed to further understand the pathophysiological mechanisms involved in cardiac allograft rejection by examining the production of these cytotoxic mediators. Specifically, detection and quantitation of the cytotoxic products tumor necrosis factor-α (TNFα) and -β (lymphotoxin, (LT)), and the T cell proteases, granzymes A and B (Hanukkah Factor (HF) and C11, respectively), were measured during the process of rat cardiac allograft rejection. Determination of which cytotoxic cytokine products are present post-transplantation and when during the time course of rejection, may lead to more effective therapies aimed at promoting graft success.

Furthermore, the achievement of successful cardiac transplantation is, in fact, critically dependent on immunosuppressive agents that can effectively prolong graft survival and prevent allograft rejection. The current protocol of administering potent immunosuppressive drugs to transplant recipients dramatically increases the chances of graft survival, accompanied frequently,
however, by toxic side effects. One approach to alleviating adverse side effects associated with high dose immunosuppression employs the administration of a combination of low dose immunosuppressive agents that may act additively or synergistically to inhibit alloactivation. Studies in this dissertation were designed to examine the use of a low dose cyclosporine A (CSA)/methotrexate (MTX) combination therapy following cardiac transplantation. Specifically, the ability of low dose CSA/MTX combination treatment to prolong cardiac allograft survival was determined. Additionally, the expression of cytotoxic genes, TNFα, LT, HF, and C11, in the transplanted cardiac grafts was measured in combination CSA/MTX-treated recipients. Studies such as these may provide opportunities to promote graft survival by using combination therapies which may minimize adverse side effects.

Historical Background

The first half of the twentieth century was characterized by a debate between proponents of pure cellular participation and proponents of the antibody and complement system as agonists in graft rejection. In the early 1940s the cellular nature of the alloimmune response was reported, and the allogeneic rejection reaction was largely attributed to cellular immune responses (Gibson & Medawar, 1943; Medawar et al., 1944). By the mid 1950s, the transplant rejection response was characterized as the result of cell-mediated (Billingham et al., 1954) and antigen-specific (Gorer, 1938) events. Despite the fact that the allograft rejection response has been shown to be a result of antigen-specific cellular mechanisms, the pathophysiological events which lead to the destruction of foreign donor tissue have yet to be elucidated. There are a number of mechanisms which have been proposed as effectors of graft rejection, including
cytotoxic alloantibodies (Carpenter et al., 1976), delayed-type hypersensitivity (Loveland & McKenzie, 1982), cytotoxic T cells (Lowry et al., 1985a; Hall et al., 1978), natural killer (NK) cells (Strom et al., 1977), and macrophages (MacPherson & Christmas, 1984). It is not known, however, to what extent each of these effectors may contribute to rejection of the transplanted graft. Therefore, it is clear that a more detailed examination of discrete cellular mechanisms and subpopulations involved in graft rejection needs to be performed.

Mechanisms of Allograft Rejection

Role of Genetics and the MHC

Although the immunological basis for transplant rejection has been well established, the detailed cellular mechanisms responsible for the destruction of donor tissue by the host's immune system are not yet fully understood. Early studies have indicated that grafts which were transplanted between genetically identical individuals or isografts were accepted, whereas grafts transplanted between genetically non-identical individuals or allografts were rejected (Little & Tyzzer, 1916). In fact, it has been demonstrated that successful transplantation depends on the donor and recipient sharing a number of independently segregating alleles, known as the histocompatibility genes (Gorer et al., 1948). Genetic studies on the segregation of histocompatibility genes have shown a large number of independently segregating loci in all vertebrate species. However, there is one gene region or cluster, the major histocompatibility complex (MHC), also referred to in humans as the human leukocyte antigen complex (or HLA), which elicits stronger allogeneic reactions than the others (Dausset et al., 1965; Hart & Fabre, 1981). The MHC maps to a single
chromosome, located on the short arm of chromosome 6 in humans (Robinson 1989), and encodes four classes of genes, the first two of which provide the major antigenic stimulus in allograft rejection. MHC class I products are constitutively expressed in humans by most tissues and cells except erythrocytes. Class II products are much more limited in distribution and are normally expressed on B lymphocytes, dendritic cells, monocytes and macrophages, as well as most endothelia, but may be induced on other cell types by gamma interferon (IFNγ) (Fuggle et al., 1986).

The MHC products, which are commonly referred to as the MHC antigens, consist of heterodimeric cell surface molecules that are highly polymorphic. MHC is essential for reactions involving the immune response because they are responsible for antigen presentation and self-recognition (Benacerraf, 1981). Although non-MHC antigens may contribute to graft rejection (Witherspoon & Storb, 1989), the strongest rejection responses appear to be directed against the products of the MHC, specifically class I and class II antigens (Dausset et al., 1965; Hart & Fabre, 1981). In fact, the immune system is commonly referred to as being "MHC-restricted" (Zinkernagel & Doherty, 1974).

Mature T cells express receptors (TCR) that recognize foreign antigens which are generally in the form of short peptides bound to self MHC molecules (Jorgenen et al., 1992) (Figure 1A). In normal host defense mechanisms, exogenous antigens are presented in the context of Class II, whereas intracellularly derived antigens such as viral proteins are processed and presented in association with MHC class I. The transplantation of MHC-mismatched tissue presents an interesting variation in T cell recognition of
antigen because the foreign antigens themselves are MHC molecules. Initiation of allograft rejection is thought to involve recognition of donor allogeneic MHC antigens expressed on the allograft and/or on "passenger leukocytes" within the graft, by receptors of CD4+ T helper cells of the recipient. Activated CD4+ T cells, along with antigen presenting cells, produce cytokines that support lymphocyte proliferation and maturation of CD8+ cytotoxic T cells which are specific for donor class I MHC. Infiltrating T cells may recognize processed endogenous peptides in the context of self MHC (Figure 1C), or processed allogeneic MHC antigens presented in association with self-MHC (Figure 1D). Additionally, direct recognition of epitopes on native alloantigen can also occur (Figure 1B). Support for these mechanisms of T cell recognition of alloantigen exists, but is not discussed here (Reviewed in Krensky et al., 1990).

Cells Mediating Allograft Rejection

The host cells infiltrating into acutely rejecting tissues include T and B lymphocytes, macrophages, and NK cells (Tilney 1975 and Mason 1986). Progressive infiltration of allografted tissues by host mononuclear cells is characteristic of the rejection process. Following transplantation, host lymphocytes infiltrate perivascular areas and subsequently scatter throughout the graft parenchyma. As inflammatory reactions proceed, macrophages become increasingly prevalent, with disruption of peri-capillary tissues, progressive interstitial inflammation, and eventual tissue necrosis. At the same time, both mature and blastic lymphocytes increase within recipient lymphoid compartments (Tilney 1991).
Fig. 1. Possible mechanisms of T cell recognition of alloantigen. T cell receptors (TCR) typically recognize foreign antigens in the form of short processed peptides bound to self MHC (A). However, during transplantation of an allograft, infiltrating T cells may recognize allogeneic MHC antigens as foreign (B), TCR may recognize self-peptides in the context of allogeneic MHC located on the allograft or passenger leukocytes (C), or processed allogeneic MHC peptides may also be recognized by the TCR when presented in association with self-MHC (D).

T-Cell Activation and Proliferation

As mentioned earlier, the early T cell response to alloantigen is primarily by the CD4⁺/T helper cells that detect foreign MHC class II antigens on the allografted tissue. It has also been shown, however, that much of the initial
immunogenicity of the transplanted graft may be a response by the recipient T cells against passenger leukocytes, which are dendritic leukocytes within the graft that express high levels of MHC class II antigen (Larsen, et al., 1990). Once recognition of foreign or allogeneic antigen occurs, transmembrane signals lead to intracellular activation and subsequent expansion and differentiation of the recipient's T lymphocytes. Early intracellular events in this process include the phosphophatidylinositol and the tyrosine kinase pathways, which act as second messengers during T cell activation.

In the phosphophatidylinositol pathway, activation of intracellular phospholipase C cleaves phosphatidylinositol 4, 5-biphosphate (PIP$_2$) into two potent second messengers, inositol 1, 4, 15-triphosphate and diacyl glycerol, which results in a 10-fold increase in intracellular calcium levels and activation of protein kinase C, respectively (Krensky et al., 1990). Calcium-activated kinases, in turn, modify DNA binding proteins, thereby affecting gene expression (in particular interleukin-2 (IL-2)) and ultimately T cell activation and differentiation. The tyrosine kinase pathway is directly activated by membrane receptor recognition of foreign or allo-antigen. Tyrosine kinase phosphorylates phospholipase C which in turn activates PIP$_2$, again leading to T cell activation.

Activated CD$_4^+$ T cells, along with antigen presenting cells, produce cytokines that support lymphocyte proliferation and the maturation of CD$_8^+$ cytotoxic T cells specific for donor class I MHC. The primary responsibility of T cells in the acute destructive process relies on their ability to lyse donor target cells without the need for antibody (Hayry & Defendi, 1970).
Macrophage Response During Allograft Rejection

Following TCR-antigen recognition, host macrophages are thought to provide the essential second signal, IL-1, which serves to trigger the proliferation of CD4+ T cells (Halloran et al., 1989). IL-1 also possesses many other proinflammatory effects, including promoting B cell growth and differentiation, increasing the cytotoxicity of NK cells, potentiating platelet activating factor by macrophages, and possessing chemotactic effects on macrophages (Halloran et al., 1989). In addition to IL-1, activated macrophages also have the ability to produce the cytokine, tumor necrosis factor alpha (TNFα). Like IL-1, TNFα possesses many proinflammatory effects which may contribute to the allograft rejection response. These effects include: potent induction of chemotactic factors for polymorphonuclear leukocytes and monocytes (Baggiolini et al., 1989), which may cause infiltration of these effector cells to the site of acute rejection, augmentation of class I MHC antigen expression (Tracey et al., 1987), stimulation of endothelium-derived platelet activating factor, which may promote ischemia within the graft, and induction of macrophage activation.

In fact, the macrophage has been implicated as an effector cell in first-set (acute) allograft rejection (MacPherson & Christmas, 1984), and their numbers have been shown to dramatically increase during rejection (Leendert et al., 1992; Payne et al., submitted). Specifically, macrophages have been described to be the predominant infiltrating cell at the time of rejection and are present in intimate relationship to myocardial cells (Christmas & MacPherson, 1982; Forbes & Guttmann, 1983). Furthermore, macrophages have the ability to act both as
antigen presenting cells that initiate an immune response and as cytotoxic effector cells that contribute to graft destruction (Nathan et al., 1980).

Other Immune Cell Populations Found in Rejecting Grafts

In addition to T lymphocytes and macrophages, other cell populations infiltrate rejecting grafts. B lymphocytes, when stimulated by alloantigen, differentiate into antibody-producing plasma cells. B cells have been demonstrated to appear late during the first-set acute allograft response and are detected within graft infiltrates by the time rejection is complete (Payne et al., submitted). B cells from graft infiltrates have the ability to secrete both nonspecific antibodies and those directed against donor cells (Garovoy et al., 1982). However, despite their obvious activity, their actual role in acute cell-mediated rejection remains unknown and is not well defined.

NK cells are a population of cytotoxic leukocytes that play a central role in the host defense against some malignancies. Although they infiltrate organ allografts rapidly, deletion experiments in graft recipients using specific anti-NK cell antibodies show that they are not critical in the acute rejection process (Heidecke et al., 1985). However, NK cells have been shown to mediate resistance to allogeneic and semi-allogeneic bone marrow grafts. Therefore the possibility exists that NK cells may effect rejection of allografts. In fact, it has been suggested that NK cells present at the graft site may develop into alloimmune cytotoxic T lymphocytes, or that NK cells may be functioning to promote the allograft response; perhaps by their ability to secrete lymphokines, such as IL-1 or IFNγ, and/or cytotoxic serine proteases (Hoffman et al., 1988).
Other cell types, including neutrophils and eosinophils, are present within the rejecting allografts, but their contribution, if any, to the rejection process is poorly defined.

**Effector Arms of Graft Destruction**

After placement of an allograft, a leukocyte infiltrate accumulates at the graft site. It is presumed that there are two principal means by which these infiltrating cells promote allograft rejection: either directly, through cell-mediated injury or lysis of the graft target cells (Figure 2A), or indirectly, through local production of soluble mediators of the immune response (i.e., cytokines), which in turn lead to graft target cell death (Figure 2B).

![Fig. 2](image-url)

Fig. 2. Two examples in which infiltrating cells may mediate graft target cell destruction. Foreign MHC class II antigens on graft stimulate host helper T cells to help cytotoxic T cells to destroy graft target cells; cytotoxic T cells can also recognize MHC class I foreign antigens, leading to direct cell lysis of graft target cells (A). Helper T cells and macrophages, for example, can respond to foreign graft cells by producing certain soluble mediators of the immune response, or cytokines, which can either directly injure graft target cells themselves, or do so by recruiting and activating nonspecific components of graft destruction (B).
Direct Cell Lysis

As mentioned earlier, activated CD4+ cells, along with antigen presenting cells, such as the macrophage, have the ability to produce certain cytokines that aid in the maturation and subsequent activation of CD8+ specific cytotoxic T cells. These activated cytotoxic T cells are then able to lyse foreign graft target cells through direct cell contact. Direct graft cell lysis is thought to result from the release of a preformed membrane-disrupting protein called "perforin," which shares structural homology to the C5b-C9 "membrane attack complex" of the complement cascade (Shinkai et al., 1988). The released perforin monomers bind to the membrane of graft target cells and polymerize to form pores that subsequently allow ion fluxes and entrance of cytolytic proteases, thus leading to cell death (Reviewed in Berke, 1991).

Furthermore, although the importance of specific anti-graft antibodies in acute rejection is uncertain, B lymphocytes are also known to be activated by T cell products in the presence of antigen to produce high levels of anti-class I antibodies. These antibodies are thought to be important in hyper acute rejection, accelerated vascular rejection, and antibody-dependent-cell-mediated cytotoxicity (Morris et al., 1990).

Production of Inflammatory Mediators

Promotion of allograft rejection by infiltrating cells may also occur indirectly, through effects resulting from the local release of inflammatory mediators which cause subsequent graft cell death. Production of these mediators may either directly injure graft target cells themselves, or do so by
recruiting and activating nonspecific components of graft destruction. Halloran et al (1989) summarized the relevance some of these inflammatory mediators have to the rejection process. These include: 1) the activation of macrophages as well as inhibition of their migration from the graft (primarily by IFNγ), leading to increased macrophage release of cytokines (IL-1 and TNFα), eicosanoids, and reactive oxygen intermediates; 2) the recruitment (by IL-1, IL-8, and other chemokines) of polymorphonuclear leukocytes; 3) secondary effects of platelet and coagulation activation which leads to graft ischemia; and, 4) the direct, nonspecific cell necrosis capability of TNF, and perhaps IL-1 and IFNγ, at concentrations found within acutely rejecting grafts (Reviewed in Halloran et al., 1989).

Taken together, both specific and nonspecific components of allograft rejection leads, in the absence of immunosuppression, to an intense immune cell infiltrate and subsequent graft cell destruction observed in acute rejection.

The focus of this dissertation is the investigation of the role of certain soluble mediators of the immune response produced during rat cardiac allograft rejection is investigated. A subset of these proteins which are known to be toxic to cells, including the cytokines TNFα and LT, and the granzymes A and B (HF and C11, respectively), were evaluated for their role(s) in the host's response to transplanted rat cardiac tissue. Insight into which of these cytotoxic proteins are produced, and when during the course of allograft rejection, may provide opportunities for both the detection of early markers of graft rejection and the
design of improved immunosuppressive therapies which may be used to prolong graft survival.

**Cytokines and Granzymes in Allograft Rejection**

**Historical Background**

As previously stated, the immune process in response to alloantigen is in part due to the production by different cell types of soluble endogenous biological mediators, among which are cytokines. Cytokines are soluble proteins produced by a variety of hematopoietic cells that have the ability to regulate the local development of alloreactivity. Cytokines can promote graft rejection by increasing MHC class II antigen expression (Hall et al., 1984; Fuggle et al., 1987), inducing adhesion molecule expression (Reviewed in Pober & Cotran, 1990), providing growth and differentiation signals to effector cells, and in some cases, directly damaging the graft parenchyma (Halloran et al., 1989).

Initial studies investigating the relevance of cytokines to allograft rejection have taken advantage of the development of specific immunoassays and bioassays that allow measurement of the cytokines of interest in biological fluids. Generally, the strategy has been to measure specific cytokines in the blood or urine of transplant recipients prior to, and following, graft transplantation, and to attempt to correlate changes in cytokine levels with clinical status. Identification of a given cytokine during rejection episodes suggests but does not prove its participation in the rejection process.
Several investigators have reported increased serum or plasma levels of TNFα (Maury & Teppo, 1987; Hoffman et al., 1991), IL-1β (Tilg et al., 1990; Hoffman et al., 1991), and IL-6 (Caillat-Zuckman et al., 1991) in patients at the time of acute rejection episodes. Additionally, elevated levels of TNFα have also been detected in the urine of rejecting human transplant recipients (McLaughlin et al., 1991). In patients receiving renal allografts, increased IL-2 (McKenna et al., 1988) and IL-2 receptor (Simpson et al., 1989) levels were observed in the periphery during acute rejection episodes. However, IL-2 and IL-2 receptor, along with TNFα, were also found to be significantly increased during bacterial or viral infection (Maury & Teppo, 1987; McKenna et al., 1988; Simpson et al., 1989; Imagawa et al., 1990a). Therefore, although changes in peripherally-measured cytokines often accompany allograft rejection and may provide information concerning the events occurring within the graft, the specificity of the data recovered, especially regarding their role in the pathogenesis of the acute rejection process, is very difficult to interpret.

In fact, systemic or circulating cytokine levels may not accurately reflect events occurring in the local intragraft environment. Although elevated circulating proinflammatory cytokines are found during acute rejection, infections in general and viral infections in particular, may elicit the same pattern of cytokine expression. In addition, it has previously been shown that cytokines may circulate bound to other proteins, such as natural inhibitors (Larrick, 1989), soluble receptors (Dinarello et al., 1993), preformed auto antibodies (Bendtzen et al., 1990), and α2-macroglobulin (James, 1990), which inhibit their biological activity but do not affect their immunoreactivity. Detection of an abnormally
elevated cytokine in the systemic circulation does not necessarily prove that the mediator is biologically active. Therefore, *in situ* measurement of these immune mediators at the graft site is a far more accurate and reliable method of identifying the cytokines elaborated during allograft rejection.

**Cytokine Production Within Rejecting Allografts**

During the last few years, the development of sensitive molecular techniques that allow identification of cytokine mRNA in small amounts of tissue or in limited numbers of cells has been paramount in characterizing the local cytokine pathways that are elaborated during allograft rejection. For example, *in situ* hybridization, as well as polymerase chain reaction (PCR) amplification have recently been employed to address this issue. Recently, IL-6, TNFα, and IFNγ gene expression was examined by *in situ* hybridization techniques in human renal allografts (Vandenbroeke *et al.*, 1991; Noronha *et al.*, 1992). It was found that the majority of samples taken from rejecting kidneys contained elevated levels of IL-6 gene transcripts, which were localized within glomerular cells, endothelium, tubular epithelia, and the infiltrate; IFNγ and TNFα mRNA, however, were not found to be associated with rejection. These findings implicate a role for IL-6 in the pathogenesis of human kidney allograft rejection.

Krams, *et al.* analyzed cytokine gene expression by PCR amplification in both rejecting and rejected renal allografts (Krams *et al.*, 1992). IL-1β, IL-6, and TNFα gene transcripts were prominent in rejected allografts, (supporting the concept that end-stage transplants are sites of ongoing inflammation); IL-6 and TNFα mRNA transcripts were detected less frequently in rejecting allografts than rejected allografts, while IL-1β mRNA was not evident. Conversely, gene
transcripts for the immune mediators IL-2, IL-4, and IL-5 were more prevalent in rejecting allografts than in end-stage transplants, suggesting that different immunologic processes may predominate depending on the progression of rejection. Wu et al (1992), also utilized the methodology of PCR amplification to detect cytokine mRNA transcripts in biopsies obtained from heterotopic abdominal cardiac allografts. IFNγ mRNA transcripts were detected in all transplants two days after surgery, before evidence of rejection was demonstrable by histopathologic analysis. IL-1β, IL-2, and IL-6 gene transcripts were detected when minimal rejection was noted, and IL-1α, IL-1β, IL-2, IL-6, IL-8, TNFβ (LT), and IFNγ mRNA transcripts were detectable in end-stage transplants (Wu et al., 1992).

Recently, Lipman, et al (1992) investigated cytokine gene expression for IL-1β, IL-2, IL-6, TNFα, and the cytotoxic T cell-specific serine protease, C11, in biopsies obtained from human renal allografts. The core biopsies were classified into three different categories based on histology: no evidence of rejection, acute cellular rejection, and equivocal rejection. IL-1β, IL-2, IL-6, and TNFα mRNA transcripts were detected in all biopsy classifications, with no difference in the frequency of gene expression of any of the aforementioned cytokines in relation to histology. However, C11 gene expression notably correlated with rejection. These results support a primary role for cytotoxic T lymphocytes in the cellular mechanisms of renal allograft rejection.

Although specific mechanisms have not yet been elucidated, there is enough data to indicate that cytokines play an important role in the pathogenesis
of allograft rejection, and may prove to be a useful target to disrupt the rejection process. In the present dissertation, employment of the previously mentioned methodology, that is, intragraft measurement of specific cytokines in transplanted rat cardiac tissues, is utilized to detect cytotoxic cytokine levels during the evolution of graft rejection. Specifically, TNFα and LT, as well as two cytotoxic T cell-specific serine proteases of the granzyme family, HF and C11, will be examined for their putative roles in the rejection process of heterotopically transplanted rat cardiac allografts.

Tumor Necrosis Factors-α and -β

TNFα and -β (LT) are two closely related cytokines that are endogenous mediators of immunological and inflammatory events. TNFα is a small peptide cytokine with a molecular weight of 17 kD which is produced primarily by activated macrophages (Beutler et al., 1985a), but recently has also been shown to be synthesized by activated peripheral T lymphocytes (Steffen et al., 1988). LT is a glycoprotein with a molecular mass of 25 kD originally identified as a cytolytic factor produced by cultured T lymphocytes in response to challenge with an antigen to which the cells had previously been exposed in vivo (Ruddle & Waksman, 1967), or in response to nonspecific mitogenic stimuli, (i.e., lectins) (Granger & Williams, 1968). TNFα and LT share 36% and 51% structural homology in overall nucleotide and amino acid sequences, respectively (Pennica et al., 1984), and also appear to have common receptor-binding domains (Aggarwal et al., 1985a).

Although TNFα and LT are distinct biochemically, they exert similar immunological effects in vitro (Ruddle, 1985; Beutler & Cerami, 1986). One of
the many biological activities of TNFα and LT includes activation of macrophages and enhancement of their cytotoxic potential in vitro (Aggarwal et al., 1985b). In addition to their cytotoxic effects, TNFα and LT have been shown to activate T lymphocytes, B lymphocytes, and NK cells (Yokota et al., 1988; Kehrl et al., 1987), and to elicit an increase in IL-1 and IL-6 synthesis and IL-2 receptor expression by cultured macrophages and T cells (Beutler et al., 1985b).

The specific roles of TNFα and LT in allograft rejection, however, are less well defined. As previously mentioned, TNFα has many proinflammatory actions which have been implicated to contribute to the allograft rejection response. These effects include: potent induction of chemotactic factors for polymorphonuclear leukocytes and monocytes (Baggioolini et al., 1989), which may cause infiltration of these effector cells to the graft site, augmentation of class I MHC antigen expression (Tracey et al., 1987), stimulation of endothelium-derived platelet activating factor, promoting ischemia within the graft, and induction of macrophage activation.

Macrophages, in fact, have been shown to be major components of the cellular infiltrate in transplanted organs undergoing rejection (Strom et al., 1977), and have been implicated as an effector cell in first-set (acute) allograft rejection (MacPherson & Christmas, 1984). Macrophage numbers have been shown to dramatically increase during rejection (Leendert et al., 1992), and have been described to be the predominant infiltrating cell at the time of rejection (Christmas & MacPherson, 1982; Forbes & Guttmann, 1983). Furthermore, macrophages can act as antigen presenting cells that have the ability to induce
immune responses, and as effector cells able to cytotoxically damage and cause eventual necrosis of graft target cells (Nathan et al., 1980). For these reasons, the present dissertation investigates TNFα, primarily an activated macrophage cell marker, for its possible role during the process of acute allograft rejection of rat cardiac transplants. Additionally, because TNFα and LT are known to exert similar immunological effects (Ruddle, 1985; Beutler & Cerami, 1986), and in fact, bind to common receptor domains (Aggarwal et al., 1985a), the studies contained within this dissertation also examined the putative role LT may contribute to acute rejection of rat cardiac allografts.

It has previously been reported that a marked elevation of serum TNFα levels exists during rejection episodes in human renal allograft recipients (Maury & Teppo, 1987). In addition, serum TNFα levels in cardiac allograft recipients have been shown to correlate with severity of rejection based on histological assessment of endomyocardial biopsies (Jordan et al., 1993). TNFα activity has also been demonstrated in homogenates from rejecting rat cardiac allografts (Lowry & Blais, 1988), and localized to mononuclear cell infiltrates in increased amounts during rejection (Hancock et al., 1991). Cytotoxic activity, attributed to LT, has been reported in both rejecting human (Moy & Rosenau, 1981) and rat (Lowry et al., 1985b) renal allografts. Recent studies have reported successful use of anti-TNFα and LT antibodies in prolonging the survival of transplanted grafts (Imagawa et al., 1990b; Scheringa et al., 1991; Teramoto et al., 1991), as well as reducing cellular infiltrates and edema (Saito et al., 1993) in rat models of rejection, indicating the contribution of TNF in mediating acute rejection. These
data strongly suggest a role for TNFα and/or LT in the pathogenesis of allograft rejection.

Granzymes A and B (HF and C11)

Granzymes are a family of highly homologous serine proteases present in secretory granules of cytolytic T lymphocyte cells. Seven serine proteases have been identified (granzymes A through G) (Reviewed in Masson & Tschopp; Jenne & Tschopp, 1988) from cytolytic granules of murine cytotoxic T lymphocytes and NK cells, and four serine proteases have been identified from human cytolytic lymphocytes (granzymes A, B, 3, and H) (Reviewed in Griffiths & Mueller, 1991a). Besides granzymes, perforin and proteoglycan molecules of the chondroitin sulfate A type, are the other two major constituents of these cytolytic granules (Krahenbuhl & Tschopp, 1991). Granzymes have been implicated in cell-mediated lysis by the observation that lysis by cloned T cells and isolated granules is inhibited by treatment with serine protease inhibitors (Chang & Eisen, 1980; Acha-Orbea et al., 1983). However, the physiologic role of granzymes is unknown. There is considerable evidence that the cytolytic granular components of cytotoxic T cells are released upon effector-target cell binding (Bykovaskaja et al., 1978). In fact, granzymes and proteoglycan molecules have been detected in the culture medium of cytotoxic T lymphocyte-target cell conjugates (Pasternack et al., 1986; Takayama et al., 1987). Granzymes by themselves, however, do not have cytolytic activity (Masson & Tschopp, 1985; Podack et al., 1985). Yet in combination with purified perforin, granzymes can affect the release of target cell DNA, an event which occurs during cell-mediated lysis (Hayes et al., 1989).
An attractive model for "lethal hit delivery" has been proposed describing the events leading to target cell lysis. This granule-exocytosis model suggests that, upon appropriate effector-target cell conjugation, the effector cells deliver dense cytoplasmic granules and their contents into the intercellular space at the site of contact between the target and effector cell (Sitkovsky, 1988). This process of degranulation releases perforin, which, upon polymerization and in the presence of Ca\(^{2+}\), induces transmembrane, ring-like pores in the target cell membrane. The formation of pores in the target cell membrane subsequently allows ion fluxes and access to the released granular contents, including granzymes, which leads to target cell apoptosis and eventual cell death (Berke, 1991) (summarized in Figure 3).

![Diagram of the proposed granule-exocytosis model](image)

**Fig. 3.** Proposed granule-exocytosis model for "lethal hit delivery," leading to target cell lysis.
As stated earlier, cytotoxic T lymphocytes are believed to be important effector cells in the destruction of transplanted tissue during allograft rejection (Loveland & McKenzie, 1982; Lowry et al., 1985b). After recognition of alloantigens on the surface of MHC-nonidentical target cells, cytotoxic effector cells bind to them and induce target cell lysis (Hall et al., 1978). Although various steps in this process have been analyzed in considerable detail, most studies have not provided insight into the mechanism(s) by which the cytolytic effector cell administers the "lethal hit" to the target cell. Recently, the genes encoding the serine esterases HF and C11 (Granzymes A and B, respectively), which have been demonstrated to be selectively expressed in cytotoxic cells including cytotoxic T lymphocytes and NK cells, have been cloned (Gershenfield & Weissman, 1986; Lobe et al., 1986). The kinetics of HF and C11 gene expression closely parallel the cytolytic responses in vitro, in that the observed kinetics of serine esterase release and of the delivery of the lethal hit are consistent with the hypothesis that both events occur simultaneously (Gershenfield & Weissman, 1986; Lobe et al., 1986). Therefore, a strong correlation exists between granzyme gene expression and cytotoxic potential. For these reasons, this dissertation investigated the expression of both HF and C11 serine proteases for their putative role in the process of acute allograft rejection of rat cardiac transplants.

One of the first studies demonstrating the importance of granzyme-expressing lymphocytes in vivo, involves the detection of transcripts encoding serine proteases in situ, during murine cardiac allograft rejection (Mueller et al., 1988). In this study, the time course of serine protease expression closely paralleled the rejection process. Recently, both granzymes A and B have been
described as putative markers for rejection in patients following cardiac transplantation (Griffiths et al., 1991b; Clement et al., 1991), as well as in experimental animal models of cardiac (Mueller et al., 1991) and renal (Lipman et al., 1992) allograft rejection. Therefore, these data would suggest that cytotoxic cell-specific serine proteases, such as HF and C11, may play a role in the pathogenesis of rat cardiac allograft rejection.

Immunosuppression to Prevent Allograft Rejection

Historical Background

Therapeutic attempts to prevent allograft rejection have gradually evolved from nonspecific suppression of immune responsiveness to the modulation of specific components of rejection. In the late 1950s, a single incidence of long-term success was achieved with total body irradiation for a human renal allograft transplanted into a fraternal twin recipient (Murray et al., 1960). In the early 60s, Calne, et al (1962) demonstrated the usefulness of mercaptopurine, a purine analog antimetabolite that nonspecifically inhibits gene replication therefore affecting T cell proliferation and antibody synthesis. In this study, mercaptopurine was found to be successful in prolonging canine renal allografts, and soon after, its parent compound, azathioprine, became commonly used in clinical settings. Subsequently, the beneficial synergistic effect of steroids with azathioprine was realized, and the introduction in the 1960s of antilymphocyte serum in conjunction to this combination therapy, especially for the reversal of acute rejection, came into use (Reviewed in Murray et al., 1981). However, adverse side effects, including leukopenia, thrombocytopenia, and allergic
reactions to heterologous serum as well as anti-lymphocyte serum antibody production limited its long-term use.

The decade of the 1980s was highlighted by the introduction and application of cyclosporine A (CSA) in the field of organ transplantation. CSA was first used clinically in 1978 (Calne et al., 1978), and because it was demonstrated to be a potent immunosuppressive agent acting at the level of the T lymphocyte, CSA was subsequently used therapeutically following organ transplantation.

Cyclosporine A

CSA is a cyclic endecapeptide derived from the soil fungus, *Tolypocladium inflatum*, and as stated earlier, has been demonstrated to be a potent immunosuppressive agent and inhibitor of helper T cell function (Borel et al., 1976; Orosz et al., 1983). The profound, reversible suppression of cell-mediated immunity caused by CSA was first described by Borel et al (1976), and since those landmark studies, it has been the focus of numerous investigations to examine its exact mechanism(s) of action. CSA has been shown to consistently inhibit IL-2 gene transcription (Kronke et al., 1984; Elliot et al., 1984), and therefore, the direct effects of CSA upon CD4+ T helper lymphocytes have become the central focus of most efforts attempting to elucidate CSA's immunosuppressive action, since one functional characteristic of the CD4+ T helper lymphocyte population is the biosynthesis and release of an array of cytokines, including IL-2.

IL-2 is a T helper cell product that functions as a second signal in the presence of appropriate antigen to promote the differentiation and clonal
expansion of mature T lymphocytes and appears to be an important mediator in allograft rejection, since a positive correlation between IL-2 levels and rejection episodes has been described (Yoshimura & Kahan, 1985; Vie et al., 1985; McKenna et al., 1988). Anti-IL-2 receptor antibodies have also been shown to prevent allograft rejection in experimental models, thus supporting the central role IL-2 plays in the rejection process (Kirkman et al., 1985). Although CSA's action on IL-2 gene expression in T cells is currently taken as the prototype for all its immunosuppressive effects, CSA also has effects on many other cell types, and on their cytokine production. In mast cells, CSA has been demonstrated to block degranulation as well as transcriptional activation of several cytokine genes, including IL-3 and IL-5 (Cirillo et al., 1990; Hultsch et al., 1991). In stimulated THP-1 cells, a leukemic monocyte cell line, CSA has been shown to inhibit the in vitro production of both IL-1α and IL-1β (Reisman et al., 1991). CSA has also been reported to inhibit in vitro-stimulated cytokine production by peripheral blood mononuclear cells; these cytokines include IL-2, IFNγ, IL-4, IL-5, and TNFα (Andersson et al., 1992).

As stated earlier, most studies investigating the actions of CSA have concentrated on T cells as the targets for CSA immunosuppression. However, recent attention has also focused on the role of the macrophage. Benson et al (1989) demonstrated that decreased IL-2 production observed with CSA treatment is actually caused by inhibition of macrophage antigen presentation. Additionally, in vitro studies have shown that CSA inhibits production of TNF (Espevik et al., 1987) without depressing mRNA or intracellular levels of TNF (Remick et al., 1989). CSA has also been shown to block the extracellular
production of TNF in response to lipopolysaccharide (LPS) challenge, both *in vitro* and *in vivo*, without decreasing TNF mRNA (Nguyen et al., 1990). Therefore, although CSA is generally accepted to act as an immunosuppressive agent through potent inhibition of IL-2 production by T cells, CSA may also exert its immunosuppressive actions through inhibition of additional cytokines produced by different leukocyte populations.

While CSA effects upon cytokine biosynthesis and release have been reproducibly demonstrated, the exact mechanism of CSA action remains to be characterized. CSA is thought to enter target cells through passive transport by diffusing through the plasma membrane lipid bilayer (LeGrue & Munn, 1986a), concentrating on the inner membrane surface, and presumably gaining intracellular access from that point (Rossaro et al., 1988). Investigation has revealed that CSA binds to a number of intracellular proteins in extremely small quantities with two notable exceptions. Calmodulin, a calcium binding protein which undergoes a conformational change upon receptor-ligand interaction, binds to CSA; however, the interaction is of relatively low affinity and has not been linked to the biologic effects mediated by CSA (Hess et al., 1988; LeGrue et al., 1986b). At high concentrations, CSA does exert some effects upon calmodulin, but the collective data have argued against the interaction representing a primary component of CSA's mechanism of action (Drugge & Handschumacher, 1988).

Recently, the binding of CSA to an intracellular protein, cyclophilin, has been reported (Handschumacher et al., 1984). Subsequent protein purification
and sequence determination has revealed cyclophilin to represent a novel molecule 17 kD in size and characterized by its ability to bend or fold proteins at proline groups, from the "cis" to the "trans" position, and therefore described as a peptidyl-prolyl cis-trans isomerase (Harding et al., 1986; Takahashi et al., 1989; Fischer et al., 1989). CSA is thought to inhibit the rotomase enzyme activity of cyclophilin. It has been postulated that CSA's immunosuppressive effect is due to the inhibition of cyclophilin's isomerase activity, needed for signal transduction (Lorber et al., 1990).

More recent evidence has shown that CSA specifically inhibits the DNA binding activities of several nuclear factors important in transcriptional activation of the genes for IL-2 and several other cytokines (Emmel et al., 1989; Mattila et al., 1990). Specifically, the transcription factors NF-AT, AP-3, NF-AL, and NF-κB, were found to be affected by CSA; each of these transcription factors have a common distinguishing feature in that all require Ca\(^{2+}\) mobilization for activation. Therefore, the aforementioned observations have led to a working hypothesis proposed by Schreiber et al (1992) by which CSA blocks IL-2 gene activation, and possibly other cytokines, in T cells and other immune cell populations (summarized in Figure 4).

In addition to its immunosuppressive capabilities, CSA has also been shown to mediate a number of physiologic changes; the clinical manifestations of these effects often resulting in toxic side effects. Therefore, although CSA has been used with considerable success clinically to prolong organ graft survival, significant adverse side effects, such as nephrotoxicity (Myers et al./, 1984),
Fig. 4. Proposed model of CSA inhibition of cytokine (IL-2) gene activation. Binding of antigen to TCR leads to intracellular Ca\(^{2+}\) release. Calcium activates the phosphatase, calcineurin which, in the absence of CSA, dephosphorylates NF-AT\(_C\) (cytosolic subunit) thus facilitating its translocation into the nucleus. NF-AT\(_C\) can then combine with NF-AT\(_N\) (nuclear cofactor) to form an activated transcription factor with the ability to activate RNA polymerase, thereby regulating gene transcription. This process is blocked when CSA binds to its receptor protein, cyclophilin. The CSA-cyclophilin complex can inhibit calcineurin, which blocks nuclear translocation of NF-AT\(_C\), therefore inhibiting cytokine gene activation.
hepatotoxicity (Vine et al., 1988), hypertension (Munoz et al., 1988), as well as neurological toxicities (deGroen et al., 1987) have been reported with its use. Consequently, a need remains for implementing specific therapies directed at achieving transplant success with a minimum of undesirable side effects.

As newer agents with immunosuppressive capabilities appear on the horizon, the possibility of utilizing combination treatments has offered hope for improved therapeutic management of organ transplantation. For example, the introduction of both FK506 (Tanaka et al., 1987), an antibiotic tacrolimus isolated from the soil fungus Streptomyces tsukubaenis, and rapamycin (Seghal et al., 1975), another macrolide similar in structure to tacrolimus, have demonstrated potent immunosuppressive activity (Beck & Akiyama, 1989; Morris et al., 1990). Rapamycin, in particular, has been shown to exhibit marked synergy when combined with CSA in inhibiting various parameters of in vitro immunological performance by human peripheral blood lymphocytes (Kahan et al., 1991). Monoclonal antibodies, such as OKT3, a murine monoclonal antibody against the CD3 complex, have also shown promising results in the clinical management of organ transplantation (Millis et al., 1989). Although these immunosuppressive agents have demonstrated initial clinical success, their mechanisms of action as well as their use in organ transplantation therapy will not be discussed in detail within the present dissertation, but are reviewed elsewhere (Metcalf & Richards, 1990; Schreiber & Crabtree, 1992). It may be said, however, that despite their promising immunosuppressive effects, FK506, rapamycin, and OKT3, similar to CSA, all have certain adverse side effects associated with their use at therapeutic doses. One possible approach to forego high dose toxicities is the utilization of
combination treatments, administered at lower therapeutic doses, to achieve increased survival time of organ transplants while minimizing adverse side effects.

Methotrexate

One agent which may provide promising results for combination therapy is methotrexate (MTX). MTX is classified pharmacologically as an antimetabolite because of its antagonistic effect on folic acid metabolism and function (Mitchell et al., 1969). MTX has been shown to bind dihydrofolate reductase which results in the inhibition of DNA synthesis; this antiproliferative action has made MTX a useful drug for cancer chemotherapy (Jolivet et al., 1983). Compared to CSA, little is known about the immunosuppressive actions of MTX. However, the importance of MTX as an immunosuppressive agent was first considered when anti-inflammatory effects were noticed with its utilization in the treatment of rheumatoid arthritis, wherein MTX demonstrated both long term and short term efficacy (Weinblatt et al., 1985; Perhala & Wilke, 1991). The mechanism(s), however, by which MTX suppresses disease activity in inflammatory rheumatoid arthritis is still poorly understood and adverse side effects, such as severe leukopenia and gastrointestinal upset, have been associated with its use (Weinblatt et al., 1985).

It has been postulated that MTX may exert its immunosuppressive action by deactivation of macrophages. This would result in decreased IL-1 production (Hu et al., 1988), as well as inhibition of macrophage migration, resulting in the decreased accumulation of inflammatory cells and/or immune mediators (Johnson et al., 1988). Despite these findings, the administration of MTX has also
been shown to restore both T cell function (Kourounakis & Kapusta, 1976) and IL-2 production (Combe et al., 1985) in rheumatoid arthritis, thereby counteracting the immunosuppressive effects demonstrated by Hu et al (1988).

MTX, however, has recently shown favorable results in treating persistent mild (Olsen et al., 1990), as well as recurrent moderate to severe cardiac allograft rejection (Costanzo-Nordin et al., 1988). Success has also been demonstrated using CSA at doses of 10-15 mg/kg/day combined with either high or low dose MTX in the prevention of graft-versus-host disease after bone marrow transplantation in experimental animal models (Deeg et al., 1982; Deeg et al., 1984), as well as in human clinical studies (Storb et al., 1986; Truog & Wozniak, 1990).

Taken together, immune processes in response to alloantigen is the result of both direct cell-mediated events and the production of, by different cell types, soluble biological mediators, including cytokines and granzymes. In the absence of immunosuppression, these specific and non-specific components of allograft rejection lead to intense immune cell infiltration and subsequent graft cell destruction. Previous observations strongly suggest that administration of CSA/MTX combination therapy may serve as a possible alternative treatment in promoting allograft survival. Figure 5 summarizes the postulated mechanisms of CSA/MTX immunosuppression on effector cells and cytokine/granzyme production during allograft rejection. For these reasons, the focus of the present dissertation is to examine the effects of a low dose CSA/MTX combination therapy in prolonging rat cardiac allografts. Specifically, low dose CSA/MTX
combination therapy will be evaluated for its ability to prolong rat cardiac allograft survival time, as well as for its effect on cytotoxic cytokine expression, including TNFα, LT, and granzymes A and B (HF and C11).

Therefore, the major objectives of this dissertation project are: 1) to quantitate the expression of the aforementioned cytotoxic cytokine gene products during the evolution of graft rejection, and 2) to evaluate and compare the efficacy of a low dose CSA/MTX combination therapy for its ability to prolong
survival of rat cardiac allografts. These objectives will be fulfilled by accomplishing the following specific aims: the expression of the genes encoding the cytotoxic cytokines, TNFα and LT (Chapter II) and the serine proteases HF and C11 (granzymes A and B, respectively) (Chapter IV), are quantitated during rat cardiac allograft rejection. In Chapter III, the aims are: 1) to evaluate the efficacy of a low dose CSA (1.0 mg/kg/day)/MTX (450 µg/kg/wk) combination therapy in prolonging rat cardiac allograft survival, and 2) to determine the effects of low dose CSA/MTX on IL-2 gene expression in rat cardiac allografts. Finally, in Chapter IV, the effects of low dose CSA/MTX on the expression of the cytotoxic cytokines, TNFα, LT, as well as the serine proteases HF and C11, are determined in rat cardiac allografts during rejection.

Insight into which of the cytotoxic mediators are synthesized, and when post-tx, may provide opportunities for early detection of molecular "markers" that signal impending rejection. Assessment of the cytokine profile during allograft rejection may also aid in the development of more specifically targeted immunosuppressive therapies designed to prolong graft survival. Therefore, the overall goal of these studies is to expand our knowledge of the immunopathological mechanisms involved in graft rejection and to evaluate more effective strategies aimed at prolonging graft survival while minimizing adverse side effects often associated with the use of immunosuppressive agents.
CHAPTER 2

INDUCTION OF TNFα AND TNFβ GENE EXPRESSION IN RAT CARDIAC TRANSPLANTS DURING ALLOGRAFT REJECTION

Abstract

The expression of the cytotoxic cytokines, TNFα and LT, was assessed in rat cardiac transplants during rejection. Newborn rat cardiac grafts placed in adult rat ear pinnae were retrieved on days 1 through 10 post-transplantation (post-tx); the average time to rejection, assessed by the absence of detectable electrocardiographic (ECG) activity, was determined to be 7 days. Total cellular RNA and tissue homogenates were prepared from cardiac transplants in order that relative levels of TNFα and LT mRNA and TNF protein could be determined. A biphasic pattern of TNFα gene expression was consistently seen in cardiac allografts. TNFα mRNA transcripts were detected as early as day 2 post-tx, with peak levels appearing on day 3 post-tx. Although transcript levels decreased by day 4, a significant increase appeared again on day 6 post-tx, coincident with the onset of rejection. Similar to TNFα gene expression, LT transcripts demonstrated a biphasic pattern of induction. LT mRNA transcripts also reached peak levels on day 3 post-tx, with a second increase in transcript levels coincident with rejection. TNF protein levels in allografts displayed a biphasic pattern, similar to that shown by the cytokine mRNAs. Peak levels of TNF protein were detected on day 3 post-tx, with a second increase again coinciding with rejection. In contrast to TNF expression found in allografts,
TNFα and LT mRNA transcripts were not detected in isografts on days 1 through 10 post-tx. TNF protein levels in cardiac isografts were consistently at or below the standard limits of detection, and on days 3 through 7 post-tx were significantly reduced ($p \leq 0.001$) when compared to time-matched allografts. Increased expression of the cytotoxic cytokines, TNFα and LT, therefore, appears to be allograft-specific and is an early event during rat cardiac allograft rejection. In conclusion, induction of TNF gene expression may be an important early indicator of transplant rejection.

**Introduction**

Although the immunological basis for transplant rejection has been well established, the cellular mechanisms responsible for allograft rejection have not yet been fully elucidated. After transplantation of a tissue allograft, a cellular infiltrate consisting of leukocytes of several phenotypes appears within the graft (Hanto et al., 1982). Infiltration of both helper and cytotoxic T lymphocytes precedes the destruction of the allograft by several days, and it is believed that these cells play a major role in the process of allograft rejection (Loveland & McKenzie, 1982; Lowry et al., 1983). Macrophages and NK cells have also been found to be major components of the cellular infiltrate in organ allografts undergoing acute rejection (Strom et al., 1977; MacPherson & Christmas, 1984; Nemlander et al., 1983). These cells proliferate and release a range of soluble mediators of the immune response (i.e., cytokines) - which, among many other effects, have been shown to damage blood vessels within the graft (Dvorak et al., 1980), cause an increase in graft infiltration by leukocytes (Cox et al., 1984), and stimulate the differentiation of graft-specific T cells at the graft site (Heidecke et al., 1984). These events precipitate allograft damage and eventual necrosis of the
transplanted tissue. There are two principal mechanisms by which cell-mediated injury may occur: through direct lysis of the graft target cells, or indirectly, through local production of cytokines which, in turn, contribute to allograft destruction.

TNFα and LT are two closely related cytokines that are endogenous mediators of immunological and inflammatory events. TNFα is a small peptide cytokine with a molecular weight of 17 kD which is produced primarily by activated macrophages (Beutler et al., 1985a), but recently has also been shown to be synthesized by activated peripheral T lymphocytes (Steffen et al., 1988). LT is a glycoprotein with a molecular mass of 25 kD originally identified as a cytolytic factor produced by cultured T lymphocytes in response to challenge with an antigen to which the cells had previously been exposed in vivo, or in response to nonspecific mitogenic stimuli, (i.e., lectins) (Ruddle & Waksman, 1967; Granger & Williams, 1968). TNFα and LT share 36% and 51% structural homology in overall nucleotide and amino acid sequences, respectively (Pennica et al., 1984), and also appear to have common receptor-binding domains (Aggarwal et al., 1985a).

Although TNFα and LT are distinct biochemically, they exert similar immunological effects in vitro (Beutler & Cerami, 1986; Beutler, 1990). One of the many biological activities of TNFα and LT includes activation of macrophages and enhancement of their cytotoxic potential in vitro (Aggarwal et al., 1985b). In addition to their cytotoxic effects, TNFα and LT have been shown to activate T lymphocytes, B lymphocytes, and NK cells (Yokota et al., 1988;
Kehrl et al., 1987), and to elicit an increase in IL-1 and IL-6 synthesis and IL-2 receptor expression by cultured macrophages and T cells (Beutler et al., 1985b).

The specific roles of TNFα and LT in allograft rejection, however, are less well defined. It has been reported that a marked elevation of serum TNFα levels exists during rejection episodes in human renal allograft recipients (Maury & Teppo, 1987). Similarly, serum TNFα levels have been found to be significantly elevated in patients experiencing acute rejection following orthotopic liver transplantation (Imagawa et al., 1990a). TNFα activity has also been demonstrated in homogenates from rejecting rat cardiac allografts (Lowry & Blais, 1988) and localized to mononuclear cell infiltrates in increased amounts during rejection (Hancock et al., 1991). Cytotoxic activity, attributed to LT, has been reported in both rejecting human (Moy & Rosenau, 1981) and rat (Lowry et al., 1985b) renal allografts. Recent studies have reported successful use of anti-TNFα and LT antibodies in prolonging the survival of transplanted grafts in experimental animals, indicating the contribution of TNF in mediating acute rejection (Imagawa et al., 1990b; Scheringa et al., 1991; Teramoto et al., 1991). These data strongly suggest a role for TNFα and/or LT in the pathogenesis of allograft rejection.

The purpose of this study was to quantitate the expression of the genes encoding these two cytokines during rat cardiac allograft rejection. Insight into which of these two cytotoxic proteins are synthesized, and when post-tx, may provide opportunities for early detection of cytokine "markers" that signal impending rejection. Assessment of the cytokine profile during allograft
rejection may also aid in the development of more specifically targeted immunosuppressive therapies designed to prolong graft survival.

Materials and Methods

Animals

Pregnant Brown Norway (BN) (RTln) and 6-8-week-old male Lewis (RT1l) rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Dark Agouti (DA) (RTla) rats were obtained from an inbred rat colony maintained at Loyola University Medical Center. All animals received water and food ad libitum, and were housed in accordance with institutional animal care and use guidelines. Two isograft groups were used in these studies: adult male Lewis or DA rats served as recipients of donor cardiac tissues obtained from 1-3 day-old Lewis or DA rats, respectively. The two allograft groups consisted of adult male Lewis rats serving as recipients of either 1-3 day-old BN or DA rat cardiac transplants. The donor ages used have been previously reported to result in optimum isograft viability (Fulmer et al., 1963) and were of appropriate size so that whole heart grafts might be accommodated by the recipient's ear.

Cardiac Transplantation

The procedure utilized was a modification of the heterotopic method of cardiac transplantation into the mouse ear as described by Fulmer et al (1963) (Figure 6). Cardiac graft recipients were anesthetized with ketamine/xylazine (20:1) (Fort Dodge Laboratories, Fort Dodge, IA and Rugby Laboratories, Inc., Rockville Centre, NY) at 105 mg/kg body weight i.p. prior to surgery. The dorsal surface of the recipient's ear pinna was nicked at the proximal end with a
sterile scalpel, and a subcutaneous pouch was prepared using blunt forceps. Whole newborn hearts were harvested promptly at the time of donor sacrifice and gently inserted into the ear pouches of the recipients. In order to prevent graft loss and promote revascularization, skin flaps surrounding the graft site were apposed by applying gentle pressure with forceps. Each recipient was grafted bilaterally, one whole heart per recipient ear, with two isografts or two allografts per animal. Cardiac graft recipients were anesthetized and monitored from day 1 to day 10 post-tx by visual inspection for pulsatile activity and by electrocardiography using pin electrodes placed on either side of the graft with leads connected to a polygraph recorder (Grass Instruments Model 79D, Quincy, MA) (Figure 7). Grafts were removed on days 1 through 10 post-tx and processed as described below for RNA blot analysis or protein assays.

Fig. 6. Schematic diagram of the heterotopic heart graft technique used in the present studies. The dorsal surface of the recipient's ear pinna is nicked at the proximal end (1). A subcutaneous pouch is prepared to accommodate grafts (2). Whole newborn donor hearts are inserted into ear pouches (3). Cardiac grafts are monitored daily (4).
Fig. 7. Illustration of physiograph recordings. Input from graft leads to determine graft ECG activity (a). Input from intrinsic heart leads to determine intrinsic heart's ECG activity (b).

Cell and Tissue Culture

All cells were cultured in RPMI-1640 media supplemented with 2% penicillin-streptomycin, 5% L-glutamine, and 5% fetal bovine serum (Gibco, Grand Island, NY) at a cell density of 1 X 10^6 cells/ml at 37°C in a 5% CO₂ atmosphere, with the exception of the RNK-16 cell line, which was also
supplemented with 1% nonessential amino acids and 5 X 10^{-5} \text{M} 2\text{-b-mercaptoethanol (Gibco). The murine macrophage cell line P388D1 (ATCC, Rockville, MD), was incubated for 4 hr with 10 \mu \text{g/ml LPS (Difco, Detroit, MI) and used as a positive TNF}\alpha \text{ mRNA control. The rat NK cell line RNK-16 (a gift from Dr. C. Reynolds, NIH, Frederick, MD), was cultured for 18 hr in the presence of 20 ng/ml phorbol myristate acetate (PMA) and 1000 U/ml IL-2, (Sigma, St. Louis, MO) and used as a negative TNF}\alpha \text{ mRNA control. Splenic mononuclear cells (SMNCs) were isolated as previously described (Schneider 1984) from adult male Lewis rats and were cultured for 18 hr in the presence or absence of 20 ng/ml PMA and 2 \mu \text{g/ml phytohemagglutinin (PHA), and used as postive and negative LT mRNA controls, respectively. After incubation with respective mitogens, cells were collected and processed for RNA as described below.}

**RNA Blot Analysis**

For Northern blot analysis, total cellular RNA was prepared from cells and tissues by homogenization using a Brinkmann polytron (Brinkmann Instruments, Inc., Westbury, NY) in the presence of guanidinium isothiocyanate (Chirgwin et al., 1979). RNA was quantitated spectrophotometrically using O.D. 260 nm determinations (Maniatis et al., 1989). After electrophoretic separation in a 1.0% formaldeyde agarose gel, RNA was applied by Northern transfer (Thomas, 1980) to Nytran filters (Schleicher & Schuell, Keene, N.H.). Filters were baked at 80^\circ \text{C} for 1 hr and prehybridized in a solution containing 50\% formamide and 10\% dextran sulfate for 4 hr at 42^\circ \text{C}. Filters were then hybridized overnight at 42^\circ \text{C} in a solution containing either a TNF}\alpha \text{ cDNA probe prepared using the 1.7 kb murine TNF}\alpha \text{ sequence cloned into the PstI/BamHI site of
pUC9 vector, (a gift from Chiron Corporation, Emeryville, CA) (Pennica et al., 1985), or an LT probe prepared using the 0.71kb KpnI/HincII fragment of the 1.42 kb murine LT cDNA sequence (a gift from Dr. N. Ruddle, Yale University, New Haven, CT) (Li et al., 1987). Probes were radiolabeled with [\textsuperscript{32}P] \( \alpha \)-dATP as described elsewhere (Feinberg & Vogelstein, 1983), using the Prime-a-Gene labeling system (Promega, Madison, WI), and used at \( 2 \times 10^6 \) cpm/ml of hybridization solution. Hybridized filters were subsequently washed at room temperature (2 X 15 min) in 2X SSC and 0.1% sodium dodecyl sulfate, and then at 55°C (2 X 15 min) in 0.2X SSC and 0.1% sodium dodecyl sulfate, after which the filters were air-dried and autoradiographed using Kodak XAR-5 x-ray film with intensifying screens at -70°C.

Protein Assays

Cardiac graft tissue homogenates were prepared using a Brinkmann polytron (Brinkmann Instruments) in 1.5 ml phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Sigma) for analysis of TNF protein. Tissue homogenates were briefly centrifuged in a microcentrifuge to remove excess particulate matter. Supernatants were then concentrated approximately 10-fold using Centricon-10 microconcentrators (Amicon, Beverly, MA). The samples were kept on ice for the duration of the assay and subsequently stored at -70°C. Total protein levels were quantitated using a modification of the Lowry colorimetric procedure (Sigma). Tissue samples were standardized to 150 µg total protein per well and assayed in duplicate for TNF immunoreactive protein using an ELISA (enzyme linked immunosorbent assay) system containing a hamster antimouse TNF\(\alpha\) antibody (Genzyme, Boston, MA). Results were analyzed
spectrophotometrically using a MR600 microplate reader (Dynatech Laboratories, Chantilly, VA) and TNF levels were expressed as pg/ml and U/ml ± S.E.M., using recombinant mouse TNFα as the standard (Genzyme).

Statistical Analysis

Experimental groups were compared using a two way analysis of variance for the existence of significant interaction between experimental group and day post-tx. Differences were considered to be statistically significant at $p \leq 0.05$.

Results

In order to determine cardiac graft viability, both isograft and allograft tissues were monitored daily for ECG and pulsatile activity. Representative ECG recordings from isografts at six different time points (days 2, 7, and 10, and at 1, 4, and 12 months) post-tx are shown in Figure 8. Isograft tissue demonstrated regular and progressively stronger ECG activity when measured on days 2, 7, and 10 post-tx, with the average heart rate increasing by almost two-fold from day 2 to day 10 post-tx (Figure 8). In addition, an increase in heart rate up to one month post-tx was observed in isografts, at which time they tended to reach a steady rate of ECG activity. In isografts monitored up to 1 year post-tx, graft heart rate appeared to approach approximately 50% of the intrinsic heart rate of the animals (Figure 8). The average intrinsic heart rate of a rat was found to average 350 bpm, and differences in the two isograft combinations used, DA-into-DA and Lewis-into-Lewis were not significant (data not shown).
In comparison, ECG recordings from cardiac allograft tissue on days 2, 7, and 10 post-tx demonstrated diminished electrical activity with time post-tx, indicating graft failure as defined by absence of detectable bpms (Figure 9). On day 2 post-tx, the average heart rate for allografts was 77.97 ± 10.3 bpm, which was not statistically significant when compared with the average isograft rate on the same day (Table 1). However, by day 7 post-tx the allograft rate decreased to 64.83 ± 9.1 bpm, in contrast to an increased heart rate of 121.43 ± 16.7 observed in isografts. These findings represented a statistically significant difference in allograft heart rate on day 7 post-tx, as compared with control isografts \( p \leq 0.002 \). Isograft heart rates remained relatively constant at day 10 post-tx at 143.14 ± 19.9, while allograft rates continued to decrease with time, such that by day 10 post-tx, ECG and pulsatile activity of cardiac allografts were virtually undetectable. In all but one case, the heart rate of allografts on day 10 post-tx were 0 bpm (Table 1). It was determined in the course of these studies that the average time to rejection of cardiac transplants in allograft recipients was 7 days. Both allograft combinations used, DA-into-Lewis and BN-into-Lewis, yielded similar results (data not shown).

<table>
<thead>
<tr>
<th>TABLE 1. Cardiac graft viability post-transplantation</th>
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<tr>
<td><strong>Day post-TX</strong></td>
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<tr>
<td>2</td>
</tr>
<tr>
<td>Allograft (bpm)</td>
</tr>
<tr>
<td>(n = 32)</td>
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<tr>
<td>Isograft (bpm)</td>
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<td>(n = 25)</td>
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\( ^a P \leq 0.002 \)

\( ^b \) All values except for 1 (n = 22) = 0.00.
Fig. 8. Long-term cardiac isograft viability as determined by ECG recordings. Representative ECG recordings from six different time points post-tx are shown. All recordings were performed at a constant chart speed of 5.0 mm/sec; bpm, beats per minute.
Fig. 9. Comparison of cardiac isograft and allograft viability. Representative ECG recordings from three different time points post-tx are shown. All recordings were performed at a constant chart speed of 5.0 mm/sec; bpm, beats per minute.
Expression of the cytotoxic cytokine genes TNFα and LT was examined in cardiac transplants at various times post-tx. Cardiac graft tissue was analyzed for the presence of TNFα mRNA on days 1-10 post-tx by Northern blot analysis (Figure 10). Expression of TNFα in cardiac allografts appeared to be biphasic (Figure 10A). Peak levels of TNFα mRNA appeared on day 3 post-tx, and although levels decreased by day 4 post-tx, an increase in TNFα mRNA appeared again on day 6 or 7 post-tx, coinciding with graft rejection as measured by ECG activity (Figure 9). Detectable levels of TNFα gene expression were absent in RNA samples prepared from cardiac isografts (Figure 10B).

Cardiac transplants were also analyzed for LT mRNA levels on days 1 through 10 post-tx by Northern blot analysis (Figure 11). Similar to TNFα gene expression in cardiac allografts, LT demonstrated a biphasic pattern of expression (Figure 11A). Peak levels of LT mRNA in cardiac allografts were observed on day 3 post-tx, with a second increase in LT transcript levels occurring later, coincident with graft rejection. No detectable levels of LT gene expression were found on days 1 through 10 post-tx in RNA samples prepared from cardiac isografts (Figure 11B).
Fig. 10A. TNFα mRNA levels in cardiac allografts. Total RNA isolated from cardiac allografts retrieved on days 1-10 post-tx were examined by Northern blot analysis for TNFα gene expression. Representative Northern blot is shown above. As with Fig. 10B, the murine monocyte cell line, P388D1, stimulated in vitro with LPS (10 µg/ml) for 4 hr was used as a positive TNFα mRNA control.
Fig. 10B. TNFα mRNA levels in cardiac isografts. Total RNA isolated from cardiac isografts retrieved on days 1-10 post-tx were examined by Northern blot analysis for TNFα gene expression. Representative Northern blot is shown above. The rat NK cell line, RNK-16, stimulated in vitro with a PMA (20 ng/ml)/IL-2 (1000 U/ml) combination served as a negative control for TNFα mRNA expression. As with Fig. 10A, blots were prepared using 15 μg of total RNA per lane; autoradiogram exposure time was 18 hr. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits is included to illustrate standardized lane loading and intact, nondegraded cellular RNA. Kb, kilobases.
Fig. 11A. LT mRNA levels in cardiac allografts. Total RNA isolated from cardiac allografts retrieved on days 1-10 post-tx were examined by Northern blot analysis for LT gene expression. Representative Northern blot is shown above.
Fig. 11B. LT mRNA levels in cardiac isografts. Total RNA isolated from cardiac isografts retrieved on days 1-10 post-tx were examined by Northern blot analysis for LT gene expression. Representative Northern blot is shown above. SMNCs isolated from adult male Lewis rats and stimulated in vitro with a combination of PMA (20 ng/ml) and PHA (2 µg/ml) for 18 hr were used as a positive control, and untreated SMNCs were used as a negative control for LT mRNA. As with Fig. 11A, blots were prepared using 15 µg of total RNA per lane; autoradiogram exposure time was 5 days. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits is included to illustrate standardized lane loading and intact, nondegraded cellular RNA. Kb, kilobases.
In order to determine whether TNF protein levels paralleled the observed changes in mRNA, an ELISA method was used to quantitate TNF protein in transplanted cardiac tissues. TNF protein levels in cardiac allografts were significantly increased over cardiac isografts as early as day 2 post-tx (Figure 12). TNF protein demonstrated a biphasic pattern, similar to that shown for TNFα and LT mRNA in cardiac allograft tissues on day 3 post-tx, with 4-fold greater levels in allografts (approximately 400 pg/ml or 16.5 U/ml; 1 U = approx. 24.3 pg) compared to isografts, and a second increase in TNF appeared in allografts on day 6 post-tx. In contrast, TNF protein levels in cardiac isografts were at or below standard limits of detection (50 pg/ml) at all other times post-tx, and were significantly decreased on days 3 through 7 when compared with time-matched cardiac allografts ($p \leq 0.001$).
Fig. 12. TNF protein levels in cardiac transplants. Cardiac isograft and allograft tissues were retrieved on days 1-10 post-tx and analyzed for TNF protein, expressed as pg/ml and U/ml (1 U = approx. 24.3 pg) ± SEM. Data shown are mean values of four experiments performed with different allograft and isograft tissue samples. TNF protein levels in cardiac allografts were significantly increased when compared to cardiac isografts ($p \leq 0.001$).
Discussion

In the present study using a heterotopic method of cardiac transplantation into the rat ear, we have demonstrated that cardiac allografts, but not isografts, undergo acute rejection by day 10 post-tx. Serial recordings of ECG activity indicate that the average day to rejection of cardiac allografts is Day 7 post-tx, at which time the average allograft heart rate has significantly decreased by two-fold, when compared to the average heart rate of day-matched isografts. These findings are consistent with earlier reports assessing rat cardiac allograft rejection by cessation of palpable pulsatile activity (Tilney et al., 1978; Hancock et al., 1991; Scheringa et al., 1991). Furthermore, average allograft heart rates are found to continually decrease, such that by day 10 post-tx ECG activity is undetectable. In contrast to allograft activity, average isograft heart rates demonstrate regular ECG and pulsatile activity up to 1 year post-tx. Although palpable cardiac contractility may determine day of rejection, monitoring ECG activity allows for qualitative and quantitative analysis of relative graft viability.

Rat cardiac allografts, but not isografts, indicate significant increases in TNFα and LT mRNA and protein levels between days 1 through 10 post-tx. We have found that both TNFα and LT gene expression reach peak levels in situ early after allografting, usually on day 3 post-tx. Levels of these cytotoxic cytokines decrease subsequently and rise again later post-tx, coincident with graft rejection. The relative contributions of TNFα and LT proteins could not be assessed since the TNFα antibody used in the ELISA procedure can not distinguish between these two cytokine proteins (Sheehan et al., 1989). Since the TNFα antibody was elicited using recombinant mouse TNFα (Sheenan et al.,
1989), and results were obtained using a recombinant mouse TNFα standard, values of TNF protein in rat cardiac transplants reported in these studies may therefore be underestimated.

Interestingly, the second increase in both TNFα and LT expression is consistently of lesser magnitude when compared with the first peak of expression, but it appears to be allograft-specific. The significance of the early rise in these cytotoxic cytokines post-tx is unknown. However, since this elevation in TNF gene expression is noted in both allograft and isograft tissues, we speculate that at least part of the first rise may be attributed to postsurgical inflammatory (i.e., nonallospecific) events. Furthermore, the significantly greater early TNF response noted in allografts may reflect allospecific events, such as endothelial cell activation (Collins et al., 1986) and increased migration and adhesion of leukocytes (Shalaby et al., 1985).

These results demonstrating the presence of TNF in rat cardiac allografts are in accordance with recent observations reporting marked elevations in circulating TNFα in patients undergoing allograft rejection (Maury & Teppo, 1987; Chollet-Martin et al., 1990), as well as in situ in allografted tissues (Arbustini et al., 1991; Krams 1989). Likewise, increased circulating levels of LT have also been detected in patients (McKenna et al., 1988; Dallman et al., 1991) and animal models of allograft rejection (Lowry et al., 1985b). However, our attempts to measure circulating levels of TNF in the transplant model used in the present study have consistently indicated levels at or below the limits of detection (50 pg/ml), even in allograft recipients. Results obtained from the
present study, however, indicate that both TNFα and LT levels are significantly elevated post-tx in allograft tissues relative to isograft controls.

Although the present study and others report elevated levels of TNFα and LT during allograft rejection, the actual roles of these two cytotoxic cytokines in rejection are less well defined. They may participate in the rejection process by indirect activation of leukocyte subpopulations or by direct lytic mechanisms. One possible source of TNF may be activated mononuclear phagocytes in the heart graft, since it has been reported that TNFα is released by cells of the monocyte/macrophage lineage in infiltrating rejecting rat cardiac allografts (Lowry & Blais, 1988). Another contributing source, although possibly not the primary source, may be activated peripheral T lymphocytes, which have recently been shown to synthesize TNFα (Steffen et al., 1988).

At least one report indicates that elevated TNF levels in serum coincides with clinical rejection (Maury & Teppo, 1987). The present studies, however, indicate significant elevations in graft TNF levels well in advance of transplant rejection. It has been reported that rat cardiac allografts elicit a biphasic cellular immune response, with the second phase coinciding with the time of acute graft rejection (Tilney et al., 1978); these findings are consistent with the increases in TNF levels observed in the present study.

The present study supports a role for both TNFα and LT cytotoxic gene products in the pathogenesis of allograft rejection and indicate that elevated levels of TNFα/LT may act as early indicators of incipient graft rejection. Studies
such as these are designed to elucidate the pathophysiological mechanisms involved in graft rejection and may lead to more effective strategies aimed at promoting graft success.
CHAPTER 3
USE OF LOW DOSE CYCLOSPORINE A/METHOTREXATE TO PROLONG RAT CARDIAC ALLOGRAFT SURVIVAL

Abstract

CSA is the standard immunosuppressive treatment used in human cardiac transplantation to prevent rejection; however, adverse side effects have been reported at therapeutic doses. Therefore, a need remains for the implementation of specific therapies designed to achieve transplant success with a minimum of undesirable side effects. The aims of the present study were: 1) to evaluate the efficacy of a low dose CSA (1.0 mg/kg/day) / MTX (450 µg/kg/wk) combination therapy in prolonging rat cardiac allograft survival, and 2) to determine the effects of low dose CSA/MTX on IL-2 gene expression in rat cardiac allografts. The average time to rejection of newborn donor BN rat hearts transplanted into the ear pinnae of CSA/MTX-treated adult Lewis recipients, measured by the absence of ECG activity, more than doubled from day 8 post-tx to day 18 post-tx when compared to allografts in untreated control recipients (p < 0.01). Northern blot analysis demonstrated that IL-2 mRNA transcripts in cardiac allografts treated with low dose CSA/MTX were detected as early as day 1 post-tx, and at increasing levels as rejection progressed post-tx. When IL-2 gene expression in allografts from CSA/MTX treated recipients was compared to levels in allografts from untreated recipients, no significant difference in the pattern of IL-2 induction was observed. In contrast, IL-2 mRNA transcripts were
not detected post-tx in allografts from recipients treated with high-dose (15 mg/kg/day) CSA or in cardiac isografts. The presence of IL-2 gene transcripts, therefore, appears to be allograft-specific. Furthermore, low dose CSA/MTX therapy does not appear to prolong transplant survival by altering steady-state levels of IL-2 gene transcripts. We conclude that low dose CSA/MTX treatment may serve as a possible alternative immunosuppressive therapy for promoting allograft survival while minimizing high dose CSA side effects. Further studies are required to elucidate the mechanism(s) by which low dose CSA/MTX combination therapy increases the time to rat cardiac allograft rejection.

**Introduction**

Using a heterotopic method of cardiac transplantation into the rat ear (Fulmer *et al.*, 1963), we have previously demonstrated that cardiac allografts but not isografts undergo acute rejection by day 10 post-tx, with the average time to rejection being day 7 post-tx (Pizarro *et al.*, 1993a). In this model, we have found that significant increases in TNFα and LT mRNA and protein levels occur between days 1 through 10 post-tx in rat cardiac allografts but not isografts (Pizarro *et al.*, 1993a). These studies support a role for both TNFα and LT cytotoxic gene products in the pathogenesis of allograft rejection. The objective of the present study is to determine if a combination low CSA/MTX immunosuppressive therapy administered to allograft recipients has the ability to prolong survival of rat cardiac transplants compared to untreated controls, using the aforementioned model.
CSA is a cyclic endodecapeptide derived from the soil fungus, *Tolypocladium inflatums*, and has been demonstrated to be a potent immunosuppressive agent and inhibitor of helper T function (Borel et al., 1976; Orosz et al., 1983). CSA was first introduced in human transplantation over a decade ago (Calne et al., 1978), and since that time has become the immunosuppressive agent of choice following organ replacement. Although CSA has been used with considerable success clinically to prolong organ graft survival (Calne et al., 1978; Costanzo-Nordin et al., 1990), significant adverse side effects, such as nephrotoxicity (Myers et al., 1984), hepatotoxicity (Vine et al., 1988), hypertension (Munoz et al., 1988), as well as neurological toxicities (deGroen et al., 1987) have been reported with its use.

Since the landmark findings which first described CSA's ability to suppress cell-mediated immunity (Borel et al., 1976), numerous investigations have examined the mechanism(s) of action of CSA and have consistently demonstrated the inhibition of IL-2 gene transcription by CSA (Kronke et al., 1984; Elliot et al., 1984). The main focus in attempting to elucidate CSA's immunosuppressive action has centered on the direct effects of CSA upon CD4+ helper T lymphocytes; one functional characteristic of CD4+ helper T cells is the synthesis and subsequent release of a range of several cytokines, including IL-2.

IL-2 has been demonstrated to induce the differentiation and clonal expansion of mature T lymphocytes and appears to be an important mediator of allograft rejection, since a positive correlation between IL-2 levels and rejection episodes has been described (Yoshimura & Kahan, 1985; Vie et al., 1985;
McKenna et al., 1988). In addition, anti-IL-2 receptor antibodies have been shown to prevent allograft rejection in experimental models (Kirkman et al., 1985). CSA is thought to act as an immunosuppressive agent through potent inhibition of IL-2 production by T cells (Kronke et al., 1984; Elliot et al., 1984).

Since CSA at therapeutic doses may exert profound physiologic changes in addition to its immunosuppressive properties, these changes often result in toxic side effects (Myers et al., 1984; Vine et al., 1988; Munoz et al., 1988; deGroen et al., 1987). Therefore, a need remains for implementing specific therapies directed at achieving transplant success with a minimum of undesirable sequelae. One approach is the utilization of combination treatments at lower therapeutic doses, which may result in increased survival time of organ transplants, while minimizing adverse side effects.

MTX is classified pharmacologically as an antimetabolite because of its antagonistic effect on folic acid metabolism and function (Mitchell et al., 1969). MTX binds to dihydrofolate reductase which results in the inhibition of DNA synthesis; this antiproliferative action has made MTX a useful drug for cancer chemotherapy (Jolivet et al., 1983). Immunosuppressive effects have also been attributed to MTX (Mitchell et al., 1969), and its utilization in the treatment of rheumatoid arthritis, for example, has demonstrated both long term and short term efficacy (Weinblatt et al., 1985; Perhala & Wilke, 1991). The mechanism(s), however, by which MTX suppresses disease activity in inflammatory rheumatoid arthritis is still poorly understood and adverse side effects, such as severe leukopenia and gastrointestinal upset, have been associated with the use of MTX.
(Weinblatt et al., 1985). It has been suggested that MTX may exert its immunosuppressive action by deactivation of macrophages, resulting in decreased IL-1 production (Hu et al., 1988), as well as by inhibition of macrophage migration, resulting in decreased accumulation of inflammatory cells and/or immune mediators (Johnson et al., 1988). Conversely, the administration of MTX restored both T cell function (Kourounakis & Kapusta, 1976) and IL-2 production (Combe et al., 1985) in rheumatoid arthritis.

Recently, MTX has also shown favorable results in treating persistent mild (Olsen et al., 1990), as well as recurrent moderate to severe cardiac allograft rejection (Costanzo-Nordin et al., 1988). Success has also been demonstrated using CSA at doses of 10-15 mg/kg/day combined with either high or low dose MTX in the prevention of graft-versus-host disease after bone marrow transplantation in experimental animal models (Deeg et al., 1982; Deeg et al., 1984), as well as in human clinical studies (Storb et al., 1986; Truog & Wozniak, 1990). These data strongly suggest that administration of a low dose CSA/MTX combination therapy may serve as a possible alternative treatment in promoting cardiac allograft survival while minimizing both high dose CSA and MTX side effects.

The purpose of the present study was to evaluate the efficacy of low dose CSA (1.0 mg/kg/day) / MTX (450 µg/kg/wk) combination therapy in prolonging rat cardiac allograft survival and to determine the effects of low dose CSA/MTX on IL-2 gene expression in rat cardiac allografts. Studies such as these may provide a basis for the implementation of combination
immunosuppressive therapies leading to improvements in post-transplantation management.

**Materials, Methods and Experimental Design**

**Animals**

Pregnant BN and Lewis, and six to eight-week-old male Lewis rats were purchased from Harlan Sprague Dawley (Harlan Sprague Dawley, Indianapolis, IN). All animals received water and food *ad libitum*, and were housed in accordance with institutional animal care and use guidelines. Adult male Lewis rats served as recipients of donor cardiac tissue obtained from either one to three-day-old Lewis or BN rats, which served as isograft and allograft groups, respectively. The donor ages used have been previously reported to result in optimum isograft viability (Fulmer *et al.*, 1963) and were of appropriate size so that the whole heart grafts might be accommodated by the recipients' ears.

**Cardiac Transplantation**

The procedure utilized was a modification of the heterotopic method of cardiac transplantation into the mouse ear as described by Fulmer *et al.* (1963) (Figure 6). Cardiac graft recipients were anesthetized with i.p. injection of ketamine/xylazine (20:1) (Fort Dodge Laboratories, Fort Dodge, IA and Rugby Laboratories, Inc., Rockville Centre, NY) at 105 mg/kg body weight prior to surgery. The dorsal surface of the recipient's ear pinna was nicked at the proximal end with a sterile scalpel and a subcutaneous pouch was prepared using blunt forceps. At the time of donor sacrifice, whole newborn hearts were harvested promptly and gently inserted into the ear pouches of the recipients. In
order to prevent graft loss and promote revascularization, skin flaps surrounding the graft site were apposed by applying gentle pressure with forceps. Each recipient was grafted bilaterally, one whole heart per recipient ear, with either two isografts or allografts per animal. Cardiac graft recipients were anesthetized with ketamine and monitored daily by visual inspection for pulsatile activity and by electrocardiography using pin electrodes placed on either side of the graft with leads connected to a polygraph recorder (Grass Model 79D, Grass Instruments Co., Quincy, MA) (Figure 7).

Animal Treatment Groups

Lewis cardiac allograft recipients were injected i.p. with CSA in olive oil vehicle once daily, beginning on the same day of transplantation (day 0), and/or MTX in saline vehicle once weekly, beginning one week prior to the day of transplantation, as follows. Allograft recipients were placed in one of five treatment groups consisting of either high dose CSA (15 mg/kg/day), low dose CSA (1 mg/kg/day), low dose MTX (450 µg/kg/wk), combination low dose CSA/MTX treatment (1 mg/kg/day and 450 µg/kg/wk, respectively), or control treatment group (saline and olive oil vehicles according to the aforementioned protocol). All allograft recipients received treatment through day 7 post-tx. Cardiac transplants were monitored daily for pulsatile and ECG activity as described above for graft survival, with retrieval of one graft per recipient occurring on days 3 through 8 post-tx for each treatment group; cardiac grafts were processed for total cellular RNA as described below. Subsequent to these studies, a second treatment group consisting of low dose CSA/MTX combination therapy was prepared to permit graft retrieval at eight time points, days 1 through 8 post-tx, for RNA analysis.
Cell Culture

SMNCs were isolated from adult male Lewis rats by centrifugation of spleen tissue suspensions through a Ficoll density gradient (Lymphocyte Separation Medium (LSM), Organon Teknika, Durham, NC), as described in an earlier report (Pizarro et al., 1993a). SMNCs were cultured in RPMI-1640 medium supplemented with 2% penicillin-streptomycin, 5% L-glutamine and 5% fetal bovine serum (GIBCO, Grand Island, NY) at a cell density of 1 X 10^6 cells/ml at 37°C in 5% CO2 in air, either in the presence or absence of a 20 ng/ml PMA and 2 µg/ml PHA combination for 18 hr and used as positive and negative controls for IL-2 mRNA, respectively. After incubation with or without mitogen stimulation, cells were collected and processed for RNA as described below.

RNA Blot Analysis

For Northern and slot blot analysis, total cellular RNA was prepared from cells and tissues by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987), and blots were prepared as described previously (Pizarro et al., 1993a). Filters were hybridized with an IL-2 cDNA probe prepared using a 900 b.p. murine IL-2 insert cloned into the BamHI site of pCD vector, (a gift from Dr. Ken-iichi Arai, DNAX Research Inst., Palo Alto, CA) (Kashima et al., 1985) and prepared for autoradiography as previously described (Pizarro et al., 1993a).

Statistical Analysis

Experimental groups were compared using analysis of variance and multiple regression analysis for the existence of significant differences between
treatment and control groups. Differences were considered to be statistically significant at $p \leq 0.05$.

**Results**

In order to determine cardiac graft viability, tissues transplanted in allograft recipients from the four experimental treatment groups (N=6) and control group (N=7) were monitored daily for ECG and pulsatile activity (Figure 7). Allograft rejection was defined by the absence of detectable ECG activity. In untreated allograft recipients, absence of ECG activity was noted on either day 6 or 7 post-tx in five of the seven cardiac allografts. ECG activity was undetectable in the two remaining grafts by day 10 post-tx; the average time to allograft rejection in the control group was determined to be day $7.4 \pm 0.68$ post-tx (Table 2).

<table>
<thead>
<tr>
<th>TABLE 2. Cardiac allograft survival time post-transplantation</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>RECIPENT #</th>
<th>RANGE</th>
<th>MEAN ± S.E.M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH CSA$^1$</td>
<td>18* 12* 36</td>
<td>21* 36</td>
<td>32.3 ± 3.75</td>
</tr>
<tr>
<td>CSA/MTX$^2$</td>
<td>18 17 18 18 18 18 18</td>
<td>17-18</td>
<td>17.8 ± 0.16</td>
</tr>
<tr>
<td>LOW CSA$^3$</td>
<td>14 14 12 14 14 14 12</td>
<td>12-14</td>
<td>13.3 ± 0.42</td>
</tr>
<tr>
<td>MTX$^4$</td>
<td>10 10 10 14 8 8 8</td>
<td>8-14</td>
<td>10.0 ± 0.89</td>
</tr>
<tr>
<td>CONTROL$^5$</td>
<td>6 6 10 7 6 10 7</td>
<td>6-10</td>
<td>7.4 ± 0.68</td>
</tr>
</tbody>
</table>

$^*$ Expired due to anesthesia complications.

$^1-4 N = 6$

$^5 N = 7$
High dose CSA-treated recipients demonstrated the longest survival time of cardiac allografts when compared to control and other treatment groups (day 32.3 ± 3.75). Figure 13 summarizes these data in graph form by depicting the proportion of surviving cardiac allografts in control and experimental treatment groups, and comparing transplant survival time (day post-tx) among the different groups. The incidence of death due to failure to recover from anesthesia administered for ECG measurements, however, was increased in the high dose CSA-treated group (three of the six recipients expired while under anesthesia during graft monitoring on days 12, 18, and 21 post-tx). Cardiac allograft tissue transplanted in combination low dose CSA/MTX-treated recipients demonstrated significantly increased survival time post-tx (average time to rejection was determined to be day 17.8 ± 0.16), when compared to untreated controls (p ≤ 0.01), without apparent complications due to anesthesia during graft monitoring (Table 2, Figure 13). In fact, all but one (rejection by day 17 post-tx) of the combination low dose CSA/MTX-treated recipients demonstrated rejection of cardiac allografts at day 18 post-tx, a two and one-half-fold increase in allograft survival time over untreated controls.

Furthermore, cardiac transplants in low dose CSA alone-treated recipients showed a significant increase in allograft survival time post-tx when compared to control (average time to rejection was 13.3 ± 0.42 days); however, this increase was found to be comparatively less than that found in heart allografts transplanted into low dose CSA/MTX-treated recipients (p ≤ 0.05) (Table 2, Figure 13). Cardiac allografts in MTX alone-treated recipients did not show a
significant difference in graft survival time post-tx (average time to rejection was 10.0 ± 0.89 days) compared to control (Table 2, Figure 13).

![Graph showing survival curves for cardiac allograft control and experimental treatment groups. All control (N=7) and treatment (N=6) groups were assessed for graft viability by serial ECG recordings post-tx. Allograft rejection was defined by the absence of detectable ECG activity. Results are reported as the proportion of surviving cardiac allografts in control and experimental treatment groups with time post-tx. Cardiac allograft survival was significantly prolonged in recipients treated with high dose CSA (p < 0.001), low dose CSA/MTX (p < 0.01), and low dose CSA (p < 0.05), when compared to untreated allograft recipients.]

Fig. 13. Survival curves for cardiac allograft control and experimental treatment groups. All control (N=7) and treatment (N=6) groups were assessed for graft viability by serial ECG recordings post-tx. Allograft rejection was defined by the absence of detectable ECG activity. Results are reported as the proportion of surviving cardiac allografts in control and experimental treatment groups with time post-tx. Cardiac allograft survival was significantly prolonged in recipients treated with high dose CSA (p < 0.001), low dose CSA/MTX (p < 0.01), and low dose CSA (p < 0.05), when compared to untreated allograft recipients.

In addition to daily monitoring of allograft viability, the intrinsic heart rates of control and treated recipients were also measured (Figure 7). As shown in Table 3, the intrinsic heart rate of all cardiac allograft recipients was significantly greater than graft heart rates at all times monitored, regardless of treatment of the recipient. The intrinsic heart rate of the recipient was found to
average approximately 425 bpm, with no significant differences among treatment groups (Table 3).

In comparison to intrinsic heart rates, a decrease in the average heart rate of cardiac allografts was observed with time post-tx, which was dependent on the treatment given to the recipient. All cardiac allografts demonstrated similar heart rates up to day 3 post-tx, after which gradually diminishing as well as irregular ECG activity was demonstrable, indicating incipient graft rejection (Table 3). Allografts from untreated recipients showed more than a two-fold decrease in heart rate from day 3 to day 6 post-tx, from $107.14 \pm 11.5$ to $41.82 \pm 8.9$ bpm (Table 3). As mentioned previously, absence of detectable ECG activity was noted on either day 6 or 7 post-tx in five of the seven untreated control cardiac allografts, with the two remaining grafts losing ECG activity by day 10 post-tx (Table 2).

Heart rates measured in cardiac allografts transplanted into low dose CSA/MTX-treated recipients on days 3, 6, 8, and 10 post-tx were found to have distinct and regular heart rates of $82.72 \pm 8.2$, $130.00 \pm 20.7$, $133.33 \pm 15.6$, and $122.33 \pm 11.6$, respectively (Table 3). However, by day 14, a decrease in allograft heart rate ($62.00 \pm 19.3$, Table 3) was noted in low dose CSA/MTX-treated recipients. A further decrease was reported with time, such that in all but one case, ECG and pulsatile activity were virtually undetectable by day 18 post-tx (Table 2). This finding, however, represented more than a two-fold increase in cardiac allograft survival in low dose CSA/MTX-treated recipients when compared to untreated controls ($p \leq 0.01$).
Graft heart rate in cardiac allografts transplanted into low dose CSA-treated recipients decreased by day 10 post-tx (data not shown), with no detectable ECG activity by day 14 post-tx (Table 3). Although graft survival time was shorter than that found in low dose CSA/MTX-treatment, administration of low dose CSA alone also showed prolongation of graft survival time by almost one week post-tx, when compared to untreated controls ($p \leq 0.05$). Conversely, in MTX alone-treated animals, graft heart rates showed diminished ECG and pulsatile activity by day 6 post-tx (47.5 ± 11.1), which continued until day 10 or 11 post-tx, at which time no detectable ECG activity was measured (Table 3). No statistically significant difference in allograft survival time was demonstrated in cardiac allografts in MTX alone-treated recipients, as compared with untreated controls.

Cardiac allografts in high dose CSA-treated recipients showed regular but gradually diminishing heart rates through day 29 post-tx, at which time the remaining three allografts demonstrated markedly diminished heart rates (Table 3, Figure 13); graft failure was noted by day 36, when no detectable ECG and pulsatile activity were recorded (Table 3).
### TABLE 3. Cardiac heart rate post-transplant

<table>
<thead>
<tr>
<th>DAY POST-TX</th>
<th>DAY 3</th>
<th>DAY 6</th>
<th>DAY 8</th>
<th>DAY 14</th>
<th>DAY 18</th>
<th>DAY 36</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TREATMENT</strong></td>
<td><strong>GRAFT RATE</strong></td>
<td><strong>INTRINSIC RATE</strong></td>
<td><strong>GRAFT RATE</strong></td>
<td><strong>INTRINSIC RATE</strong></td>
<td><strong>GRAFT RATE</strong></td>
<td><strong>INTRINSIC RATE</strong></td>
</tr>
<tr>
<td>HIGH CSA (N = 6)$^2$</td>
<td>85.00 ± 9.6</td>
<td>400.00 ± 11.5</td>
<td>130.00 ± 18.3</td>
<td>406.67 ± 29.6</td>
<td>155.00 ± 12.6</td>
<td>430.00 ± 25.2</td>
</tr>
<tr>
<td>CSA/MTX (N = 6)</td>
<td>82.72 ± 8.2</td>
<td>457.70 ± 21.7</td>
<td>130.00 ± 20.7</td>
<td>412.50 ± 12.5</td>
<td>133.33 ± 15.6</td>
<td>490.00 ± 29.2</td>
</tr>
<tr>
<td>LOW CSA (N = 6)</td>
<td>90.00 ± 12.9</td>
<td>433.00 ± 24.0</td>
<td>125.00 ± 18.5</td>
<td>376.66 ± 43.3</td>
<td>122.50 ± 6.3</td>
<td>426.66 ± 17.6</td>
</tr>
<tr>
<td>MTX (N = 6)</td>
<td>67.50 ± 14.9</td>
<td>466.67 ± 24.0</td>
<td>47.50 ± 11.1</td>
<td>426.67 ± 17.6</td>
<td>24.28 ± 9.0</td>
<td>483.33 ± 37.6</td>
</tr>
<tr>
<td>CONTROL (N = 7)</td>
<td>107.14 ± 11.5</td>
<td>400.00 ± 28.9</td>
<td>41.82 ± 8.9</td>
<td>420.00 ± 41.6</td>
<td>*</td>
<td>446.67</td>
</tr>
</tbody>
</table>

* No detectable ECG activity
1 Heart rate measured in bpm
2 For all treatment groups, N = number at Day 3 post-tx
Representative ECG recordings from cardiac allograft tissue from control and experimental treatment groups at nine different time points (days 3, 6, 8, 10, 12, 14, 18, 29, and 36) post-tx are shown in Figure 14. Diminished electrical activity with time post-tx was observed for transplanted cardiac allografts by day 8 in untreated control recipients, day 10 in MTX alone-treated recipients, day 14 in low dose CSA alone-treated recipients, day 18 in combination low dose CSA/MTX-treated recipients, and day 36 in high dose CSA-treated recipients. Diminished ECG and pulsatile activity, as well as a decrease in the amplitude and regularity of heart rates, occurred a few days prior to actual rejection indicating impending graft failure.
Fig. 14. Comparison of cardiac allograft viability in treated and untreated recipients. Representative ECG recordings from nine different time points (day 3, 6, 8, 10, 12, 14, 18, 29, and 36) post-tx are shown (A on this page, B, and C on the following pages). All recordings were performed at a constant chart speed of 5.0 mm/sec; bpm, beats per minute.
Fig. 14B.
Fig. 14C.
IL-2 mRNA levels were examined in cardiac allografts from untreated control recipients and combination low dose CSA/MTX-treated recipients at various times post-tx. IL-2 mRNA levels were also examined in cardiac isografts transplanted into untreated recipients. Northern blot analysis of IL-2 gene expression in untreated cardiac allografts appeared to increase with time post-tx (Figure 15A). Although detectable transcripts were observed as early as day 1 or day 2 post-tx, a significant increase in IL-2 mRNA levels appeared on day 3 post-tx, as well as on days 7 and 8 post-tx, at which time graft rejection usually occurs (Table 2, Figure 15A). IL-2 mRNA levels were also examined on days 1 through 8 post-tx in cardiac allografts transplanted into combination low dose CSA/MTX-treated recipients (Figure 15B). Similar to IL-2 gene expression in cardiac allografts from untreated control recipients, IL-2 transcripts in low dose CSA/MTX-treated cardiac allografts were detected at early times post-tx, with increased IL-2 mRNA levels by day 3 post-tx. These levels of IL-2 mRNA remained high, with the exception of day 6, through day 8 post-tx, coincident with graft rejection in untreated allograft recipients. In contrast to untreated and low dose CSA/MTX-treated allografts, Northern blot analysis of cardiac isografts did not demonstrate detectable levels of IL-2 mRNA on days 1 through 8 post-tx (Figure 15C). Similarly, IL-2 gene transcripts were not detected in rat cardiac allografts transplanted into high dose CSA-treated recipients (Figure 15D). However, no apparent differences, including a decrease in IL-2 transcript levels on day 6 post-tx, were seen at the steady state level of IL-2 mRNA in rat cardiac allograft tissues transplanted into untreated and low dose CSA/MTX-treated recipients.
Fig. 15A. IL-2 mRNA levels in cardiac allografts transplanted into untreated control recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for IL-2 gene expression. Representative Northern blot is shown above. As with Fig. 14B, 14C, and 14D, blots were prepared using 15 µg of total RNA per lane; autogradiogram exposure time was 18 hr. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits is included to illustrate standardized lane loading and intact, non degraded cellular RNA. Kb, kilobases.
Fig. 15B. IL-2 mRNA levels in cardiac allografts transplanted into low dose CSA/MTX-treated recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for IL-2 gene expression. Representative Northern blot is shown above. SMNCs, spleen mononuclear cells.
Fig. 15C. IL-2 mRNA levels in cardiac isografts transplanted into untreated control recipients. Total RNA isolated from cardiac isografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for IL-2 gene expression. Representative Northern blot is shown above.
Fig. 15D. IL-2 mRNA levels in cardiac allografts transplanted into high dose CSA-treated recipients. Total RNA isolated from cardiac allografts retrieved on days 3-8 and 36 post-tx were examined by Northern blot analysis for IL-2 gene expression. Representative Northern blot is shown above. Blot was subsequently stripped and reprobed for GAPDH housekeeping gene expression 1.3 Kb, (middle panel).
Discussion

Both beneficial as well as adverse effects of CSA have been well documented since its initial use as a potent immunosuppressive agent in clinical transplantation (Calne et al., 1978). Adverse side effects, in particular nephrotoxicity (Myers et al., 1984) and hepatotoxicity (Vine et al., 1988), have restricted its use at therapeutic doses (between 10-15 mg/kg/day) and have initiated attempts into exploring combination low-dose therapies. In fact, concurrent administration of CSA with azathioprine, corticosteroids, polyclonal antilymphocyte globulins, and monoclonal antibodies, such as OKT3, are combination immunosuppressive regimens currently used in patients to promote cardiac allograft survival (McGoon & Frantz, 1992). However, along with CSA, the aforementioned immunosuppressive agents when administered at therapeutic doses, also possess side effects with potentially undesirable consequences (McGoon & Frantz, 1992).

Immunosuppressive properties have been attributed to the chemotherapeutic drug, MTX (Mitchell et al., 1969; Weinblatt et al., 1985; Perhala & Wilke, 1991). High dose MTX has long been used for treatment of neoplastic diseases and works by indirectly inhibiting DNA synthesis (Jolivet et al., 1983). The primary clinical concern with the administration of therapeutic doses of MTX (conventionally 10 mg/every other day), is bone marrow suppression, which may result in leukopenia as well as initiate the impending risk of infection (Weinblatt et al., 1985). Low dose MTX has been also used in the treatment of rheumatoid arthritis and its efficacy appears to depend on immunosuppressive activities (Weinblatt et al., 1985; Perhala & Wilke, 1991). Recently, low dose MTX
has also been used in patients to control persistent mild, as well as recalcitrant cardiac allograft rejection (Costanzo-Nordin et al., 1988), and in canine recipients to prevent rejection of pulmonary allografts (Blumenstock et al., 1988). Therefore, low dose MTX treatment (450 µg/kg/wk in the present study), maintains immunosuppressive efficacy and has a markedly lower incidence of toxicity.

The present study, using a heterotopic method of cardiac transplantation into the rat ear, has indicated that low dose CSA/MTX treatment, where average day to rejection is day 18 post-tx, significantly prolonged cardiac allograft survival over day-matched allografts transplanted into untreated control recipients, where average day to rejection is day 7 post-tx. Treatment of cardiac allograft recipients with combination low dose CSA/MTX immunosuppressive therapy, therefore, more than doubled allograft survival time post-tx when compared to untreated controls. No apparent complications due to anesthesia administration at the time of cardiac monitoring were noted in this treatment group, in contrast to high dose CSA-treated recipients. Contrary to cardiac allograft viability, average isograft heart rates were previously shown to possess regular ECG and pulsatile activity up to one year post-tx (Pizarro et al., 1993a).

The present study also showed that combination low dose CSA/MTX treatment significantly increased graft survival compared to low dose CSA alone as well as MTX alone-treatments. Cardiac allografts transplanted into MTX alone-treated recipients did not show significant differences in graft survival time post-tx when compared with allografts from untreated recipients. Although cardiac allografts transplanted into low dose CSA-treated recipients
demonstrated an increase in transplant survival time over untreated controls ($p \leq 0.05$), this increase was found to be less than that found in combination low dose CSA/MTX-treated recipients ($p \leq 0.01$).

Low dose CSA treatment (2.0 mg/kg/day) of allograft recipients reduces T cell proliferative responsiveness, but is unable to sustain long-term cardiac allograft survival (Stepkowski et al., 1989). Studies such as these suggest that low dose CSA administration has the ability to prolong cardiac graft survival post-tx, by activation of T cell suppressor function(s) and/or by altering the T cell's ability to proliferate. The present studies indicate that low dose CSA treatment, while able to delay allograft rejection, does not have the efficacy of combination low dose CSA/MTX in prolonging rat cardiac allograft survival. The possibility exists that MTX may provide additional immunosuppressive capabilities by affecting T cell function and/or regulating other cells participating in the immune response.

Although the mechanism(s) by which MTX appears to exert immunosuppression is still poorly understood, it is believed that MTX may possess primarily anti-inflammatory activity due to rapidly occurring palliative effects (Weinblatt et al., 1985). Indeed, animal studies support an anti-inflammatory mechanism of action for MTX. In a murine model of adjuvant-induced arthritis, MTX inhibited hindpaw edema, the activation of macrophages by PGE$_2$, the influx of MHC class II and macrophages into synovial tissue, and IL-1 production (Hu et al., 1988). The findings that low dose MTX prevents occurrence of arthritis induced by Freund's adjuvant in the rat, but fails to inhibit
cyclo-oxygenase or 5-lipoxygenase, has been interpreted as evidence for immunomodulation (Stepkowski et al., 1989). Furthermore, production of IL-1 and induction of primary delayed-type hypersensitivity were inhibited in MTX-treated rodents (Hu et al., 1988; Gibbons & Lucas, 1989). Therefore, in the present study, the possibility exists that significant prolongation of cardiac allografts transplanted into combination low dose CSA/MTX-treated recipients may be due, in part, to the combined immunosuppressive actions of low dose CSA primarily at the T cell level and MTX primarily at the macrophage effector cell level.

In the present study, IL-2 mRNA levels in cardiac tissue was examined to determine whether combination low dose CSA/MTX treatment, like high dose CSA therapy, exerts its immunosuppressive action by altering steady state levels of IL-2 mRNA. IL-2 gene transcripts were not detected in isografts or in rat cardiac allografts transplanted into high dose CSA-treated recipients on any day post-tx, when compared to allografts from untreated recipients. These findings support previous reports that treatment with high dose CSA suppresses IL-2 gene transcription (Kronke et al., 1984; Elliot et al., 1984). No apparent differences in IL-2 mRNA transcript levels were observed, however, between rat cardiac allograft tissues transplanted into untreated and low dose CSA/MTX-treated recipients. IL-2 gene expression occurred early during the rejection process, with IL-2 transcripts detected as early as day 1 or day 2 post-tx in heart tissues transplanted in both low dose CSA/MTX-treated and untreated recipients. Peak levels of IL-2 mRNA in cardiac allografts appeared early after transplant, usually by day 3 post-tx, in both treated and untreated recipients. IL-
2 mRNA levels remained elevated through day 8 post-tx, by which time allograft rejection occurs in untreated recipients, as measured by the absence of detectable ECG and pulsatile activity.

The present study reports an increased efficacy of low dose CSA/MTX combination therapy in prolonging rat cardiac allograft survival when compared to low dose CSA alone, low dose MTX alone, as well as untreated control recipients. Unlike high dose CSA therapy, the mechanism(s) by which low dose CSA/MTX prolongs allograft survival does not appear to be mediated by the inhibition of IL-2 gene transcription. The possibility exists that low dose CSA/MTX combination therapy may act on different effector cell populations engaged in the allogeneic immune response. Further studies must address the effects of low dose CSA and low dose MTX treatment alone on T-cell and macrophage cytokine gene expression in rat cardiac allografts. Studies such as these may provide new insight into the mechanisms of action of combination therapies designed to promote transplant survival with minimal adverse side effects.
CHAPTER 4
LOW DOSE CYCLOSPORINE A/METHOTREXATE COMBINATION THERAPY LOWERS CYTOTOXIC GENE EXPRESSION IN RAT CARDIAC TRANSPLANTS

Abstract
We have previously shown that administration of a combination low dose CSA (1.0 mg/kg/day) / MTX (450 µg/kg/wk) treatment significantly increases the survival of rat cardiac allografts, and may therefore potentially serve as an alternative immunosuppressive therapy designed to promote transplant survival while minimizing high dose CSA side effects. In contrast to high dose CSA, low dose CSA/MTX treatment does not appear to alter IL-2 gene expression, since similar patterns of IL-2 gene transcripts were found in both low dose CSA/MTX-treated and untreated control allografts on Days 1 through 8 post-tx. The mechanism(s) by which low dose CSA/MTX therapy increases the time of allograft survival remains to be elucidated. The aim of the present study was to determine the effects of low dose CSA/MTX on the expression of the cytotoxic cytokines, TNFα, LT, and the serine proteases HF and C11 (granzymes A and B, respectively) in rat cardiac allografts during rejection. RNA blot analysis showed significant suppression of TNFα, LT, HF, and C11 gene expression on days 1 through 8 post-tx in cardiac allografts from low dose CSA/MTX-treated recipients compared to untreated allograft controls. TNF protein levels in cardiac allografts from low dose CSA/MTX-treated recipients
were also found to be significantly reduced on days 1 through 8 post-tx when compared to time-matched untreated allograft controls \((p \leq 0.001)\). We conclude that low dose CSA/MTX treatment, while effective in prolonging cardiac transplant survival, appears to act at the mRNA level to downregulate cytotoxic gene expression. Such trials aimed at evaluating low-dose combination therapy may afford new insight into mechanisms underlying improvement in immunosuppressive treatment.

**Introduction**

We have previously shown that low doses of both CSA and MTX, when used in combination, significantly increases the survival of rat cardiac allografts (Pizarro *et al.*, 1993b) and therefore may serve as an alternative immunosuppressive therapy directed at promoting transplant survival. In contrast to high dose CSA administration, combination low dose CSA/MTX treatment does not appear to effect IL-2 gene expression. Similar levels of IL-2 gene transcripts have been found in cardiac allografts from both low dose CSA/MTX-treated and untreated transplant recipients on Days 1 through 8 post-tx (Pizarro *et al.*, 1993b). In addition, significant increases in TNFα and LT mRNA and protein levels have been shown to occur between days 1 through 10 post-tx in rat cardiac allografts transplanted into untreated recipients (Pizarro *et al.*, 1993a), supporting a role for both TNFα and LT in the pathogenesis of allograft rejection. The need remains, therefore, to determine the mechanism(s) by which low dose CSA/MTX combination therapy increases allograft survival time compared to controls.
After transplantation of a tissue allograft, an infiltrate consisting of several cell phenotypes appears within the graft (Hanto et al., 1982). Both helper and cytotoxic T lymphocyte infiltration precede the destruction of the transplanted tissue by several days, and it is believed that these cells are paramount in the process of allograft rejection (Loveland & McKenzie, 1982; Lowry et al., 1983). Macrophages and NK cells have also been found to be components of the cellular infiltrate in organ allografts undergoing acute rejection (Strom et al., 1977; MacPherson & Christmas, 1984; Nemlander et al., 1983). These cells proliferate and release cytokines which have been shown to damage blood vessels within the graft (Dvorak et al., 1980), cause an increase in graft infiltration by leukocytes (Cox et al., 1984), and stimulate the differentiation of graft-specific T cells at the graft site (Heidecke et al., 1984). These local events mediated, at least in part, by cytokines contribute to allograft damage and eventual rejection of the transplanted tissue.

TNFα and LT are two closely related cytokines which are endogenous mediators of immunological and inflammatory events. TNFα and LT share 36% and 51% structural homology in overall nucleotide and amino acid sequences, respectively (Pennica et al., 1984), appear to have common receptor binding domains (Aggarwal et al., 1985a), and also exert similar immunological effects in vitro (Beutler & Cerami, 1986; Beutler, 1990). With regard to transplant rejection, elevated levels of TNFα have been reported during rejection of rat cardiac allografts (Lowry & Blais, 1988) as well as in human renal and liver allografts (Maury & Teppo, 1987; Imagawa et al., 1990a). Additionally, enhanced cytotoxic activity, attributed to LT, has been reported during renal allograft
rejection (Hancock et al., 1991; Moy & Rosenau, 1981). We have recently demonstrated elevated biphasic patterns for both TNFα and LT gene expression post-tx in rat cardiac allografts (Pizarro et al., 1993a). This allospecific increase in TNF levels noted within the first few days post-tx may act as an early indicator of graft rejection. Furthermore, recent studies have reported successful use of anti-TNFα and LT antibodies in prolonging the survival of transplanted grafts in experimental animals, indicating a contribution of TNF in mediating acute rejection (Imagawa et al., 1990b; Scheringa et al., 1991; Teramoto et al., 1991). These data strongly suggest a role for TNFα and/or LT as putative effector molecules in allograft rejection.

Cytotoxic T lymphocytes are believed to be important effector cells in the destruction of transplanted tissue during allograft rejection (Loveland & McKenzie, 1982; Lowry et al., 1983). Cytotoxic T cells recognize alloantigens on the surface of MHC-nonidentical target cells, bind to them, and induce target cell lysis (Hall et al., 1978). Although various steps in this process have been analyzed in considerable detail, most studies have not provided insight into the mechanism(s) by which the killer cell administers the "lethal hit" to the target cell. Recently, two genes encoding the serine esterases HF and Cl1 (Granzymes A and B, respectively), which are selectively expressed in cytotoxic cells including cytotoxic T lymphocytes and NK cells, have been cloned (Gershenfeld & Weissman, 1986; Lobe et al., 1986). The kinetics of HF and Cl1 gene expression closely parallel the cytolytic responses in vitro (Gershenfeld & Weissman, 1986; Lobe et al., 1986), in that the observed kinetics of serine esterase release and of the delivery of the lethal hit are consistent with the hypothesis that both events occur.
simultaneously. There exists, therefore, a strong correlation between granzyme gene expression and cytotoxic potential. Detection of transcripts encoding cytotoxic T cell serine proteases have been recently described in situ during murine cardiac allograft rejection (Mueller et al., 1988). These data would therefore suggest that cytotoxic cell-specific serine proteases, HF and C11, may play a role in the pathogenesis of rat cardiac allograft rejection.

The objectives of this study were to assess the degree of expression of the genes encoding the serine proteases, HF and C11, during rat cardiac allograft rejection and to determine the effects of a combination low dose CSA/MTX therapy on the expression of TNFα and LT, as well as HF and C11, during rejection. We report herein that combination low dose CSA/MTX immunosuppressive treatment of rat cardiac recipients significantly decreases TNFα, LT, HF, and C11 gene expression on days 1 through 8 post-tx in transplanted allografts when compared to allografts retrieved from untreated control recipients. Therefore, low dose CSA/MTX therapy prolongs cardiac transplant survival and appears to act at the transcriptional level to downregulate cytotoxic cytokine gene expression. Studies of cytotoxic cytokine gene expression during allograft rejection may aid in the development of more specifically targeted immunosuppressive therapies designed to prolong graft survival.
Materials and Methods

Animals

Pregnant BN and Lewis, and 6 to 8-week-old male Lewis rats were purchased from Harlan Sprague Dawley (Harlan Sprague Dawley, Indianapolis, IN). All animals received water and food ad libitum, and were housed in accordance with institutional animal care and use guidelines. Adult male Lewis rats served as recipients of donor cardiac tissue obtained from either 1 to 3 day-old Lewis or BN rats, which served as isograft and allograft groups, respectively. The donor ages used have been previously reported to result in optimum isograft viability (Fulmer et al., 1963) and were of appropriate size so that whole heart grafts might be accommodated by the recipients' ears.

Cardiac Transplantation

The procedure utilized was a modification of the heterotopic method of cardiac transplantation into the mouse ear as described by Fulmer et al (1963) (Figure 6). Cardiac graft recipients were anesthetized with an i.p. injection of ketamine/xylazine (20:1) (Fort Dodge Laboratories, Fort Dodge, IA and Rugby Laboratories, Inc., Rockville Centre, NY) at 105 mg/kg body weight prior to surgery. The dorsal surface of the recipient's ear pinna was nicked at the proximal end with a sterile scalpel and a subcutaneous pouch was prepared using blunt forceps. Whole newborn hearts were harvested promptly at the time of donor sacrifice and gently inserted into the ear pouches of the recipients. In order to prevent graft loss and promote revascularization, skin flaps surrounding the graft site were apposed by applying gentle pressure with forceps. Each recipient was grafted bilaterally, one whole heart per recipient ear, with either two isografts or allografts per animal. Cardiac graft recipients were anesthetized
and monitored daily by visual inspection for pulsatile activity and by electrocardiography using pin electrodes placed on either side of the graft with leads connected to a polygraph recorder (Grass Model 79D, Grass Instruments Co., Quincy, MA) (Figure 7).

Animal Treatment Groups

Lewis cardiac allograft recipients were injected i.p. with CSA in olive oil vehicle once daily, beginning on the same day of transplantation, and/or MTX in saline vehicle once weekly, beginning 1 week prior to the day of transplantation (day 0), as follows. Allograft recipients were placed in one of five treatment groups consisting of either high dose CSA (15 mg/kg/day), low dose CSA (1 mg/kg/day), low dose MTX (450 µg/kg/wk), combination low dose CSA/MTX treatment (1 mg/kg/day and 450 µg/kg/wk, respectively), or control treatment group (saline and olive oil vehicles according to the aforementioned protocol). All allograft recipients received treatment through day 7 post-tx. Cardiac transplants were monitored daily for pulsatile and ECG activity as described above for graft survival, with retrieval of one graft per recipient occurring on days 1-8 post-tx for each treatment group; cardiac grafts were processed for RNA blot analysis as described below. Subsequent to these studies, a second treatment group consisting of low dose CSA/MTX combination therapy was prepared to permit graft retrieval at eight time points, days 1-8 post-tx.

Cell Culture

All cells were cultured in RPMI-1640 media supplemented with penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), 2 mM L-glutamine and 5% fetal bovine serum (GIBCO, Grand Island, NY) at a cell density of 1 X 10^6
cells/ml at 37°C in 5% CO₂, with the exception of the RNK-16 cell line which was also supplemented with 1% nonessential amino acids and 5 X 10⁻⁵ M 2-β-mercaptoethanol (GIBCO). The murine macrophage cell line, ANA-1 (a gift from Dr. Luigi Varesio, NIH, Frederick, MD), was incubated for 4 hr with 10 µg/ml LPS (Difco, Detroit, MI), and used as a positive TNFα mRNA control. The rat NK cell line, RNK-16 (a gift from Dr. C.W. Reynolds, NIH, Frederick, MD), was cultured for 18 hr in the presence of 20 ng/ml PMA and 1000 U/ml IL-2, (Sigma Chemical Co., St. Louis, MO), and used as a negative TNFα mRNA control.

SMNCs isolated from adult male Lewis rats as described previously (Pizarro et al., 1993a), were cultured for 18 hr with or without a combination of 20 ng/ml PMA and 2 µg/ml PHA, and used as positive and negative controls, respectively, for LT and granzyme mRNA expression. After incubation with respective mitogens, cells were collected and processed for RNA as described below.

RNA Blot Analysis

For Northern and slot blot analysis, total cellular RNA was prepared from cells and tissues by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi, 1987), and blots were prepared as described previously (Pizarro et al., 1993a). Filters were hybridized overnight at 42°C in a solution containing either a TNFα cDNA probe prepared using the 1.7 kb murine TNFα sequence cloned into the PstI/BamHI site of pUC9 vector, (a gift from the Chiron Corp., Emeryville, CA) (Pennica et al., 1985), an LT probe prepared using a 0.71kb KpnI/HincII insert of the 1.42 kb murine LT cDNA fragment cloned into the EcoRI/BamHI site of pBR322 vector (a gift from Dr. N. Ruddle, Yale University, New Haven, CT) (Li et al., 1987), an HF probe synthesized from a 950 b.p. murine HF cDNA fragment cloned into the EcoRI site of PBS KS⁻ vector (a
gift from Dr. I. Weissman, Stanford University School of Medicine, Palo Alto, CA) (Gershenfeld & Weissman, 1986), or a murine C11 probe prepared using an 800 b.p. C11 cDNA sequence cloned into the EcoRI/BamH1 site of pGEM3Z vector (a gift from Dr. R.C. Bleackley, University of Alberta, Edmonton, ALB) (Lobe et al., 1986), and prepared for autoradiography as described in an earlier report (Pizarro et al., 1993a). Relative quantitation of mRNA signals on slot blot autoradiographs were measured by transmission densitometry (Model EC910, E-C Apparatus, Corp., St. Petersburg, FL) and reported as arbitrary units representing integrated areas of densitometric tracings.

Protein Assays

Cardiac graft tissue homogenates were prepared using a Brinkmann polytron (Brinkmann Instruments, Inc.) in 1.5 ml phosphate buffered saline containing 2 mM PMSF protease inhibitor (Sigma Chemical Co.) for analysis of TNF protein. Tissue homogenates were briefly centrifuged in a microcentrifuge to remove excess particulate matter. Supernatants were then concentrated approximately 10-fold using Centricon-10 microconcentrators (Amicon, Beverly, MA). The samples were kept on ice for the duration of the assay and subsequently stored at -70°C. Total protein levels were quantitated using a modification of the Lowry colorimetric procedure (Sigma Diagnostics, St. Louis, MO). Tissue samples were standardized to 150 µg total protein per well and assayed in duplicate for TNF immunoreactive protein in an ELISA system using a hamster anti-mouse TNF antibody (Genzyme Corp., Boston, MA), which recognizes both mouse TNFα and LT. Results were analyzed spectrophotometrically using a MR600 microplate reader (Dynatech Laboratories, Inc., Chantilly, VA) and TNF levels were expressed as pg/ml and
U/ml ± SEM, relative to a recombinant mouse TNFα standard (Genzyme Corp., Boston MA).

Statistical Analysis

Experimental groups were compared using analysis of variance and multiple regression analysis for the existence of significant differences between treatment and control groups. Differences were considered to be statistically significant at \( p \leq 0.05 \).
Results

Cardiac allograft tissues were serially monitored for ECG and pulsatile activity in order to determine graft viability post-tx. Allograft rejection was defined by the absence of detectable ECG activity. The average time to allograft rejection of cardiac transplants in untreated control recipients was determined to be Day 7.4 ± 0.68 post-tx, with absence of detectable ECG and pulsatile activity noted in all untreated control allografts by Day 10 post-tx (Pizarro et al., 1993a; Pizarro et al., 1993b).

Combination low dose CSA/MTX-treated recipients demonstrated a two and one-half fold increase in survival time post-tx of transplanted cardiac allografts when compared to cardiac allografts transplanted into untreated control recipients (p ≤ 0.01) (Pizarro et al, 1993b). Cardiac transplants in low dose CSA alone-treated recipients also demonstrated a less, but significant increase in allograft survival time when compared to control (p ≤0.05), while no statistically significant difference in cardiac allograft survival times post-tx between MTX-alone and untreated recipient groups were found (Pizarro et al., 1993b). Cardiac allograft survival time post-tx in high dose CSA-treated recipients represented the longest survival time of the four treatment groups; however, the incidence of anesthesia-related death was increased in this experimental group (Pizarro et al., 1993b).

Expression of the cytotoxic cytokine genes, TNFα and LT, was examined in cardiac allografts transplanted into untreated control and combination low dose CSA/MTX-treated recipients on days 1 through 8 post-tx. As shown in
Figure 16, cardiac graft tissues were analyzed for the presence of TNFα mRNA by Northern blot analysis. Expression of TNFα in cardiac allografts from untreated control recipients appeared to be biphasic (Figure 16A). Peak levels of TNFα mRNA appeared on day 3 post-tx, and although levels decreased by day 4 post-tx, an increase in TNFα mRNA appeared again on day 6 (5/10) or 7 (3/10) post-tx, coinciding with graft rejection as measured by ECG activity. TNFα transcript levels appeared to be significantly decreased in allograft heart tissues from combination low dose CSA/MTX-treated recipients when compared to untreated recipients on all days assayed post-tx (Figure 16B). Although TNFα transcript levels were significantly decreased in cardiac allografts from CSA/MTX-treated animals, these samples also indicated a biphasic pattern of TNFα expression with time post-tx. Similar to untreated control allografts, an increase in TNFα transcripts was detected on day 3 post-tx, and again just prior to rejection in low dose CSA/MTX-treated allografts (Figure 16B). TNFα transcript levels were undetectable in cardiac isografts on days 1-8 post-tx (Pizarro et al., 1993a).
Fig. 16A. TNFα mRNA levels in cardiac allografts transplanted into untreated control recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for TNFα gene expression. A Representative Northern blot is shown above. As with Fig. 16 B, the murine monocyte cell line, ANA-1, stimulated in vitro with LPS (10 µg/ml) for 4 hr was used as a positive TNFα mRNA control, while the rat NK cell line, RNK-16, stimulated in vitro with a PMA (20 ng/ml)/IL-2 (1000U/ml) combination served as a negative control for TNFα mRNA expression. Kb, kilobases.
Fig. 16B. TNFα mRNA levels in cardiac allografts transplanted into low dose CSA/MTX-treated recipients. Total RNA isolated from cardiac allografts on days 1-8 post-tx were examined by Northern blot analysis for TNFα gene expression. Representative Northern blot is shown above. As with Fig. 16A, blots were prepared using 15 µg of total RNA per lane; autoradiogram exposure time was 18 hr. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits is included to illustrate standardized lane loading and intact, non degraded cellular RNA.
Fig. 17A. LT mRNA levels in cardiac allografts transplanted into untreated control recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for LT gene expression. Representative Northern blot is shown above. As with Fig. 17B, SMNCs isolated from adult male Lewis rats and stimulated in vitro with a combination of PMA (20 ng/ml) and PHA (2 µg/ml) for 18 hr were used as a positive control, and untreated SMNCs were used as a negative control for LT mRNA expression. SMNCs, spleen mononuclear cells; Kb, kilobases.
Fig. 17B. LT mRNA levels in cardiac allografts transplanted into low dose CSA/MTX-treated recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for LT gene expression. Representative Northern blot is shown above. As with Fig. 17A, blots were prepared using 15 µg of total RNA per lane; autogradiogram exposure time was 5 days. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits is included to illustrate standardized lane loading and intact, non degraded cellular RNA.
Cardiac allograft transplants from untreated and low dose CSA/MTX-treated recipients were also analyzed for LT mRNA levels on days 1 through 8 post-tx by Northern blot analysis (Figure 17). Similar to TNFα, LT demonstrated a biphasic pattern of gene expression in allografted heart transplants from untreated recipients (Figure 17A). Peak levels of LT mRNA in untreated cardiac allografts were observed on day 3 post-tx, with a second increase in LT transcript levels occurring later, coincident with graft rejection. In contrast, LT mRNA levels were significantly reduced in low dose CSA/MTX-treated cardiac allografts when compared to untreated controls (Figure 17B); low to undetectable levels of LT transcripts were found in low dose CSA/MTX-treated allografts on Days 1 through 8 post-tx (Figure 17B). Similar patterns of TNFα and LT mRNA levels were observed in slot blot analysis (Figure 18A and 18B), where all cardiac allograft RNA samples were exposed to identical hybridization and autoradiography conditions, with different exposure times. LT mRNA levels were undetectable in cardiac isografts on days 1-8 post-tx (Pizarro 1993a).
Fig. 18. Densitometric measurement of TNFα and LT transcript levels from representative slot blots of cardiac allograft total cellular RNA. mRNA levels for TNFα (A) and LT (B) are expressed as arbitrary units representing integrated areas of densitometric tracings obtained by transmission densitometry. Slot blots were prepared by loading 8 µg total RNA of sample per slot; autoradiogram exposure time was 1 day for TNFα and 3 days for LT.

In order to determine whether TNF protein levels paralleled the observed changes in mRNA, an ELISA method using an antibody which recognizes both murine TNFα and LT was used to quantitate TNF protein in transplanted cardiac tissues from untreated and low dose CSA/MTX-treated recipients. TNF protein levels in cardiac allografts from untreated recipients as a group were significantly increased over cardiac allografts in low dose CSA/MTX-treated recipients (p ≤ 0.001) (Figure 19). TNF protein from untreated allografts demonstrated a biphasic pattern, similar to that shown for TNFα and LT mRNA from cardiac allograft tissues in untreated recipients. Two and one-half-fold greater levels of TNF were measured in untreated allografts.
(approximately 400 pg/ml or 16.5 U/ml; 1 U = approx. 24.3 pg) compared to low dose CSA/MTX-treated allografts on day 3 post-tx ($p \leq 0.001$). A second increase in TNF levels appeared in untreated allografts on day 6 post-tx, coincident with allograft rejection, as measured by the absence of ECG activity. TNF protein levels in cardiac allografts in combination low dose CSA/MTX-treated recipients did not vary significantly, measuring approximately 150 pg/ml or 6.0 U/ml at all time points examined post-tx (Figure 19). In contrast to TNF protein levels measured in cardiac allografts, TNF levels in cardiac isografts were at or below standard limits of detection (50 pg/ml) at all times except day 3 (100 pg/ml) post-tx (Pizarro et al., 1993a), and were significantly decreased compared with time-matched cardiac allografts from either control-untreated ($p \leq 0.001$) or low dose CSA/MTX-treated ($p \leq 0.01$) recipients.

Expression of granzymes A and B, HF and C11, respectively, were examined in cardiac transplants at various times post-tx. Cardiac transplants from untreated allograft recipients were analyzed for the presence of HF (Figure 20A, top panel) and C11 (Figure 20A, middle panel) on days 1 through 8 post-tx by Northern blot analysis. Expression of HF and C11 mRNA occurred at later time points during the rejection process compared to TNFα and LT. Granzyme A and B transcripts were detected on days 5 through 7 and days 5 and 6 post-tx, respectively, in cardiac transplants from untreated allograft recipients (Figure 20A). Granzyme mRNA levels were therefore elevated just prior to allograft rejection, with HF and C11 transcript levels undetectable at earlier time points post-tx.
Fig. 19. TNF protein levels in cardiac transplants from low dose CSA/MTX-treated and untreated control recipients. Cardiac allograft tissues were retrieved on days 1 through 8 post-tx and analyzed for TNF protein, expressed as pg/ml and U/ml (1 U = approx. 24.3 pg) ± S.E.M. Data shown are mean values ± S.E.M. of four experiments performed with different allograft tissue samples (p ≤ 0.001).

Cardiac allografts from combination low dose CSA/MTX-treated recipients were also analyzed on days 1 through 8 post-tx by Northern blot analysis for HF and C11 mRNA levels. Transcript levels for both HF and C11 were undetectable in allografts from low dose CSA/MTX-treated recipients on all days assayed post-tx (Figure 20B and 20C, respectively). Furthermore, no detectable levels of HF or C11 transcripts were found on days 1 through 10 post-tx in RNA samples prepared from cardiac isografts (Figure 20D). When cardiac
allograft samples from untreated and low dose CSA/MTX-treated recipients were assayed simultaneously and compared by slot blot analysis, HF (Figure 21A) and C11 (Figure 21B) mRNA levels were detected only in tissue from untreated recipients, at times and levels similar to those demonstrated in Northern blot analysis (Figures 20A).
Fig. 20A. HF and C11 mRNA levels in cardiac allografts transplanted into untreated control recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for HF (top panel) and C11 (middle panel) gene expression. A representative Northern blot is shown above. As with Fig. 20B, 20C, and 20D, blots were prepared using 15 μg of total RNA per lane; autogradiogram exposure time was 2 days for both HF and C11 probes. Ethidium bromide staining of 18S and 28S ribosomal RNA subunits (bottom panel) is included to illustrate uniformity of lane loading and intact, nondegraded cellular RNA. Kb, kilobases.
Fig. 20B. HF mRNA levels in cardiac allografts transplanted into low dose CSA/MTX-treated recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for HF gene expression. Representative Northern blot is shown above. Autogradiogram exposure time was 5 days.
Fig. 20C. C11 mRNA levels in cardiac allografts transplanted into low dose CSA/MTX-treated recipients. Total RNA isolated from cardiac allografts retrieved on days 1-8 post-tx were examined by Northern blot analysis for C11 gene expression. Representative Northern blot is shown above. Autogradiogram exposure time was 5 days.
Fig. 21. Densitometric measurement of HF and C11 transcript levels from representative slot blots of cardiac allograft total cellular RNA. mRNA levels for HF (21A) and C11 (21B) are expressed as arbitrary units representing integrated areas of densitometric tracings obtained by transmission densitometry. Slot blots were prepared by loading 8 µg total RNA of sample per slot; autoradiogram exposure time was 3 days for both HF and C11 probes.
Fig. 20D. HF and C11 mRNA levels in cardiac isografts transplanted into untreated control recipients. Total RNA isolated from cardiac isografts retrieved on days 1-7 and 10 post-tx were examined by Northern blot analysis for HF (top panel) and C11 (middle panel) gene expression. Representative Northern blots are shown above. Autogradiogram exposure time was 5 days for both HF and C11 probes.
Discussion

Using a heterotopic method of cardiac transplantation into the rat ear, we have previously reported that treatment of cardiac transplant recipients with combination low dose CSA/MTX immunosuppressive therapy resulted in allograft survival times which more than doubled when compared to survival of allografts transplanted into untreated control recipients (p \leq 0.01) (Pizarro et al., 1993b). The average time to rejection of untreated control allografts was previously found to be Day 7 post-tx (Pizarro et al., 1993a), whereas combination low dose CSA/MTX-treated recipients undergo acute rejection by day 18 post-tx, at which time ECG and pulsatile activity was virtually undetectable (Pizarro et al., 1993b). In addition, combination low dose CSA/MTX treatment increased graft survival over cardiac allografts transplanted in either low dose CSA or MTX alone-treated recipients (Pizarro et al., 1993b).

In order to elucidate the possible mechanism(s) by which combination low dose CSA/MTX prolongs rat cardiac allograft survival, we have previously examined IL-2 production at the mRNA level (Pizarro et al., 1993b). IL-2 mRNA levels were examined in cardiac allografts to determine if low dose CSA/MTX therapy, like high dose CSA treatment, exerted its immunosuppressive action through inhibition of IL-2 gene expression. IL-2 appears to be an important mediator of allograft rejection since a positive correlation between IL-2 levels and rejection episodes has been described (Yoshimura & Kahan, 1985; McKenna et al., 1988). CSA is believed to exert its immunosuppressive actions through potent inhibition of IL-2 production by T cells (Kronke et al., 1984; Elliot et al., 1984). We
found that, in contrast to high dose CSA administration, combination low dose CSA/MTX treatment (1.0 mg/kg/day and 450 µg/kg/week, respectively) did not alter IL-2 gene expression. Similar patterns of IL-2 gene expression were found in cardiac allografts from both low dose CSA/MTX-treated and untreated transplant recipients on days 1 through 8 post-tx (Pizarro et al., 1993b). Therefore, cardiac allografts in both low dose CSA/MTX-treated and untreated control recipients indicated comparable increases in IL-2 mRNA levels. Unlike high dose CSA treatment, the mechanism(s) by which low dose CSA/MTX combination therapy increases allograft survival time post-tx does not appear to be mediated by the inhibition of IL-2 gene expression.

We, and others, have reported the presence of elevated levels of TNFα and LT during allograft rejection (Pizarro et al., 1993a; Lowry & Blais, 1988; Moy & Rosenau, 1981). Importantly, results of anti-TNFα and LT antibody therapy increasing allograft survival time imply that TNF is a mediator of acute rejection (Imagawa et al., 1990b; Scheringa et al., 1991; Teramoto et al., 1991). Taken together, these data strongly suggest a role for TNFα and/or LT in the pathogenesis of allograft rejection. We therefore chose to investigate the effects that combination low dose CSA/MTX therapy might have on the expression of these cytotoxic cytokines.

In the present study, rat cardiac allografts transplanted into combination low dose CSA/MTX-treated recipients indicate significant decreases in TNFα and LT mRNA and protein levels between days 1 through 8 post-tx when compared to untreated allograft controls. Although TNFα transcript levels were
significantly decreased in cardiac allografts from CSA/MTX-treated animals, low
dose CSA/MTX treatment did not abrogate TNFα gene transcription. Low but
detectable levels of TNFα mRNA were seen on day 3 post-tx, and again on days 7
or 8 post-tx with combination treatment, similar to the pattern in control
allografts from untreated recipients. In contrast to TNFα gene expression, LT
mRNA levels in cardiac allograft transplants from low dose CSA/MTX-treated
recipients were virtually undetectable on days 1 through 8 post-tx.

TNF protein levels in cardiac allografts transplanted into untreated
recipients were significantly increased over cardiac allografts from low dose
CSA/MTX-treated recipients (p \leq 0.001). TNF protein in allografts from
untreated recipients demonstrated a biphasic pattern, similar to that shown for
TNFα and LT mRNA, while TNF protein levels in transplants from low dose
CSA/MTX-treated recipients remained low and did not significantly fluctuate on
days 1 through 8 post-tx. In contrast to cardiac allografts transplanted into either
control or low dose CSA/MTX-treated recipients, TNF protein levels in time-
matched cardiac transplants from isograft recipients have been shown to be at or
below standard limits of detection on all measured time points except day 3 post-
tx (Pizarro et al., 1993a). Therefore, TNF protein levels in cardiac isografts were
found to be significantly decreased when compared to cardiac allografts from
either control or low dose CSA/MTX treated recipients, indicating that TNF
expression appears to be allograft specific.

In addition to TNFα and LT levels, expression of granzymes A and B was
also examined in cardiac transplants. The HF (granzyme A) gene encodes a
trypsin-like serine esterase, while C11 (granzyme B) encodes a chymotrypsin-like serine protease with apparent specificity for acidic residues (Lobe et al., 1986). The HF protein is promptly secreted by both cytotoxic CD4+ and CD8+ T cells and NK cells after stimulation in vitro, while C11 has been detected in activated cytotoxic T lymphocytes and NK cells (Velotti et al., 1989; Griffiths & Mueller, 1991a). Granzymes are considered to be essential mediators in cell-mediated cytolysis by both cytotoxic T cells and NK cells, and it has been postulated that they may serve as diagnostic markers for cardiac allograft rejection (Griffiths et al., 1991b).

In the present study we report that expression of HF and C11 occurs at later times during rejection compared to TNF. Granzyme mRNA levels were elevated just prior to allograft rejection, with HF or C11 transcript levels undetectable at earlier time points post-tx. Granzyme mRNA levels in cardiac allografts from combination low dose CSA/MTX-treated recipients were undetectable in low dose CSA/MTX-treated allografts on all days assayed post-tx. Furthermore, no detectable levels of either HF or C11 transcripts were found in day-matched cardiac isografts. Therefore, although granzymes may be indicators of rat cardiac allograft rejection, TNFs may serve as important early marker of cardiac allograft rejection.

The present study reports a decreased expression of the cytotoxic cytokines TNFα and LT, and granzymes A and B, (HF and C11), in cardiac allografts transplanted into low dose CSA/MTX-treated recipients when compared to untreated recipients. The actual functional significance of
combination low dose CSA/MTX treatment, however, is less well understood. It has been demonstrated that low dose CSA therapy (2.0 mg/kg/day) of allograft recipients reduces T cell proliferative responsiveness, but is unable to sustain long-term cardiac allograft survival (Stepkowski et al., 1989). The possibility exists that low dose CSA may also affect TNFα production from T lymphocytes since activated peripheral T cells have been shown to be a contributing source of TNFα (Steffen et al., 1988). In addition, the immunosuppressive effect of low dose CSA may not be limited to T lymphocytes; in fact, low dose CSA treatment (1.0 µg/ml) has been described to also affect monocyte cell function in vitro by inhibiting IL-1α and β, as well as PGE2 production (Reisman et al., 1991). However, in vivo, different populations of human monocytes have been reported to possess varying sensitivities to CSA (Esa et al., 1988). The possibility therefore exists that low dose CSA may affect production of TNF as well as IL-2.

Low dose CSA therapy, while prolonging cardiac allograft survival, does not have the efficacy of combination low dose CSA/MTX. In addition, the mechanism(s) by which MTX appears to exert immunosuppression is still poorly understood, it is believed that MTX possesses primarily anti-inflammatory activity (Weinblatt et al., 1985). Direct evidence of MTX immunomodulation is supported by reports describing the effects of MTX in experimental models of arthritis, in which the production of IL-1 and induction of primary delayed-type hypersensitivity were inhibited in MTX-treated rats (Hu et al., 1988; O'Callaghan et al., 1986).
The present study explores the mechanism of action of combination low dose CSA/MTX therapy in prolonging cardiac allograft survival. The possibility exists that low dose CSA/MTX combination therapy may act on two different effector cells of the immune response, i.e., cytotoxic T cells and macrophages, by inhibiting production of immune mediators, such as granzymes and TNF. The present study also supports a role for the cytotoxic cytokines, TNFα and LT, and granzymes A and B in the pathogenesis of allograft rejection. Experiments are currently underway in our laboratory to examine levels of these cytotoxic gene products just prior to rejection time in combination low dose CSA/MTX treated recipients of cardiac allografts. Studies such as these are designed to elucidate the pathophysiological mechanisms involved in graft rejection and may lead to more effective immunosuppressive therapy using synergistic combination treatments aimed at promoting graft survival while minimizing adverse side effects.
CHAPTER V
SUMMARY

One of the major problems associated with cardiac transplantation is rejection of the allografted heart. The immune response to alloantigen involves the production of, by different cell types, soluble endogenous mediators, which include cytokines and granzymes. The present dissertation has investigated possible pathophysiological mechanisms involved in cardiac allograft rejection by examining the production of these cytotoxic mediators in a heterotopic model of rat cardiac transplantation. In particular, detection and quantitation of the cytotoxic products TNFα, LT, HF, and C11, were measured during the process of cardiac allograft rejection. Identification of which cytotoxic mediators are present during the course of rejection, and when post-tx, may aid in designing more specifically targeted therapies aimed to promote graft success.

Additionally, success of the allografted heart is critically dependent on the availability of powerful immunosuppressive agents that have the ability to effectively prolong graft survival and prevent allograft rejection. Currently, administration of potent immunosuppressive drugs to transplant recipients not only dramatically increases the chances of allograft survival, but also with the possibility of decreasing toxic side effects. An alternative approach to reduce these side effects associated with high dose immunosuppression utilizes the
administration of low dose combination therapies that may act synergistically to inhibit alloactivation. Studies contained in this dissertation examined the use of a low dose CSA/MTX combination therapy during cardiac transplantation. Specifically, low dose CSA/MTX combination therapy was investigated in order to determine its ability to prolong rat cardiac allograft survival. In addition, modulation of the cytotoxic mediators, TNFα, LT, H, and C11, was measured in cardiac allografts obtained from low dose CSA/MTX-treated recipients. Studies such as these are designed to improve allograft success by administering synergistic combination therapies which prolong graft survival and minimize the number of adverse side effects.

It has previously been shown that donor MHC-incompatible antigens have the ability to precipitate a host allograft rejection response in small animal models (McKenzie & Henning, 1977; Lowry et al., 1985a). Therefore, MHC-incompatibility between transplant donor and recipient has become an experimental parameter when investigating allograft rejection. The studies contained within this dissertation employed a modification of a heterotopic method of cardiac transplantation into the mouse ear, originally described by Fulmer et al (1963), and applied it to a rat system as a model of allograft rejection using the MHC-mismatched BN or DA donor hearts into Lewis recipients as allograft pairs and Lewis donor hearts into Lewis recipients as isograft pairs.

The heterotopic transplantation of cardiac grafts into the rat ear provides many advantages when investigating the processes of allograft rejection. Using this method, cardiac graft recipients are subject to minimal trauma compared to
those undergoing vascular transplantation techniques, and therefore have an increased chance of survival. Furthermore, because of the ease of graft placement and subsequent graft retrieval, it is possible to evaluate a large number of animals for consistent trends characteristic of graft rejection. Additionally, graft success is more conveniently monitored. Visual transillumination of the recipients' ears is possible to determine size and swelling, patterns of vascularization, and pulsatile activity of the cardiac grafts. ECG recordings via direct placement of pin electrodes into the ear pinnae on either side of the transplanted heart enables quantitative as well as qualitative information when monitoring cardiac graft viability. ECG recordings taken from heterotopically transplanted cardiac grafts into rat pinnae allow quantitative comparative analyses between experimental groups, which may be problematic in vascular-connected transplants due to the possible interference of the recipient's own intrinsic heartbeat. In fact, using the heart into ear model, the ability to distinguish between graft and recipient heart rate is easily determined. Therefore, although a nonvascular method of cardiac transplantation into the rat ear pinnae may not exactly parallel events occurring in human transplant rejection, this model provides multiple advantages when examining the pathogenic events leading to allograft rejection.

Using the rat heart into ear transplantation model, it was established that cardiac allografts, but not isografts, undergo rejection by day 10 post-tx, at which time ECG activity is undetectable. Additionally, the average day to rejection of rat allografted hearts was determined to be 7 days post-tx, whereas isografted hearts demonstrated regular ECG and pulsatile activity up to 18 months post-tx.
These results are in accordance with a heterotopic method for accessory cervical heart transplantation in rats using the same allograft and isograft pairings, which has been subsequently established in our laboratory. Using this transplantation method of direct vascular anastomoses, the average day to rejection of allografted hearts was determined to be 8 days post-tx, while isografted hearts displayed viability for at least one year post-tx (Payne et al., submitted). These findings are also consistent with earlier reports by other investigators which assessed rat cardiac allograft rejection by cessation of palpable pulsatile activity (Tilney et al., 1978; Hancock et al., 1991; Scheringa et al., 1991). Taken together, these data suggest that the rat heart into ear graft technique may provide a useful model to investigate the mechanisms of allograft rejection in cardiac transplantation.

Detection and quantitation of soluble endogenous mediators produced during inflammatory processes in response to alloantigen were measured during rat cardiac allograft rejection using the heart into ear model. The studies contained within the present dissertation focused on the production of cytotoxic mediators, including TNF and granzymes. Specifically, TNF-α and β (LT) were examined for reasons mentioned earlier (Chapter 1), including their many proinflammatory actions which may contribute to allograft rejection responses. These effects include potent induction of chemotactic factors causing effector cell infiltration, augmentation of class I MHC antigen expression, stimulation of platelet activating factor (PAF) promoting ischemia within the graft, and induction of macrophage activation (Reviewed in Halloran et al., 1989). In addition, it had previously been shown that a strong correlation exists between
granzyme gene expression and cytotoxic potential (Gershenfield & Weissman, 1986; Lobe et al., 1986). In fact, one of the first studies demonstrating their importance involved the detection of granzyme-expressing lymphocytes in vivo during murine cardiac allograft rejection (Mueller et al., 1988). For these reasons, including those previously mentioned (Chapter 1), HF and C11 (granzymes A and B) were measured, using the aforementioned heart into ear model, for their putative role in the process of rat cardiac allograft rejection.

TNF and granzyme expression was examined in a window of time consisting of days 1 through 8, and in some cases through day 10, post-tx. This time frame was established based on the findings that using the rat heart into ear graft technique, the average time to rejection of cardiac allografts was 7 days post-tx, with no detectable ECG activity measured by day 10 post-tx. In fact, both TNFα and LT mRNA and protein levels in cardiac allografts consistently demonstrated a biphasic pattern of expression where peak levels were reached on day 3 post-tx with a second increase on day 6 or 7 post-tx which subsequently decreased through day 10 post-tx. In contrast to TNF expression found in allografts, TNFα and LT mRNA transcripts were undetectable in isografts on days 1 through 10 post-tx, while TNF protein levels were consistently undetectable or significantly reduced when compared to time-matched allografts. Therefore, these data indicate that increased expression of TNF appears to be allograft-specific and occurs early during rat cardiac allograft rejection, possibly serving as an important early indicator of transplant rejection.
Compared to TNF, granzyme expression occurred at later time points during the rejection process. Granzyme mRNA levels were elevated just prior to rejection with HF and C11 transcripts detected on days 5 through 7 and days 5 and 6 post-tx, respectively, in rat cardiac allografts. Furthermore, no detectable levels of HF or C11 transcripts were found on days 1 through 10 post-tx in rat cardiac isografts. Taken together, these data suggest that although granzymes may be indicators of rat cardiac allograft rejection, TNF may serve as an important early marker of cardiac allograft rejection.

As mentioned earlier, the significance of the early rise in TNF (TNFα and LT) levels post-tx is unknown. Although the second increase in TNF expression appears to be solely allograft-specific, the first peak is noted in both allograft and to a lesser extent, in isograft tissues. Speculation regarding this early elevation in TNF gene expression may be, in part, due to postsurgical inflammatory (nonallospecific) events. In addition, this significantly greater early TNF response present in allografts may reflect allospecific events, including endothelial cell activation (Collins et al., 1986) and augmented migration and adhesion of leukocytes (Shalaby et al., 1985). Furthermore, since TNF is known to have angiogenic properties, the possibility exists that an increase in TNF production following transplantation may be due, in part, to the graft's need for vascularization. One possible source of TNF may be activated mononuclear phagocytes in the heart graft, since it has been reported that TNFα is released by infiltrating macrophages which are present early in the rejection process (Lowry & Blais, 1988; MacPherson & Christmas, 1984; Payne et al., submitted). Another contributing source may be activated peripheral T lymphocytes, which have also
been shown to synthesize TNF (Steffen et al., 1988). Therefore, results obtained from the present studies indicate significant elevations in graft TNF levels well in advance of transplant rejection.

The second increase in TNF levels observed in cardiac allografts appears to coincide with the time of graft rejection. Although the actual roles of TNFα and LT in rejection are not clearly defined, they may participate in the rejection process by indirect activation of leukocyte subpopulations or by direct lytic mechanisms of the graft parenchyma. The biphasic pattern of TNF expression demonstrated in the present studies is also consistent with previous reports demonstrating that rat cardiac allografts elicit a biphasic cellular immune response, with the second phase coinciding with the time of acute graft rejection (Tilney et al., 1978). More recent studies also showed similar trends of bimodal TNF expression in the pathogenesis of rat lung allograft rejection, with significant attenuation in those recipients pretreated with neutralizing TNF antisera (DeMeester et al., 1993). Therefore, activated mononuclear leukocytes, including macrophages, infiltrating the graft parenchyma may be responsible for the early rise in TNF levels which subsequently may serve as an early marker of incipient rejection, while the second increase in TNF levels is more coincident with graft rejection and may represent an accumulation of events leading to eventual tissue graft destruction.

These data are also supported by recent findings in our laboratory using a heterotopic method for accessory cervical heart transplantation, wherein immunohistochemical characterization of the cellular phenotypes present in
rejecting rat cardiac allografts was performed (Payne et al., submitted). The results from these studies demonstrated significantly elevated staining in cardiac allografts for the macrophage/monocyte population as early as day 2 post-tx, which remained sustained through day 8 post-tx. Moreover, macrophages appeared to precede T cells in transplant infiltrates; immunohistologic staining for the CD8+ T cell subset was markedly increased by day 4 post-tx and appeared to be allograft-specific (Payne et al., submitted). This is consistent with the expression of the granzymes, HF and C11 in the present dissertation which was present between days 5 through 7 post-tx in cardiac allografts. Therefore, as stated earlier, granzymes may indicate rejection, however, TNF appears to be an earlier marker of rat cardiac allograft rejection.

Once the time course for TNF and granzyme elaboration was determined in the rat heart into ear transplantation model, it appeared as though the crucial window of time for studying TNF and granzyme expression was on days 1 through 8 post-tx. A low dose CSA/MTX combination immunosuppressive therapy was then investigated as a possible approach to prolong allograft survival and to examine how this treatment would modulate TNF and granzyme expression. As stated earlier, low dose CSA/MTX treatment was considered as an alternative immunosuppressive therapy to promote allograft survival while minimizing high dose CSA side effects. Furthermore, immunosuppressive effects have been attributed to MTX (Mitchell et al., 1969), which has shown some success in the treatment of rheumatoid arthritis (Weinblatt et al., 1985; Perhala & Wilke, 1991).
The results from the present studies have indicated that low dose CSA/MTX treatment significantly prolonged rat cardiac allograft survival over day-matched allografts transplanted into untreated control recipients, where the average day to rejection was day 18 and day 7 post-tx, respectively. Therefore, treatment of cardiac allograft recipients with combination low dose CSA/MTX therapy more than doubled allograft survival time post-tx when compared to untreated controls. Combination low dose CSA/MTX therapy also significantly increased graft survival compared to low dose CSA alone as well as MTX-alone treated allograft recipients. High dose CSA-treated recipients had the longest allograft survival time (36 days); however, high mortality was noted in this treatment group following anesthesia administration during cardiac monitoring. Although the reasons for this phenomenon are unknown, the possibility exists that apparent complications may be due to known toxicities associated with high dose CSA administration.

The mechanism(s) by which low dose CSA/MTX combination therapy produces its effect is unknown. Low dose CSA treatment (1.0 mg/kg/day) of allograft recipients has been shown to reduce T cell proliferative responsiveness but was unable to sustain long-term cardiac graft survival (Stepkowski et al., 1989). This suggests that low dose CSA has the ability to prolong cardiac graft survival post-tx by activation of T cell suppressor function(s) and/or by altering T cell proliferation. In fact, the present studies indicate that low dose CSA treatment, while able to delay allograft rejection, does not have the efficacy of combination low dose CSA/MTX in prolonging rat cardiac allograft survival. Therefore, the possibility exists that MTX provides additional
immunosuppressive capabilities by affecting T cell function and/or regulating other cells participating in the immune response.

Although it is unclear by which mechanism(s) MTX exerts its immunosuppressive effects, animal studies support an anti-inflammatory mode of action. In a murine model of adjuvant-induced arthritis, MTX has been reported to inhibit hind paw edema, the activation of macrophages by PGE2, the influx of MHC class II and macrophages into synovial tissue, and IL-1 production (Hu et al., 1988). These data support the possible role MTX may have in inhibiting macrophage effector cell function. Therefore, in the present study, significant prolongation of cardiac allografts transplanted into combination low dose CSA/MTX-treated recipients may be due, in part, to the combined immunosuppressive actions of low dose CSA primarily at the T cell level and MTX primarily at the macrophages effector cell level.

IL-2 mRNA levels in transplanted cardiac tissues was also examined to determine whether combination low dose CSA/MTX treatment, like high dose CSA, exerts its immunosuppressive action by altering steady state levels of IL-2 mRNA. IL-2 gene transcripts were not detected in isografts or in allografts transplanted into high dose CSA-treated recipients on any day post-tx when compared to allografts from untreated recipients, supporting previous reports demonstrating suppression of IL-2 gene transcription with high dose CSA administration (Kronke et al., 1984 and Elliot et al., 1984). No apparent differences in IL-2 mRNA transcript levels were observed, however, between allografts transplanted into untreated and low dose CSA/MTX-treated
recipients. IL-2 mRNA transcripts appeared early after transplant and remained elevated through day 8 post-tx. Therefore, unlike high dose CSA therapy, the mechanism(s) by which low dose CSA/MTX prolongs allograft survival does not appear to be mediated by the inhibition of IL-2 gene transcription.

The effects of low dose CSA/MTX on the expression of the cytotoxic mediators, TNFα, LT, and granzymes A and B (HF and C11, respectively) were then examined in an attempt to elucidate possible mechanism(s) by which low dose CSA/MTX therapy increases the time of allograft survival. Significant suppression of TNFα, LT, HF, and C11 gene expression was seen on days 1 through 8 post-tx in cardiac allografts from low dose CSA/MTX-treated recipients compared to untreated allograft controls. TNF protein levels in cardiac allografts from low dose CSA/MTX-treated recipients were also found to be significantly reduced on days 1 through 8 post-tx when compared to time-matched untreated allograft controls. This data indicate that low dose CSA/MTX treatment is effective in prolonging rat cardiac transplant survival and appears to act at the transcriptional level to downregulate cytotoxic gene expression. Table 4 describes the studies contained within this dissertation by summarizing the cytokine and granzyme levels in low dose CSA/MTX-treated and untreated control cardiac grafts. Table 4 is a qualitative description comparing relative levels of mediators among experimental groups; quantitative measurements can not be extrapolated from this information.
TABLE 4. Summary of cytokine levels in low dose CSA/MTX-treated and untreated control cardiac grafts.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>DAY POST-TX</th>
<th>CYTOKINE MEASURED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 8</td>
<td></td>
</tr>
<tr>
<td>CSA/MTX</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>ALLO. CONT.</td>
<td>+ +++ + ++ ++</td>
<td>TNFα mRNA</td>
</tr>
<tr>
<td>ISO. CONT.</td>
<td>+++ ++</td>
<td>LT mRNA</td>
</tr>
<tr>
<td>CSA/MTX</td>
<td>+ +++ + ++ ++</td>
<td>TNF protein</td>
</tr>
<tr>
<td>ALLO. CONT.</td>
<td>+ + + + + + +</td>
<td>HF mRNA</td>
</tr>
<tr>
<td>ISO. CONT.</td>
<td>+</td>
<td>C11 mRNA</td>
</tr>
<tr>
<td>CSA/MTX</td>
<td>+ + ++++++++ ++++</td>
<td>IL-2 mRNA</td>
</tr>
<tr>
<td>ALLO. CONT.</td>
<td>+ + ++++++++ ++++</td>
<td></td>
</tr>
<tr>
<td>ISO. CONT.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The actual functional significance of combination low dose CSA/MTX treatment is less well understood. As mentioned earlier, the possibility exists that low dose CSA may affect T cell responsiveness (Stepkowski et al., 1989) as well as TNF production from T lymphocytes since activated peripheral T cells have also been shown to be a contributing source of TNFα (Steffen et al., 1988). In the same manner, CSA may also have an effect on granzyme production since granzymes have been shown to be primarily a T cell product. Additionally, as stated earlier, the possibility exists that MTX may play a role in downregulating macrophage effector cell function. Therefore, TNFα production by macrophages may be affected by MTX administration. In the present studies, significant prolongation of cardiac allografts transplanted into combination low dose CSA/MTX-treated recipients may be due to combined immunosuppressive actions of both low dose CSA and MTX, acting on both T cells and macrophage effector cells.

Alternatively, the combination of low dose CSA plus low dose MTX may act in a synergistic manner. Further studies are needed to examine the effects of both low dose CSA alone and low dose MTX alone treatment on the aforementioned cytotoxic mediators in order to determine if a single immunosuppressive agent is regulating a specific cytokine or if the combination low dose CSA/MTX is acting synergistically. In addition, it would be interesting to perform dose-response experiments in which the dose of each individual immunosuppressive agent (CSA or MTX) would vary, and their effect on each cytotoxic mediator measured. In this way, an ideal dosage of each immunosuppressive drug can be established to downregulate a specific cytotoxic
mediator. Furthermore, an optimal dosage of combination CSA/MTX which elicits the greatest decrease in one or more mediators may be determined.

The studies contained within this dissertation have explored the possibility of using combination low dose immunosuppressive therapy, specifically low dose CSA/MTX, as an alternative treatment aimed at prolonging rat cardiac graft survival while minimizing adverse side effects often associated with use of high dose immunosuppression following allograft transplantation. Low dose CSA/MTX combination treatment has been shown to decrease the expression of the cytotoxic mediators TNFα, LT, HF, and C11 during allograft rejection. Figure 22 summarizes the postulated effects that low dose CSA/MTX combination therapy may have on TNF and granzyme production during rat cardiac allograft rejection.

The need remains for CSA, MTX, and other immunosuppressive drugs to be evaluated for their efficacy in downregulating TNFα, LT, HF, C11, and other proinflammatory mediators involved in allograft rejection. Insight into which cytotoxic mediators are synthesized, and when post-tx they appear, may provide opportunities for early detection of molecular "markers" that signal impending rejection. Assessment of the cytokine profile during allograft rejection may also aid in the development of more specifically targeted immunosuppressive therapies designed to prolong graft survival.
Fig. 22. Postulated effects of low dose CSA/MTX administration on TNF and granzyme production during rat cardiac allograft rejection.
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The dissertation is therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

4/15/94  
Date  
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