Immunological Mechanisms in Cardiac Disease

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Abstract

The aim of the work presented in this thesis was to examine cell and antibody-mediated immune mechanisms in heart muscle disease. The two conditions studied were acute rejection of cardiac allografts as an example of cell-mediated immune damage and alcoholic heart muscle disease as a potential example of antibody-mediated disease.

In the first study experiments were performed on 94 endomyocardial biopsies (EMB) from 73 patients to determine the relationship between the ability to grow lymphocytes from EMB in culture and (i) grade of concurrent or future rejection, (ii) the presence of endocardial lymphocytic infiltrates (ELI) and (iii) donor/recipient HLA mismatches. These studies showed that outgrowth of lymphocytes was not closely related to the degree of rejection, not influenced by the use of polyclonal activators in the medium but it was affected by the presence of two HLA mismatches at the DR locus. The presence of ELI was related to rejection but not to outgrowth of lymphocytes. It was concluded that, contrary to predictions in the literature, the ability to grow lymphocytes from EMB did not indicate presence of impending rejection.

In the second study experiments were performed to test the hypothesis that alcoholic heart muscle disease may be caused by autoantibodies to acetaldehyde-modified myocardial proteins. Sera from 61 subjects were tested by Western analysis for antibodies against acetaldehyde treated human myocardial proteins. No such antibodies were detected in 11 healthy control subjects or 28 patients with non-alcoholic heart diseases. In contrast, 4 out of 14 patients with alcoholic heart disease (28%) had detectable antibodies, suggesting a role for these antibodies in alcohol-induced heart muscle disease.

To isolate potential antigenic myocardial proteins from small samples of myocardium a novel technique was developed for rapidly electroeluting proteins separated on a polyacrylamide gel. This system was validated by electroeluting endothelin receptors from human myocardium.

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Finally I would like to absolve these people from any responsibility for any errors or omissions I have committed.

Dedication

To Joanne

without whom it

would not have been worthwhile

Declaration

This thesis was composed by myself and the work contained within it was performed entirely by myself, except for a few instances where stated otherwise.

This thesis has not been submitted for any other degree, diploma or professional qualification.

Alun Andrew Harcombe

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Abbreviations

ACM	alcoholic cardiomyopathy
APC	antigen presenting cells
BSA	bovine serum albumin
CD3	cluster of differentiation antigen 3
CIM	cytoimmunological monitoring
CML	cell mediated lympholysis
CTLp	cytotoxic T cell precursors
DCM	idiopathic dilated cardiomyopathy
DNA	deoxy-ribonucleic acid
EBV	Epstein-Barr virus
ECACC	European Collection of Animal Cell Cultures
ECG	electrocardiogram
ELI	endocardial lymphocytic infiltrates
ELISA	enzyme linked immunosorbent assay
EMB	endomyocardial biopsy
ER	endoplasmic reticulum
ET1-3	endothelin 1 to 3
ET_BR	endothelin type B receptor
GM-CSF	granulocyte-macrophage colony stimulating factor
HPLC	high performance liquid chromatography
HLA	human leucocyte antigens
ICAM	intercellular adhesion molecule
IFNγ	interferon gamma
IHD	ischaemic heart disease
IL2	interleukin-2
IL2R	interleukin-2 receptor
Ig	immunoglobulin
IgA	immunoglobulin A
ISH	in-situ hybridisation
kDa	kilo-Daltons
LAK	lymphokine activated killer cells
LDA	limiting dilution assays
LFA1	leucocyte functional antigen 1
LV	left ventricle
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction

NIAA	National Institute on Alcohol Abuse and Alcoholism
NIH	National Institutes of Health
NK	natural killer cells
OD	optical density
PBLs	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PLT	primed lymphocyte testing
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
S6b	Sarafotoxin S6b
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCR	T cell receptor
TH1, TH2	T helper type 1 or 2 cells
TNFα ,β	Tumour necrosis factor (alpha, beta)
VCAM	vascular cell adhesion molecule
WHO	World Health Organisation

1. General Introduction

The aim of the work of this thesis was to examine the role of autoimmune mechanisms in non-ischaemic cardiac disease. Two forms of cardiac disease were selected for study: acute rejection of cardiac transplant allografts and alcoholic heart muscle disease.

The detection of acute rejection relies upon histological examination of endomyocardial biopsies (EMB), particularly during the early post-transplant period. Histologically, EMB are graded using a system based upon the degree of cellular infiltration and amount of myocyte damage detected. Unfortunately, no histological or other features predict those patients who will subsequently develop rejection and so EMB are performed routinely. When acute rejection is detected patients are normally treated with intensive courses of additional immunosuppressive drugs.

Several groups have cultured the cells infiltrating EMB by means of in-vitro culture systems employing interleukin-2 supplemented media. These studies have shown a correlation between the ability to culture activated T-lymphocytes from EMB and histological evidence of acute rejection. It has also been claimed that growth of lymphocytes from biopsies without histological evidence of rejection predicts subsequent episodes of rejection. These studies did not take account of the use of different supplements to culture media, the effect of the presence of endocardial lymphocytic infiltrates or the role of HLA mis-matches in influencing culture results.

In addition to the histological features characteristic of rejection, endocardial lymphocytic infiltrates (ELI) have been observed. These are mixed infiltrates, predominantly of T lymphocytes, together with macrophages. They may be associated with histological evidence of myocyte damage but are thought to be distinct from acute rejection. From their anatomical location in the area of the sub-endocardium, it is possible that lymphocytes from such foci could preferentially grow from EMB.

The study of EMB was therefore set up with the following aims:

1) to determine the effect of medium supplements on the ability to grow lymphocytes from EMB.
2) to examine the relationship between lymphocyte growth in vitro and histological acute rejection together with its predictive value for subsequent episodes of rejection.
3) to determine whether the presence of ELI has any influence on the ability to grow lymphocytes from EMB.
4) to determine if in vitro outgrowth of lymphocytes is related to the number of HLA antigen mismatches between graft recipient and donor.

Alcoholic cardiomyopathy was selected for study because alcohol has many effects on the heart, both directly and through it's major metabolite, acetaldehyde. Patients with alcoholic liver disease have abnormalities of humoral immune function such as increased levels of immunoglobulin A and autoantibodies to acetaldehyde adducts of liver proteins. Studies using immunofluorescence have demonstrated immunoglobulin binding to the heart in patients with both idiopathic dilated cardiomyopathy and alcoholic heart muscle disease. There are therefore grounds to believe that alcohol, or a metabolite, may play a part in causing humorally mediated autoimmune organ damage after prolonged alcohol abuse. Accordingly the hypothesis tested in this part of the thesis, using Western immunoblotting, was that patients with alcoholic heart muscle disease have circulating antibodies to acetaldehyde-modified cardiac proteins as a result of chronic alcohol ingestion.

In order to isolate the proteins detected by Western blotting for further analysis it was necessary to develop a method for electroeluting proteins from a polyacrylamide gel whilst maintaining electrophoretic separation. In addition, studies of T cell responses to fractionated human myocardium might allow the development of culture supplements to improve the proliferation of cells cultured from EMB. A device to allow such experiments was designed, constructed and tested using endothelin receptors, which are known to be expressed in human myocardium.

This thesis is therefore presented in three sections. The first deals with the lymphocyte culture technique, the second with immunoblotting and alcoholic cardiomyopathy and the third with electroelution of myocardial proteins.

Part I

Cardiac Transplantation

2. Cardiac Transplantation

The first human heart transplant was performed in 1967 (Barnard, 1967). The growth of heart transplantation since then has been such that the sixth report of the Registry of the International Society for Heart Transplantation, which included data from nearly all heart and heart-lung transplants, numbered orthotopic heart transplants at 9,139 as of March 1989 (Heck, 1989). As the main indications for transplantation were cardiomyopathy and ischaemic heart disease, this procedure is the only realistic chance of long term survival, since, even with optimal medical therapy for end stage cardiac failure, the one year mortality was around 39% (Consensus Trial Study Group, 1987). By 1988, nearly 2,500 heart transplantations were being performed annually. From these data, the five-year survival for patients on triple therapy (cyclosporin, azathioprine and steroids) was 81.9% and the ten year survival 73.3%.

Most cardiac transplants have been orthotopic, where the recipients diseased heart is removed leaving the great vessels and posterior atrial walls, to which the donor organ is connected. A much smaller number of heterotopic transplants, in which the donor organ is anastomosed to the great vessels and the native organ is left in situ, have been performed (including the original operation in 1967). The technical aspects of the surgical procedures involved will not be discussed further here.

The availability of cardiac allografts has been limited and it is only recently that cardiac transplantation has expanded beyond the two centres, Harefield and Papworth Hospitals, where the current UK program began in 1979. The UK transplant program was not activated in earnest until two key developments occurred. Firstly, the emergence of improved immunosuppressive regimes (including the use of cyclosporin A) and secondly, a safe and effective method for taking endomyocardial biopsy samples from the right ventricle transvenously (Caves, 1973). By 1985 these two centres had performed 186 transplants in 179 recipients (Pomerance, 1985).

The survival of cardiac allografts is particularly crucial since, as opposed to renal transplants where graft failure can be treated with dialysis, graft failure will mean death unless re-transplantation is possible. The three major hazards to survival of cardiac allografts are rejection, infection and coronary occlusive disease (Sharples, 1991). There is an inevitable connection between these hazards since the risk of rejection is reduced by using immunosuppression which in turn puts patients at risk of opportunistic infections. There is thus a balance of risk whereby sufficient immunosuppression is used to avoid rejection but not so much as to make infection highly likely. Coronary occlusive disease is perceived as a form of chronic rejection and hence it too may be related to immunosuppression protocols.

In order to detect rejection and tailor immunosuppressive treatment appropriately endomyocardial biopsy (EMB) is performed routinely and frequently in the early post-transplant period; with biopsies being performed less frequently as patients survive beyond the early high-risk period for rejection (Pomerance, 1985). There is no other reliable means of detecting potentially fatal rejection events. Thus a great deal of reliance is placed on the histological interpretation of the morphological changes observed in serial EMB.

2.1. The Histology of Endomyocardial Biopsies

In the early years of cardiac transplantation various grading systems for endomyocardial biopsy histology were described, most based on the proposals of Billingham and colleagues (Caves, 1973a and Billingham, 1981). However, each centre had its individual interpretation (for example: Pomerance, 1985; Kemnitz, 1987; Kottke-Marchant, 1990). This made it difficult to determine the absolute and comparative incidence of significant rejection as well as preventing comparison of immunosuppression regimes between centres.

To address these problems several grading systems have been proposed culminating in the most recent formulation of the Heart Rejection Study Group (Billingham, 1990). This is intended to standardise the reporting of EMB, to provide a framework for further refinements of description of histological rejection and to permit comparative research between centres. Acute rejection is histologically graded 0 to 4 (Table 1). The grading system, in common with all previous systems, is based on the degree of cellular infiltration and associated myocyte damage or death. Though cellular infiltration is important the main feature of acute rejection is cellular damage. Hence degree of myocyte injury is central to this system. It is assumed that there must be a connection between myocyte damage and graft dysfunction.

This grading system, as with all others, does not represent an inevitable progression through grades 1 to 4 for any individual. Each grade of rejection may occur without there having been previously observed rejection at the next lowest grade. It has been shown that progression of rejection from one biopsy to the next may follow particular patterns but not a logical sequence through the grades (Gallo, 1992). It is also felt that grade 2 (or focal moderate) rejection is a distinct entity which rarely signifies incipient severe rejection and which usually resolves despite the presence of myocyte damage (Gallo, 1992).

Table 1. Working Formulation of Heart Rejection Study Group

This table shows the working party formulation for the description of rejection on histological slides of EMB. Alongside the new grades in column one are the new terms in the central column. The old, or equivalent terms are in the third column. The table is divided half-way down by a double line. This indicates the grade (IIIA) at or above which enhanced immunosuppression is likely to be used to treat acute rejection. Additional information required by this system is whether there were less than 4 biopsy fragments, evidence of humoral rejection, presence of "Quilty effect" (with or without myocyte encroachment), ischaemia (within or beyond three weeks of transplantation), infection or lymphoproliferative disorder and any other features.

Grade	"NEW" Nomenclature	"OLD" Nomenclature
0	No rejection	*No rejection*
IA	Focal (perivascular or interstitial infiltrate)	*Mild rejection*
IB	Diffuse but sparse infiltrate	
II	One focus only with aggressive infiltration and/or focal myocyte damage	*"Focal" moderate rejection*
IIIA	Multifocal aggressive infiltrates and/or myocyte damage	*"Low" moderate rejection*
IIIB	Diffuse inflammatory process	*"Borderline/Severe"*
IV	Diffuse, aggressive polymorphous +/-oedema,+/-haemorrhage,+ /-vasculitis	*"Severe acute " rejection*

Denoted by a lesser grade: "Resolving rejection"
Denoted by Grade 0: "Resolved rejection"

Grade 3a rejection is commonly used by clinicians as the level at which additional immunosuppression would be utilised (Cary, 1994 - personal communication). However, there are no data from controlled clinical trials to support this arbitrary cutoff point. Histopathologists point out that the grading system does not necessarily reflect a continuum of rejection and that is it merely a descriptive tool. Despite this, the histology report is used to guide therapy, in a fashion based on Billinghams original work. The clinical relevance of the morphological changes described by Billingham were confirmed by observations in transplanted dogs (Caves, 1973b; Caves, 1975 and Billingham, 1978). In addition, it was found at autopsy that patients who suffered sudden cardiac death after transplantation had rejection (Chomette, 1985). Since lesser degrees of rejection were rarely noted it was assumed that this histological appearance had pathological significance. The UK experience has reflected this. Histopathological examination of hearts from patients dying suddenly,

who were not suffering from infection, has shown the presence of rejection as defined histologically. However, the degree of rejection was not always severe and other pathologies were also noted, including coronary thrombosis, pulmonary embolism, tamponade and changes of chronic rejection (Pomerance, 1985).

Since transplanted hearts are generally denervated the earliest indication of acute rejection might be death, though careful clinical examination might reveal a third heart sound suggestive of left ventricular dysfunction and reduced voltages may be observed on the electrocardiogram (ECG). These clinical indications are relatively insensitive and hence screening of the state of the myocardium in transplanted patients was instituted using endomyocardial biopsy. Thus, subsequently, patients were treated empirically when higher grades of rejection were observed and apparently fewer suffered sudden cardiac death. Again, there are no controlled studies to support these decisions. In the early days of transplantation there were few patients on which to base adequate trials and patient survival was justifiably held to be vital. Any manouevre likely to ensure survival was embraced.

Now that transplant programmes are larger it is not possible to perform these trials. They would not be ethical. Comparisons among different immunosuppressive agents are made but observational studies of untreated rejection at higher grades could not be performed. Transplantation of other organs involves similar questions but it must be remembered that the immunological behaviour of the kidney and the liver for example is not identical to that of the heart.

A central question about the relationship between the nature of the rejection process in relation to cells infiltrating the myocardium cannot therefore be satisfactorily answered. Hence, studies in this area use the histopathological appearance of the transplanted heart as a surrogate "gold standard" to judge the well being and likely prognosis of individual patients, even though this standard has not been validated. This situation is not satisfactory. This thesis in turn is obliged to use this system for comparison with results obtained from the various experiments performed.

A newer, objective and more predictive approach is required if the treatment and prevention of acute rejection is to be formalised and studied in multi-centre trials. It is also of concern that current treatment regimes, based as they are on the empirical observations of small numbers of observers, may not be optimum or worse still may be contributing to patient morbidity and mortality. The role of routine histological examination of EMB therefore requires to be re-examined.

2.1.1. Endocardial Lymphocytic Infiltrates

The "Quilty effect" was named after the first patient in whom it was described (Imakita, 1988). It refers to endocardial and sub-endocardial cellular infiltrates consisting predominantly of T cells but including B cells and macrophages. The term "endocardial lymphocytic infiltrates" (ELI) is synonymous. The significance of ELI is uncertain, although a number of suggestions have been made. Radio and others suggested that, although ELI may be associated with myocyte damage histologically, they are distinct from acute rejection (Radio, 1991). Kottke-Marchant et al have suggested that ELI are consistent with an early lymphoproliferative lesion, such as those associated with Epstein-Barr virus (EBV) and cyclosporin therapy (Kottke-Marchant, 1989; Suit, 1989). In one study, comparing the incidence of ELI among patients treated with different immunosuppression protocols, ELI appeared to be related only to cyclosporin therapy, being very rare in patients taking azathioprine and steroids (Forbes, 1990). In addition, other workers have disputed the association of ELI with EBV. Nakleh et al studied biopsy material from 19 patients using in-situ hybridisation for EBV genomic sequences and obtained negative results. They also found no serological evidence of post-transplant infection with EBV (Nakhleh, 1991).

After transplantation some patients develop more florid lymphocytic infiltration of their allograft - post-transplantation lymphoproliferative disorder. In cardiac transplant recipients this was associated with the use of the monoclonal antibody OKT3 in immunosuppressive therapy (Swinnen, 1990). A range of studies have examined patients with post-transplantation lymphoproliferative disorders and reported an association with EBV (Cleary, 1984a; Cleary, 1984b; Wreghitt, 1989). Abu-Farsakh et al used in-situ hybridisation (ISH), the polymerase chain reaction (PCR) and dot-blots for EBV genomic sequences to confirm the presence of EBV in lymphocytes obtained from the heart of a patient who died from post-transplantation lymphoproliferative disorder (Abu-Farsakh 1992).

Thus there is evidence to associate lymphoproliferative disorder with EBV but Nakleh's study indicates that ELI do not appear to have this association. The proposal that ELI are somehow a precursor of later lesions and that proliferation is EBV driven is not supported by this work. However, Naklehs study was small and used ISH, rather than PCR or southern blot analysis which would have been more sensitive for small copy numbers of viral genome per cell. The earliest lesions might not be associated with a strong presence of EBV and so serological responses would also be lacking. In studies on patients with liver allografts Randhawa et al found expression of RNA for an EBV gene (EBER-1) expressed early on in latent infection, before the development of clinical or histological evidence of lymphoproliferative disorder (Randhawa,1992).

Because of the anatomical location of ELI, they could easily be included in EMB and therefore could influence culture results. Previous studies have not addressed this question and since the reporting of ELI may not previously have been uniform, their effect on such studies cannot be assessed.

2.2. Other Methods of Monitoring Cardiac Allografts

Standard non-invasive methods of monitoring cardiac transplant recipients include clinical assessment, routine biochemical and haematological tests, and radiology (including chest X-rays and regular coronary angiography to look for coronary occlusive disease). Other methods of monitoring patients for rejection of heart transplants have included more traditional means of non-invasively studying the heart such as the surface ECG (Schroeder, 1974), echocardiography (Valantine, 1987) and even phonocardiography (Caves, 1974). The ECG was the mainstay of allograft monitoring in the early days of cardiac transplantation. With the twelve lead ECG it was found that rejection was associated with a reduction in R wave voltages which reversed after treatment. Other changes such as right axis deviation and atrial arrhythmias were also observed (Wallwork, 1984). However, the ECG changes probably lag behind histologic abnormalities by a period of days and may not be sensitive enough to detect early rejection. Echocardiography may also detect graft dysfunction but this has to be quite severe to be detected by this technique. Phonocardiography is primarily of historical interest in this context.

In addition to these routine clinically indicated tests a number of research tools have been used to determine if there are non-invasive alternatives to performing frequent EMB.

Cytoimunological monitoring (CIM) developed in Munich in 1983 is concerned with detecting activated lymphocytes in peripheral blood samples from cardiac transplant patients. This method is highly sensitive for rejection (as diagnosed by histology) but has a false positive rate of 28% (Ertel, 1985 and Hanson, 1988). However a multi-centre trial of this method found that of 12 centres involved only six could reliably apply the method (Schubel, 1990).

Reader et al used limiting dilution assays (LDA) in experiments where peripheral blood lymphocytes from recipients of cardiac transplants were co-cultured in a range of dilutions for 10 days with irradiated donor splenocytes (Reader, 1990). These cells were then tested for cytolytic activity against donor cell targets. The reasoning behind LDA, put simply, is that the series of dilutions allows calculation of the frequency of cytotoxic T cell precursors (CTLp) present in the original sample. Complex statistical methods are involved and stringent conditions apply to processing the data obtained. In this case 12 cardiac transplant recipients were studied. The

preoperative CTLp frequency did not predict patients likely to undergo postoperative rejection but when measured longitudinally it appeared that the frequency of CTLp was higher in patients undergoing rejection compared with those who were not. The authors concluded that this method would be of value in monitoring cardiac transplant patients. The small numbers of patients involved and the nature of the method leave this conclusion open to question. Since the culture period alone takes ten days, any patients with severe acute rejection would have suffered irreversible damage before a result from this assay could be available. In addition, without data on the variability of CTLp frequencies in a larger number of individuals with and without rejection, over a long period of time it is difficult to see how fluctuations could easily be interpreted. Thus, this labour intensive method appears to be unlikely to find widespread application in transplant centres in any application other than as a useful research tool.

In conclusion, to date there are no completely effective non-invasive markers of acute rejection and so EMB continue to be monitored closely in the early months after transplantation in order to detect and treat acute rejection. At present no method offers the ability to predict the likelihood of future rejections episodes.

2.3. The Immunology of Acute Rejection

The relationships between the various cells involved in rejecting solid organ transplants are complex. A large range of cells and responses involving many molecules results in the "immune response" to an allograft and it is difficult to detail them all in the space available. Halloran has recently reviewed the molecular immunology of transplant rejection (Halloran, 1993). The molecules involved include: the immunoglobulin (Ig) gene superfamily of receptors, adhesion molecules and cytokines (and their receptors). These molecules can be divided into:

- Antigen presenting structures
- Antigen recognition structures
- Molecules involved in cellular interactions during antigen recognition

Molecules in the third group can be thought of as 'adhesion molecules', which can be further grouped into the Ig superfamily, the selectin family and the integrin family. The cytokines involved in acute rejection are numerous but include interleukins, interferons, growth hormones and colony stimulating factors.

In terms of cardiac allograft rejection, at a cellular level, these molecules are found in association with, or elaborated by, T cells, B cells, monocyte/macrophages, natural killer cells, dendritic cells, endothelial cells and cardiac myocytes. Thus a large range of cells are involved in potential and actual interactions culminating in the process

recognised histologically and clinically as "rejection". The relationships of these various cells and molecules are briefly described below.

2.4. The Major Histocompatibility Complex

In the 1940s Medawar showed that a second skin graft from one rabbit to another was rejected quicker than the first. If the second graft was accompanied by one from a different donor than the first, then the additional graft did not suffer accelerated rejection (Medawar 1944 and 1945). Thus the first graft sensitises the immune system of the recipient to donor antigens and it therefore responds faster and more powerfully on re-challenge. Larger grafts were rejected sooner and more aggressively. These experiments showed that rejection of skin grafts displayed latency, dose effect, specificity and memory - all key features of an acquired immune response. In addition, it was subsequently shown that the ability to reject grafts could be transferred with lymphocytes from a previously grafted individual (Mitchison, 1954). Thus the functional ability to reject allografts is found in the lymphocyte population.

Further experiments in mice showed that responses against skin grafts were related to inheritance of H-2 antigens controlled by the genes of the mouse major histocompatibility complex (MHC) on chromosome 17 (Klein, 1979). The major histocompatibility antigens on the surface of cells are those against which an immune response may be mounted. There are also many other "minor" histocompatibility antigens which do not map to chromosome 17 and which can be responsible for strong rejection responses against allografts (reviewed by de Bueger, 1993). Medawar's group showed that similar responses to those demonstrated in mice against skin grafts occurred in man (Gibson, 1943).

The central process in rejection involves the recognition of graft proteins by the recipients immune system. Whole proteins are not recognised; rather they are broken down and processed into peptides before recognition can take place. The means by which recognition occurs is via MHC presentation of peptides to T cells via T cell receptors (TCR). The various ways by which peptides can be brought to this interaction are numerous. There are a number of antigen presenting cells types which may present peptide in the context of class I or class II MHC molecules. In addition there are various other factors which may strengthen the cellular interactions involved.

The human MHC gene locus on chromosome 6 has approximately 4 million base pairs encoding genes for class I, class II and class III antigens (Trowsdale, 1991). Because lymphocytes were used in initial typing experiments of class I and class II gene products they are also known as "Human Leucocyte Antigens" (HLA). Class I HLA molecules are involved in cytotoxic T cell responses and Class II HLA molecules in T helper cell responses and some cytotoxic functions. Class III proteins are involved

in the complement system. Genes for tumour necrosis factor, an important cytokine, also lie in this region of chromosome 6 (fig.1).

The genetic organisation of the MHC is complex. A rudimentary and highly simplified map is shown (fig.1) but a more detailed and up to date map has been published (Campbell, 1993). There are three class I genes, A, B and C, though there are a number of class I-like genes in addition. The gene for β_2-microglobulin lies outside the MHC region (on chromosome 15). The class II region includes genes for DP (four), DN(one), DO(one), DQ(five) and DR(variable), with a number of pseudogenes. The MHC genes are highly polymorphic and have hypervariable regions which correspond with the peptide binding areas on class I and class II molecules.

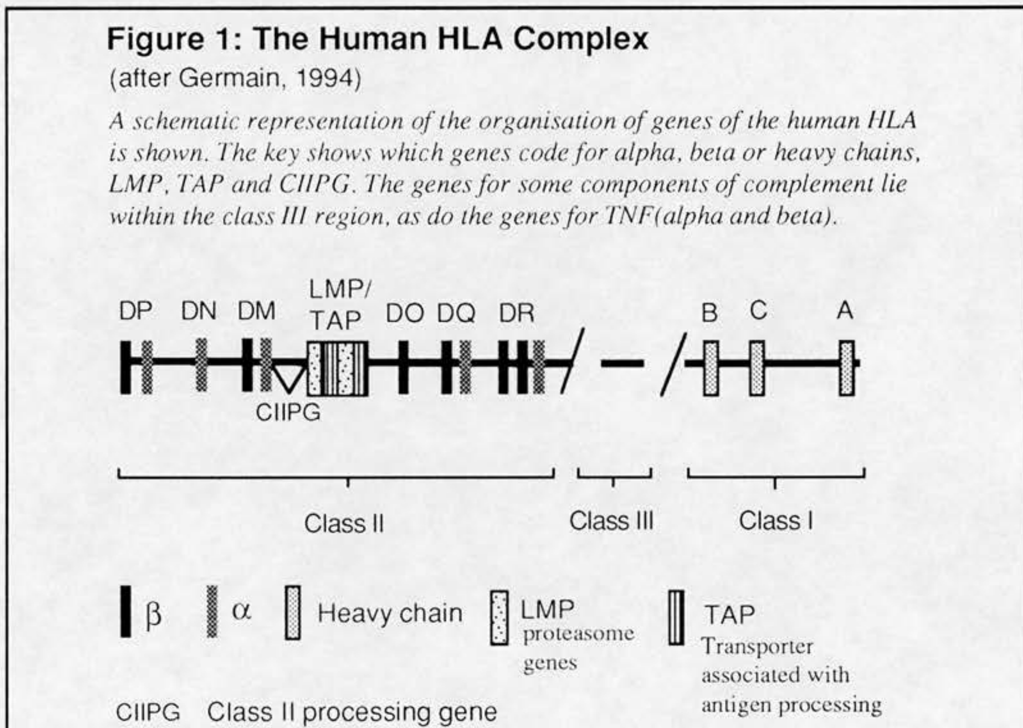


Figure 1: The Human HLA Complex
(after Germain, 1994)

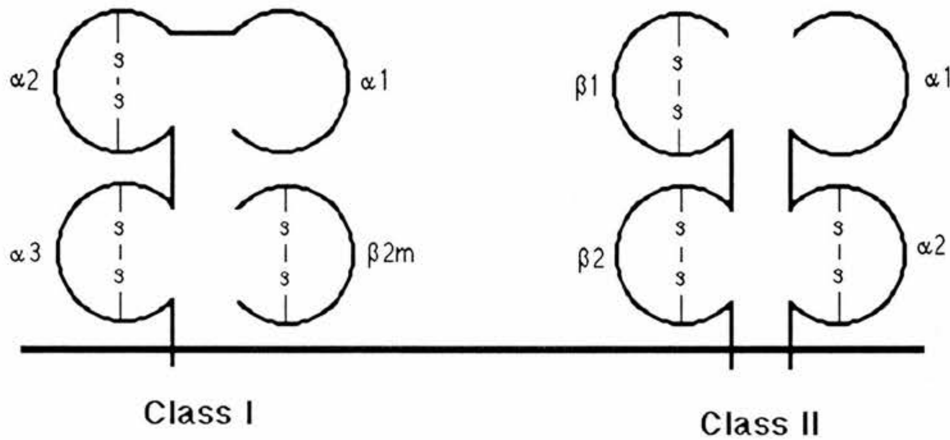
A schematic representation of the organisation of genes of the human HLA is shown. The key shows which genes code for alpha, beta or heavy chains, LMP, TAP and CIIPG. The genes for some components of complement lie within the class III region, as do the genes for TNF(alpha and beta).

MHC molecules are part of a family with related structures - the Immunoglobulin (Ig) gene superfamily, which has evolved through economical use of the gene for the Ig domain (Williams, 1987). The Ig domain has a molecular weight of around 12kD and consists of approximately 90 amino acids. Apart from immunoglobulins, a number of molecules contain Ig domains: the T cell receptor (TCR), CD8, CD4, Class I and Class II molecules, CD3ε, CD2 and a range of adhesion molecules (e.g. ICAM-1, ICAM-2, VCAM, LFA3). β_2-microglobulin, a component of class I, essentially consists of a single Ig domain with a disulphide bond (fig.2).

Class I and class II molecules each have four Ig domains (fig.2). Class I consists of an α-chain (with three Ig domains) along with β_2-microglobulin. Class II is made up of an α-chain and a β-chain. These are arranged in each type of molecule such that two of the Ig type domains are aligned so as to form a 'peptide binding groove'. Class I and II molecules also have binding sites for CD8 and CD4 respectively.

Figure 2: Class I and Class II Molecular Domains

This cartoon shows the organisation of the Ig domains in class I and II molecules (after Germain, 1994). These membrane bound glycoproteins are very similar. Class I has three alpha chains (α1 and so on) with two disulphide bonds in the second and third (represented as -s-s-). The fourth domain is made up of beta-2 microglobulin (β2m) which also has a disulphide bond. Class II molecules have two alpha and two beta chains with a total of three disulphide bonds.



Recently the structure of HLA-DR1 from human B cell membranes was determined using X-ray crystallography and was shown to be very similar to class I molecules, as had been predicted (Brown, 1993). It appears that class II forms heterodimers which themselves may dimerise to form a "dimer of dimers" which might be able to interact with two TCR complexes. This complex may play a role in T cell signalling and might stabilise the interaction between antigen presenting cell and T lymphocyte (Ploegh, 1993). It would appear that a significant difference between class I and II is in the size of peptides they may bind. Class I has a peptide binding pocket blocked at either end such that peptides of 8 to 10 amino acids in size can be bound. Class II on the other hand has an open pocket which can allow peptides to protrude beyond it, allowing peptides of 15 to 18 residues to be bound (Ploegh, 1993).

Individuals express one or two HLA class I and class II molecules on the relevant cells, according to whether they are homo- or heterozygous at the MHC locus. Most nucleated cells, as well as platelets and erythrocytes, express class I antigens, but class II is found mainly on cells of the immune system and probably some epithelial cells. Rose et al found little evidence for class I expression on normal myocytes but

after transplantation demonstrated induction of expression of MHC molecules on myocytes, particularly class I, which implies a role in the rejection process (Rose, 1986).

It has been shown that class I MHC presents endogenously derived peptides and class II MHC presents peptides from exogenous sources (Brodsky, 1991; Malnati, 1992). Thus CD8+ T cells recognise virally infected cells which express proteins encoded by viral genes on their cells surface whereas CD4+ T cells recognise peptides originating from extracellular organisms which have been appropriately processed. This results from the different means by which class I and II are assembled and transported to the cell surface. Class I is assembled from component chains in the endoplasmic reticulum (ER) in the presence of peptide, with which it associates. The class I-peptide complex is then transported to the cell surface where presentation of the peptide can occur. The source of the peptide is from cytoplasmic proteins which are broken down and then transported into the ER. Class II molecules are made in the ER and then transported to the endosome, an acidic membrane bound compartment. Once there the class II molecule is able to bind peptides which have been formed by the breakdown of proteins which have entered the endosome. The source of these proteins may be from the cell surface via endocytic vesicles, the cell membrane and also from the cytosol (which is how class II can present endogenously derived peptides). Once peptide is bound the class II-peptide complex is transported to the cell surface.

Allograft rejection may be thought of as an inevitable consequence of a normally functioning immune system which has evolved to defend against infectious agents. The cellular immune system identifies and kills infected cells by recognising foreign peptides in association with MHC molecules found on the cell surface. The MHC antigens are the means by which the body recognises its own cells as "self" and unfortunately they provide a means by which tissue, such as an allograft, may be recognised as foreign and destroyed.

The immune system normally attacks its own cells only when they are expressing novel proteins at the cell surface. This may happen, for example, in the case of cancer cells. In viral infections cells may be programmed by the virus to produce certain proteins, which are then presented by the cell in association with MHC class I molecules. Cytotoxic T cells recognising this combination would kill these and any other cells with the same class I molecules presenting the peptides in question. They would not recognise cells with different MHC antigens; a process known as MHC restricted killing (Zinkernagel, 1974; Zinkernagel, 1979; Rosenthal, 1973). In the same way the immune system may recognise peptides presented by foreign MHC molecules and initiate a response against the cells in question. It is not known whether rejection involves initial destruction of foreign cells and the processing of their MHC molecules

into peptides to be presented in association with recipient MHC molecules or whether these molecules are recognised intact on the surface of the allograft cells.

Allogeneic responses are stronger than syngeneic responses and, in this respect at least, transplant immunology differs markedly from fundamental immunology (Paul, 1993). Antigen specific syngeneic CTL recognise peptide in the context of self-MHC molecules but is not clear whether alloreactive CTL use a similar mechanism. They may recognise foreign MHC alone with no peptide bound ("empty MHC") (Elliot, 1990), they may recognise a conformational change induced in the MHC by the bound peptide, or they may recognise both the peptide and the foreign MHC together (Alexander-Miller, 1993). Thus the exact nature of alloreactivity is difficult to determine.

The alloresponse may be engendered by dendritic cells within the allograft as stated above. However, in the cellular infiltrate in an allograft there are also B cells which are capable of presenting antigen to CD4+ cells, further complicating the picture. B cells may bind solublised donor MHC, take it up, process it and then present it in the peptide binding groove of their own class II MHC molecules. The interaction between these cells being strengthened by adhesion molecules (Halloran, 1994). Thus the alloresponse may involve a range of presenting cell types of donor or recipient origin, which may present different donor peptides in the context of their MHC molecules.

Certain HLA molecules are associated with particular "autoimmune" diseases, where the immune system appears to be involved in causing tissue damage. For example, HLA DR3 is associated with dermatitis herpetiformis (Svejgard, 1983), HLA DR4 with insulin dependent diabetes mellitus (Svejgard, 1983) and idiopathic dilated cardiomyopathy (Carlquist, 1991) and HLA B27 with ankylosing spondylitis and Reiters disease (Svejgard, 1983). This may reflect the efficiency of these molecules at presenting certain processed proteins to the immune system and may relate to why particular individuals are more prone to develop acute rejection than others.

The role of HLA antigens in transplantation has been extensively studied. It is known that prospective matching of HLA type between donor and recipient has a beneficial effect on organ survival. This was shown for renal transplants some years ago, particularly with regard to matching for HLA DR (Ting, 1978; Gilks, 1987; Thorogood, 1990). A large study of HLA matching for 1386 cadaveric renal transplants confirmed this benefit (Takemoto, 1992). There was a one year survival rate of 88% for matched first grafts compared with 79% for mismatched grafts. More recently a large collaborative study of cardiac transplants has demonstrated a beneficial effect of matching for HLA A, B and DR on graft survival at three years (Opelz, 1994). The survival of the 128 donor hearts which had no HLA mismatches with the recipient was 83% at three years, compared with 71% for hearts with three to six mismatches. As a result of this study the authors and others have suggested that prospective

matching should eventually be introduced to cardiac transplantation in a similar fashion to that employed in renal transplant centres. It is also proposed that less immunosuppression may then be necessary since well matched grafts may not require such intensive regimes (Morris, 1994). The HLA system is so polymorphic that Opelz et al's study would not have the power to determine the relationship of individual HLA types to rejection or graft loss.

2.5. Antigen Presenting Cells

In order to respond to antigen, naive CD4 T cells (primary response) require appropriate presentation of antigen in the context of MHC class II molecules along with certain co-stimulatory signals. Cells which are able to function as antigen presenting cells (APC) in this context are macrophage/monocytes, dendritic cells and B cells (because these three cell types express class II molecules and can be present in the allograft). The APC for early rejection responses may be of donor origin but recipient APC may be involved in subsequent episodes. The route whereby antigen is presented in the context of donor APC is sometimes said to be "direct presentation" and for recipient APC to be "indirect presentation".

In secondary responses any cell which bears MHC class II can function as an antigen presenting cell. There is evidence that dendritic cells are still potent in this regard.

Maximal stimulation of CD4+ T cells occurs when antigen is presented along with the appropriate costimulatory signal (the so called "second signal"). It appears that the main molecules involved in costimulation are those known as B7 and B7.2, which bind to CD28 and CTLA-4 receptors on T cells. In the absence of a co-stimulatory signal on the APC, T cells which recognise antigen may become anergic and fail to proliferate (Janeway, 1994).

There is evidence that donor APC may be responsible for the initial presentation of antigen to host T cells. The most likely candidates for this function are dendritic cells which are very potent stimulators of T cell responses (Austyn, 1991). These leucocytes are present in most tissues of the body and are able to present antigens along with signals for T cell activation. Dendritic cells are able to stimulate the mixed lymphocyte reaction (MLR) and can activate both CD4+ and CD8+ cells (Inaba, 1987). Dendritic cells have therefore been described as 'passenger leucocytes', present in donor allografts and able to elicit proliferative responses among recipient T cells (Austyn, 1993). In a murine cardiac transplant model, migration of donor leucocytes from cardiac allografts into the recipient's spleen has been demonstrated (Larsen, 1990). Dendritic cells may acquire antigen in the allograft and then migrate to lymph nodes (or the spleen) where they may lose some cell surface markers, upregulate class II

expression and begin associating with clusters of T cells (Knight, 1993). Tyerman et al showed in a rat cardiac allograft model that pretreatment of the graft to reduce dendritic cell content contributed to prolongation of graft survival (Tyerman, 1990).

Another possible interface between the allograft and recipient lymphocytes is the vascular endothelium. Endothelial cells are able to function as antigen presenting cells (Pober, 1991), can be induced to express class II molecules and may rapidly change following transplantation from being of donor to recipient origin through re-endothelialisation (O'Connell, 1991). There is evidence for an antibody response to the endothelium in cardiac transplants and immunoctyochemical evidence of endothelial damage in rejecting cardiac allografts has been shown (Dunn, 1992; Hengstenberg, 1990). Jutte et al have shown cytotoxic T cell responses against donor heart endothelial cells among allograft infiltrating cells cultured from EMB taken from four patients (Jutte, 1993).

Thus there is more than one candidate for stimulating allogeneic responses against the allograft. Dendritic cells may present donor peptides in the context of foreign MHC or host dendritic cells may infiltrate the graft and subsequently act as APC. Vascular endothelial cells may present donor peptides in the context of self-MHC and may themselves suffer immune mediated injury as a result, which might provide a favourable environment for amplification of the immune response to the graft.

2.6. Cytokines, TH1 and TH2 Cells

Cytokines are proteins which bind to specific receptors and provide cellular signals. The range of cytokines is diverse and they are involved in controlling the functions of haemopoietic cells, lymphopoietic cells and cells involved in immune responses. They include the interleukins (IL-1 to 10), the interferons (α, β and γ) tumour necrosis factor (TNFα and β) and the colony stimulating factors (CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF). These substances are produced by and act on T cells, B cells, macrophages, monocytes, endothelial cells, fibroblasts and bone marrow stem cells.

CD4 cells are the lymphocyte subset most closely associated with cytokine production though many cell types can elaborate these substances. In the mouse there are patterns of cytokine expression which differentiate in vitro cultured CD4+ T helper cells into two groups: TH1 and TH2 cells (Mosmann 1989). TH1 cells secrete IFNγ, IL-2 and TNFβ. TH2 cells secrete IL-4, IL-5, IL-6 and IL-10. Both TH1 and TH2 can produce IL-3, TNFα and GM-CSF. TH1 cytokines can be produced by large granular lymphocytes and CD8+ cells. There are no monoclonal antibodies which mark out TH1

or TH2 cells as distinct from among CD4+ cells. In addition, these activities are yet to be described in man.

The roles of these cells appear to centre on regulating B cell responses but they may also have effects on CD8+ cells. TH1 cells are able to induce delayed type hypersensitivity responses whereas TH2 cells are not. The effects of TH1 cells on B cells are to selectively promote IgG2a responses, but they can also elicit IgM and IgG3 (Coffman,1988). TH2 cells on the other hand can promote IgG1 and IgE responses effectively, as well as IgM, IgG3 and IgA (Kuhn, 1991). Another feature of TH1 and TH2 cells is that they can crossregulate each other, each tending to inhibit or antagonise the effects of the other (Mosmann, 1991a).

In transplantation, the stimulation of TH1 type responses could result in rejection processes along the lines of a delayed type hypersensitivity response. The regulation of the response to allograft antigens by CD4+ T cells would be through cytokine production and it may be that the murine pattern of TH1/TH2 is reflected in the analogous situation in man. Janeway and Bottomly suggest that the presence of MHC class II dimers may influence the type of response produced as a result of differing ligand density on cells. They argued that MHC class II dimers presenting self-peptide would not stimulate CD4+ cells whereas the presence of foreign peptide on one or other member of the dimer would result in a TH2 type response. TH1 type responses might be seen with higher concentrations of foreign peptide such that it is bound to both members of the class II dimer (Janeway, 1994).

Ruan et al (Ruan, 1992) demonstrated, using immunohistochemical techniques, the presence of IL-2 in lymphocytes observed in rejecting EMB. They showed that IFNγ expression increased with degree of rejection and they occasionally detected IL-6. This study suggests that both TH1 and TH2 type responses were occurring and supports a role for cytokines in the rejection process in human heart transplants.

2.7. Adhesion Molecules

The interactions between lymphocytes binding to MHC associated with peptide are assisted by proteins present at the cell surface of both the antigen presenting and the responding cell. As stated above these consist of the selectins, the integrins and Ig superfamily members (reviewed in Moody, 1992).

The selectins consist of L-selectin, found on leucocytes; E-selectin found on endothelial cells and P-selectin which is present on platelets and endothelial cells. They are involved in weak early binding interactions between lymphocytes and endothelial cells. The selectins have transmembrane and intracytoplasmic segments as well as an N-terminal lectin domain, an epidermal growth factor like domain and then several 'short consensus repeats'. The ligand for L-selectin is E-selectin. L-selectin is the

ligand for the other two selectins. E-selectin is induced on endothelial cells by IL-1 and TNF.

The integrins are probably involved in secondary binding interactions and their adhesion interactions are stronger than those of the selectins. The integrins are made up of α and β chains and they are found expressed on a range of leucocytes, platelets and other cells. They divide into $\beta 1$, $\beta 2$ and $\beta 3$ sub-groups. The $\beta 1$ subgroup consists of very late antigens (VLA) 1 to 6, the $\beta 2$ group includes leucocyte functional antigen-1 (LFA1), and the $\beta 3$ group includes gpIIb and IIIb as well as 'VNR' (Halloran, 1993). These molecules are probably involved in cellular interactions once activation has occurred. Their ligands include laminin, collagen, fibronectin, vascular cell adhesion molecule-1(VCAM-1), intercellular adhesion molecule-1 (ICAM-1) to 3, vitronectin and fibrinogen.

The third group of adhesion molecules, the Ig superfamily members, includes ICAM-1, ICAM-2, ICAM-3, LFA-2, LFA-3 and VCAM-1. These are found on endothelial cells and a range of leucocytes. Their ligands are mostly integrins with which they form fairly strong interactions and their expression tends to be increased by IFNγ, TNF and IL-1.

The complexity of the total number of possible interactions between these molecules cannot easily be represented as there is such a large number. One important aspect of these molecules is that they may have a role in cellular signalling in addition to cell adhesion.

In human cardiac transplant recipients the expression of ICAM-1, VCAM-1 (vascular cell adhesion molecule) and ELAM-1 (endothelial leucocyte adhesion molecule) has been studied using immunohistochemistry. Briscoe et al found that ICAM-1 and VCAM-1 increased in capillaries in the presence of cellular rejection but they did not find evidence of ELAM-1 expression with rejection (Briscoe, 1991). In a study involving 83 patients, Qiao et al found little evidence of ELAM-1 expression. They did however find ICAM-1 staining in capillary endothelial cells and lymphocytes in EMB from patients with rejection (Qiao, 1992). There was also ICAM-1 staining in lymphocytes from ELI in 9 of 10 cases in this study. Though no causal inferences can be made merely from the presence of these adhesion molecules, their presence in the context of rejection does hint at a potential role.

2.8. The T Cell Receptor

T cells bear on their surface receptors which belong to the Ig gene superfamily and which strongly resemble membrane-bound Fab fragments of immunoglobulin. Designed to recognise antigen in the context of MHC molecules, the T cell receptor (TCR) subserves an analogous function in T cells as bound immunoglobulin does in B

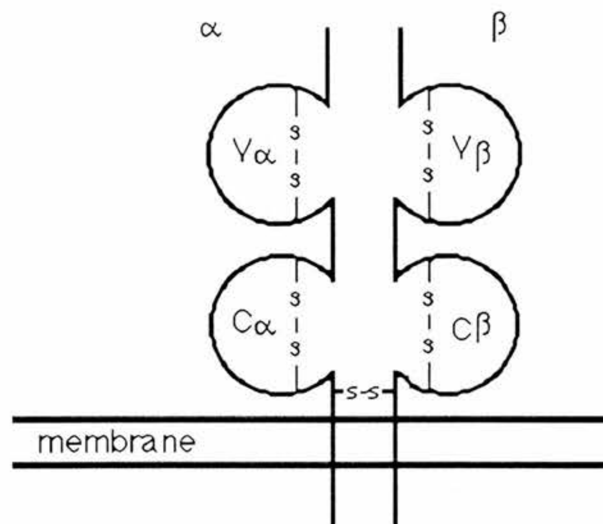
cells. The human T cell receptor in health and disease has recently been reviewed by Moss et al (Moss, 1992).

There are two different TCRs consisting of heterodimers of different chains. The $\alpha\beta$ TCR performs most of the functions required by T cells and is the most common form of TCR, observed on 95% of T cells. The $\gamma\delta$ TCR has unknown function and constitutes a minority of T cells (5%). The structure of the human TCR is illustrated below (fig.3). The molecule is found in association with the CD3 molecule which itself is composed of five different chains (γ, δ, ε, ζ and η in the mouse).

The structure of the TCR relates to its function in that it may allow alignment with the MHC molecule so as to bring the more variable regions of each molecule to interact with the MHC presented peptide. This interaction was modelled by Davis and Bjorkman (Davis, 1988). The complex would involve CD3 molecules flanking the TCR along with the relevant CD4 or CD8 molecule according to the T cell phenotype (Collins, 1993). CD4 and CD8 molecules probably function as co-receptors, binding to structures on the antigen presenting cell and enhancing activation of the T cell. CD4 binds to class II MHC molecules and CD8 to class I (Collins, 1993).

Figure 3. Structure of The Human T Cell Receptor

The cartoon shows the structure of a typical human TCR (after Roitt, 1989). The molecule is a heterodimer of these two chains which have variable (V), constant (C), diversity (D, in the β and δ chains) and joining (J) regions in an analogous fashion to immunoglobulin. The chains have intra-chain disulphide bonds (shown) as well as an inter-chain disulphide bond. The molecule also has a membrane spanning and intracytoplasmic portion. TCR are found associated with CD3 molecules (see text).



The gene for the α chain of the human TCR lies on chromosome 14, for β and γ chains on chromosome 7. The δ chain gene complex lies within the α chain complex.

T cell receptor genes are organised into segments of V (variable), D (diversity), J (joining) and C (constant) genes. These genes combine in developing T cells to produce V(D)J exons which are then spliced together with the C region at the RNA level (Moss, 1992). This organisation, with a large number of gene segments, gives the potential for a great deal of genetic diversity. This is contributed to by the addition of "N region" nucleotides (at V-D-J junctions or Jα-Cα junctions) which are not encoded in the germline. For $\alpha\beta$ and $\gamma\delta$ TCR there are something like 10^{16} possible junctional combinations. Somatic hypermutation, in a fashion analogous to that observed for immunoglobulins, does not occur (Moss, 1992). Thus, of the three potential mechanisms of generating diversity (as seen in Ig): possessing a large number of genes, somatic hypermutation and somatic recombination, the first and third appear to occur with TCR. Presumably the absence of somatic hypermutation helps prevent the generation of T cell clones which have already been selected out at the thymic level.

There are two ways in which the TCR can interact with the MHC. The first is the predominant form of interaction which involves recognition by the TCR of processed peptide presented by MHC molecules. The second involves so-called superantigens which bind to MHC class II molecules and by interacting with specific Vβ elements of the TCR bring about proliferation of T cells. There appear to be endogenous and exogenous superantigens. Endogenous superantigens were detected in mice initially, being predominantly coded for by murine mammary tumour virus sequences and as yet they have not been demonstrated in man (May, 1989). Exogenous superantigens include substances such as staphylococcal enterotoxins (Choi , 1989), toxic shock syndrome toxin-1 (Choi, 1990) and streptococcus pyogenes toxins (Abe, 1991). These antigens do not require MHC matching of APCs and effector cells in order to exert their effects. The clinical effects of these toxins are dramatic and consistent with their ability to activate approximately 50% of T cells. However, the role of superantigens in human disease remains unclear.

2.9. T Cell Activation and Interleukin-2 Receptors

T lymphocytes are normally in a resting state. When they come into contact with antigen presenting cells (APCs) bearing processed foreign antigen, or directly with foreign cells, they become activated. The cells become metabolically active and enlarge prior to proliferation. Cell to cell interaction between T cells and antigen presenting cells involves adhesion molecules. CD2 (or LFA-2) molecules on T cells may interact with LFA-3 (CD58) (Takai, 1987), CD59 (Hahn, 1992) and CD48 (Kato, 1992) on the cell surface of antigen presenting cells. As well as functioning as an adhesion molecule, CD2 may also play a role in T cell activation by transmitting intracellular second

messengers (reviewed in Bromberg, 1993). Intercellular Adhesion Molecule 1 (ICAM-1, CD54) and LFA-1 (CD11a/CD18) may be present on both the APC and the T cell and interact in either direction (Suthanthiran, 1993).

The process of activation proceeds via binding of antigen, which is presented bound to MHC (class I or II), with the T cell receptor (TCR), found on the T cell surface in association with the CD3 complex. The CD4 or CD8 molecule is also involved in this complex and may help to stabilise the cell to cell interaction.

Binding of antigen to the complex triggers a series of intracellular events culminating in phosphorylation of cytoplasmic and membrane proteins. These changes regulate transcription of genes involved in proliferation. A number of receptors which regulate the proliferative response are subsequently expressed on the cell surface. One of the key receptors is the interleukin-2 receptor (IL2R). Interleukin-2 (IL-2) is a 15kD polypeptide produced by T helper (CD4+) cells. Antigen activated T lymphocytes will not divide unless IL-2 is bound (Cantrell, 1984). The effects of the potent immunosuppressive agent, cyclosporine, appear to involve inhibiting the transcription of the IL-2R gene. These effects also seem to apply to other immunosuppressive drugs (FK-506, rapamycin) though these are not the only effects these drugs have (reviewed in Sigal, 1992).

The IL2R has two main polypeptide chains, α (Leonard, 1983) and β (Hatakeyama, 1989) and a third, γ, has recently been described (Takeshita, 1992). At rest mostly α-chains are present on the cell surface. On activation more β-chains are produced than α-chains. There are high affinity receptors for IL-2, made up of α, β, and γ chains. Low affinity receptors for IL-2 are α-chains alone, intermediate affinity receptors being β- and γ-chain heterodimers. Intermediate affinity receptors can mediate cell activation in the presence of high concentrations of IL-2. This may occur with natural killer cells which, at rest, possess more cell surface α-chains than T cells. Concentrations of IL-2 of the order of 500 - 2000 units/ml can activate natural killer cells to produce Lymphokine Activated Killer (LAK) cells, though not all LAK cells derive from NK cells (Philips, 1986). LAK cells may be tested for anti-neoplastic cell activity for example. IL-2 also has effects on other cells bearing IL2R, including B cells and monocytes.

On activation IL2R are present at the cell surface for 7 days and then disappear unless further expression is triggered. In-vitro culture of T lymphocytes therefore requires periodic re-stimulation in order to maintain proliferation and functional activity. Activated T cells, expressing IL2R, will respond to interleukin-2 with rapid proliferation.

2.10. Rejection in Cardiac Transplant Recipients

As with other solid organ transplants, the major obstacle to successful heart transplantation is the host's immune defences, which recognise an allograft as foreign tissue. In the immediate post-transplant period "hyperacute rejection" may occur, mediated by pre-formed cytotoxic antibodies (Weil, 1981). This process is dependent on activation of the complement system and the clotting cascade following binding of antibodies to the allograft and results in rapid destruction of the allograft (Forbes, 1982). Once initiated this process cannot be halted. The presence of antibodies to donor blood group antibodies would result in this type of response which is why cardiac allografts must be matched for blood group (blood group antigens are expressed on endothelial cells). Other types of pre-formed antibodies include those against endothelial antigens of other species as well as anti-MHC antibodies in individuals previously exposed to allogeneic tissues. In xenogeneic transplants (between species) hyperacute rejection is the major process by which organs are rejected, with pre-formed antibodies to glycoproteins on the vascular endothelium being responsible. Depletion of complement using cobra venom factor can reduce the severity of this response (Forbes, 1978).

Subsequently there may be "acute rejection" mediated by the cellular immune system as discussed above. At later stages "chronic rejection" may occur, which manifests as vascular disease, known as "coronary occlusive disease" in cardiac transplant recipients (Hunt, 1983). This is a form of atherosclerosis which may affect both small and large vessels and which is rarely amenable to coronary artery bypass grafting. At four years after transplantation 25% of cardiac transplant recipients were shown to have this form of disease (Baumgartner, 1979). Chronic rejection, as well as being related to cellular mechanisms of rejection, might also be mediated by the humoral immune system (Dunn, 1992). Cramer et al showed in a rat cardiac allograft model that more intensive immunosuppression with FK-506 was able to reduce the atherosclerotic changes consistent with chronic rejection (Cramer, 1990).

Hyperacute rejection is avoided by adequate cross-matching and lymphocytotoxicity testing pre-transplantation, although it may still occur despite satisfactory results from these tests. Immunosuppressive therapy is required to prevent destruction of the allograft by acute rejection. This therapy puts the recipient at increased risk of opportunistic infection, which may be fatal. The risks of immunosuppression must be balanced against the risks of acute rejection, particularly in the early months when rejection is most common. In the first three months after transplantation approximately two-thirds of patients will have at least one rejection episode (requiring augmented immunosuppression) (Rose, 1989). Since no specific

anti-rejection agents are available, the therapy used is of a broad nature and does not solely affect those cells responsible for acute rejection. Chronic rejection, on the other hand, cannot be treated, would not necessarily be detected on EMB histology and is a major source of graft loss and mortality in the later stages after transplantation. Regular coronary angiography is required to monitor the development of this complication, which may warrant re-transplantation.

2.11. Mechanisms of Cardiac Transplant Rejection

There are a number of possible mechanisms by which a cardiac allograft might undergo rejection including antibody mediated damage (Weil, 1981), lymphocyte mediated cytotoxicity, DTH-type reactions and natural cytotoxicity (discussed in Cramer, 1987). The precise effector cells of allograft rejection are not known but a number of possibilities exist. Cytotoxic CD8+ T cells, macrophages and natural killer cells have all been implicated in cardiac transplant rejection. T cells of CD4+ subtype may be involved with any or all of these mechanisms and it may be that rejection consists of a balance of these processes affected by various recipient and donor factors.

The phenotype of the cells involved has been studied by Hoshinaga et al who used an immunoperoxidase method to stain EMB for cell surface markers (Hoshinaga, 1984). They found that in the early phase of acute rejection, CD4+ cells showed the greatest influx into the EMB whereas at later stages CD8+ cells and macrophages predominated. Cells more usually associated with acute inflammation and other immune reactions (neutrophils, basophils and eosinophils) can also be present when rejection is severe (Kemnitz, 1987).

It has been shown that cytotoxic T cells with specificity directed against myocytes can be obtained from the myocardium of transplanted hearts in animals and in man (Bradley, 1992). Once a recipient is sensitised to an allograft further responses of CD8+ T cells can become independent of CD4+ help and these cells can then effect rejection on their own account (Andersson, 1975; Gurley, 1986). However, it is known that CD8+ cells alone are not sufficient to cause cardiac allograft damage. Allograft specific cytotoxic CD8+ T cells given to heart transplanted animals depleted of native T cells could not cause rejection alone (Hall, 1987). The precise role of CD8+ cytotoxic T cells in cardiac transplant rejection is therefore not clear.

The means by which CD8+ cells may damage cardiac cells is not known. Granzyme A and perforin are proteins produced by activated cytotoxic T cells and they appear to be involved in the functional activity of these cells (Young, 1987; Tschopp, 1988). At least one study has shown evidence of myocyte damage due to perforin in humans (Young, 1990). Griffiths et al used ISH to look for expression of RNA for granzyme A and perforin in EMB from recipients of cardiac transplants. RNA for

granzyme A and perforin was found in patients undergoing rejection and in those who went on to develop rejection (Griffiths, 1991). Thus one means by which CD8+ CTLs may damage human myocytes in rejecting allografts is via the delivery of pore forming proteins to the surface of myocytes.

CD4+ cells are known to be able to mediate cytotoxic responses, mediated by class II, but probably they are mostly concerned with providing help for CD8+ cells, monocytes and possibly natural killer cells. Macrophages can certainly be observed in the monocellular infiltrate within cardiac allografts (Hoshinaga, 1984) and hence the components of a delayed hypersensitivity type response are present. Whether this type of response is causing myocyte damage in cardiac transplants is not known.

There must be other mechanisms capable of damaging cardiac transplants as well as cellular immune responses since electrical and mechanical dysfunction can occur in transplanted hearts in the absence of significant myocytolysis (Myles, 1987; Schroeder, 1974). There is evidence pointing towards an intrinsic defect of myocyte function, despite normal high energy phosphate stores, in patients with cardiac transplants (DiSesa, 1991). Soluble factors that might influence cardiac function are many, but in mice that have undergone cardiac transplantation mRNA for IL-1, -2, -3, -4, -5, -6, TNFα and IFNγ was expressed by infiltrating cells (Dallman, 1991). In addition toxic effects of leucotrienes, which may be produced by monocytes, eosinophils and neutrophils, were shown against rat cardiac myocytes by Maisch et al (Maisch, 1987).

Cytotoxic T cells require some of these cytokines for their activation, particularly IL-1, IL-4 and IFNγ (Mizel, 1989). In addition, IFNα, IFNγ, and TNFα can have effects on allograft cells such as inducing MHC class I and II expression (David-Watine, 1990), which might enhance their susceptibility to T lymphocyte mediated responses. In situations where CD8+ cells are observed yet myocyte damage does not occur, such as in Halls study (Hall, 1987), it may be that there are limiting amounts of cytokine present, not allowing growth and effector function to develop.

A number of studies in animals, chiefly in rats and mice, have shed some light on the mechanisms of cardiac allograft rejection. It has been shown that soluble class I antigen of donor type infused into rats with cardiac allografts suppresses rejection via a mechanism which involves blocking CTL recognition of graft antigens (Sumimoto, 1990). Heidecke et al were able to show that it was possible to separate alloreactive T cells from "bystander cells" in rat cardiac allografts (Heidecke, 1990). This supports the idea that not all the infiltrating cells observed in the myocardium of cardiac transplant recipients are involved in rejecting the graft, providing further reasons why histology of EMB is not as accurate as it ought to be.

Hancock et al studied accelerated rejection as a model of hyperacute rejection (Hancock, 1990). They found that, in addition to the known antibody mediated damage, there was a cellular response. This was evidenced by graft infiltration by mononuclear cells producing IL-2, IFNγ and TNF, which was able to be reduced by cyclosporine therapy or treatment with IL-2R monoclonal antibody. In another study, Imagawa et al demonstrated the ability of anti-TNFα and β antibodies to prolong cardiac allograft survival in rats (Imagawa, 1990). These studies provide evidence of both CD8+ and CD4+ T cell involvement with cardiac allograft rejection and suggest potential therapies for use in man in the future.

Two highly simplified traditional models by which an allograft might be damaged are illustrated below (fig. 4). In the first, T helper cells recognise class II molecules on allograft cells and are then stimulated to produce cytokines which act on macrophages to promote cytotoxicity against the graft. This is similar to a delayed-type hypersensitivity reaction (Brent, 1958). In the second, T helper cells recognise class II molecules and then provide help to cytotoxic T cells which recognise class I molecules on allograft cells and bring about cytotoxic T cell mediated killing (Hayry, 1970 and Cerottini, 1974).

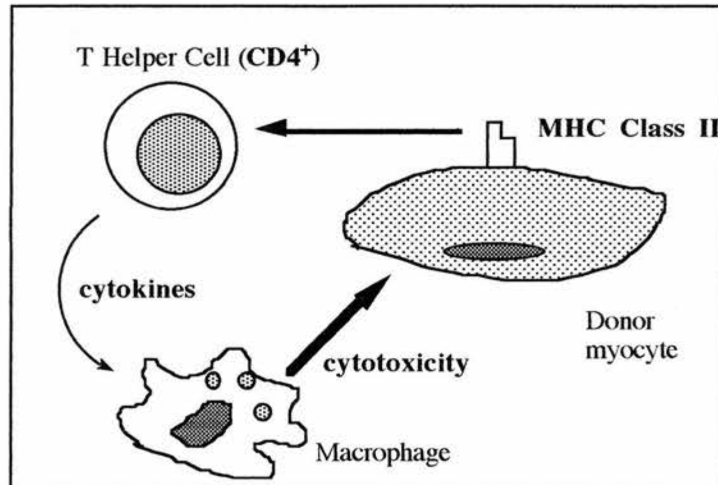
Rose et al demonstrated induction of expression of MHC molecules on myocytes, particularly class I, after transplantation (Rose, 1986). Suitters et al demonstrated the induction of class I expression on cardiac myocytes in cardiac transplant recipients which appeared to correlate with the presence of rejection (Suitters, 1987). They also found evidence for class II expression on interstitial structures in these patients. Caforio et al used a double labelling technique (with antibodies to human factor VIII, specific for endothelium, as well as class II) and found that class II expression on cardiac endothelium in the transplanted human heart is upregulated in the face of impending rejection (Caforio, 1990a). These studies suggest that donor myocardium and endothelium can express the MHC molecules required to sustain cellular immune responses.

Neither of the mechanisms illustrated takes account of the possible role of vascular endothelium as a target for T cell responses yet there is evidence for such a role (Hengstenberg, 1990; Caforio, 1990a; Dunn, 1992; Jutte 1993). Also not illustrated is the role of antigen presenting cells (APCs). Donor APCs may present donor peptides directly to recipient lymphocytes or recipient APCs may take up and process donor proteins, for example class I, and present these in the context of their own class II molecules.

Figure 4. Two Simplified Mechanisms of Allograft Rejection

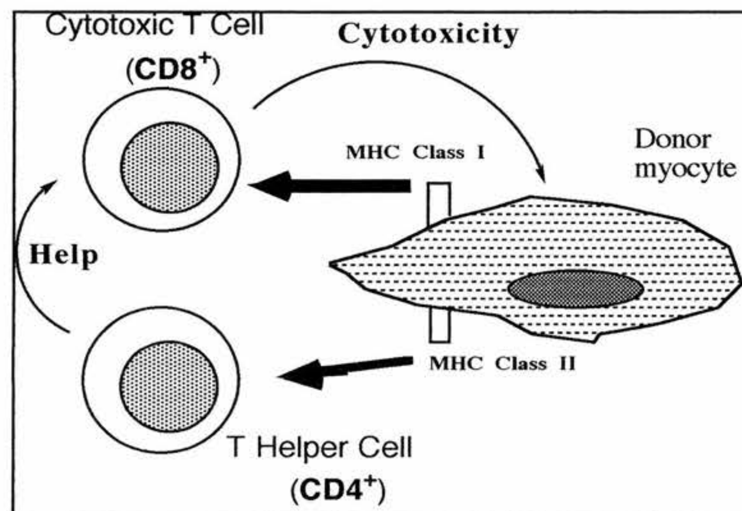
a. Similar to delayed-type hypersensitivity

This figure represents the possible interactions between the key cells involved with this mechanism. The transplant cell is recognised by the T-helper (CD4) cell which is then activated to secrete cytokines which in turn activate macrophages. Here, cell killing is non-specific.



b. Cytotoxic T Cell Killing

In this model recognition of the transplant cell occurs via cytotoxic (CD8) and T-helper (CD4) cells, each seeing class I and class II molecules respectively. Specific destruction of myocytes occurs, with CD4+ cells providing "help". The CD8 cells deliver a lethal hit to individual cells and hence killing is specific.



2.12. The Phenotype of Cells Infiltrating Cardiac Allografts

In early studies of animal models of cardiac allograft rejection, cell suspensions were harvested from explanted allografts. T lymphocytes and monocytes were predominant in these preparations (Tilney, 1975). Subsequently, the major cellular components of the myocardial infiltrate in rejecting hearts were shown to be: CD4+ T cells, CD8+ T cells and monocyte/macrophages, though cells with the natural killer phenotype were also described (Yacoub, 1983; Marboe, 1983; Hoshinaga, 1984).

These cells may be involved in low grade rejection, with perhaps a perivascular infiltrate not associated with myocyte damage (grade 1a), or alternatively there may be larger numbers of cells aggressively infiltrating the myocardium and injuring adjacent myocytes (grade 3a). This implies a developing process whereby lymphocytes marginate on blood vessels of the allograft and then begin to infiltrate the parenchymal structures, proliferating as acute rejection develops. The proximity of these proliferating cells to the endothelial surface presumably relates to how they may be cultured from EMB. There is no study which assigns a particular phenotype to any of these functions or anatomical locations. In addition it is difficult to show that particular grades of rejection are associated with particular phenotypes.

Ahmed-Ansari et al performed phenotyping of cells infiltrating EMB using monoclonal antibodies and an immunoperoxidase method (Ahmed-Ansari, 1988). They found few B cells, monocytes or macrophages but a small number of biopsies (8 to 16% depending on rejection grade) contained cells bearing the NKH-1 marker (found on natural killer and other cells). The majority of cells observed were T cells, with CD4+ cells predominating (about 65 to 74% of infiltrating cells compared with 23 to 33% being CD8+). Using antibodies to HLA-DR they found evidence of activation in 75 to 83% of the infiltrating cells. In this study EMB were also placed in culture. The resulting lymphocyte cultures were phenotyped and comparison made with results obtained from the in situ immunoperoxidase staining. The data in this paper were presented poorly (percentages of cells positive for each marker were quoted along with standard deviations, which were often very large) and the overall patient numbers were rarely stated. However, it appeared that the phenotype of the infiltrating cells observed histologically and by immunoperoxidase staining correlated broadly with the cells subsequently cultured from EMB taken at the same time. It was stated that some individuals EMB grew predominantly CD4+ cells when the in situ phenotype was predominantly CD8 and vice versa, but these data were not shown.

2.13. Lymphocyte Culture from Endomyocardial Biopsies

One approach to the problem of elucidating the nature of cells infiltrating allografts, originally applied to renal transplants in 1985, has been to culture graft-infiltrating T-cells from small biopsy fragments. The culture medium employed was supplemented with interleukin-2 in order specifically to enhance proliferation of cells bearing IL-2 receptors, which would include activated T cells, but also natural killer cells and monocyte/macrophages. Several groups using this method have described phenotypic and functional aspects of cells cultured from EMB. Some of the chief conclusions from these studies were that: cultured cells were of a particular phenotype at particular times after transplantation, ability to culture lymphocytes was correlated positively with presence of histological rejection in concurrent biopsies and that culture of lymphocytes in the absence of rejection predicted subsequent rejection. It therefore appeared that this approach allowed culture of T cells which related to the in-vivo rejection process.

2.14. Methods Used to Study Cultured Lymphocytes from EMB

Many of the studies relating to lymphocyte culture from EMB have used similar experimental conditions. To inform discussion of these studies a table summarising the methods used in the majority of these studies is given below (table 2). In addition some of the conditions and methods used are described in more detail.

A. Lymphocyte Culture

In most studies small (approximately $1mm^3$) fragments of tissue biopsies (kidney, heart) were placed in tissue culture wells in culture medium. The culture medium was invariably based on the Roswell Park Memorial Institute Medium-1640 (RPMI-1640) with various additional supplements, detailed below.

(i) Interleukin-2

The most important supplement was IL-2, quantified in terms of international units(iu). Most studies used concentrations of 5 to 1000iu/ml. Some studies used tissue culture supernatants rich in IL-2 and therefore describe a percentage of IL-2 present according to how much of the supernatant was added (Zeevi, 1986; Pfeffer, 1988; Farrel, 1990, Jutte, 1990, Ouwehand, 1991). The usual source of IL-2 is now recombinant IL-2 and most studies made use of this form (Kaufman, 1990 for example). The various studies differed in how often and how much IL-2 is added to cultures once they are in progress.

Table 2. Studies of Lymphocyte Culture from Endomyocardial Biopsies

Author	Patient (&Biopsy) Nos.	Medium	IL-2	Serum	PHA	Feeder Cells	Results (Positive cultures)	Phenotype
Zeevi 1986	4 (13)	RPMI-1640 (200μl wells)	lectin depleted supernatant - 50%	10% human	-	Yes	13/13	CD4 - 4, CD8 - 3 Both - 1
Duquesnoy 1987	5 (7)	See Zeevi 1986	-	-	-	-	-	-
Weber 1988	73 (520)	See Zeevi 1986	-	-	-	Yes	Complex	-
Pfeffer 1988	15 (91)	RPMI-1640 2ml wells	20%	10% human	-	Yes, when positive	20/35 rejection 17/56 none.	-
Carlquist 1988	14 (125)	RPMI-1640 2mls	160u/ml (purified)	5% human AB	1μg/ml (to positive cultures)	-	49% (61) - correlated with rejection	12 of 61 typed. CD8:12/12, CD4:5/11 NK:4/10,Mphage:1/11
Ahmed-Ansari 1988	39 (238)	RPMI-1640, 3mls	1000u/ml rIL-2	-	-	-	43% (102)	**See text
Weber 1989	79 (201)	RPMI-1640 200μl	20iu/ml	10% human AB	-	Yes	51/63 - rejection. 55/138 - none	-
Kaufman 1989	33 (231)	RPMI-1640	20iu/ml rIL-2	10% human AB	-	Yes	-	-
Carlquist 1989	Abstract	Not given				-	-	-
Suitters 1989	Not given	RPMI-1640	28iu/ml rIL-2	-	-	-	-	CD8 > CD4
Ouwehand 1990	87 (535)	No detail	-	-	-	Yes	As for Ouwehand 1991	
Farrel 1990	29 (54)	RPMI-1640	20% supernatant	20% human A	-	-	-	-
Kaufman 1990	55	RPMI-1640 200μl	30iu/ml rIL-2	See Zeevi 1986 for other details	-	Yes	-	-
Suitters 1990	86 (121)	RPMI-1640 200μl	28u/ml rIL-2	10% human AB	-	-	-	-
Frisman 1990	14 (88)	No details	10-100iu/ml	-	No detail	-	-	-
Jutte 1990	2 (3)	RPMI-1640 200μl	10% lymphocult (lectin-free)	10% human AB	-	Yes	-	-
Carlquist 1990	41 (289)	RPMI-1640	160iu/ml (purified)	5% human AB	-	-	-	-
Ouwehand 1991	87 (535)	RPMI-1640	10% lymphocult (lectin free)	10% human	-	After 3 weeks - variable	53%(37% grade I, 62% grade II 82% gradeIII)	90% CD3, CD4 29, CD8 15, mixed 38, mostly:CD4 70,CD8 42
Carlquist 1993	17 (28)	RPMI-1640 2mls	160iu/ml	5% human AB	-	-	Efficiency not given	10 CD8,17 CD4, both 1.

(ii) Phytohaemagglutinin (PHA)

This plant lectin (a plant protein which can bind to and cross-link cell surface glycoproteins) activates all T cells non-specifically via the CD2 molecule on the cell surface. Only a few workers have used PHA in their studies (Carlquist, 1988 and Frisman, 1990).

(iii) Feeder Cells

These are peripheral blood mononuclear cells (PBMC) which may originate from the donor, the recipient or an unrelated third party. The cells are treated to prevent them from being able to proliferate (with irradiation or chemically with mitomycin-C). The rationale for their use is that they provide cell to cell contact, local co-factors for growth and they produce various lymphokines which help to maintain an appropriate environment for cultured T cells.

Recent studies have used oxidised irradiated PBLs as described by Fleischer (Fleischer, 1988). Fleischer's method involves rendering a sample of lymphocytes from a given subject incapable of proliferation by exposing them to radiation. The cells are then oxidised although the role of oxidation in this process is unclear. In this condition they provide an appropriate milieu for dividing lymphocytes, with cell to cell contact, cytokine production and other ill-defined effects.

Briefly, cells were suspended in RPMI-1640 medium and then subjected to irradiation. Cells were then spun down and resuspended in "oxidation mix", containing neuraminidase and galactose oxidase, and then incubated. The cells were then washed in a solution containing galactose to remove any residual galactose oxidase activity. Approximately 5×10^4 per ml oxidised feeder cells were added per tissue culture well.

In 9 of the 19 studies listed in the table feeder cells were used. It was most common to use irradiated autologous feeder cells but allogeneic cells were also used.

B. Phenotyping

(i) Immunocytochemistry

Once sufficient cells have been cultured many groups have proceeded to phenotype the cell population (Zeevi, 1986; Carlquist, 1988; Ahmed-Ansari, 1988; Ouwehand, 1991; Carlquist, 1993). CD markers are functional molecules on the surface of cells (particularly T cells) which allow them to be identified and which may also indicate the function of a given population of cells. Listed below are some of the cell surface markers against which monoclonal antibodies have been raised for use in such experiments (table 3).

Table 3. Antibodies to Cell Surface Markers

This table lists some of the various cell surface markers against which antibodies have been raised. In the first column the table gives the cluster designation number according to the international workshop definition. In the middle column is given any other designation. A brief description of the cells carrying the specified marker appears in the third column.

Surface Marker	Other Designation	Cell Population
CD2	LFA-2	T cells
CD3	OK-T3	T cells
CD4	Leu 3, OK-T4	T helper cells
CD8	Leu 2, OK-T8	Cytotoxic T cells
CD16	Fc receptor (FcγRIII) low affinity	Large granular lymphocytes
CD19		B cells
CD25	TAC, IL2R	Activated T cells
CD68		Monocyte/Macrophages
CD71	Transferrin receptor	Activated T cells
W6/32	Class I MHC	All cells
HLA-DR	Class II MHC	B cells, Monocytes Activated T cells

There are two methods of staining routinely used in these studies: immunoperoxidase and immunofluorescence. Both may utilise monoclonal antibodies to cell surface markers in order to stain the cells of interest. A secondary antibody may then be employed to detect the primary antibody. This may be conjugated to an enzyme (horse radish peroxidase, alkaline phosphatase) or a flourescent molecule (fluorescein isothiocyanate, phycoerythrin). In some experiments a third antibody is used against the secondary antibody in order to increase sensitivity and it would be this final antibody which would bear the conjugated molecule. Some protocols, particularly with immunoperoxidase staining, use avidin-biotin systems to increase sensitivity.

Immunofluorescence staining can be used in association with flow cytometry, whereby cells are detected according to granularity, size and fluorescence, to quantify the cell populations stained with a particular fluorescent molecule. Alternatively preparations of cells may be made by spinning them in a centrifuge at high speed onto a microscope slide (cytospin). The slides are subjected to the staining protocol and visualised microscopically to be counted. Either immunoperoxidase or immunofluorescence can be used with cytospin preparations.

(ii) HLA Typing

In order to determine which HLA class I and class II molecules are present on an individual's lymphocytes, and sometimes on cultured cells, defined anti-sera to particular HLA antigens are added to cells along with complement molecules (Terasaki, 1965). If the antibodies in the sera bind to the cells and fix complement then lysis occurs. Thus where cells are lysed they bear the antigen known to be recognised by the test serum. Some workers used the information gained from HLA typing to select third party cells as targets for cytotoxicity experiments. Other methods for performing HLA typing involve restriction fragment polymorphisms. Also, the polymerase chain reaction can be used to amplify DNA from lymphocytes for molecular identification of their HLA phenotype (Joysey, 1993).

C. Functional Testing

(i) Response to IL-2

A crude measure of the ability of cultured lymphocytes to grow is to test their ability to proliferate in response to exogenous IL-2. This is based on the observation that activated T cells express increased numbers of IL2R on their surface. Thus, some workers took aliquots of lymphocyte cultures and added defined amounts of IL-2 (Weber, 1989; Kaufman, 1989). Proliferation was measured by tritiated thymidine(^{3}H-thymidine) incorporation and compared with the rate of proliferation in IL-2 with other cultures and sometimes with PBMC from the recipient. The uptake of ^{3}H-thymidine depends upon the rate of cellular DNA synthesis. Thymidine is one of the bases used in the construction of DNA and proliferating cells use it when replicating DNA for subsequent cell division. The uptake of ^{3}H-thymidine from culture medium is therefore an indirect measure of proliferation.

(ii) 2 Day Mixed Lymphocyte Reaction(MLR)

This assay consists of adding cultured lymphocytes to irradiated (or mitomycin-C treated) PBMC from a relevant subject (donor, recipient, third party) and then incubating them for 48 hours. If the cultured cells have been 'primed', that is they have already undergone a primary response to the antigens borne by the target cells in-vivo, they will proliferate rapidly when re-exposed to the same antigen. Significant incorporation of tritiated thymidine will only occur if this is the case.

(iii) Primed Lymphocyte Testing(PLT)

This is essentially the same as the two-day MLR except that the incubation period is extended to three days, allowing greater time for a response to develop. The original method (Frandelizi, 1975) was adapted for use with cells cultured from EMB.

The majority of workers in this field have used this form of the experiment (Zeevi, 1986; Weber, 1988; Ahmed-Ansari, 1988 and Kaufman, 1989).

(iv) Cell Mediated Lympholysis (Cytotoxicity)

This involves testing T cell populations (effector cells) for ability to kill target cells (European CML Workshop, 1980). When positive it demonstrates that the population tested has previously been exposed to and developed activity against the antigens borne by the target cells. In these experiments target cells, usually donor or third party PBMC, would be labelled with ^{51}Cr beforehand. The test cells would then be added and co-incubated for four hours. A range of effector to target cell ratios would normally be employed. Where cell killing occurs ^{51}Cr is released into the medium. Cultures are then filtered onto paper and the amount of ^{51}Cr in the culture medium counted by scintillation counting.

Calculations are facilitated by measuring spontaneous release(S) from a culture to which no effector cells are added. In addition 100% lysis(M) is produced, often using a detergent such as Triton X-100. A figure (specific lysis) is derived which reflects the ability of the effector cells to kill the targets. The equation used to calculate specific lysis for each test is as follows:

$$\% \text{ specific lysis} = \frac{(E - S)}{(M - S)} \times 100$$

(where E = experimental value, S = spontaneous release, M = maximum lysis)
Specific lysis >10% is usually taken as a significant result.

Several studies used these methods to test for cytotoxicity in cultured cells (Pfeffer, 1988; Kaufman, 1989).

(v) Natural Killer Activity

This is tested by measuring cytotoxic activity against a cell line known to be sensitive to this kind of cellular killing (K562 cells), (Ahmed Ansari, 1988 and Pfeffer, 1988).

2.15. Phenotypic and Functional Studies, Relation to Rejection

The phenotype of cells cultured from small numbers of renal biopsies in the two original reports of this technique was mixed, with CD8 and CD4 positive T cells being present(Mayer, 1985; Miceli, 1985). CD8+ cells predominated in Mayer's study of 8 biopsies. When the phenotype of infiltrating cells cultured from cardiac biopsies was examined it was also found that CD4 and CD8 cells were present in various

proportions. Monocyte/macrophages and B cells were also observed (Ahmed-Ansari, 1988; Carlquist, 1988; Suitters, 1989).

Mayer demonstrated evidence of cytotoxic function among cells cultured from renal allografts, directed against cells bearing class I antigens identical to those of the donor (Mayer, 1985). There was very little cytotoxicity against third party cells hence this activity was donor-specific. In experiments on cells grown from human cardiac allograft biopsies, Zeevi et al demonstrated proliferative responses to cells which shared MHC molecules with the donor but not to third party cells (Zeevi, 1986). Ahmed-Ansari et al showed that there was both proliferative and cytotoxic T cell activity in cells grown from EMB (Ahmed-Ansari, 1988). This study also found some natural killer activity. Pfeffer et al also showed, in similar experiments, that there was donor specific cytotoxicity but not natural killer activity among cultured lymphocytes (Pfeffer, 1988).

Ahmed-Ansari demonstrated that the phenotype of lymphocytes grown from EMB in bulk culture, as determined by flow cytometry and immunofluorescence staining, generally correlated with the phenotype observed directly in EMB taken at the same time (the in situ phenotype), using immunoperoxidase staining (Ahmed-Ansari, 1988). This suggests that the culture method used produces cell populations which, in their phenotype at least, are representative of the cells present in vivo. There were some deficiencies in the presentation of data in this paper and so only the most general conclusions can be drawn. This study also found no relationship between circulating IL-2R or cyclosporin levels and phenotype of cultured cells. Interestingly, there was no correlation between the in-situ phenotype and the grade of rejection. This might suggest that any phenotype (CD4 or CD8 predominantly) can be associated with, or mediate, acute rejection. This might support there being more than one rejection mechanism at work. Alternatively, it may represent the inability of histology to accurately reflect in vivo pathophysiological processes.

A number of other authors have sought to relate the ability to culture lymphocytes from allograft biopsies to the presence or absence of rejection on histological examination of simultaneously obtained biopsies. Some authors have not addressed this at all. Mayer obtained biopsies only from renal transplant patients who were known to be undergoing rejection in the first place (Mayer, 1985). Carlquist et al reported in one study that there was a relationship between ability to culture lymphocytes from EMB and rejection but in another study they were unable to find such a relationship (Carlquist, 1988; Carlquist, 1989). Pfeffer found that 20 of 35 (57%) biopsies with histological evidence of rejection grew lymphocytes whereas only 17 of 56 (30%) without rejection produced positive cultures (Pfeffer, 1988).

The ability to culture lymphocytes from histologically negative biopsies may be used to predict subsequent rejection. Weber showed that there was an increased

cumulative incidence of subsequent rejection within 30 days (Weber, 1989). Carlquist showed that the overall incidence of subsequent rejection was greater for those patients with positive culture but negative histology, compared to those with negative culture and negative histology (Carlquist, 1989).

2.16. Cardiac Transplantation and EMB: Summary

Cardiac transplantation is an important therapy for patients with end-stage cardiac failure. However, the occurrence of acute rejection significantly affects survival, having its greatest effect in the early post-transplant period. Rejection is diagnosed histologically on EMB, taken at regular intervals, and is treated with additional immunosuppression. The grading system for rejection is based on the degree of cellular infiltration and myocyte damage but includes observations of other features such as endocardial lymphocytic infiltrates (ELI). There are several potential mechanisms by which allografts may be damaged and which of these operate in individual patients is not clear. The use of IL2-supplemented media to culture activated T cells from EMB has allowed in-vitro studies of the nature of graft infiltrating cells but has also been investigated with a view to possible use as a prognostic tool.

This study was undertaken to test the hypothesis that supplementing a basic culture medium containing IL-2 with polyclonal stimulators of T cell proliferation such as phytohaemagglutinin or anti-CD3 antibodies, would increase the number of positive cultures compared with a medium supplemented with IL-2 only. Previous studies have utilised culture medium supplemented with IL-2 in various concentrations but have also included phytohaemagglutinin, irradiated feeder cells (allogeneic and autologous) and different types of serum supplements.

The study was also designed to observe the incidence of subsequent rejection in patients with positive culture results but no rejection on histological examination of associated biopsies. This was done to confirm the notion that T cell culture might have potential clinical use in monitoring cardiac transplant recipients.

In addition it was planned to compare the effects of presence or absence of ELI in cases with positive or negative culture results. If ELI were contributing to positive culture results, but were not related to rejection then they could easily distort the usefulness of this technique in predicting subsequent rejection. The hypothesis was therefore that ELI were confusing the results of lymphocyte culture from EMB.

Previous studies have also not addressed the question of MHC antigen mis-matches at Class I or Class II loci. Rejection is not an all or none phenomenon; it is possible to have grade 2 rejection on biopsy and remain entirely well with no cardiac dysfunction, despite the presence of cells infiltrating the myocardium. There may be patients whose immune response to the allograft is stronger than other patients and

whose target is Class I or Class II bound peptides recognised more strongly for certain HLA molecules than for others. If this were the case then some patients with mis-matched allografts would have more infiltrating cells and be more likely to have a positive culture result from EMB. Therefore data were collected on HLA A, B and DR mis-matches among these patients to determine if patients with mis-matches at one or more loci were more likely to have positive lymphocyte cultures obtained from their EMB.

3. Lymphocyte Culture from Endomyocardial Biopsies: Patients, Materials and Methods

3.1. Patients

Endomyocardial biopsies (n=94) were obtained from 73 cardiac transplant recipients (7 female, average age 47 years, range 16-63 years) and from 5 patients who were not transplanted (age range 20-49 years, 1 female), 4 of whom had idiopathic dilated cardiomyopathy and 1 had alcoholic cardiomyopathy. The pre-transplant diagnosis was ischaemic heart disease (IHD) in 45 (62%) patients and idiopathic dilated cardiomyopathy (DCM) in 28 (38%) patients.

Transplant patients were receiving routine immunosuppression with azathioprine, cyclosporin and prednisolone at the time of biopsy. Multiple biopsies (average 10) were obtained at routine diagnostic biopsy in each patient and one of these was used in the culture studies described. Informed consent was obtained and the study was approved by local ethical committee.

3.2. Endomyocardial Biopsy

Biopsies were obtained from the right ventricle via the right internal jugular vein, under local anaesthesia, using a standard bioptome with fluoroscopic guidance at a median of 8 months following transplantation (range 1 week to 96 months). Thirty-one biopsies were taken within three months of transplantation. A total of 94 biopsies were obtained for culture studies from 73 cardiac transplant recipients (17 patients were biopsied twice and 2 three times). Biopsies were obtained over a fourteen month period commencing May 1990.

3.3. Lymphocyte Culture

Each biopsy sample was collected into the basic medium used in these studies which contained the substances listed below:

- RPMI-1640 medium (ICN-Flow, High Wycombe, Bucks),
- Penicillin 100i.u. per ml (ICN-Flow),
- Streptomycin 100μg per ml (ICN-Flow),
- L-glutamine 2mM (ICN-Flow),
- Sodium bicarbonate 0.2% (ICN-Flow),
- 10% human AB-negative serum (ICN-Flow)
- 5i.u. per ml recombinant interleukin-2 (IL2, Human recombinant DNA IL2, National Institute for Biological Standards and Control, South Mimms, Herts)

Samples were collected in a sterile universal container at room temperature and transported to the laboratory within three hours.

Under sterile conditions in a tissue culture hood the biopsy was placed on a petri dish and finely divided into up to three approximately equal fragments using a scalpel. Each fragment was placed in a single well of a round bottomed 96-well tissue culture plate containing 200µl of medium as below. Biopsies were cultured at 37^0C in 5% CO_2 in air. Irradiated feeder cells were not used. Fragments from each biopsy were cultured in at least one of the following media for the first three days:

1) basic medium plus 4 µg/ml phytohaemagglutinin (PHA, ICN-Flow);
2) basic medium with monoclonal antibody to the CD3 cell surface marker (1:10,000 final concentration of cell culture supernatant from cell line CRL 8001 OKT3 obtained from ECACC. The optimum concentration was determined by assays for proliferation of PBMC, see below)
3) IL2 alone.

After three days, and every third day thereafter, half the medium in each well was removed and replaced with the basic medium alone.

Fifty-seven biopsies were sufficiently large for a fragment to be grown with each of the three media (PHA+IL2, CD3+IL2 and IL2 alone); 73 biopsies were large enough for pieces to be grown in medium with PHA+IL2 and in IL2-alone; 18 biopsies were cultured in medium with PHA+IL2 only. Wells were observed daily for outgrowth of cells on an inverted stage microscope. A biopsy was considered to be positive when lymphocytes could clearly be seen exuding from it and proliferating, as previously described (Carlquist, 1988). Effete red cells were observed and discounted and fibroblast-like cells were sometimes observed, forming a monolayer as previously described (Mayer, 1985).

3.4. Immunophenotyping

Cells from a group of twelve consecutive cultures were counted and subjected to indirect immunostaining with a panel of monoclonal antibodies to confirm their phenotype. Cells were suspended in RPMI-1640 medium and an aliquot was counted on a haemocytometer by trypan blue exclusion to ensure that cell viability was greater than 95%. After counting, the cells were resuspended in serum-free medium and spun on to microscope slides in a cyto-centrifuge for 10s at 1000 rpm. The slides were left to dry in air overnight and were then fixed in acetone at 4^oC for 15 minutes. After drying, the cells were then stained with a panel of monoclonal antibodies to the following cell surface markers: CD8 and CD4 (Dept. of Immunology. Royal Free Hospital, London), CD16 (Dako, High Wycombe, Bucks), CD19 (Serotec) and CD68 (Dako) at room

temperature for 60 minutes. After washing with phosphate-buffered saline and 0.1% Tween, the cells were incubated with fluorescein isothiocyanate conjugated Fab'2 fragments of mouse anti-human immunoglobulins (Dako) for 60 minutes at room temperature. At least two hundred cells were stained using each antibody. Control slides were incubated in serum-free medium in place of primary antibody. Positive staining was observed using ultra-violet light microscopy (Zeiss Axioskop. Carl Zeiss, Oberkochen, Germany. HBO 50 high pressure mercury lamp with red attenuating filter).

3.5. Histology

Routine histology was performed on haemotoxylin and eosin-stained serial sections of the remaining nine formalin-fixed biopsies for each subject by the Histopathology Laboratory at Papworth Hospital. Biopsies were graded, without knowledge of the culture result, according to the International Society for Heart and Lung Transplantation working formulation, by Dr N Cary (Billingham, 1990).

3.6. Separation of Peripheral Blood Mononuclear Cells (PBMC)

Venous blood was obtained (10mls) into a sterile universal container with 100 units of preservative-free heparin. Under sterile conditions in a tissue culture hood the blood was layered onto 7.5mls of Lymphoprep solution (Nycomed Pharma AS, Oslo, Norway) in a fresh sterile universal container. This was centrifuged in an IEC Electra-8 centrifuge at 2000 r.p.m. for 15 minutes with no brake applied. The lymphocyte layer, at the interface between serum and the solution, was aspirated using a Pasteur pipette into another sterile universal container. The cells were resuspended in 10mls of phosphate buffered saline (PBS) and spun at 2,000rpm (with brake). The PBS was aspirated and the cells were twice more resuspended in PBS and spun at 1200rpm. After the final step cells were counted by trypan blue exclusion. A 50µl aliquot was mixed 1:1 with trypan blue solution and counted on a haemocytometer using an inverted stage microscope. Dead cells were recognised by blue staining and preparations were always assessed to ensure greater than 95% viability of cells. Finally cells were resuspended in the appropriate solution and concentration, depending on the requirements.

3.7. Proliferation Assays

Peripheral blood mononuclear cells were prepared as under **3.6.** After the final washing step cells were resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (ICN-Flow) at 2 x 10^6 viable cells per ml. From this solution 100µl was placed into each of 54 wells of a sterile 96 well tissue culture plate with "U"

bottomed wells giving 18 sets of triplicate wells. The supernatant from cell line CRL 8001 OKT3 (obtained from the European Collection of Animal Cell Cultures) was added to each triplicate in the following dilutions:

1:100	1:200	1:400	1:800	1:1200	1:1600
1:2000	1:4000	1:5000	1:6000	1:8000	1:9000
1:10000	1:12000	1:14000	1:16000	1:2000	

The plate was sealed in cling film and cultured in a humidified incubator in 5%CO_2 in air at 37^0C. On the evening of the third day in culture, tritiated thymidine (Amersham International plc, Amersham, Bucks) was added to each well (1μCi per well). After 16 hours incubation the cells were harvested using a Skatron cell harvester (Flow) onto discs of filter paper. These were dried by incubation at 37^0C for an hour. The discs were then placed into scintillation vials (Beckmann, High Wycombe, Bucks) for counting along with 0.5ml "Optiscint" scintillation cocktail (Amersham). All vials were then counted in a scintillation counter (Packard Tri-Carb 1500 Liquid Scintillation Analyser. Canberra Packard Instrument Co.,Pangbourne, Berks) for 1 minute per vial. Three vials containing filter discs and scintillation fluid but no cells were counted as a background control. Results were produced in the form of counts per minute and were corrected for the background count.

3.8. HLA Typing

HLA typing was performed by the tissue typing laboratory at Addenbrookes Hospital, Cambridge. For routine tissue typing the NIH (National Institutes of Health) method was used as originally published by Terasaki (Terasaki, 1965). Instead of staining cells with eosin however, ethidium bromide and acridine orange were used with ultra-violet light examination to determine cell viability (dead cells fluoresce red and live ones green). Typing was performed using a reliable panel of antisera used for transplant analysis.

3.9. Statistical Methods

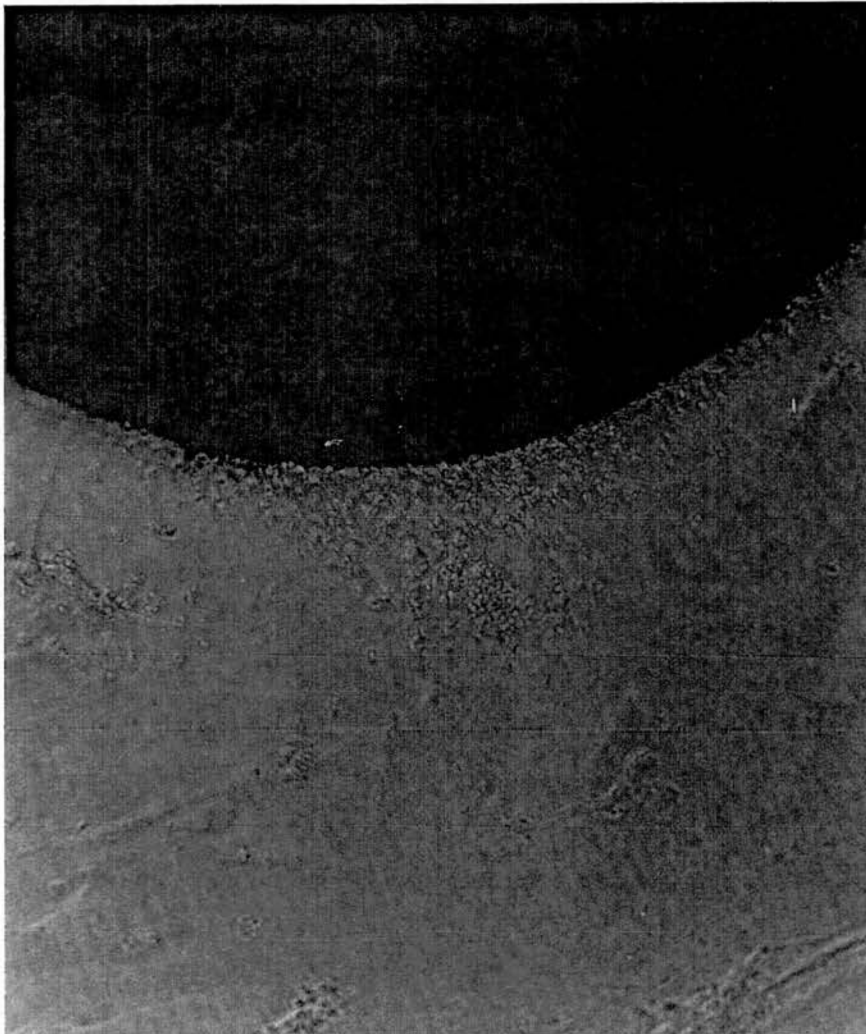
To compare time to observed outgrowth between different media the log rank test was used, with the fragments which failed to produce lymphocytes being excluded. The Mann-Whitney U Test was used to compare two median times, for example time since transplantation was performed, for patients with and without ELI. Logistic regression was used to assess the relationship between culture supplement and rejection, time since transplantation was performed and so on. Results for the likelihood ratio test are provided. Other tests used were the Chi-square test and Fishers exact test.

4. Results: Endomyocardial Biopsies

4.1. Lymphocyte Culture Results

A total of 94 biopsies from 73 cardiac transplant patients were obtained in order to study the ability to culture lymphocytes. 17 patients underwent biopsy twice and two patients had three biopsies each in this study. No lymphocytes grew from biopsies taken from the 5 control (non-transplanted) patients, and histology in these showed no evidence of T cell infiltrates. Biopsy fragments remained in the wells until cultures were discarded. An example of a positive culture is shown below (fig. 5).

Figure 5. Lymphocytes Exuding from Endomyocardial Biopsy
This photomicrograph, (Nikon 35mm camera on Nikon inverted stage microscope, film speed ASA 400, magnification X40, exposure 1/15sec), shows a large dark area, which is the bulk of the endomyocardial biopsy, from which many small cells can be seen exuding. This picture was taken at an early stage (day five) in culture.



From the culture conditions chosen and their similarity to previous studies it was assumed that T-lymphocytes would be grown from EMB, as opposed to any other cell type. The morphology of the cells, their behaviour in culture and immunophenotyping confirmed that this was the case. Only cultures consisting of significant numbers of T-lymphocytes were accepted as positive, as described earlier in the methods section. Pure cultures of monocytes or fibroblast-like cells were occasionally observed and discounted. The photomicrographs in figures 5 (above) and 6 (below) show typical T lymphocytes exuding from biopsy fragments. The appearance of these cells contrasts with a culture consisting of monocytes as shown in figure 7.

Figure 6. Lymphocytes Exuding from Endomyocardial Biopsy
This picture, (ASA400, X 100, 1/15sec), was taken at day eight. A larger number of lymphocytes can clearly be seen. Again the biopsy is seen only as a dark mass to one side of the picture.

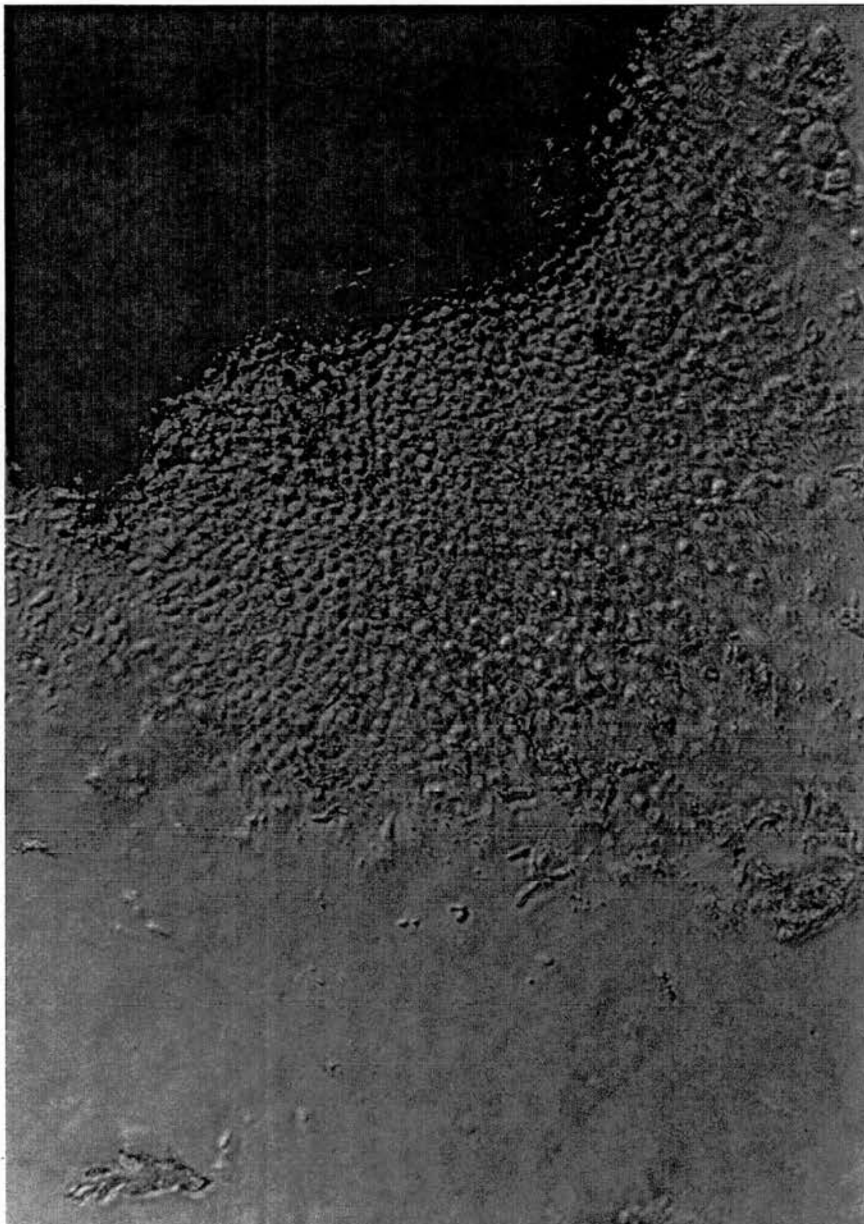
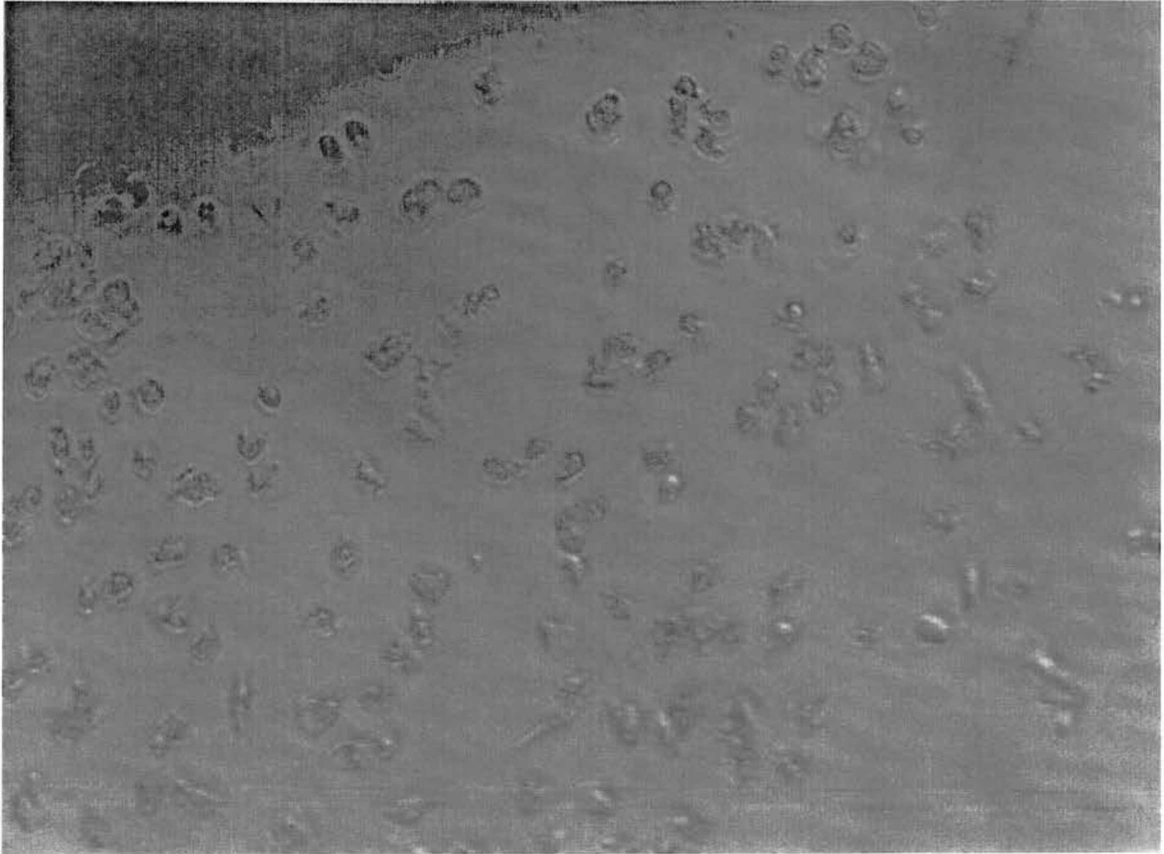


Figure 7. Monocytes cultured from an Endomyocardial Biopsy
This photomicrograph, (ASA400, X 100, 1/8sec), shows a culture consisting almost entirely of monocytes, as was later confirmed by immunophenotyping with the CD68 marker. These cells are larger than T lymphocytes and have a more granular 'spiky' appearance.



Lymphocytes grew from 60 of 94 (64%) biopsies in medium supplemented with PHA and IL-2, 37 of 57 (65%) in medium with CD3 and IL-2 and 36 of 76 (47%) in medium with IL-2 alone. The individual results are shown in the table which follows (table 4). For biopsy fragments taken within three months of transplantation, cultures were positive in 19 of 31(61%) in medium with PHA and IL-2, in 12 of 20(60%) in medium with CD3 and IL-2 and in 15 of 25(60%) in medium with IL-2 alone (table 5).

Table 4. Lymphocyte Culture from EMB: Results

The table shows a list of patients whose biopsies were subjected to culture. They are placed in order of those who only had one biopsy fragment cultured proceeding to those who had three cultured. The time since the recipient underwent transplantation (in months) is shown in the second column. The results of attempts to culture lymphocytes in each of the three media used are in the third to fifth columns (with the number of days to positive culture being observed shown for positive results and 'NEG' indicating failure of lymphocytes to grow). In column six the histology grade of the biopsies taken at the same time as the EMB used for culture experiments is shown, along with the total number of biopsy fragments taken at that time in brackets. Also denoted is the absence of ELI (as -) or presence of ELI (+) or ELI plus myocyte injury (++). Column seven shows the histology (where available) on the next biopsy obtained from that individual. The final column shows the patients diagnosis (1=ischaemic heart disease, 2=dilated cardiomyopathy).

Patients (& repeats)	Months from Tx	Growth in: PHA	IL-2	CD3	Histology on initial biopsy	Histology on next biopsy	Diagnosis
1, 59	4	NEG			3a+ (8)	2+ (10)	1
2, 42	7.5	10			0- (9)	2- (10)	1
3, 21	3	8			1a- (8)	3a- (10)	1
4	7.75	8			1a++ (9)	1a+ (8)	1
5, 22	1.5	8			1a- (9)	1a- (9)	1
6, 31, 79	2.25	10			1a- (8)	2- (8)	2
7, 33	24	8			1a- (8)	1a+ (8)	2
8, 60	24	NEG			2- (8)	1a+ (10)	2
9	1.5	NEG			2- (10)	1a- (10)	2
10, 29	1.5	NEG			0- (10)	0- (12)	1
11	3.5	7			1a- (5)	0- (10)	2
12	35	7			1a+ (10)	3a+ (8)	1
13	3	7			1a- (8)	3a- (7)	1
14	62	7			1a- (10)	0- (8)	2
15	37	NEG			1a+ (14)	1a+ (9)	1
16	96	7			0- (10)	0- (10)	1
17	72	7			0- (10)	0- (10)	1
18, 24, 49	14	7			1a- (10)	3a- (9)	2
19, 32	8	8			3a+ (10)	1a+ (10)	1
20, 43	9	NEG			0- (9)	1a- (7)	1
21, 3	4	NEG			3a- (10)	1a- (11)	
22, 5	2.5	6	6		1a- (9)	3a- (9)	
23	12	NEG	NEG		0- (9)	3a- (7)	
24, 18, 49	11	6	6		3a- (9)	1a+ (10)	
25	5.5	14	5		3a- (8)	2+ (9)	1
26	13	NEG	NEG		0+ (9)	0++ (9)	1
27, 46	0.5	14	14		1a- (13)	0- (9)	1
28	0.5	14	14		0- (10)	1a- (12)	1
29, 10	1.5	14	14		0- (10)	1a- (10)	
30	24	NEG	NEG		2+ (8)	2- (12)	1
31, 6, 79	3.5	NEG	NEG		2- (8)	3b- (10)	
32, 19	8.75	7	NEG		1a++ (10)	0+ (10)	
33, 7	25.75	7	NEG		3a- (7)	2- (8)	2
34	48	7	NEG		1a++ (10)	0- (9)	2

Patients (& repeats)	Months from Tx	Growth in: PHA	IL-2	CD3	Histology on initial biopsy	Histology on next biopsy	Diagnosis
35	6.25	12	NEG		1a- (8)	1a- (11)	2
36	5.25	NEG	NEG		1a+ (9)	*	2
37	0.25	NEG	NEG		0- (8)	3a- (11)	1
38, 53	3.25	7	7	7	0- (8)	1a- (9)	1
39	16	7	NEG	NEG	1a- (7)	2++ (10)	1
40, 67	6	NEG	NEG	NEG	3a++ (7)	1a- (12)	1
41, 58	0.75	NEG	NEG	NEG	0- (8)	1a- (12)	1
42, 2	14	NEG	NEG	NEG	1a- (7)	3a- (8)	
43, 20	22	NEG	NEG	NEG	2++ (8)	2+ (8)	1
44	11	NEG	NEG	NEG	0- (7)	2- (9)	
45	62	11	7	7	0++ (7)	0++ (7)	2
46, 27	3	NEG	NEG	NEG	0- (8)	1a- (7)	
47	24	7	12	7	1b+ (8)	3b+ (10)	1
48	2.25	12	NEG	NEG	1a- (10)	0- (12)	2
49, 18, 24	25	12	12	12	1a- (9)	0+ (9)	
50, 56	0.25	6	6	6	1a- (10)	2- (10)	1
51, 64	1	NEG	NEG	NEG	1a- (12)	1a- (9)	1
52, 86	22	6	6	6	0++ (12)	0++ (8)	1
53, 38	4	9	NEG	NEG	1a- (9)	0- (8)	
54	8	6	6	NEG	3b- (10)	3a- (11)	2
55	15	NEG	6	6	3b- (10)	1a- (10)	2
56, 50	0.5	6	6	6	2- (10)	1a- (10)	
57	1	NEG	NEG	NEG	1a- (9)	1a- (10)	1
58, 41	1	NEG	NEG	NEG	1a- (8)	1a- (10)	
59, 1	7.5	2	2	6	3b++ (8)	1a++ (8)	
60, 8	29	6	2	3	3a- (9)	1a- (9)	
61	13.5	5	8	NEG	1a- (11)	0++ (6)	2
62	1.5	8	5	5	3b- (8)	1a+ (9)	1
63	24	1	1	1	3b++ (8)	2++ (11)	2
64, 51	1.25	NEG	NEG	NEG	1a- (9)	1b- (10)	
65	14	10	10	2	1a- (7)	1a+ (8)	1
66	37	10	NEG	10	1a++ (12)	0+ (8)	2
67, 40	7	10	10	10	1a- (12)	1a- (9)	
68	0.5	NEG	10	10	2- (11)	0- (9)	1
69	5.5	10	10	10	3b+ (11)	3b+ (10)	1
70	12	10	14	10	1a- (8)	3a- (7)	2
71	2	10	10	10	1b- (12)	1a++ (10)	1
72	2	NEG	13	9	2- (9)	1a- (9)	1
73	72	NEG	9	9	U (10)	3a- (9)	1
74	15	NEG	NEG	NEG	1a- (8)	2- (6)	2
75	0.25	9	9	9	3b- (9)	3a- (12)	2
76	0.5	9	9	9	3b- (10)	3b- (10)	1
77	72	NEG	NEG	13	1a- (10)	0- (6)	2
78, 85	0.25	NEG	NEG	13	0- (11)	0- (9)	2

Patients (& repeats)	Months from Tx	Growth in: PHA	IL-2	CD3	Histology on initial biopsy	Histology on next biopsy	Diagnosis
79, 6, 31	7.75	6	6	6	0- (9)	*	
80	1	6	6	6	3a+ (11)	1a- (12)	1
81	12	1	6	1	3a++ (11)	1a+ (9)	1
82	1	6	6	6	3a+ (10)	3a- (10)	2
83	12	8	NEG	8	0+ (7)	1a+ (9)	1
84	26	5	NEG	1	1a+ (7)	3a++ (6)	1
85, 78	1.5	5	5	NEG	3a++ (8)	3a+ (11)	
86, 52	26	NEG	NEG	NEG	0+ (8)	0- (6)	
87	12	NEG	NEG	5	0- (8)	3a- (7)	2
88	18	7	NEG	7	2- (10)	0- (10)	1
89	15	7	NEG	2	3a+ (15)	1a+ (12)	2
90	72	13	NEG	13	3a+ (13)	3a++ (8)	2
91	19	NEG	NEG	NEG	1a- (9)	3a+ (9)	1
92	3	6	NEG	6	3a- (9)	0+ (10)	1
93	12	1	NEG	1	1a+ (7)	0- (8)	2
94	10	NEG	NEG	NEG	0- (9)	1a- (6)	1

Table 5. Lymphocyte Culture in Relation to Medium Used and Time from Transplantation

The table shows the numbers of biopsies which produced positive cultures. This is broken down by each of the three different supplements to the basic medium. Results are shown as number of positive cultures against total number of biopsies placed in culture in that particular supplement. The patients are divided into those within and those beyond three months of transplantation. The overall figures for positive culture are also shown in the bottom row.

Time from Transplant	**PHA + IL2** (medium 1)	**CD3 + IL2** (medium 2)	**IL2 Alone** (medium 3)
<3 months	19/31 (61%)	12/20 (60%)	15/25 (60%)
>3 months	41/63 (65%)	25/37 (68%)	20/48 (42%)
All biopsies	60/94 (64%)	37/57 (65%)	35/73 (48%)

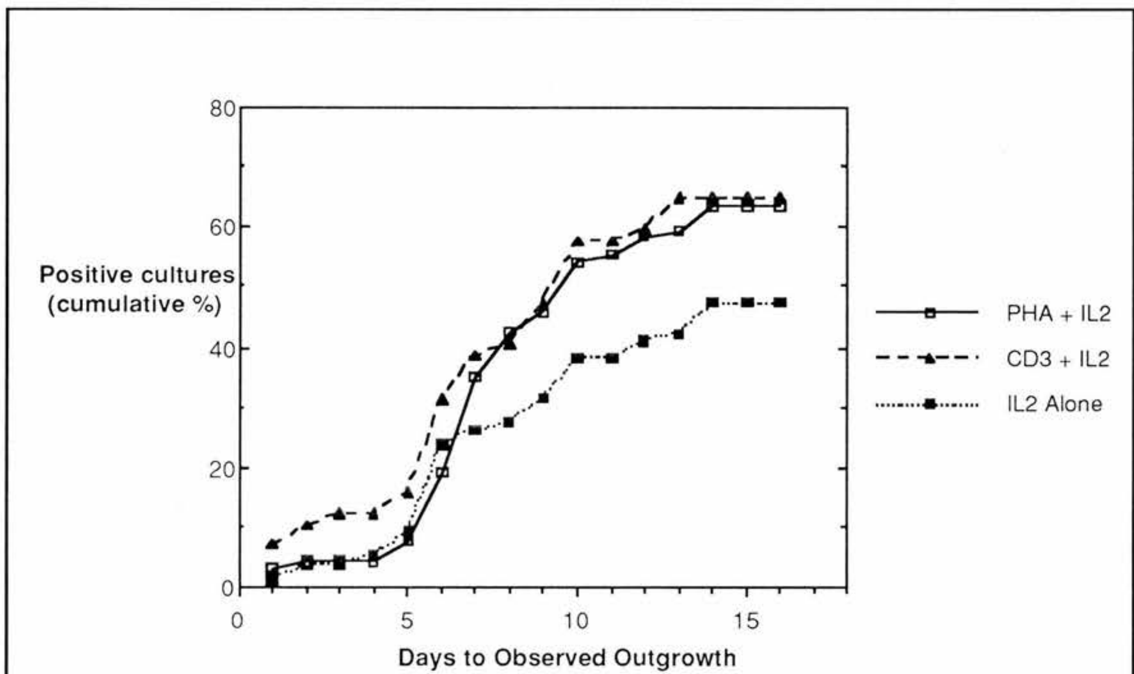
The median time to positive culture for each biopsy fragment grown in medium with PHA and IL-2 was 7 days (range 1-14); 7 days (1-14) in medium with CD3 and IL-2; and 6.5 days (1-14) in medium with IL-2 alone. The overall median time to

positive culture for all fragments (n=224) was 7 days (1-14). Because no new cultures were observed after 14 days the study was ended at this point.

The rate of appearance of lymphocyte outgrowth was similar for each medium (fig.8). Variation between media in time to observed outgrowth using the Log-Rank test was close to significant at traditional levels (p=0.077). Comparing lymphocyte cultures grown in IL-2 alone (medium 3) with the other two media separately gave similar results when adjusted for multiple testing (medium 3 vs. medium 1, p=0.132 and medium 3 vs. medium 2 p=0.082).

Figure 8. Rate of Appearance of Positive Cultures in Different Media Supplements

This cumulative frequency graph shows the accumulation of positive culture results for biopsy fragments grown in each of the three media supplements. The first day on which a culture was deemed to be definitely positive has been plotted for all the positive cultures. It can be seen that by day 14 all positive cultures have occurred.



4.2. Effect of T Cell Activators on Endomyocardial Culture

Fragments from 57 biopsies were grown in all three media. In 36(63%), the culture result was identical irrespective of the culture conditions. Lymphocytes grew from 22 of these 36 cultures and no growth was seen in 14. In the remaining 21 biopsies, concordant results were observed in media 1 and 3 in 6 (3 positive, 3 negative); media 1 and 2 in 8 (all positive) and media 2 and 3 in 7 (3 positive, 4 negative).

4.3. Rejection Grades

The distribution of rejection grades amongst all the biopsies studied was as follows: grade 0 in 23 (24%) biopsies, grade 1a in 37 (39%), grade 1b in 2 (2%), grade 2 in 9 (10%), grade 3a in 15 (16%) and grade 3b in 8 (8%). No biopsies showed grade 4 rejection.

Positive culture results for fragments grown with each of the three supplements are shown below (table 6) in relation to the histological grade of rejection found on biopsies taken at the same time. The distribution of positive culture results among the rejection grades follows what would be predicted if the distribution occurred by chance.

Table 6. Culture Result by Medium Used and Rejection Grade

This table shows the results of lymphocyte culture for fragments grown in each of the three media broken down by the grade of rejection observed on biopsies taken at the same time. The figures given are the number of positive cultures obtained against the total number of fragments grown in that medium, also expressed as a percentage in brackets.

Rejection Grade	**PHA+IL2** (medium 1)	**CD3+IL2** (medium 2)	**IL2 Alone** (medium 3)
0	10/23 (43%)	8/12 (67%)	7/19 (37%)
1a	27/37 (73%)	9/20 (45%)	8/26 (31%)
1b	2/2 (100%)	2/2 (100%)	2/2 (100%)
2	2/9 (22%)	4/5 (80%)	3/7 (43%)
3a	12/15 (80%)	7/9 (78%)	8/14 (57%)
3b	7/8 (88%)	7/8 (88%)	8/8 (100%)
4	0	0	0
All Grades	60/94	37/57	36/76

The series of results for fragments grown in IL-2 alone is also shown as a bar graph (fig. 9). The 1b group of rejection results is too small (n=2) to be meaningful and so this is amalgamated with 1a rejection in the figure.

Figure 9. Rejection Grade and Positive Culture in IL2 alone

The bar graph shows the percentage of positive cultures resulting from fragments grown in medium supplemented with IL-2 only. The apparent trend is towards more positive cultures with higher grades of rejection. Rejection grades 1a and 1b have been amalgamated into grade 1 alone.

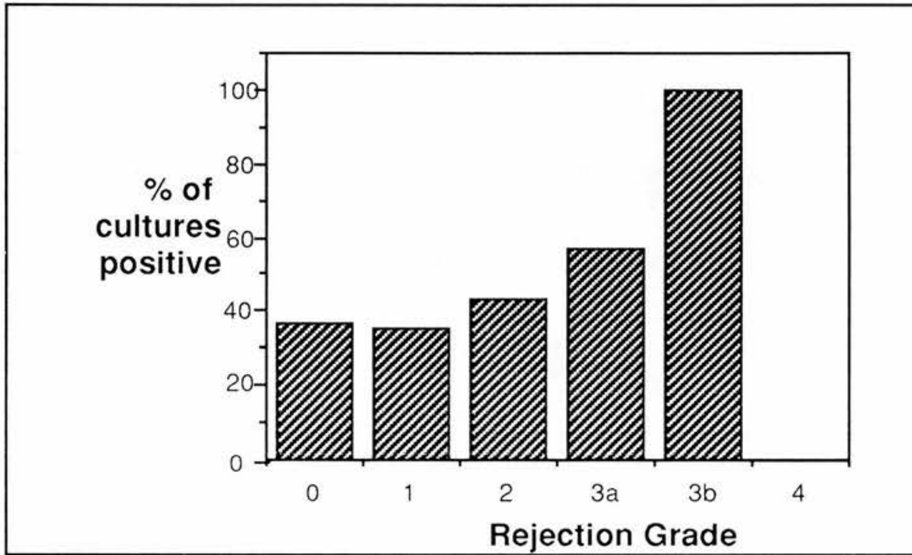
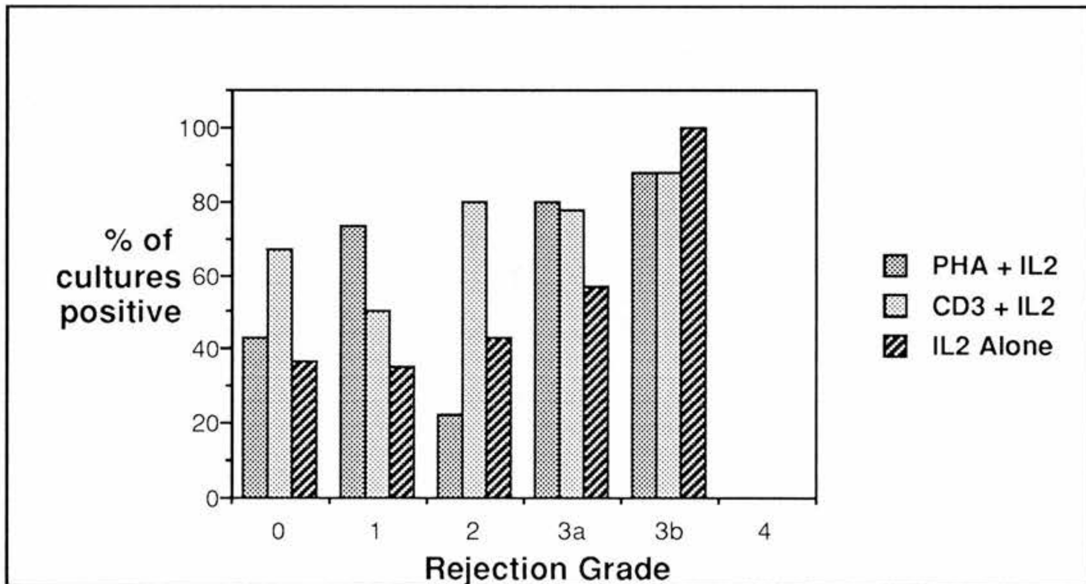


Figure 10. Rejection Grade and Positive Culture for all three Supplements

This bar graph contains similar information to fig.9. Results for fragments grown in each of the three media supplements are shown side-by-side. Here grades 1a and 1b are also amalgamated.



Results for all three media supplements used are shown in figure 10. Here comparison is made between culture results at each rejection grade for each of the three supplements used. The graph shows that the trend towards increasing ability to culture lymphocytes with higher grades of rejection is not so apparent with media 1 and 2.

4.4. Comparison of Culture Results with Rejection

There was a tendency to a higher percentage of positive cultures with higher grades of rejection (approximately 90% for grade 3b versus 50% for grade 1), but this was not significant (for example p=0.08 for medium 1, also see fig. 10). However, if patients whose biopsies revealed grade 3a rejection or higher were designated "rejecting" and those with grades less than 3a as "non-rejecting", then a significant relationship between positive culture and rejection was found for biopsies grown in medium 1 (p=0.025) and medium 3 (p=0.004, see Table 7).

Table 7. Culture Result and Presence or Absence of Rejection

This figure shows the number of positive cultures for each fragment grown in each of the three media according to whether the biopsy was taken from patients whose accompanying biopsies showed rejection or not. It can be seen that for IL-2 alone and for PHA+IL-2, there is a significant difference between culture result in the presence or absence of rejection. The results for CD3+IL-2 are close to significance.

Histology	**PHA + IL2** (medium 1)	**CD3 + IL2** (medium 2)	**IL2 Alone** (medium 3)
Rejection (grade≥3a)	19/23 (83%)	14/17 (82%)	15/20 (75%)
No rejection (<3a)	41/71 (58%)	23/40 (58%)	20/53 (38%)
All	60/94 (64%)	37/57 (65%)	35/73 (48%)
	(p=0.025)	(p=0.062)	(p=0.004)

4.5. Prediction of Rejection

The ability to predict rejection was examined by determining how many patients without rejection on biopsy histology had biopsies which were able to produce a lymphocyte culture. In previous studies patients with such "culture positive, histology negative" biopsies were more likely to be found to have clinically significant rejection when next biopsied (Weber, 1989; Carlquist, 1989). This study found no such correlation, with a low predictive value for subsequent rejection in having a "culture

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positive, histology negative" biopsy (17.5% predictive value for biopsies grown in medium with PHA - see table 8).

Overall, 25% of the patients studied had rejection (grade ≥3a) diagnosed on their next biopsy. Of those patients with rejection on the initial biopsy, 40% still had rejection at subsequent biopsy. Approximately 21% of those patients without rejection at initial biopsy went on to develop it on subsequent biopsy. The ability to grow lymphocytes from biopsies with no evidence of rejection did not predict subsequent rejection for any group (Table 8). In the two tables that follow (8 and 9) only the results for patients with no rejection at initial biopsy are shown. This is because it is this group in which the early detection of rejection would be useful. Patients with rejection at first biopsy receive additional immunosuppressive drugs which would alter the subsequent incidence of rejection. The lowest row in the tables represent the results for all patients (including both positive or negative culture results) for comparison purposes. The subsequent biopsies available for this comparison were performed at a median of 31 days (range 6-400 days) after the first biopsy.

Table 8. Incidence of Rejection at Next Biopsy according to Original Culture and Histology Results

This table shows data for those patients whose original biopsy was not found to show rejection (that is less than grade 3a). The figures are divided according to whether lymphocytes were able to be cultured from associated biopsies taken at the same time. These patients all underwent repeat biopsy at a later date. The figure given is the number of patients developing rejection as observed on this later biopsy as a % of number at risk. No figures are given for patients with rejection and this is why the total figures for positive and negative culture do not add up to the number under the heading "all subjects".

No Rejection	**PHA+IL2** (medium 1)	**CD3+IL2** (medium 2)	**IL2 alone** (medium 3)
Positive Culture	7/40 (18%)	5/22 (23%)	4/19 (21%)
Negative Culture	6/29 (21%)	2/17 (12%)	7/33 (21%)
All Subjects	20/92 (22%)	14/56 (25%)	18/73 (25%)

In order to reduce errors in the figures introduced by those patients who had been transplanted a long time ago, and who therefore were not re-biopsied soon, data are also presented for patients re-biopsied within one month of the original biopsy. The results for these patients are shown below. These data apply to half of the patients studied.

Table 9. Rejection at Next Biopsy for Patients Re-biopsied within One Month

This table, similar to table 8, shows how many patients, of those re-biopsied within one month, had rejection on their second biopsy. The fnumber developing rejection as % of no. at risk is given.

No Rejection	PHA+IL2 (medium 1)	CD3+IL2 (medium 2)	IL2 alone (medium 3)
Positive Culture	4/11 (36%)	0/6 (0%)	0/8 (0%)
Negative Culture	2/15 (13%)	1/6 (17%)	3/10 (30%)
All Subjects	10/45 (22%)	5/25 (25%)	7/36 (20%)

A comparison of the incidence of rejection found on original biopsy with the incidence on the next biopsy is shown below (table 10) for all patients and for those re-biopsied within one month of the original biopsy.

This comparison should determine the efficacy of treating ≥3a grade rejection since subsequent incidence should be reduced in those re-biopsied soon, as a result of treatment with additional immunosuppressive therapy.

Table 10. Original and Subsequent Rejection

This table shows numbers of patients with rejection on original and subsequent biopsies. These are divided into groups according to whether the patients were re-biopsied within one month of the original biopsy and compared with the whole group of patients as a whole. It can be seen that patients treated for rejection and re-biopsied soon have a greater change in incidence of rejection compared with the whole group, for each of the three media supplements used.

		PHA+IL2	CD3+IL2	IL2 alone
All patients	Rejection originally	23 (n=92)	17 (n=56)	22 (n=73)
	Rejection next biopsy	20	14	18
	% Change	**-13**	**-18**	**-18**
Patients re-biopsied ≤1 month	Rejection originally	19 (n=45)	13 (n=25)	18 (n=36)
	Rejection next biopsy	10	5	7
	% Change	**-47**	**-54**	**-61**

4.6. Patients Within Three Months of Transplantation

As seen above (table 5), 31 patients had a biopsy within three months of transplantation which was subjected to lymphocyte culture study with PHA+IL-2 as the supplement, 20 with CD3+IL-2 and 25 with IL-2 alone. The overall ability to culture lymphocytes from these biopsies was similar to that obtained for biopsies taken from recipients beyond the three month period (see table 5). Where the medium was supplemented with IL-2 alone, there appears to be an increase in the ability to culture lymphocytes in patients within three months of transplantation, compared with beyond three months, but this is not significant. Below is a table showing the incidence of rejection on next biopsy (table 11).

Table 11. Prediction of subsequent rejection for patients within three months of transplantation.

This table shows data for those patients within three months of transplantation who did not have rejection, in much the same way as table 7. Again, the figure given is the number of patients developing rejection as observed on this later biopsy as a % of number at risk. No figures are given for patients with rejection. The numbers given are too small for statistically meaningful calculations.

	PHA+IL2 (medium 1)	CD3+IL2 (medium 2)	IL2 (medium 3)
Positive Culture	3/12 (25%)	0/6 (0%)	1/9 (11%)
Negative Culture	1/12 (8%)	0/7 (0%)	1/9 (11%)
All Subjects	8/31 (26%)	4/20 (20%)	6/25 (24%)

4.7. Endocardial Lymphocytic Infiltrates

ELI were found in 29 of the 94 biopsies (31%). In 13 (45%) of these the ELI were associated with encroachment onto or damage of myocytes (designated as 'injury' in table 12). The median time from transplantation in patients with ELI was 13 months (range: 1-72 months) compared with a median of 4 months (range: 1 week to 96 months) for patients with no ELI, (p=0.002). There was no association between the presence of ELI and outgrowth in-vitro in any of the three media used (table 12). However, rejection (grade 3a or greater) was present in 12 of 29 biopsies with ELI as opposed to 11 of 65 without (p=0.013). Adjusting for time from transplantation, using logistic regression, there was still a significantly larger number of subjects with rejection among patients with ELI compared to those without ELI (p=0.011).

The apparent discrepancy between the ability to grow lymphocytes from EMB taken from patients with and without ELI when grown in medium with IL-2 alone, as compared with EMB cultured in the polyclonally stimulating media, does not achieve statistical significance. For example, for EMB grown in medium supplemented with IL-2 alone, the Chi-square value for the difference between culture results in the presence or absence of ELI (without injury) was 2.795 (5/14 versus 12/36). If those EMB grown in polyclonal media were compared with those grown in IL-2 alone, the apparent lower rate of cultures in IL-2 alone is not statistically significant (5/14 versus 18/25, Chi-square value 3.499).

Table 12. Culture Result according to Presence of ELI with or without Myocyte Injury

This table shows the culture results for fragments grown in each media according to presence of ELI and myocyte damage. Each figure is the number of positive cultures grown from the biopsies which were associated with the given histological findings. The figures are broken down by medium supplement as previously. There appear to be more positive cultures with the polyclonally stimulating media but no effect of IL-2 alone. This is not statistically significant.

Histology	PHA+IL2 (medium 1)	CD3+IL2 (medium 2)	IL2 alone (medium 3)
No ELI	39/65 (60%)	22/38 (58%)	24/36 (67%)
ELI	10/16 (62%)	8/9 (89%)	5/14 (36%)
ELI + Injury	11/13 (85%)	7/10 (70%)	6/12 (50%)
Totals	60/94 (64%)	37/57 (65%)	35/73 (48%)

4.8. Phenotyping

Phenotyping of lymphocytes grown from 12 biopsies showed that all contained CD8+ cells. CD4+ cells were present in seven cases and there were no CD19 positive cells observed. In seven patients CD16+ cells were present and in two, where monocytes had been noted on direct microscopy, the CD68 stain was positive. This stain was also used on cultures not thought to contain monocytes where it proved negative. Cell numbers in individual cultures averaged 1.27×10^5 (range $0.36 - 4.3 \times 10^5$) at two weeks.

A table showing the phenotyping results is shown below (table 13).

Table 13. Phenotyping Results

This table shows which cell surface markers were detected by monoclonal antibodies incubated with cytospun preparations (see methods). Twelve consecutive positive cultures were studied and the presence or absence of cells bearing each markers listed is noted.

Subject	CD2	CD4	CD8	CD16	CD68
1	+	-	+	+	-
2	+	+	+	-	+
3	+	-	+	-	-
4	+	+	+	+	-
5	+	+	+	+	+
6	+	+	+	+	-
7	+	-	+	+	-
8	+	+	+	+	-
9	+	+	+	-	-
10	+	-	+	-	-
11	+	+	+	+	-
12	+	-	+	-	-
Totals	12	7	12	7	2

4.9. Ability to Culture Lymphocytes and HLA Mis-matches

Data on HLA antigens were available for 84 patients. The average number of mismatches between HLA antigens of the donor and the recipient at the A locus was 1.4, at B 1.5 and at DR 1.3. The culture results among these patients conformed to the overall results for the whole group as shown by the totals in the table below (table 14). For HLA typing, testing was performed for 12 HLA A antigens, 18 B and 10 DR antigens.

Table 14. Positive Culture Results in relation to Donor and Recipient HLA Mis-matches

This table shows the results for biopsy fragments grown in each of the three media supplements. The table is in three sections arranged horizontally according to number of mis-matches at class I loci(A or B), class II loci(DR) or all three. The figure given is the % of positive cultures obtained from fragments taken from patients with the specified number of mis-matches. The data are broken down according to whether the patients from whom the biopsies were taken had 2 HLA mis-matches at these loci or less.

	HLA mis-matches at given loci	PHA+IL2 (medium 1)	CD3+IL2 (medium 2)	IL2 Alone (medium 3)
Class I	≤1 at A or B	56% (14/25)	50% (8/16)	33% (7/21)
	2 at A or B	68% (40/59)	73% (24/33)	44% (20/46)
Class II	≤1 at DR	63% (32/51)	*59% (19/32)	51% (21/41)
	2 at DR	58% (19/33)	*88% (14/16)	42% (10/24)
All loci	≤1 at All Three	46% (6/13)	44% (4/9)	18% (2/11)
	2 at All Three	67% (12/18)	80% (8/10)	43% (6/14)
	All patients	62% (52/84)	64% (30/47)	50% (42/84)

* = $p<0.05$ (Fishers exact test)

4.10. Patients Undergoing Repeat Biopsy

Of the 73 patients studied, nineteen had two biopsies of whom two went on to have a third biopsy. These biopsies were performed at a median of 21 days apart (range 6 to 365 days). The table shows the results for each of these biopsies along with the histological grade of rejection (table 15). Among the 17 patients who had two biopsies performed, all had both biopsies cultured in medium with PHA, 7 in medium with CD3 and 8 had both biopsies cultured in medium with IL-2.

Table 15: Initial and subsequent culture results in patients who underwent two biopsies

This table shows the culture results at initial and subsequent biopsy for the patients who were biopsied twice in this study. The results are given as initial results followed by a hyphen and then the subsequent result (pos = positive culture result, neg = negative culture result) for each of the three possible media supplements used.

	Pos - Pos	Neg - Neg	Pos - Neg	Neg - Pos	Total
PHA	5	3	4	5	17
CD3	1	2	3	1	7
IL-2	1	3	3	1	8

Two patients underwent three biopsies each, one male and one female. The results for these two patients are shown (table 16).

Table 16. Results for two patients who each underwent three biopsies

This table shows the culture results along with the histology observed on concurrent biopsy for the two patients who were biopsied three times during this study. There appears to be no consistent pattern of lymphocyte growth in patient A but EMB from patient B produced lymphocyte cultures each time.

Biopsy: Patient A	Culture Result	Histology	Time between biopsies (days)
1	Pos	1a	-
2	Neg	2	36
3	Pos	1a	21
Patient B			
1	Pos	1a	-
2	Pos	3a	20
3	Pos	1a	14

4.11. Extension of Culture Period

As noted above, no new cultures were observed beyond 14 days. However, attempts were made to extend the culture period thereafter. Overall, most cultures were maintained for approximately one month before they began to die off. Outgrowth into round bottomed wells of 96 well culture plates was limited by the number of cells which could grow in the space available. Therefore cultures were split. However when this was done no growth occurred in the daughter wells, though cells left in the original well were able to be maintained for longer. It was concluded that the specific antigenic stimulus of the biopsy fragment was required to promote growth. It was not clear whether any (normal) heart would serve this purpose or whether donor heart would be required. If donor specific antigens were required it was felt the closest solution would be donor originated fibroblasts - which would not be heart specific, as cardiac myocytes could not readily be cultured. Therefore a means of separating cardiac antigens was required which might be applied to EMB fragments.

5. Cardiac Transplantation and EMB: Discussion

5.1. Lymphocyte Culture and Phenotype of Cultured Cells

This study compared the effects of adding polyclonal stimulators of T cell proliferation, PHA and anti-CD3 antibodies, to a basic medium containing IL-2 alone. This comparison was made primarily to see if it was possible to enhance the ability to culture lymphocytes from EMB in order to increase the sensitivity of this method as a diagnostic tool. Part of this work has been published (Harcombe, 1993).

Rejecting myocardium would contain activated lymphocytes expressing IL-2 receptors, which should proliferate in the presence of IL-2 (Smith, 1986). IL-2 has been used in all the previous studies of T cell growth from EMB (table 1). Media containing polyclonal activators might favour outgrowth of any T lymphocytes present in the biopsy, even if they were not activated and by implication not participating in the rejection process. T lymphocytes possess the cell surface marker CD2 (reviewed in Bierer, 1993) and it is through this that PHA predominantly exerts its proliferative effects (Nowell, 1960). Furthermore, all T cells express CD3, and anti-CD3 antibodies are known to mediate proliferative signal transduction through this molecule (Van Wauwe, 1980).

Natural killer cells possess CD16 cell surface marker (the IgG Fc receptor or FcRIII) as well as low levels of CD2. They also express IL-2 receptors and might be capable of proliferation in this culture system (Nagler, 1989). They may therefore grow in all three media, but undergo greater expansion in media supplemented with PHA or CD3, making a positive culture result more likely. Against this hypothesis is published evidence showing that lectins such as PHA and Concanavalin A are not able to induce NK cells to proliferate, even in the presence of autologous accessory cells (London, 1985; Ythier, 1985). However, there are data to show that PHA may increase the ability of non T-cell large granular lymphocytes to respond to IL-2 (Hercend, 1982; Roberts, 1988), although these effects are not large when examined in terms of proliferation frequencies by LDA (Vose, 1983).

This study confirmed that it is possible to culture lymphocytes from EMB efficiently as 48 to 65% of EMB produced positive cultures, depending on medium supplement used. The study did not select biopsies in that patients who did not have rejection clinically, including those more than a year post-transplantation, were included.

Polyclonal stimulation did not significantly affect the ability to produce lymphocyte cultures but there was a trend to more positive cultures with medium 1 and 2. This comparison, made directly using the Chi-square test, does not produce a significant difference, as shown in the results section. Furthermore, analysing the data

by linear regression confirms this finding. In most previous studies IL-2 alone was used as a culture supplement; rarely have polyclonal stimulators of T cell proliferation been used and no comparisons made (table 1).

It was found that cultures were positive at a median of 7 days (range 1-14). This compares with figures varying from 10+/-6 days (mean+/-SD) to 12.9+/-9.1 days, depending on degree of rejection on histology, reported by Carlquist et al (Carlquist, 1988). Other studies of similar nature (table 1) have not commented on this aspect of lymphocyte culture from EMB. Whereas the present experiments examined 94 biopsy fragments from 73 patients at all stages after cardiac transplantation, Carlquist studied 125 biopsies from 14 patients only, between 48 and 361 days after cardiac transplantation. By beginning their study at 48 days they excluded many early aggressive episodes of rejection, when more active infiltrates of T cells would be present. This could account for the difference observed in this study.

48% of biopsies grew lymphocytes in a medium supplemented with IL-2 only, which agrees closely with Carlquists figure of 49% (61 of 125) biopsies (Carlquist, 1988), in a similar but not identical medium (RPMI-1640 supplemented with 5% human group AB serum and 160 units/ml human IL-2). Similarly, Ahmed-Ansari et al (Ahmed-Ansari, 1988) also used a high concentration of IL-2 in their study of 238 biopsies between 2 and 390 days after cardiac transplantation. Biopsies were cultured in RPMI-1640 supplemented with 10% human serum and 10% IL-2 or 1000units/ml r-IL-2 (both were used but it was not made clear for which patients). They found 43% (102/238) positive cultures, again comparable with the figure of 48% from this study. Thus, using IL-2 at such high concentrations is clearly unnecessary.

However, Suitters et al (Suitters, 1989) cultured T cells from EMB using RPMI-1640 supplemented with 28u/ml r-IL2, only 27% (28/104) of cultures grew. This cannot be explained by IL-2 concentration and it is interesting to note that Suitters group did not use human serum. The provision of hitherto undefined growth factors by serum may be important in lymphocyte outgrowth in addition to IL-2. Perhaps other lymphokines such as IL-1, IL-4, IL-6 and IL-10 are provided by human serum in limited amounts. IL-1, along with anti-CD3 antibody, is able to induce proliferation and IL-2 production in CD4+ and CD8+ cells, whereas CD3 antibody alone induced IL-2R expression but not proliferation or IL-2 production (Stein, 1992). IL-4 can function as a T cell growth factor and is able to stimulate proliferation even in the absence if IL-2 (Cherwinski, 1987; Kurt-Jones, 1987). In addition, as well as promoting T cell growth and acting as a B cell growth factor, IL-4 increases class II expression on B cells (Paul, 1987). IL-6 has many effects but may play a role in cytotoxic T cell differentiation (Van Snick, 1990) as might IL-10 (Mosmann, 1991).

In this study the results of phenotyping showed a predominance of CD8+ cells (present in all twelve cultures) with CD4+ cells in 7. There were no CD19 positive cells (B cells) observed. In seven patients CD16+ cells were present and in two, the CD68 stain(monocytes) was positive. The phenotype of cells cultured from needle biopsies of renal transplant allografts was also CD8 and CD4 positive T cells, though CD8+ cells predominated (Mayer, 1985; Miceli, 1985). These studies were performed in clinically and biochemically rejecting kidney allografts and direct comparison is difficult.

The results presented are similar to those of Carlquist, who found in 12 of the 61 cultures studied, CD8+ cells in all 12 cultures, 5 of 11 contained CD4+ cells, 4 of 10 had CD57 (Leu 11) positive cells, B lymphocytes were found in 4 of 10 and one culture had cells bearing a monocyte/macrophage marker (M1) (Carlquist, 1988). B cells were not found in the present study, but in all other respects the results are very similar. Carlquist looked for CD57+ cells, but this is a poor marker for natural killer cells. In transplant patients these cells are probably found as a subpopulation of CD8 cells. Suitters et al phenotyped 28 cultures and found CD8+ predominance in 57% and CD4 predominance in 36%, results which are similar to those presented here (Suitters, 1989).

As already stated, natural killer cells possess IL-2 receptors, may be present in EMB and might proliferate in this culture system. There are data from other studies to confirm the ability of NK cells to grow in vitro (for example: London, 1985). The phenotyping data indicate that cells positive for the CD16 marker, which include natural killer cells, are indeed present. These cells also possess the CD2 cell surface marker through which PHA exerts its proliferative effects.

Natural killer cells, though detectable, are not a dominant feature of cellular infiltrates in acute rejection (Marboe, 1983), thus cultured NK cells should not correlate with histological acute rejection. Ahmed-Ansari's group tested for NK activity and less than 5% of CD4+ cultures had any, 3 of 28 CD8+ cultures had weak NK activity whereas 2 of 5 CD3+ CD4- CD8- cultures had NK activity (Ahmed-Ansari, 1988).

Thus culture of T lymphocytes from EMB can be achieved efficiently using either IL-2 alone or IL-2 with polyclonal T cell activators. A relationship between ability to culture lymphocytes and clinically significant rejection was found when EMB fragments were cultured in PHA+Il2 and with IL-2 alone, but not with CD3+IL2. Therefore a medium containing IL-2 alone is satisfactory for these studies and it may promote culture of a population of cells which bear some relationship to the rejection process. However it is not certain that these cultured cells mediate rejection. Future studies should test for functional differences between phenotypically similar populations of cultured cells. They should also examine the relationship between the functional attributes of lymphocytes observed histologically in EMB and those cultured.

In animal models, as discussed earlier, allograft rejection is mediated by both CD8+ and CD4+ cells. Infiltrating lymphocytes of each phenotype can be grown from murine cardiac allografts by a culture system similar to that discussed here (Carlquist, 1990b). It is also known that not all cells infiltrating grafts are involved in the rejection process (Heidecke, 1990). Therefore it may be possible to perform experiments in animals which would help to answer questions which cannot be answered safely in man.

An additional aspect for further study might be the long term culture and cloning of cells. This would allow analysis of the functional repertoire of individual cells, though of course would be even further removed from the in-vivo situation. Lymphocytes grown from EMB might be bulk cultured initially and then cloned by limiting dilution. Clones with cytotoxicity against cells of donor origin could be tested for their fine specificity against proteins suspected of being involved in responses against cardiac myocytes. Target cells for LDA using these clones would be a problem and it would be best to set up a prospective study whereby donor splenocytes were available for these experiments. Experiments should be performed on clones originating from cultures obtained with and without polyclonal stimulators in the culture medium in order to determine whether there are any functional differences in these cells.

In the short term, bulk cultures could be tested using the polymerase chain reaction for expression of cytokine mRNAs to determine if particular patterns of cytokine expression are associated with particular patterns of histological rejection. The results may be rather confusing in that a range of cytokine mRNAs might be detected. This study might then be extended to cloned cells since these would produce a relatively pure pattern of cytokine expression. It may be possible to differentiate Th1 from Th2 type responses among these cells (Mosmann 1989). This could then be correlated with ISH for mRNA on EMB taken from cardiac transplant recipients to determine if the cells bulk cultured and then cloned from the EMB present a true picture of in vivo cytokine expression. The relationship to histological evidence of rejection could also be examined. It might then be possible to manipulate the immune response in patients with these patterns of rejection (by using monoclonal antibodies to the relevant cytokine for example).

Another approach to the problem of fine-tuning immunosuppressive therapy might be to use anti-sense oligonucleotides against sequences of transcribed cytokine genes. This might allow a more specific form of modulation of the immune response than current therapy and could be tailored to use only those oligonucleotides directed against cytokines expressed in the patients EMB. This would have to be carefully studied in animal models since there may be unpredictable effects on other aspects of the recipients immune system which might be adverse. There are few studies at the

molecular level conducted in the field of clinical heart transplantation and it may be that this area is going to prove fruitful in solving the problem of rejection.

5.2. Rejection, and Prediction of Rejection

The occurrence of acute rejection of cardiac transplants has always been the major source of morbidity and death, particularly in the first three months post-transplantation. Thereafter, opportunistic infection, resulting from the inevitable immunosuppression caused by the drugs used to induce tolerance of the graft, assumes a similar importance. The balance between sufficient immunosuppression to prevent graft rejection and excessive treatment leading to infection is difficult to maintain. Patients with acute rejection (grade 3a or greater) receive short (several days) pulses of high dose intravenous steroids. In this study therefore, the cut-off point for "rejection" versus "non-rejection" was selected as grade 3a, because it has direct clinical relevance.

The absence of clinical trials or experimental evidence to confirm that this policy is correct is of concern. It is crucial to the study of human cardiac transplant rejection that the histological changes observed be validated. However, it has been shown that heart failure (as opposed to sudden death) in association with histological evidence of rejection can be reversed by augmented immunosuppression, which also reverses the changes of rejection (Myles, 1987). It may be that animal studies using the working formulation for rejection histology will be the only practical way to approach this problem. Clinicians feel (informal discussions with members of the Transplant team at Papworth) that as long as there is a balance between patients succumbing to rejection and to infection then the immunosuppression regime is probably being used appropriately. This of course begs the question as to whether the regime might be used even more appropriately so that fewer people die of either complication.

Trials of augmented immunosuppression in grade 3a rejection might be performed in a comparative fashion but the very real concern that a patient might succumb to acute rejection would probably prevent this since the main endpoint would be death. However, it may be that patients are succumbing to infection as a result of excessive immunosuppression. Despite these concerns, five year survival in cardiac transplant recipients is quite high with modern management (Heck, 1989) and thus change is difficult to envision. It would appear that the traditional approach of histological examination of EMB cannot take this situation forward. The search for a more specific, truly predictive, test for acute rejection should continue.

Since acute rejection is a major cause of graft loss, its early detection is of paramount importance. It is difficult to detect rejection clinically in many cases. A patient may present with a fever, heart failure, arrhythmia or even sudden death. Therefore, routine endomyocardial biopsy remains the test performed to detect sub-

clinical rejection prior to the development of serious cardiac dysfunction. It is this biopsy material which has been studied in reports of T cell culture from these patients.

Previous studies have claimed that the ability to culture lymphocytes from EMB where the associated histology shows no rejection (so-called "culture positive, rejection negative biopsies") allows prediction of subsequent rejection (Carlquist, 1989; Weber, 1989). In these studies patients whose EMB were able to produce cultures, when rejection was not apparent on histological grounds, went on to develop rejection more frequently than those without rejection whose biopsies were not able to produce lymphocyte cultures. It was therefore suggested that the use of lymphocyte culture would allow clinicians to detect those patients at increased risk of developing rejection in the future.

In this study media 1 and 3 enabled a significantly increased number of biopsies to produce positive cultures in the presence of rejection. Thus, a relationship between ability to culture lymphocytes and rejection (grade ≥3a) was demonstrated. In the earliest studies of this nature too few samples were involved to determine if there was such a relationship (Zeevi, 1986). Carlquist described a correlation between ability to culture lymphocytes and rejection (Carlquist, 1988) but this study was performed on small numbers of subjects (14). Farrell et al also reported a correlation between ability to culture lymphocytes and rejection (Farrell, 1990). However, Ouwehand et al studied 535 biopsy fragments from 87 subjects and reported no difference in ability to grow lymphocytes from biopsies from patients with rejection compared to those without rejection (Ouwehand, 1990).

In this study, when lymphocytes were grown from a biopsy and associated histology did not show rejection, it was not possible to predict an increased likelihood of subsequent episodes of rejection. In contrast, Carlquist et al found the incidence of subsequent rejection, in patients with no histological rejection but positive cultures, was higher than for those patients whose biopsies did not grow lymphocytes (Carlquist, 1989). Among Carlquists' subjects, whose cultures were positive when an associated biopsy showed no rejection; 24 of 45 (53.3%) experienced worsening rejection (by one grade or more), 18(40%) had no change and 3(6.6%) had a reduction in histological grade. Of 24 patients with rejection, and a positive culture result, 16(66.6%) required treatment for acute rejection. The authors concluded that the culture system as described could be of value in the diagnosis and prognosis of acute rejection in cardiac allograft recipients. From the data presented in this publication, an abstract which was not followed by a more substantial publication, it is difficult to agree with this conclusion. The grading system used for rejection was not defined, the grades were not given and the histological grade at which additional immunosuppressive treatment would be used was not indicated.

Weber et al also reported that lymphocyte culture in the presence of negative histology predicted subsequent rejection episodes (Weber, 1989; Duquesnoy, 1988). In one study they examined 113 biopsies from 55 patients (who had not previously suffered rejection) taken at various time intervals post-transplantation (0 - 10, 11 - 20 or 21 - 30 days) (Weber, 1989). Of biopsies which were histologically negative for rejection, 40% were associated with positive lymphocyte culture, compared with 81% where rejection was identified. Patients who had not previously had a rejection episode and whose biopsy produced a positive culture had a higher cumulative incidence of subsequent rejection within 30 days than those whose biopsy did not grow lymphocytes. However, for patients beyond twenty days after transplantation, the incidence of subsequent rejection within 30 more days was no longer significantly different between patients with and without positive cultures. The results presented therefore confirm that after the early post-transplant period it is not possible to use positive culture results in the absence of rejection to predict for subsequent rejection. Given that small numbers of patients were involved in each sub-group (12 to 16) in Webers study, the authors reference to a statistical software package, without a description of how it was used, left the reader unfamiliar with "BDMP Statistical Software" unable to critically evaluate the data. The validity of the authors' conclusions is therefore in question. The culture period in this paper was quoted as being 10 to 14 days. This would be too long to allow any predictive value to be clinically useful, since patients would be developing rejection before they could be recalled for repeat biopsy. Finally, the key role of the histological grading system in use (Billingham's criteria) was not discussed. Histological findings at grade 2, 3 or 4 were designated as rejection, and minor differences of interpretation could result in major differences in correlation with rejection. It should also be noted that Billingham's grade 2 would not now be labelled as clinically significant rejection and would not require treatment with augmented immunosuppression.

The data presented in the current study do not show any predictive value in "culture positive rejection negative" episodes. Therefore, as far as the value of lymphocyte culture from histologically negative EMB in predicting subsequent rejection is concerned, there is little real evidence in favour. Given the time course of lymphocyte culture in these studies the likelihood of this method proving useful seems low.

5.3. Culture Media

A basic culture medium, containing IL-2 and supplemented with PHA, anti-CD3 antibodies or nothing, was used to culture lymphocytes from EMB. This study compared the effects of the different supplements to those of IL-2 alone.

In the past a variety of culture medium supplements have been used to support T-cell outgrowth from EMB. It is known however that medium supplemented with IL2- alone is able to support outgrowth of lymphocytes from such biopsies (Ahmed-Ansari,1988; Carlquist, 1990a). In this study the use of medium supplemented by polyclonal T cell activators, compared with IL-2 alone, did not significantly affect the ability to grow lymphocytes from EMB fragments. Furthermore the supplement used did not affect the time taken to detect T cell outgrowth. Thus all three media used were associated with a similar number of lymphocyte cultures grown from EMB, which were detected after the same number of days in culture.

This study did, however, find an association between T cell outgrowth and clinically significant rejection requiring treatment (≥grade 3a). This was only significant when medium with PHA+IL2 and with IL-2 alone was used, suggesting that these conditions favour the growth of a population of cells which is participating in acute rejection. Carlquist et al, using medium supplemented with human IL-2, also reported that outgrowth of T-lymphocytes from endomyocardial biopsies was associated with rejection (Carlquist, 1988). Ouwehand et al and Farrel et al reported similar findings with different media supplements; in the former irradiated autologous peripheral blood mononuclear cells(PBMC) and in the latter tissue culture supernatant from PHA stimulated normal human PBMC (Farrell, 1990; Ouwehand, 1990). Weber et al, used RPMI-1640 supplemented with 10% human group AB serum, 200 units/ml of r-IL2 and irradiated autologous peripheral blood lymphocytes (10^5 per culture well) as feeder cells (Weber, 1989). This study found a relationship with rejection. Perhaps the addition of feeder cells in some way affected these results. The presence of feeder cells would promote cell to cell contact and they might secrete undefined factors into the medium which may affect cell growth.

It would therefore seem that the results obtained in the current study are consistent with those previously published. It is not clear however why the use of anti-CD3 antibodies as a culture medium supplement is not associated with a relationship between positive culture and rejection. This supplement has not been used previously. It does not allow more positive cultures to be produced (65% versus 64% for medium with PHA) and so it is not acting in some general way to increase the efficiency of the culture system. It merely seems to reduce the specificity of culture as a test of the presence of significant rejection.

This finding might suggest that, in the presence of anti-CD3 antibodies, some cells present in an EMB fragment are inhibited in their ability to grow, and these are related to rejection. On the other hand cells which are not related to rejection might experience an enhanced proliferative stimulus. Experiments might be conducted using cells cultured in the presence of anti-CD3 antibodies. These cells could then be labelled

with fluorescent markers. The blast cells could then be separated by fluorescence activated cell sorting (FACS) and then examined for their functional activity. In such experiments cells grown in IL-2 alone and in IL-2 plus PHA must be used for comparison.

It may also be possible to clone cells from the CD3 cultures. The function of cells which have responded may then be studied, although a large number of established clones would be required. It is possible that the optimal dilution of anti-CD3 antibodies, established in proliferation assays performed on PBMC from healthy individuals, bears no relationship to that required to promote growth in an EMB fragment. This may be difficult to study but using cloned cells the proliferative response to CD3 antibodies may be measured and compared with those initiated with PHA or IL-2 alone.

In the current studies it was observed that when attempts were made to sub-culture positive cultures they died if the biopsy was not present. Therefore the antigenic stimulus of the EMB itself was required. If it were possible to determine which protein(s) produced this effect then specific antigenic stimulation could be provided to sub-cultures for cloning purposes. Further experiments could be conducted to attempt to establish the cellular components most effective at promoting proliferation of cells cultured from EMB.

Human myocardium could be fractionated and the fractions tested for differential effects on growth of cloned cells, in small concentrations. The fractions could then be further separated, perhaps by molecular size, and again examined for ability to promote proliferation. By this means, provided a stable clone was available for a series of tests, it might be possible to narrow down the identity of the molecules against which the T cell response is directed in acute rejection. This approach can be employed for T cell responses and it has been used with myocardial proteins. Paque et al fractionated myocardium in their study of murine coxsackievirus myocarditis (Paque, 1979) and Gulle et al separated mycobacterial proteins by two-dimensional electroelution in their study of T cell responses to mycobacteria (Gulle, 1990).

If a particular protein was implicated proliferation experiments, where lymphocytes matched or mis-matched at class I and class II could be used as antigen presenting cells, could be performed in an attempt to define the specificity of the T cell response. If class I responses predominated then cytotoxicity experiments might be performed. LDA might be employed to determine the fine specificity of T cell clones of CD8+ phenotype with cytotoxic activity. Target cells could be matched lymphocytes primed with the relevant peptides.

5.4. Endocardial Lymphocytic Infiltrates

In this study 31% of biopsies contained ELI. This is higher than the 5 to 17% previously reported (Imakita, 1988; Radio, 1991; Kottke-Marchant, 1989; Suit, 1989). This may in part reflect the recent requirement to document their presence (Billingham, 1990). Alternatively it may be due to sampling error in previous studies. In this study at least ten biopsy fragments were obtained from each patient and nine examined histologically. In previous studies it is seldom reported that more than four or five fragments were obtained. Thus other workers may have underestimated the frequency of ELI.

The possibility that such infiltrates might preferentially affect lymphocyte outgrowth was considered. Occupying a sub-endocardial position these cells lie in close approximation to the areas of tissue taken at biopsy. They do contain T cells and hence could be associated with positive cultures. The study specifically examined this possibility but the results showed no such effect. No data are available from other studies to compare with the absence of an effect on ability to culture lymphocytes observed in this study.

In contrast to the previous findings of Kottke-Marchant (Kottke-Marchant, 1989) and Radio (Radio, 1991), but in agreement with Forbes (Forbes, 1990) an association was found between ELI and presence of acute rejection of the deep myocardium. This finding is very different and challenges current assumptions that these infiltrates are not related to acute rejection. The relationship may reflect a role for cells contained in the ELI in mediating rejection. Alternatively the presence of these cells may be secondary to an ongoing process of rejection, with locally secreted substances recruiting these cells into areas where acute rejection is occurring.

From Forbes study, in patients taking azathioprine and steroids the occurrence of significant ELI was almost wholly limited to biopsies with rejection. An incidence of 15% of ELI was reported by Forbes et al for patients taking cyclosporine. There was a significant relationship between ELI and rejection, with more ELI being observed in patients with moderate rejection, than in those with mild or no rejection. Thus the data presented in the current study are consistent with those of Forbes et al. In any event this study suggests that ELI require further examination in terms of their relationship to rejection and a larger scale histological study should be performed concentrating on this aspect alone (Forbes studied 172 patients from 4 to 16 months after transplantation only).

Like Kottke-Marchant (Kottke-Marchant, 1989) it was found that approximately half the ELI's were associated with myocyte encroachment and injury. This lends further support to the proposition that these cells are able to damage myocardium.

Without functional data on these cells it is difficult to investigate their role. The culture system employed does not allow the isolation of these particular cells and so alternative methods are required in order to study this further.

The incidence of ELI increased with time from transplantation, as found by Radio (Radio, 1991). This suggests that ELI are a phenomenon which may be related to chronic as opposed to acute rejection. Or it may be that the infiltrates are indolent and slowly accumulate, being more readily detected after long time periods. The possibility that these cells are related to an early form of lymphoproliferative disorder also exists. The fact that they are less likely to be present at an early stage after transplantation is against their having a role in acute rejection, which is commonest in the first three months.

As alluded to in the introduction, the significance of ELI is debated. Their true role is as yet undefined, but it is tempting to speculate that further studies on ELI may yield valuable information about the nature of cells which invade the heart and yet do not appear to be related to myocyte damage. There, is in addition, work to suggest a relationship between ELI and subsequent lymphoproliferative lesions (Kottke-Marchant, 1989; Suit, 1989). Suggestions that these lesions are associated with EBV are disputed by Nakhleh et al (Nakhleh, 1991). If ELI are associated with EBV, which in turn is associated with post-transplant lymphoproliferative disorder, then the precise inter-relationship between the various factors inducing the appearance of ELI needs to be worked out carefully. Since the immunosuppressive agents most commonly employed appear to induce ELI, particularly cyclosporine, this area requires detailed study with a view to preventing myocyte damage, and in the long term, the development of lymphoma.

It may be that ELI are indeed related to EBV infection, though this remains to be shown. If they are then the lymphocyte proliferation responsible for these infiltrates would be determined by viral gene products. Studies might then examine which proteins played the most important part in stimulating proliferation and how such proliferation might be blocked. The activation of lymphocytes in ELI, though unrelated to rejection directly, may yet be deleterious since these cells may elaborate cytokines which might promote responses against the allograft in other lymphocyte populations. This would then imply a relationship between the ELI and rejection which might be of functional significance. Alternatively, ELI may not be a product of viral stimulation and may not be related to rejection. If so they would merit study since they represent a diverse population of cells present within the allograft and not responding against it to cause injury. The mechanisms by which these cells are brought to be benign in terms of rejection might be employed to produce similar effects in other cell populations. To date, the intriguing question as to why ELI are present is unanswered.

5.5. HLA Antigens and Culture Results

The work in this thesis included examination of the HLA mis-matches between donor and recipient to determine if there was a relationship between number of mismatches and ability to culture lymphocytes. The hypothesis was that recipients of hearts from donors mis-matched at major histocompatibility loci would be more susceptible to rejection and therefore more likely to be associated with biopsies able to produce lymphocyte cultures.

The results did not in general bear out this hypothesis. However, there was a significant increase in positive results in the presence of 2 HLA DR mismatches when biopsies were cultured in medium containing IL-2 and CD3 antibodies (medium 2), but not in IL-2 alone or when supplemented with PHA. It may be that a co-stimulatory effect of the anti-CD3 antibodies allows a greater proliferation of lymphocytes in response to this medium compared with the other two. This might suggest that the CD3 complex, in association with class II, plays a major role in the proliferation of graft infiltrating lymphocytes.

HLA DR molecules are usually recognised by CD4+ lymphocytes which may then proliferate and secrete cytokines which activate monocytes and perhaps CD8+ cytotoxic cells. The increase in positive results for biopsies taken from patients with this degree of mis-match, compared with those without the mis-match, may indicate that recognition of HLA DR molecules on the surface of donor myocardial cells has a role in the process of acute rejection.

Carlquist et al examined 289 EMB taken from 41 heart transplant recipients at the time of routine biopsy (Carlquist, 1990). Simultaneous biopsies were obtained for histopathology, lymphocyte culture and immunocytochemical staining for HLA DR expression. They observed two patterns of DR staining: vascular and interstitial, as previously described by Rose et al (Rose, 1986). When associated biopsies showed vascular DR staining, lymphocytes were cultured more often (65%) than when associated biopsies showed interstitial DR staining(35%). Vascular and interstitial staining were both more likely to be observed if rejection was detected.

It is not clear whether the rejection detected is the result of high levels of expression of these molecules or if expression of DR is induced by host factors as a result of, or as part of, the rejection process. If the findings of Carlquist (Carlquist, 1990) are interpreted in terms of the MHC antigens expressed by the donor then it might be that recipients with certain mis-matches with the donor at particular HLA loci would be more likely to experience rejection. This fits with the Carlquist data but of course it is not known whether DR expression on the surface of myocytes in the

biopsies in the current study was increased or not. Future studies might encompass this aspect in order to further investigate the role of DR expression.

These results are interesting in the light of recent work demonstrating a beneficial effect of matching for HLA A, B and DR on graft survival at three years (Opelz, 1994). It has been shown that HLA DR mis-matches are more likely to result in the ability to culture lymphocytes from EMB in this study and that such mis-matches are associated with poorer outcome in Opelz study. These findings lend themselves to speculation that the ability to culture lymphocytes is more related to MHC mis-matches than directly to histological rejection, though this can only be suggested for HLA DR. The relationship of ability to culture lymphocytes with rejection may be an indirect one with MHC mis-matches at HLA DR the primary influence. The HLA antigen system is highly polymorphic so that a very much larger study to test for a significant relationship between ability to culture lymphocytes and any one HLA antigen, or group of antigens would be required. The fact that this study was able to detect any difference for the DR mis-matches may indicate a very large effect. It should also be pointed out that failure of this study to detect any effect of HLA A or B antigen mis-matches does not mean that none exists.

Future studies might examine a larger number of patients with reference to HLA DR mis-matches and determine, over a longer time period, if patients with two mis-matches at this locus in comparison with those with one or no mis-matches experience: 1) more episodes of clinically significant rejection; 2) a greater ability to culture lymphocytes from their EMB and 3) whether cultured and in situ lymphocyte populations are of consistent phenotype (such as CD4+ as might be expected) in these patients. The ability of the cultured lymphocytes to proliferate when co-cultured with irradiated lymphocytes bearing donor HLA DR antigens which have been primed with peptides of myocardial origin might also be studied.

5.6. Conclusions

These studies have shown that efficient culture of T lymphocytes from 48 to 65% of EMB from cardiac transplant recipients at all stages after transplantation is possible. The ability to culture lymphocytes relates to presence or absence of clinically significant rejection when medium with IL-2 alone or IL-2 supplemented with PHA is used but not medium supplemented with anti-CD3 antibodies. The absence of rejection on histology of the EMB when lymphocytes are able to be cultured does not predict subsequent rejection. More cultures are obtained in the presence of 2 HLA DR mis-matches than in those patients with only one or no mis-matches at this locus.

The results suggest that the positive cultures would most easily be obtained from a biopsy taken from a patient undergoing acute rejection where there were 2 HLA

DR mis-matches between donor and recipient. Biopsies would best be cultured in IL-2 alone but PHA supplemented medium could be used and might produce larger cell numbers due to its polyclonal activating effect.

It is concluded that the use of polyclonal activators in culture medium confers no advantage over the use of IL-2 alone in the ability to grow lymphocytes from EMB. Furthermore anti-CD3 antibodies obscure a relationship between acute rejection and *in-vitro* outgrowth of lymphocytes from EMB. Because no association was detected between lymphocyte outgrowth and future rejection, this technique may have little to offer in clinical management. However, the use of IL-2 culture to isolate functional lymphocytes from EMB may allow characterisation of lymphocytes directly involved in the rejection process.

Future studies should examine the role of ELI in acute rejection and in particular functional testing of cultured cells from ELI should be undertaken. This might be achieved in animal experiments but would be difficult in man since the isolation of these infiltrates from cells involved in deep rejection of the myocardium would be very difficult.

There are many other difficulties involved in taking this work further. Cell numbers have not been sufficient to allow many experiments that might otherwise be planned. Flow cytometry for example requires at least 10,000 cells per sample and thus phenotyping a typical culture in these experiments would use up a significant number of the cultured cells if a full panel of antibodies were used. Sub-culturing cells might be of assistance but this resulted in death of cells deprived of antigenic stimulus from the EMB fragment. Therefore some means of artificially providing the equivalent antigenic stimulus in order to maintain functionality of these cells should be sought. This might be achieved by the experiments described above whereby fractionated human myocardium is tested for ability to induce proliferation in cell cultured from EMB.

Part II

Alcoholic Cardiomyopathy

6. Alcoholic Cardiomyopathy - Introduction

6.1. Historical Perspective

The Ancient Egyptians believed that beer was invented by the goddess Osiris (Ghalioungui, 1979) and its use, along with wine, was widespread. By the twelfth century AD the distillation of fermented alcohol was introduced and alcoholism became a social problem (Singer, 1956). By the mid-eighteenth century in England, consumption of alcohol among the general population was perceived to be excessive. The Gin Act, intended to limit the retailing of alcohol, was passed as law in 1751 and eventually it had the desired effect (Webb, 1903). The ill-effects of alcohol were thus recognised and legislative steps taken to control its use. Meanwhile it was appreciated that the problem was more than simple intoxication. In 1852 Huss attributed the effects of chronic alcoholism to tissue damage (Huss, 1852). It is now well recognised that chronic ethanol consumption in excess may affect many organs or tissues.

6.2. Alcohol Abuse and Alcoholism

Many people consume alcohol. Given the wide variation in individual habits it is not easy to define where drinking ends and alcohol abuse begins. Recommended limits of weekly alcohol consumption are 21 units for men and 14 units for women. One unit of alcohol (half a pint of normal strength beer or lager, a glass of wine or a single measure of spirits) approximates to eight grams of ethanol.

In 1981 Turner suggested that the dividing line between moderate and heavy drinking should be 0.8g/kg body weight per day (with an absolute limit of 80g or 10 units of ethanol daily) or an average of 0.7g/kg over a three day period (Turner, 1981). According to this definition, in the USA in 1987, 24% of people were moderate drinkers and 9% heavy drinkers (NIAAA, 1987). In Britain about 10-15% of the population may be considered chronic alcohol abusers (Royal College of Physicians, 1987).

Arbitrary normal limits relating specifically to consumption do not allow for those who may drink more than the proposed limit with little or no consequence. Conversely, some people may drink less than a given limit but still suffer from the syndrome recognised as alcoholism. Thus one difficulty in studying alcohol related problems is that of case ascertainment. It appears that significant consumption for a long time is required to produce most of the major ill-effects of drinking alcohol and that to achieve the necessary intake requires abnormal drinking habits. But what exactly is excess alcohol use and what is alcoholism? The population variability in the consumption of alcohol is such that the dividing line between those who are and those

who are not alcoholics tends to be placed at the point where drinking leads to social, psychological or medical problems.

There is no single pattern of alcohol misuse and rather it should be considered not as a single disease but as a variety of behaviours (Paton, 1994). There are heavy drinkers who intermittently or continuously indulge in binge drinking and these people might soon experience harm. Problem drinking is where social, domestic work or medical problems result from drinking and can be avoided or reduced if drinking can be curtailed. Dependence is where a true form of addictive behaviour occurs and it proves difficult or impossible to stop drinking. All of these patterns may overlap and an individual may exhibit all three types of behaviour. According to these definitions an estimate of the prevalence of these conditions in Britain would give 7.3 million people drinking above sensible limits (as defined above), 4 million heavy drinkers, 800,000 problem drinkers and 400,000 dependent drinkers (Paton, 1994).

In the United States the National Institute on Alcohol Abuse and Alcoholism (NIAAA) defined alcohol abusers as drinkers who had suffered one or more severe, or moderately severe, consequences of alcohol use, such as job loss, arrest or illness during the previous year (NIAAA, 1986). Alcoholics were defined as people who had suffered one or more symptoms of alcohol withdrawal or one loss of control symptom plus one other symptom of dependence in the last year.

The true incidence of alcoholism therefore will always be difficult to ascertain and prior to the NIAAA studies others had proposed criteria in order to conduct research in this area (Feighner, 1972).

After the NIAAA definitions, the Guze criteria (table 17) were published and provide a guide to symptoms that a physician should be aware of, whether using a structured approach to taking a drinking history or not (Guze, 1988).

There is little doubt that chronic alcoholics as described above may develop important medical complications as a result of their alcohol consumption. Since there are as yet no useful objective measures of alcohol consumption, the best approach to diagnosis remains a careful history. Key aspects of the Guze criteria feature in the CAGE questions (C - **C**onsidered stopping? A - **A**nger when questioned about drinking? G - **G**uilt about drinking? E - **E**ye opener or early morning drink?)

Having ascertained that a given individual is alcoholic the problem may still remain as to whether a given pathology is a result primarily of excess alcohol consumption or whether it is secondary to malnutrition, neglect or concurrent ingestion of toxic substances. Thus, confusion existed until very recently over whether there was such an entity as alcoholic cardiomyopathy since, for certain subjects, vitamin deficiency or toxic chemicals were shown to be responsible. Once it was shown that cardiac complications could develop in well nourished patients the possibility that a

specific heart muscle disease might be related to alcohol became stronger. This will be discussed further.

Table 17. The Guze Criteria

This table shows those features identified by Guze as symptoms of alcoholism or alcohol abuse. A diagnosis of "definite alcoholism" according to these criteria requires three symptoms in each of three symptom categories. "Probable alcoholism" requires two symptoms in each of two symptom groups.

Medical Problems	Social Problems
Withdrawal	Arrests
shakes	Automobile trouble
hallucinations	Lost job
withdrawal seizures	Fighting
Organ damage	Lost friends
cirrhosis	
gastritis/bleeding	**Prior Identification**
polyneuropathy	
chronic brain syndrome	Family objects to level of individuals drinking
Blackouts	Self-admitted excessive drinking
Benders	Others thought individual drank too much
Impotence	Guilt about drinking
Control Problems	
Trouble stopping	
Admits no control	
Tried to limit	
Morning drinking	
Nonbeverage alcohol	

6.3. Alcoholism and Inheritance

Alcoholism may be a multifactorial condition, partly determined by inheritance of a gene or genes which predispose the individual to alcoholism, and partly determined by environmental factors. A search for a specific alcoholism gene is likely to be difficult and, as with ischaemic heart disease for example, a large number of factors, perhaps including inheritance of a particular combination of certain genes predisposing to alcoholism coupled with a significant environmental effect, are likely to play a part. However, definition of the genetic element involved in alcoholism may be a key means of designing treatment strategies for people who suffer from alcoholism. Such studies are in progress (Cook, 1990).

Studies of twins show a significant genetic effect on susceptibility to alcoholism in males (Kaij, 1960; Hrubec, 1981), but the evidence for females is less convincing (Goodwin, 1977; McGue, 1989). Other studies of adoptees (Cadoret, 1978) and half-siblings (Schuckit, 1972) provide further support for a genetic effect. Whatever the

factors are, it is clear that younger adult males are the group at highest risk of alcoholism.

Perhaps the best evidence on this aspect of alcoholism is provided by Sweden where good records of alcohol consumption have been kept for many years. Various studies up to 1978 have shown the incidence of alcoholism to be 7% in the general population (Cloninger, 1990), rising to 12% in grandsons of alcoholics (Kaij, 1975), 20% in adopted-away sons of alcoholics (Bohmann, 1978), 33% in dizygotic twins and 70% in monozygotic twins (Kaij, 1960).

6.4. The Metabolism of Alcohol

Ethanol is the least toxic of a series of hydrocarbon based chemicals, the alcohols with general formula $C_nH_{2n+1}OH$, whose distinctive feature is the possession of a hydroxyl group. This renders the smaller molecules of this series highly polar and imparts their ability to function as a useful solvent. Ethanol has been produced since ancient times and many civilisations have made social and religious use of its intoxicating properties. Many early physicians believed it had healing powers but ethanol itself is toxic and a single carbon lost from the ethanol molecule, forming methanol, produces a highly toxic substance.

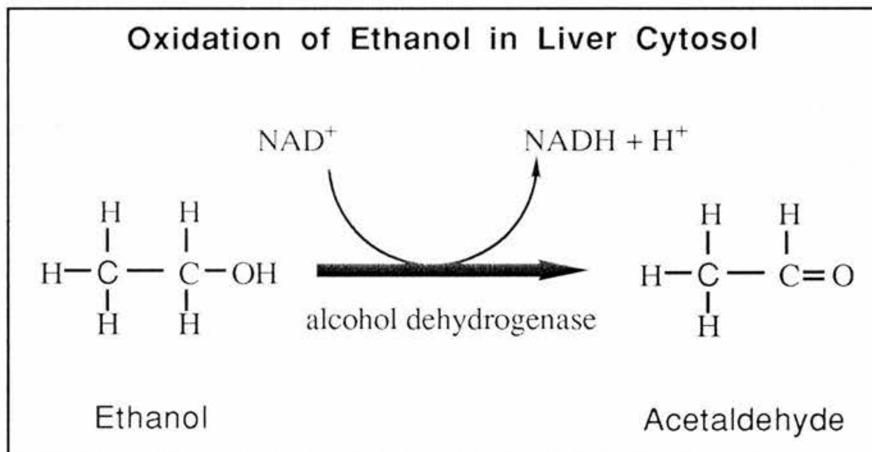
Ethanol has no known cellular receptor, its small size and polar properties ensure that it freely diffuses into cells, even across the blood brain barrier, to rapidly exert effects. It is toxic to all cells and in sufficient quantities will cause cell death. It is cleared from the circulation by the liver and to a lesser extent by the lungs, the basis of the well known "breathalyser" test for motorists. At the legal limit for driving of 80mg alcohol per 100mls blood(or 17.4mmol/l), functional testing reveals effects of alcohol already beginning to show and the risk of a road accident is more than doubled (Paton, 1994). Measured as parts alcohol per 10,000 parts blood (Blood alcohol level, BAL) this is approximately 0.05 and at this level subjects experience positive feelings such as relaxation, well-being and euphoria (Hales, 1986). At higher levels subjects experience negative feelings, including gradual loss of control of speech, balance and emotions. When the BAL reaches 0.1 a subject is drunk (with symptoms of headaches, nausea, stomach pain, fatigue, weakness, mood alteration, depression and paranoia), at 0.2 (160 to 200mg/100mls) some people become unconscious, at 0.3 some collapse and at 0.4 (400mg/100mls or 86.8mmol/l), death is possible. To put this in perspective, at 160mg/100mls (34.7mmol/l) the risk of road accident rises to tenfold.

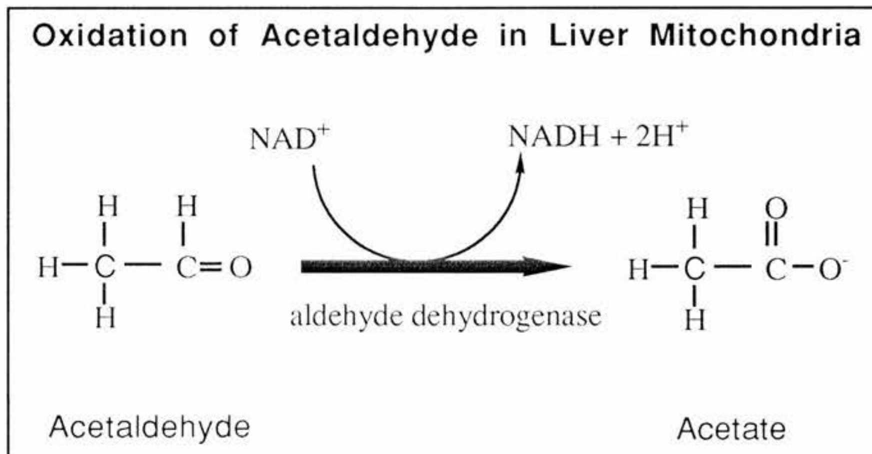
In the liver, the predominant route of alcohol metabolism is via oxidation to acetaldehyde (fig. 11; please note the equations are not balanced for simplicity), catalysed by alcohol dehydrogenase found in the cytoplasm (presumably alcohol dehydrogenase is present in man to deal with endogenously produced alcohols from

fermentation of carbohydrate). There are also microsomal NADP dependent oxidising systems. Acetaldehyde is able to enter mitochondria where aldehyde dehydrogenase can further oxidise it to acetate. Acetate is eventually oxidised to carbon dioxide and water. The initial reaction also produces NADH, which cannot be used directly and requires to be oxidised back to NAD. Thus the ability of the liver to metabolise alcohol depends on its capacity to transport reducing equivalents from the cytosol to the microsomes, via glycerol phosphate and malate-aspartate shuttles (Devlin, 1986). The NADH produced by oxidation of acetaldehyde is present in the mitochondria and can therefore be oxidized by molecular oxygen using the apparatus of oxidative phosphorylation.

Figure 11. The Oxidation of Alcohol

This figure illustrates the key reactions involved in the oxidation of alcohol which take place in the cytosol and the mitochondria. The equations are not balanced for simplicity. Alcohol is first acted on by alcohol dehydrogenase and then the acetaldehyde produced is transported to the mitochondrion where it is oxidised by aldehyde dehydrogenase.





There is much variation in individual capacity to metabolise alcohol, particularly in relation to aldehyde dehydrogenase, which has several functional isoenzymes located both in the cytosol and the mitochondria (Smith, 1973). The most widely known aspect of this type of genetic variation is aldehyde dehydrogenase deficiency among oriental populations, who have a marked intolerance of alcohol, due to excess levels of circulating acetaldehyde produced by even small amounts of alcohol consumption (Goede, 1979).

The interest in these metabolic differences lies in the possibility that they may play a role in predisposing people to become alcoholics. Despite the well characterised deficiency states in some groups, this remains to be proven.

6.5. Alcoholic Cardiomyopathy

Alcohol is clearly associated with heart failure in certain well defined circumstances. For example "Wet" Beri-Beri (Blankenhorn, 1946), heart failure due to thiamine deficiency, is correctable by restoration of a normal diet. Toxic factors present in alcoholic drinks, such as cobalt, have also been reported (Kesteloot, 1968).

In addition, alcohol is associated with hypertension (Klatsky, 1967) and may be associated with a protective effect against ischaemic heart disease in moderate amounts, but a deleterious effect at higher doses (Klatsky, 1990a). However, in some alcoholic patients heart failure is present despite an adequate diet (Steinberg, 1981). This typically presents with arrhythmias and or symptoms of heart failure in a person who has been consuming a large amount of alcohol for a long time. The precise incidence is unknown.

Since the late 1950s the literature has referred to "alcoholic cardiomyopathy". A cardiomyopathy is by definition a condition of unknown aetiology. Some authors (for example Richardson et al, 1986) prefer the term "alcoholic heart muscle disease" to "alcoholic cardiomyopathy" since it suggests a specific disorder for which the aetiology is known. Whatever term is applied, the definition of this condition is difficult and often centres on a particular level of alcohol consumption daily (or cumulative lifetime dose) in order to have a cut-off point below which subjects are not "alcoholic", for an arbitrary number of years. Turners level of 80g (or 10 units) per day is the commonest choice in these circumstances (Turner, 1981) and five years is a widely used arbitrary time period.

The condition which is labelled in clinical and haemodynamic terms as alcoholic cardiomyopathy generally differs from idiopathic dilated cardiomyopathy only in respect of the history of alcohol consumption. Both conditions are characterised by ventricular failure with subsequent dilatation and a predisposition to arrhythmias. Steady deterioration may occur resulting in death without appropriate therapy, which

may mean transplantation. Therefore it is difficult to define a group in whom alcohol is the definite cause of myocardial dysfunction. In 1980 the ISFC/WHO study group felt unable to define alcoholic cardiomyopathy and stated that they could not "define a causal versus a conditioning role of alcohol nor apply precise diagnostic criteria" (Oakley, 1980). This was despite the addition, in the Ninth Revision of the International Classification of Diseases (WHO, 1977), of the sub-category "alcoholic cardiomyopathy" (425.5) to the category of cardiomyopathy (425). Thus physicians continue empirically to attach this label to patients consuming excess alcohol in whom heart failure has been shown not to be due to ischaemic heart disease, or other specific heart muscle disorder.

There is a large body of evidence associating alcohol with ventricular dysfunction. Congestive Cardiomyopathy resulting from alcohol intake is said to have first been recognised in a case report by Walshe in 1873 (Walshe, 1873) but Wood discussed alcohol as a factor in heart failure as early as 1855 (Wood, 1855).

A clearer idea of what alcoholic cardiomyopathy might be emerged when Brigden (Brigden, 1957) and other authors began publishing papers on the subject in the late 1950's and early 1960's. Brigden discussed 13 patients with "severe alcoholism" from a series of fifty patients with isolated heart muscle disease in his 1956 St Cyres lecture (Brigden, 1957). Brigden and Robinson discussed "alcoholic heart disease" in 1964 (Brigden, 1964). These papers identified many of the key clinical features of the condition but did not define the relevant level of alcohol consumption required to produce it.

Tobin and others studied 39 alcoholic patients identified from a group of 119 patients who were less than 50 years old and who had "idiopathic primary myocardial disease" in 1967 (Tobin, 1967). This study provided early evidence that there was a significant incidence of alcoholism among patients with what is now called idiopathic dilated cardiomyopathy. Furthermore it demonstrated that cessation of drinking would lead to clinical improvement and that continuing to drink after the diagnosis is made leads to a poor prognosis.

One of the problems with the studies above, and their associated commentaries, has been the failure to exclude significant ischaemic heart disease or specific diseases of the myocardium. The widespread introduction of coronary angiography and endomyocardial biopsy has facilitated further studies in this area. In addition, epidemiological studies have lent further weight to the theory of a connection between alcohol consumption and heart disease. Klatsky found a relative risk for death from cardiomyopathy in persons drinking 6 or more drinks per day to be increased eight times compared with lifelong abstainers, though the low numbers of deaths in this group meant that statistical significance could not be detected. This study found a "J-

shaped" mortality curve, with non-drinkers having a higher mortality from cardiovascular disease than light drinkers and heavy drinkers having the highest mortality (Klatsky, 1990b).

The Kaiser-Permanente studies, based on questionnaires distributed to persons joining a health insurance scheme, have examined alcohol in relation to many forms of heart disease. One such study found that hospital admissions for cardiomyopathy and congestive heart failure, among more than a thousand subjects at risk, were significantly more frequent among those consuming six or more drinks daily (Klatsky, 1981).

Codd et al conducted a population based study of idiopathic dilated cardiomyopathy and found 46 cases (incidence of about 7.9/100,000), of whom 12 (26%) had a history of significant alcohol consumption (Codd, 1989). Smith showed that increases in alcohol consumption in Western Australia were associated with increases in mortality rates from alcoholic cardiomyopathy for males and females (Smith, 1990). He also reported that a subsequent decline in alcohol consumption was associated with a decrease in mortality from alcoholic cardiomyopathy among females, though less so among males.

A study in 1981 among patients with idiopathic dilated cardiomyopathy suggested that around 20% consumed significantly large amounts of alcohol (Fuster, 1981). In addition, studies of chronic alcohol abusers have shown sub-clinical evidence of heart dysfunction in anything up to twenty per cent of cases. Studies have also shown that in such patients, cessation of alcohol consumption leads to resolution of symptoms and signs in about thirty per cent of patients (Weishar, 1977), with stabilisation in another fifty per cent and continued deterioration in the remainder. Other follow-up studies of alcoholics with cardiomyopathy who stop drinking suggest that anything up to two-thirds of patients may survive to three years with no evidence of residual cardiac disease, whereas in those who continue to drink, the majority die (Brigden, 1964; Alexander, 1967 ; Schwartz, 1975).

In a study of prognostic features for patients with cardiomyopathy undergoing assessment for transplantation, Keogh et al described 20 of the 232 (9%) patients as having "alcoholic/idiopathic" cardiomyopathy, which meant typical clinical features of idiopathic dilated cardiomyopathy along with consumption of 60g alcohol or more per day (Keogh, 1990). This is a much lower prevalence of excess alcohol consumption among patients with idiopathic dilated cardiomyopathy than those of either Codd (Codd, 1989) or Fuster et al (Fuster, 1981). This might reflect the mortality of alcoholic cardiomyopathy since the highest prevalence is in the community based study (26%), the next highest in a hospital cardiology practice (20%) and the lowest (9%) in those who survive to be assessed for transplantation. Alternatively it may suggest that

there is a low detection rate for alcoholic cardiomyopathy with patients being undiagnosed or mis-diagnosed as idiopathic dilated cardiomyopathy.

6.6. The Heart in Alcoholic Patients

Early studies of the histopathology of hearts from patients with alcoholism found no specific features. However, using electron microscopy, Alexander described abnormalities affecting the contractile elements and the mitochondria in the majority of 45 patients studied (Alexander, 1966). These abnormalities included fragmentation of contractile elements with large numbers of swollen damaged mitochondria, but there were no inflammatory cells present. These changes were not found in a normal heart nor in hearts from patients with valvular or congenital heart disease. However, hearts from patients with idiopathic dilated cardiomyopathy were not examined.

Several studies have examined alcoholic patients to determine if alcohol consumption causes clinical appearance of heart disease. Examination of alcoholic subjects known not to have heart disease should allow detection of sub- or pre-clinical abnormalities of cardiac function. In 1977 Levi et al (Levi, 1977) used non-invasive measurements (electrocardiogram, phonocardiogram and carotid pulse tracing) in patients with chronic alcoholism, excluding those at risk of ischaemic heart disease, >40 years age and other heart disease, compared with non-drinking controls. This study found evidence of left ventricular dysfunction in the alcoholic group compared with non-drinkers.

Urbano-Marquez et al studied a group of 50 asymptomatic alcoholic men admitted to an alcohol problems unit and assessed both skeletal and cardiac muscle function (Urbano-Marquez, 1989). As well as abnormalities of skeletal muscle function, they found that alcoholic patients had a significantly lower ejection fraction compared with controls (59 versus 67%). They also found that the estimated total lifetime dose of ethanol correlated inversely with ejection fraction and directly with left ventricular muscle mass.

Bertolet et al studied "an apparently healthy alcohol abuse population" with a range of investigations (Bertolet, 1991). They found no correlation between the amount of alcohol consumed daily, or total lifetime dose, and left ventricular dysfunction. The other finding of note was that "routine" investigations, such as ECG or Chest X-Ray, were very poor at detecting those patients with left ventricular dysfunction as defined by radionuclide ventriculography.

More recently, 25 patients with chronic alcoholism, aged less than 40 years, who were being admitted to hospital for alcohol dependence treatment, were studied (Cerqueira, 1991). Using echocardiography and rest and exercise radionuclide

ventriculography the results were broadly similar compared with a group of age and sex matched controls.

Another study used radionuclide angiography in 15 otherwise asymptomatic patients who drank at least 60g alcohol daily "for several years", who were admitted for colorectal surgery (Tønnesen, 1992). Left ventricular function was reduced (pre-operatively) in patients who drank excess alcohol (54% versus 68%) compared with age, sex and operation matched controls who drank less than 25g alcohol daily. The figures for ejection fraction in this study are strikingly similar to those obtained by Urbano-Marquez (Urbano-Marquez, 1989).

6.7. Effects of Alcohol on the Heart

Ethanol may have a number of other effects on the heart. It has been described as predisposing to arrhythmias, particularly after binge drinking - the "holiday heart syndrome" (Ettinger, 1978). It also has a negative inotropic effect following acute administration (Regan, 1966).

Ethanol metabolism does not occur to a great extent in the myocardium, but studies have shown the presence of fatty acid ethyl esters in the heart in persons exposed to alcohol (Lange, 1983). Furthermore, reduced fatty acid oxidation (Kikuchi, 1970), increased myocardial triglyceride content (Kako, 1973) and impaired mitochondrial function (Cederbraun, 1974) have all been described as a result of exposure to ethanol. Therefore, attention has focused on the effects of ethanol itself. It is difficult for in-vivo studies to separate the effects of alcohol from the effects of its metabolites, such as acetaldehyde and this must be continuously borne in mind.

One of the obstacles to a clearer understanding of the effects of alcohol on the heart has been the lack of a suitable animal model. It is possible to produce alterations in function in animals consistent with sub-clinical heart disease but not a condition akin to the human disease referred to as alcoholic cardiomyopathy with myocardial dysfunction in the resting subject (Regan, 1987). However, it has been shown that ethanol may depress myocardial function acutely in various studies; the feline papillary muscle in-vitro (Spann, 1968) and in-vivo studies on dogs (Regan, 1966) as well as man (Gould, 1971).

It is known that cardiac protein synthesis may be altered by toxic substances such as alcohol (Schreiber, 1980) but in-vitro studies in the guinea pig did not have this acute effect .

The effects of acetaldehyde on cardiac function may be more important than those of ethanol. Acetaldehyde has been shown to decrease cardiac protein synthesis, by 50% (Schreiber, 1972). In a study in which guinea pigs were chronically fed ethanol a decrease in cardiac protein synthesis in-vivo was found (Schreiber, 1982). This

model was one where the right ventricle was subjected to a pressure load and the left was not and the decrease in protein synthesis was confined to the right ventricle.

Other animal studies have demonstrated reduced mitochondrial respiration, suggesting that the site of action of either ethanol or acetaldehyde, or both, is at the level of the mitochondria(Pachinger, 1973; Segel, 1979). Bing et al showed that chronic consumption of alcohol in dogs led to reduced calcium binding and uptake by mitochondria and sarcoplasmic reticulum (Bing, 1974).

In man it has been shown that coronary sinus blood of alcoholics contains higher levels of mitochondrial enzymes than controls, perhaps suggesting release of these enzymes by damaged mitochondria (Wendt, 1965). Richardson et al showed that patients consuming excess alcohol (80g daily or lifetime >250kg) had increased myocardial enzyme levels (creatine kinase, lactate dehydrogenase, α-hydroxybutyric dehydrogenase, malic dehydrogenase and aspartate aminotransferase) (Richardson, 1986).

It may be that ethanol exerts its effects on cardiac protein synthesis via acetaldehyde. In-vitro studies using homogenates of normal animal heart, where ethanol was added to the system, showed no decrease in protein synthesis (Rawat, 1979). However, in a similar system, the addition of acetaldehyde did reduce protein synthesis (Schreiber, 1974; Siddiq, 1993).

The finding of the ability of acetaldehyde to reduce cardiac protein synthesis is significant. It is known that inhibition of protein synthesis leads to the development of heart failure in rat hearts working against increased afterload (Zuchlke, 1966). However, acetaldehyde has other actions, perhaps the most important of which is its ability to bind covalently to a range of proteins. This binding occurs predominantly to lysine residues (Tuma, 1987), though it may also bind to tyrosine and valine. This is discussed further below.

The possibility that acetaldehyde may be responsible for cardiac disease does mean that therapy may be available for patients who are unable or unwilling to cease drinking once alcoholic cardiomyopathy is diagnosed. It has been shown that human blood acetaldehyde concentrations can be lowered in vitro by doses of pantethine and that liver levels of acetaldehyde can be lowered similarly in vivo in rats (Watanabe, 1985). Furthermore, another study in rats showed protection against lethal doses of acetaldehyde by combinations of thiamin, L-cysteine and L-2-methylthiazolidine-4-carboxylic acid (Sprince, 1974).

6.8. The Immune System and Antibodies in Heart Disease

Studies of immune function in patients with idiopathic dilated cardiomyopathy have suggested defective T suppressor cell function (Fowles, 1979), though Lowry et

al suggested that this was found in any form of heart failure (Lowry, 1987) and others dispute any defect (Anderson, 1981). Reduced natural killer cell activity has also been shown (Anderson, 1982; Itagaki, 1988). Gerli et al demonstrated several abnormalities including: raised CD4/CD8 ratios, greater ability to help B cell differentiation, suppressor cell defects and elevated IgM levels in comparison with patients with IHD or healthy controls (Gerli, 1986). Arbustini et al showed an apparent relationship between expression of HLA DR4 and 5 and DCM (Arbustini, 1989). This raised the possibility that the immune system plays a role in this form of heart disease. Early experiments with animal models showed that it is possible to raise anti-heart antibodies by injecting native or foreign heart tissue. It was further shown that these antibodies could have a cytopathic effect on myocytes in tissue culture (Gery, 1961).

Using direct immunofluorescence in patients with idiopathic dilated cardiomyopathy, Sanders and Ritts demonstrated gamma globulin bound adjacent to, and beneath, the sarcolemmal sheath (Sanders, 1965). Das et al found bound gamma globulin in three patients with "congestive cardiomyopathy". In two of these bound complement was also demonstrated (Das, 1971). The same group later studied 35 patients using indirect immunofluorescence and found sarcolemmal or subsarcolemmal staining in 6 patients and antinuclear antibodies (against mouse liver) in 15 (Das, 1972).

Maisch et al used indirect immunofluorescence in a study of 79 patients with dilated cardiomyopathy ("primary cardiomyopathy") and 61 with "secondary cardiomyopathy", of whom 30 had alcoholic cardiomyopathy (criteria not stated) (Maisch, 1983). They compared results with 200 healthy controls. Anti-interfibrillary antibodies were described in 67% of patients with alcoholic cardiomyopathy versus 41% with primary cardiomyopathy and 3% in healthy controls. In alcoholic cardiomyopathy these antibodies were IgG in 77% and IgM in 19%. Complement fixation occurred in 3% only. Anti-myosin antibodies were found in 17% patients with alcoholic cardiomyopathy, 20% with primary cardiomyopathy and 4% of controls. Non-organ specific antibodies were also detected: antinuclear antibodies in 30% patients with alcoholic cardiomyopathy, 24% with primary cardiomyopathy and 8% in controls.

Schultheiss examined 18 patients with dilated cardiomyopathy and found that 17 had antibodies against the adenine nucleotide translocator but none of the 7 alcoholic patients studied had these antibodies (Schultheiss, 1985).

In 1989 a Czech group used direct and indirect immunofluorescence to study 118 patients with 'congestive cardiomyopathy', of whom 38 were labelled as "definitely alcoholic" and 34 as "presumably alcoholic". They found antiheart antibodies by direct immunofluorescence, which was said to be strong, in 25% of the

"definitely alcoholic" group and 37% in the "presumably alcoholic" group. It appears that of the positive results for direct immunofluorescence, 68% had IgG bound, 51% had IgM bound and 35% IgA (Hogye, 1989).

Neumann and others used Western blotting to detect IgG antibodies against an extract of normal human heart. They studied 71 patients with idiopathic dilated cardiomyopathy, 17 patients with acute myocarditis and 15 healthy volunteers. There was no difference in the mean number of antigens recognized by serum from each group. There was an increased prevalence of antibodies to 40-49kDa and 100-109kDa antigens in patients with idiopathic cardiomyopathy. Using indirect immunofluorescence they showed high titre IgG antibodies to heart in 59% of patients with myocarditis, 20% idiopathic cardiomyopathy and none in controls. Since they did not exclude alcoholics some of these results may relate to patients consuming alcohol (Neumann, 1990).

Caforio and others excluded patients consuming excessive amounts of alcohol and, using indirect immunofluorescence found that 17 of 65 (26%) patients with idiopathic dilated cardiomyopathy had organ-specific cardiac antibodies compared with 7 of 200(3.5%) normal subjects (Caforio, 1990b).

Ansari found no serum activity against heart in alcoholic patients (by Western immunoblotting, ELISA against myosin, or immunofluorescence) except in one case who had similar results to the DCM patients (autoantibodies against the adenine nucleotide translocator and to the branched chain alpha-ketoacid dehydrogenase) (Ansari, 1991).

There may also be a role for enteroviruses in inducing autoantibodies in dilated and perhaps alcoholic cardiomyopathy. There is a large literature on the possibility that enteroviruses, such as coxsackie virus B, may cause acute myocarditis which then subsequently leads to the development of dilated cardiomyopathy (Cambridge, 1979; Woodruff, 1980; Alvarez, 1987). Hence, in patients with dilated cardiomyopathy some autoantibodies may have been virally induced. Sophisticated molecular techniques have been used to detect coxsackievirus sequences in the hearts of patients with idiopathic dilated cardiomyopathy. There were several initially promising reports (Bowles, 1986; Bowles, 1989; Jin, 1990) though subsequent studies have not confirmed the presence of coxsackievirus in these patients (Grasso, 1992). Bowles did not comment on alcohol consumption in the 1986 paper (Bowles, 1986) but at least two subjects were drinking to excess, both of whom were negative for viral RNA, in the later study (Bowles, 1989). Jin et al made no comment on alcohol consumption (Jin, 1990) and Grasso et al stated that patients with alcoholic cardiomyopathy were not included in their study (Grasso, 1992).

Rose et al discuss the possible role of coxsackieviruses in inducing autoantibody responses which become self-sustaining and go on to damage the heart chronically (Rose, 1988). They have performed studies in mice which support this contention (Neu, 1987). It may be speculated that alcohol consumption to excess during viral infection with coxsackie B viruses allows damage to myocardial cells to occur, exposing myosin at a time when antibody responses against enteroviral proteins are at their height and cross-reaction is most likely. A range of virally induced abnormalities could contribute to the development of myocardial disease under these circumstances - the mechanisms for which might include molecular mimicry, alteration of host immune responses and alteration of endogenous host antigens (Rose, 1988). Thereafter further alcohol consumption would again damage myocytes provoking a stronger antibody response. A chronic injury cycle could then be established. From the studies performed to date there is no evidence for or against this contention.

6.9. Acetaldehyde Can Render Proteins Immunogenic

Acetaldehyde has a number of interesting properties being a toxic substance with a propensity for stimulating antibody responses. It is therefore of great interest in the area of alcohol mediated disease because it is produced in significant quantities in the liver and can form antigenic compounds with a large range of cellular proteins.

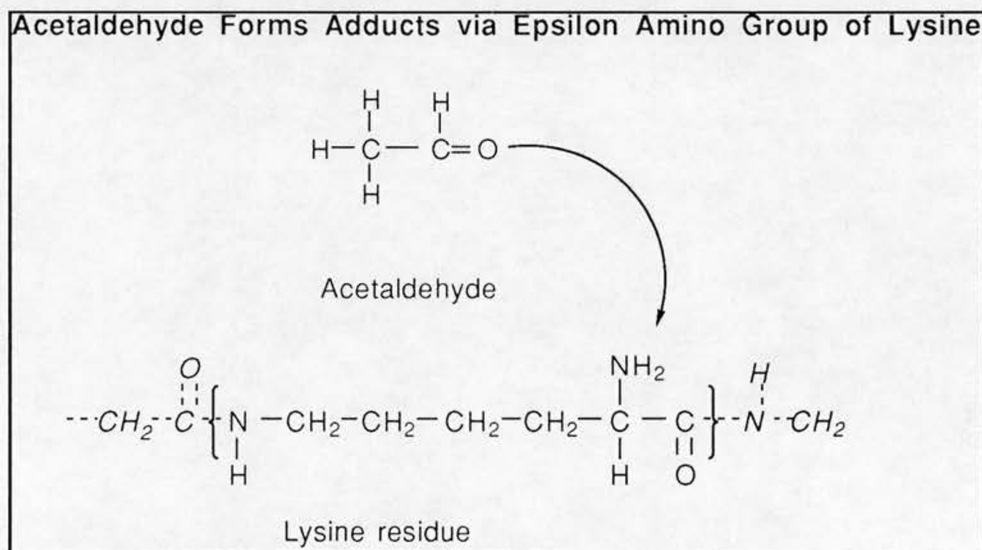
A number of studies, mostly based on analysis of liver disease, have demonstrated that acetaldehyde-modified proteins can be highly immunogenic. Acetaldehyde may bind to lysine-rich proteins (fig. 12) (Tuma, 1987) such as the enzyme ornithine decarboxylase (Rettig, 1979) to form unstable adducts. These adducts are therefore produced continuously during chronic consumption of alcohol. The formation of "acetaldehyde adducts" can occur at concentrations of acetaldehyde comparable to those found in the blood of alcoholics (Sorrel, 1985; Tuma, 1985).

Both mice and rats which have been chronically fed with alcohol have been found to produce antibodies against acetaldehyde-modified proteins (Israel, 1986; Worral, 1989). If haemoglobin or albumin protein is incubated in-vitro with acetaldehyde, so-called synthetic acetaldehyde adducts are produced. High titres of antibodies to these synthetic acetaldehyde adducts have been demonstrated in patients with alcoholic liver disease (Hoerner, 1986; Hoerner, 1988; Niemela, 1987). 37kDa and 52kDa acetaldehyde protein adducts have been reported in the livers of rats which have been chronically fed with alcohol (Lin, 1988; Lin, 1989; Behrens, 1988).

A recent study found that 70% of patients with alcoholic hepatitis had circulating IgA antibodies against a 200kDa acetaldehyde adduct in liver. Similar antibodies were found in only 20-30% of control subjects (Koskinas, 1992). In these Western blotting experiments sera were tested for antibody binding to four different

preparations made from normal healthy liver. The first of these was essentially untreated normal liver. The second was incubated with acetaldehyde alone. The third was incubated with sodium cyanoborohydride alone and the fourth with acetaldehyde and cyanoborohydride. The reducing agent, sodium cyanoborohydride, was used to prevent breakdown of the adducts, which are inherently unstable. Thus, when the electrophoresis gels were run, the sample lanes were arranged in sets of four in order to accommodate the necessary controls. The lane of most interest was lane four where stable adducts were present. The importance of this finding is that acute exposure to alcohol would presumably not be sufficient to induce antibody formation because the adducts would quickly be lost. However, chronic consumption would lead to continued production of these adducts, sufficient to produce an antibody response in particular individuals.

Figure 12. Acetaldehyde Binding to Protein via Lysine Residues
In this figure the acetaldehyde molecule is shown binding to a protein via the epsilon amino group of a lysine residue. This is a favoured site of binding for acetaldehyde but not the only possible means of forming protein adducts. The adduct so produced remains part of a polypeptide chain and is highly immunogenic.



Alcoholics have higher blood acetaldehyde levels than non-alcoholic subjects, following consumption of similar quantities of alcohol (Majchrowicz, 1970; Korsten, 1975; Lindros, 1982; Nuutinen, 1984). This is due to reduced aldehyde dehydrogenase activity, with preserved or even enhanced alcohol dehydrogenase function (Lieber, 1970; Takase, 1989). In the rat, levels of acetaldehyde in the heart exceed those in the plasma (Espinet, 1984). Comparable data are not available for man.

6.10. Alcoholic Cardiomyopathy - Summary

The evidence presented suggests that there is a distinct condition which is widely referred to as alcoholic cardiomyopathy. This is the result of an ill-defined number of years consumption probably of more than 80g alcohol daily, or more than 250kg lifetime total. It presents with symptoms and signs of ventricular failure, with or without arrhythmias, typically in a younger male. Most patients who stop drinking can expect to recover, but continued alcohol abuse may result in deterioration and death. The mechanism whereby myocardial failure is caused is unknown but one possibility is immune mediated damage resulting from circulating antibodies produced against cardiac acetaldehyde adducts.

On the basis of the evidence presented above the following hypothesis could be proposed. Subjects who consume large amounts of alcohol over a prolonged period will develop antibodies directed against acetaldehyde-adducts of myocardial proteins. Such circulating antibodies could be involved in the pathogenesis of alcoholic heart muscle disease through inhibition of enzyme function or direct damage to cells.

The work in this part of the thesis is concerned with the identification of antibodies to acetaldehyde-modified myocardial proteins in patients with high alcohol intake.

7. Alcoholic Cardiomyopathy: Patients, Materials and Methods

The aim of this study was to detect circulating antibodies to acetaldehyde adducts of normal human heart in the serum of patients with alcoholic cardiomyopathy. Serum was reacted with Western blots of appropriately treated heart tissue.

7.1. Patients

Serum samples were obtained from 12 patients with alcoholic cardiomyopathy and also from 12 patients with dilated cardiomyopathy who did not drink more than 21 units of alcohol per week (males) or 14 units per week (females), (tables 18 and 19). Sera were obtained as a kind gift from Dr Peter Richardson, Cardiac Department, Kings College Hospital, Denmark Hill, London. The samples had been collected prospectively for a separate project but sufficient residual serum was available for this study. The diagnosis of alcoholic cardiomyopathy was based on the finding of left ventricular dysfunction (ejection fraction <50% or echocardiography showing moderate or severe LV dysfunction) on a background of excess alcohol consumption (greater than 80g per day or lifetime consumption of >250kg) for longer than five years, in patients who had undergone invasive cardiac investigations to exclude ischaemic heart disease or other causes of heart failure.

Samples were also collected from patients at Addenbrookes Hospital, Cambridge via Dr PL Weissberg and Dr G Alexander. These patients included 2 with alcoholic cardiomyopathy diagnosed by the above criteria, 8 patients with dilated cardiomyopathy who did not drink excessively (as defined above), 8 patients with ischaemic heart disease and congestive heart failure who did not drink excessively and 8 patients with alcoholic liver disease who were currently drinking without clinically overt cardiac disease. In addition, sera were collected from eleven healthy laboratory personnel who did not drink to excess (less than 21 units/week in males and less than 14 units/week in females).

Tissue samples from patients undergoing transplantation who did not have alcoholic cardiomyopathy were obtained from the Transplant Unit at Papworth Hospital, Cambridge. Human ventricular tissue was obtained from the explanted heart of two different patients with ischaemic heart disease. Normal human heart was obtained from an unused donor heart. All tissues were stored at -70^{0}C until required.

Table 18. Subjects Studied by Western Blotting

The table lists all those patients included in the present study. The diagnostic group is given along with the patients age and weekly alcohol consumption at the time the serum sample was taken. Please note that patient 3(C), though consuming less than 80g alcohol per day at the time of his biopsy, had ingested a lifetime total of alcohol >250kg. Subjects 1 to 4 in the alcoholic cardiomyopathy group are also designated A to D. This identifies them in immunoblots presented later on in the results section.

Group	Age	Alcohol Units/wk
Alcoholic Cardiomyopathy		
1 (A)	46	70
2 (B) (female)	44	84
3 (C)*	49	30
4 (D)	45	120
5	28	60
6	60	70
7	48	200
8	46	>90
9	49	>50
10	23	100
11	63	100
12	54	105
13	37	140
14	35	100
Dilated Cardiomyopathy		
1	53	<21
2	46	<21
3	55	<21
4	65	<21
5	23	<21
6	44	<21
7	22	<21
8	52	<21
9	63	<21
10	28	<21
11	36	<21
12	56	<21
13	48	<21
14	20	<21
15	61	<21
16	63	<21
17	50	<21
18	28	<21
19 (female)	38	<21
20	52	<21

Group	Age	Alcohol Units/wk
Ischaemic Heart Disease		
1	73	<21
2	83	<21
3	82	0
4	57	0
5	49	7
6	58	6
7	60	0
8	86	<21
Healthy volunteers		
1(female)	47	0
2	56	1
3	30	15
4	30	4
5	24	14
6	34	2
7	29	4
8	36	1
9	34	4
10	28	16
11	26	4
Alcoholic Liver Disease		
1	44	60
2	31	84
3	40	72
4 (female)	57	56
5 (female)	55	80
6	57	42
7	50	56
8	68	50

Table 19. Further Data on Subjects with Alcoholic Cardiomyopathy

This table lists data for the subjects with alcoholic cardiomyopathy including their age, alcohol consumption, the degree of functional impairment of activity as assessed by the New York Heart Association(NYHA) criteria, the left ventricular end diastolic pressure (LVEDP), the effect of alcohol on the mean corpuscular volume (MCV) and the serum gamma glutamyl transferase level (G-GT).

Subject	AGE	Units/Wk	NYHA	LVEDP	MCV	G-GT
1 (A)	46	70	II	8	105	115
2 (B)	44	84	IV	14	100	92
3 (C)	49	21	III	12	97.3	71
4 (D)	45	120	IV	36	-	180
5	28	60	IV	26	103.4	143
6	60	70	IV	7	89.8	126
7	48	200	IV	24	96.2	-
8	46	90+	II	16	96.9	147
9	49	50+	II	8	89.9	52
10	23	100	I	8	96.8	-
11	63	100	I	10	91.7	68
12	54	105	II	23	88.5	26
13	37	140	IV	16	83.9	70
14	35	100	II	8	89	118

7.2 Materials and Methods

7.2.1. Fractionation of Myocardium

Materials:

Homogenisation Buffer	0.25M Sucrose 15mM Tris/HCl 0.1mM EDTA pH 6.8	**Microsome Buffer**	50mM $KHPO_4$-NaOH 0.1mM EDTA 10% w/v glycerol pH 7.4

Methods:

Ventricular tissue, 6-10g, was trimmed of fat, weighed and chopped into fragments with a scalpel. These were diluted roughly 1:10 with ice cold homogenisation buffer. Fragments were mechanically homogenised using several 10 second bursts in a Polytron homogeniser. The crude mixture thus produced was spun in a fixed 45^0 angle rotor (Sorvall SS-34) at 10,000g (9,000rpm) in a Sorvall RC5B centrifuge for 20 minutes. The pellet (nuclei) was discarded. The resulting supernatant was centrifuged at 100,000g (28,000rpm) in a swing-out rotor (SW41Ti) in a Sorvall ultracentrifuge for 1.5 hours. All centrifugation steps took place at 4^0C. The resulting supernatant, the cytosolic fraction, was decanted into a single 50ml Falcon tube. The remaining pellet, the microsomal fraction, was resuspended in 1ml microsome buffer, placed in an eppendorf tube and the protein concentration of an aliquot was measured. The microsomal fraction was then stored at -70^0C until use.

The cytosolic fraction was concentrated. This was performed using a 'Speed-Vac' device to evaporate water from the fraction. Samples were normally concentrated from about 60mls to around 20mls. The protein concentration was then measured using the BioRad protein assay according to the manufacturers instructions. The cytosolic fraction was stored in 1ml aliquots at -70^0C in 10% glycerol.

7.2.2.Preparation of Adducts

Materials:

Phosphate buffered saline (PBS)

Acetaldehyde 12.5mM: 66µl of stock (22.7M solution) injected into 100mls distilled water with gas tight syringe.

Sodium Cyanoborohydride 600mM: 3.77g dissolved in 100mls water

SDS-PAGE Sample Buffer:
- 1% SDS
- 1mM EDTA
- 2mM PMSF
- 10% w/v glycerol
- 50mM Tris/HCl pH 7.5
- 0.5% bromophenol blue
- 25mM dithiothreitol

(Made up in 100mls and stored in 10ml aliquots at -20^0C until required)

Methods:

Microsomal and cytosolic fractions were thawed and then incubated in the presence or absence of acetaldehyde or sodium cyanoborohydride or both, as shown in table 20 below.

Eppendorf tubes were labelled 1 to 4 and equal volumes (200-800μl) of cytosolic or microsomal fraction were placed in each one. The sample in tube 1 was treated with phosphate buffered saline (PBS), in tube 2 with acetaldehyde 2.5mM, in tube 3 with sodium cyanoborohydride 100mM and in tube 4 with acetaldehyde and cyanoborohydride. The tubes were all tightly capped and were incubated at $37^{0}C$ for four hours. The time period 4 hours was shown to be optimum in previous studies where adducts of human liver cytosol were prepared (Koskinas, 1992). Each sample was then diluted 1:1 with 2X SDS-PAGE sample buffer and boiled for 5min at $100^{0}C$. Samples were then aliquoted and stored at $-70^{0}C$ until required.

Table 20. Proportions of Reactants for Adduct Production.
The table lists typical proportions of the given substances used to produce adducts and control solutions. The amount of the given fraction used is given in microlitres followed by the amount of phosphate buffered saline (PBS), acetaldehyde and/or sodium cyanoborohydride added. The fractions are numbered in the order in which these protein solutions were run on each gel.

Tube	Fraction (μl)	PBS (μl)	Acetaldehyde 12.5mM (μl)	Cyan. 600mM (μl)	Total (μl)
1	800	400	-	-	1200
2	800	200	200	-	1200
3	800	200	-	200	1200
4	800	-	200	200	1200

7.2.3. SDS-Polyacrylamide Gel Electrophoresis

Materials:

SDS-PAGE Sample Buffer: See above

Acrylamide Stock Solution: 30% Acrylamide (150grams/500mls)
0.3% Bis-acrylamide (1.5g/500mls)

Buffer B 1.5M Tris/HCl, pH 8.8 (18.18g/100mls)

Buffer D 0.5M Tris/HCl, pH 6.8 (6.06g/100mls)

10% SDS 10g sodium dodecyl sulphate per 100mls water

10% APS 1g ammonium persulphate per 10mls water - made fresh

SDS-PAGE Running Buffer (for 2.5l):

15g Tris
72g Glycine
2.5g SDS

SDS-PAGE Gel Stain (1l):

1g Coomassie Brilliant Blue R250
0.5l Methanol (Analar)
0.1l Glacial Acetic Acid (AR)
Stirred for 30 minutes at 60^0C.

SDS-PAGE Gel De-Stain (1l):

0.5l Methanol (AR)
0.1l Glacial Acetic Acid (AR)

SDS-PAGE Gel Silver Stain

Gel incubated overnight on orbital mixer in 50% methanol
Incubated 30 min in water
Made up staining solution:

21mls 0.36% NaOH
1.4ml NH_4OH
0.8g $AgNO_3$ in 4mls water

Made up to 100mls with water - vortexing dropwise in fume cupboard.
Stain added for 15 min and mixed on orbital shaker.
Developed in 2.5ml 10% citric acid, 0.25ml Formaldehyde in water.
Rinsed in 10% methanol, 10% acetic acid.

Methods:

Polyacrylamide gel electrophoresis (PAGE) was carried out under reducing conditions in the presence of sodium dodecyl sulphate (SDS) according to the method of Laemmli (Laemmli, 1970). Either 6% or 10% polyacrylamide gels were prepared and run using BioRad's Protean IIxi casting stand and vertical slab gel electrophoresis device. Gels were 16cm in length and 16cm in width. Gels were normally cast in pairs from a single mixture made according to the table. The buffer constituents are listed below in table 21.

Gels were prepared approximately 3 hours prior to electrophoresis to allow adequate polymerisation to occur. For each preparation, 50μg protein was loaded per lane on the gel (this would correspond to 35-50μl of adduct). For molecular weight marker lanes, 10μl of a 1:20 stock of BioRad Broad range molecular weight markers was used.

Human ventricular protein samples were run on two gels, each allowing three sets of the four different samples to be run along with a single molecular weight marker (Mr) lane per gel.

Gels were cast as described and were assembled onto the Protean IIxi device as per manufacturers instructions. Buffer was placed in upper and lower tanks to cover each end of the gel(s). Electrophoresis took place overnight (approximately 16 hours) at a constant 28 volts. The following morning the voltage was increased (to 100 to 150V) until the dye front had travelled the appropriate distance into the resolving gel, approximately 8cm. Electrophoresis was then discontinued.

Table 21. Buffer Constituents for SDS-PAGE Gels

This table shows the volumes of the stock solutions used to make up SDS-PAGE gels. Two different concentrations of gel were prepared and each is shown. The stacking gel was the same in each case. The composition of the buffers is given above.

		GEL PERCENTAGE	
		6%	10%
RESOLVING GEL	Acrylamide	10.5mls	20mls
	Buffer B	12.5mls	15mls
	Water	25.6mls	23.7mls
	10% SDS	200μl	600μl
	10% APS	200μl	600μl
	TEMED	20μl	60μl
STACKING GEL	Acrylamide	4mls	4mls
	Buffer D	5mls	5mls
	Water	10.6mls	10.6mls
	10% SDS	200μl	200μl
	10% APS	200μl	200μl
	TEMED	20μl	20μl

7.2.4.Semi-Dry Electroblotting

Materials:

Dry Blot Buffer (DBB):
2.9g Glycine
5.8g Tris
0.4g SDS
200mls methanol

Made up to 1l with water. Stored at 4^0C until required. Not re-used.

Blotting Membrane: Immobilon P, polyvinylidene difluoride, Millipore, Watford, Herts

Blotting Device: Standard horizontally arranged graphite electrodes. (Constructed by Mr Adrian Boreham, Clinical School Workshops, Addenbrookes Hospital).

Filter Paper: Whatman 3MM chromatography paper.

Dialysis Membrane: Visking dialysis tubing, Medicell International, Liverpool.

Methods:

Dialysis membrane (15 x 15cm) was cut to size and was also equilibrated in blotting buffer for 15 minutes in a sandwich box on a reciprocal mixer.

After electrophoresis the gels were trimmed with a metal blade. Each set of four (or five where the Mr lane was present) lanes were clearly marked on the glass plates with a permanent marker. The gels were cut into individual pieces, three per main gel, and the six gels thus produced were all equilibrated in blotting buffer for 15 minutes in a sandwich box on a reciprocal mixer. In addition, pieces of blotting membrane were cut to the sizes of the six gels and were also equilibrated in blotting buffer for 15 minutes.

Once the gels, blotting membrane and dialysis membranes had equilibrated they were arranged in a sandwich for electroblotting as shown in the diagram.

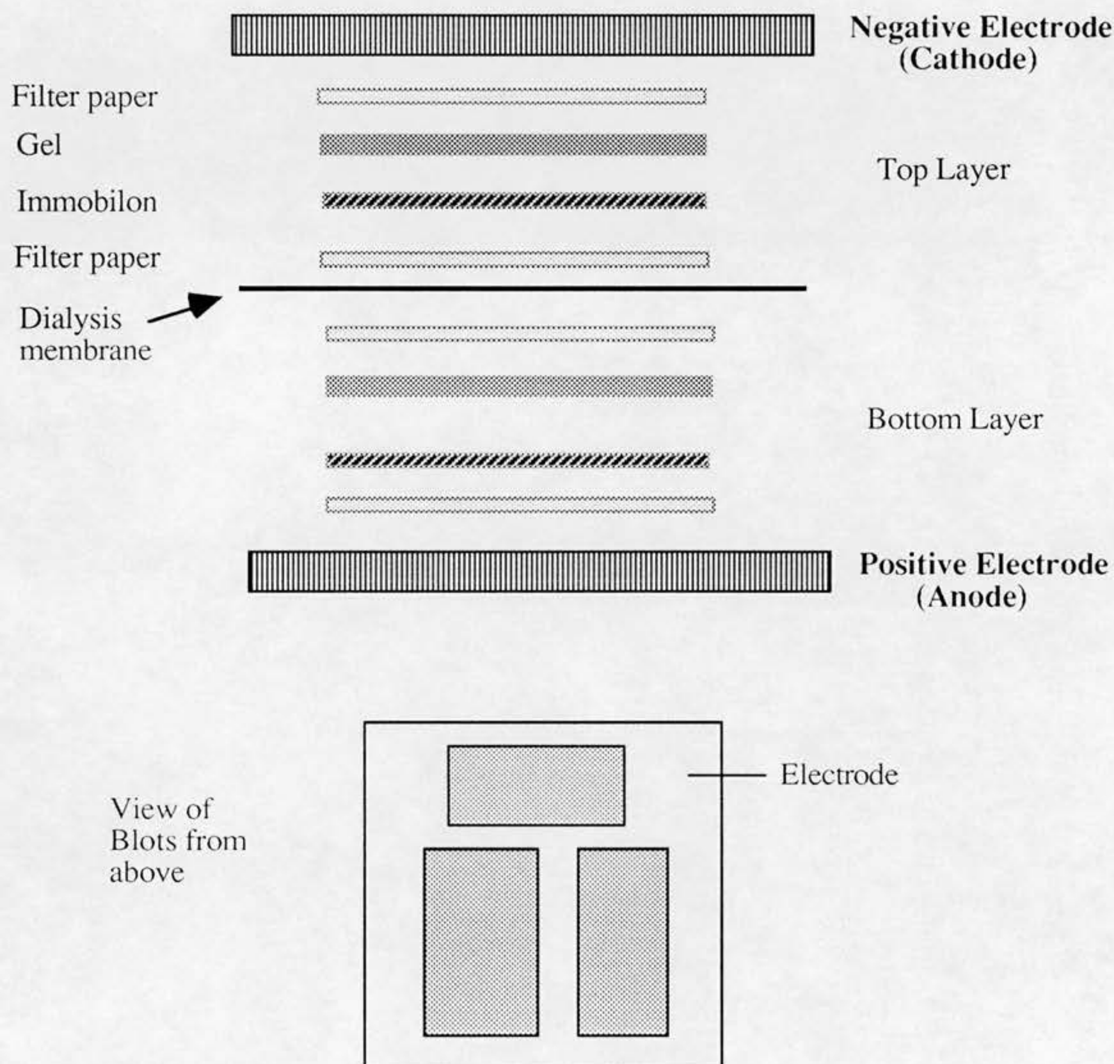
In order to accommodate six gels on a blotter with a 15 x 15 cm electrode arrangement it was necessary to blot the gels in two layers of three gels in a stack as shown in figure 13 below.

The sandwich was begun by applying two sheets of filter paper soaked in blotting buffer to the bottom electrode (anode), after first wetting the electrode with some buffer. The immobilon P membrane was then applied to the filter paper layer. Air bubbles were removed from these, and all other layers, by rolling a 10ml glass pipet over them. On top of the immobilon was placed the gel and on top of this another two pieces of filter paper. The dialysis membrane was placed on top of this sandwich and then an identical sandwich was placed directly over the first. Gels were arranged in sets of three in each layer. The two gels with the M_r lanes were of identical size and so they overlay one another. The other four gels were of similar size.

Once the double sandwich was complete and all air bubbles had been expelled the top electrode (cathode) was wet with blotting buffer and sealed down onto the sandwich with the aid of the threaded nuts provided for this purpose. The electrodes were connected to a BioRad 2.0/200 power supply and blotting proceeded for 45 minutes at 25 volts (300 to 1000mA).

Figure 13. Semi-Dry Electroblotting Arrangement

This figure illustrates the layers of the sandwich formed in performing semi-dry electroblotting. The position of each layer is important in producing adequate blotting results. Every blot was performed in this fashion. The blot arrangement is shown from a front elevation and also in an aerial view, to show how many individual sandwiches could be placed on the blotter device.



Once the blotting was complete the current was switched off and the device dismantled. The gels were placed into coomassie stain. Blots were placed into blotting buffer temporarily. The remaining constituents of the sandwich were discarded. The two gels with an M_r lane were placed on a clean surface (usually the top of a sandwich box) and the M_r lane identified (since protein bands are opaque on immobilon P and can be seen when the blot is held up to the light). These were then sliced off with a scalpel and also placed in coomassie stain. For some gels the entire gel along with its M_r lane was placed in the coomassie stain. Gels and blots or M_r lanes only were

stained in coomassie for approximately 1 hour. The blots or M_r lanes were then de-stained by washing in several successive changes of de-stain solution. Finally they were rinsed in water and left to dry in air in a dark cupboard. Dried blots/M_r lanes were placed in envelopes and stored in folders. Gels were de-stained for longer and were left in de-stain until photographed or discarded.

Meanwhile the blots were examined to ensure proteins had transferred (visible as described above). They were then placed in a folded sheet of filter paper soaked in blotting buffer, sealed in cling film and stored in a sandwich box at 4^0C until use (usually within one week).

7.2.5.Protein Assay

Materials:

BioRad Protein Assay Kit

Bovine serum albumin (BSA, SIGMA): 1mg/ml in distilled water

Methods:

All protein assays referred to were performed using a BioRad protein assay kit - based on the method of Bradford (Bradford, 1976). The microassay test was performed according to manufacturers instructions. Bovine serum albumin stock solution (1mg/ml) was used as standard in all experiments.

Briefly: dilutions of BSA stock were prepared in the range 1-25μg/ml. In a 96 well plate, 80μl test solution (BSA or unknown) plus 20μl BioRad protein assay concentrate solution was placed in each well as required. The plate was incubated for 1 hour at 37^0C. Optical density at 620nM (OD_{620})was read on a Flow Titertek Multiplate Washer (ICN-Flow). Values were corrected for those obtained from control wells containing water and protein assay concentrate only. Standard curves were produced by plotting corrected OD_{620} against concentration of BSA. The concentration of the test protein was read from this curve. Each time a protein was assayed a fresh standard curve was produced.

7.2.6.Immunostaining

Materials

Blocking Buffer:	Casein 12.5g Sodium chloride 4.5g Tris/HCl 0.605g Thimerosal 0.1g

Dissolved in 400mls water by stirring overnight at room temperature. Adjusted to pH 7.6 with addition of 5M NaOH. Made up to 500mls with water and then stored at 4^0C until use.

Washing Buffer:
Casein 20g
Sodium chloride 36g
Tris/HCl 4.84g
Thimerosal 0.8g

Dissolved in 3.5l distilled water by stirring overnight at room temperature. Adjusted to pH 7.6 with dropwise addition of c.HCl. Made up to 4l with distilled water and stored at 4^0C until use.

Detergent Buffer:
Washing buffer 200mls
Triton X-100 (SIGMA) 1ml
10% SDS 2mls
Prepared fresh immediately before use.

Tris-buffered saline:
Sodium chloride 23.38g
Tris/HCl 12.11g

Dissolved in 1.8l water and adjusted to pH 7.4 with c.HCl.
Made up to 2l with distilled water and stored at 4^0C until use.

Peroxidase Buffer
4-Chloro-1-napthol 72mg
Methanol 24mls
TBS 120mls
Hydrogen peroxide (30%) 192μl

Peroxidase conjugated Rabbit anti-human Ig: IgG, IgA and IgM (Dako)

Methods:

1. Blots were removed from storage and each one was placed in a 50ml Falcon tube with 10mls of blocking buffer per tube. Blots were then blocked for 18 hours at room temperature by mixing on a spiral mixer (12 blots fitted on this device at a time).

2. The blocking buffer was discarded and the appropriate sera were added to each tube. Sera were diluted 1:25 in washing buffer (400μl in 10 mls). The blots were then incubated at 4^0C for 18 hours on the spiral mixer.

3. The serum solution was discarded, or sometimes poured into a sterile universal container and stored at -20^{0}C for re-use. The blots were then washed with washing buffer (10mls per tube) for 5 minutes and then with detergent buffer also for 5 minutes. After two further ten minute washes in washing buffer the secondary antibody (peroxidase conjugated rabbit anti-human IgG, IgA or IgM as appropriate) was added at 1:250 (40µl per 10mls). The blots were then incubated at 4^{0}C for 18 hours on the spiral mixer.

4. The secondary antibody solution was discarded. Blots were washed four times for ten minutes each in washing buffer and then four times for ten minutes each in tris-buffered saline (TBS). The blots were then incubated in peroxidase buffer for 5 to 30 minutes (until bands appeared). They were then washed in water thoroughly and left to dry in air in a dark cupboard.

5. After drying overnight blots were placed in individually labelled envelopes.

7.3. Estimation of Relative Molecular Mass of Positive Bands

The migration of proteins in a denaturing gel is related to the logarithm of their relative molecular mass (M_r) (Hames, 1981). By calculating the distance migrated by a known protein (molecular weight marker) divided by the distance migrated by the dye front the relative mobility (Rf) is obtained. A graph of logMr against Rf produces a straight line and this allows the determination of the molecular size of an unknown protein.

Materials:

BioRad SDS-PAGE Molecular Weight Standards, Broad Range.
BioRad, Hemel Hempstead, England.

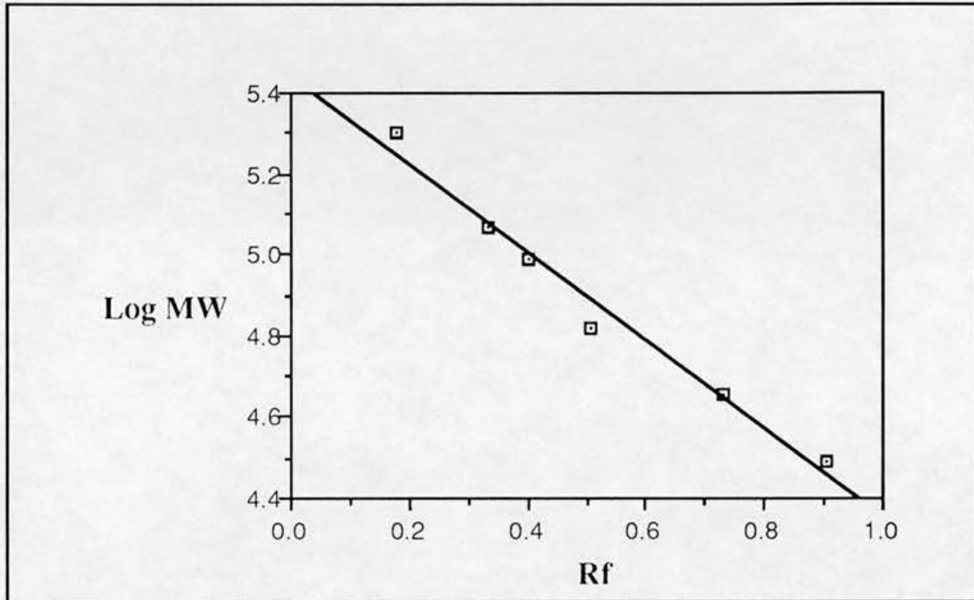
Methods:

The molecular weight standards (M_rS) were diluted 1:20 in SDS-PAGE sample buffer and stored in working aliquots at -20^{0}C until use. Prior to electrophoresis they were boiled at 100^{0}C for 5 minutes. For each lane, 10µl of stock M_rS was used.

When blots were dry, measurements were made with a ruler and plotted as described above. A sample graph is shown below (fig.14). Where bands were large, a measurement was made at the top and bottom of the band and a range of M_r was calculated.

Figure 14. Graph of Relative Mobility vs. Log Mr.

This graph shows a plot of the logarithm of the molecular size of a given molecular weight marker against its relative mobility in a polyacrylamide gel. Broad Range Mr Standards were resolved on 10% SDS-PAGE gel and blotted onto immobilon P. The blots were stained with coomassie blue. The blot was identical to that used for immunostaining in order to make comparison with any positive bands obtained. A typical plot from the actual experiments reported is shown. Log MW = logarithm of molecular weight, Rf = relative mobility.



7.4. Densitometry

Where bands were not clearly discernible as either positive or negative densitometry was performed to measure accurately the colour reaction of particular bands of interest. The principle used was that a specific band would show a colour reaction at least two times greater than that of a suitable control, a principle widely used in other enzyme immunoassay procedures such as ELISA.

Materials:

Personal Densitometer. Software: ImageQuant Version 3.2
Molecular Dynamics Ltd., Kemsing, Sevenoaks, Kent.

Methods:

The device used was a densitometer which measures reflectance from a surface and displays the resulting image on a screen, allowing manipulation of the display for optimum clarity of viewing. The software does not allow manipulation of the raw data, only the image, so that measurements were always made on the data as they were acquired and not on the altered image.

Blots were soaked in methanol for 5 seconds and then immersed in water until use (within 15 minutes).

Blots were placed on the imaging plate after the machine had performed internal calibration. The blot was scanned and the resulting image displayed on a colour screen. Once the data for any given blot were stored, the blot was left to dry in air and returned to storage in an envelope.

Bands of interest were selected. A measuring box was placed closely around these bands using the system software and the "integrated pixel volume" (IPV) calculated within this box. The same box was used to calculate the background from an area of the blot remote from the band of interest but lying within the lane being examined. Comparison with bands in adjacent lanes was made in an identical fashion. All results were corrected for background. A positive result was one where the IPV for the band of interest was more than twice the IPV of the control band.

7.5. Statistics used

The main statistical method used in this section was the Chi-square test with Yate's correction applied.

i.e.

Chi-Square Test:

$$\aleph^2 = \frac{n\,[\,(ad-bc) - {}^1/_2 n\,]^2}{(a+b)(c+d)(a+c)(b+d)}$$

8. Results: Alcoholic Cardiomyopathy

Sera from 59 subjects in five groups (see table in methods section) were examined by Western blotting. Four different preparations of human cardiac cytosolic protein fraction were resolved together on SDS-PAGE and then blotted onto a membrane. This was probed with the sera. The resulting immunoblots consist of four lanes of protein representing the four different fractions. The first lane in every blot is the native or untreated protein. This was incubated with saline only. The second lane contains protein that was incubated with acetaldehyde only, included as a control. The unstable nature of acetaldehyde adducts is such that this lane might be expected to be similar to lane one. Lane three represents protein treated with cyanoborohydride, a reducing agent intended to stabilise adducts produced by acetaldehyde, another control lane. Lane four is the lane where a specific positive result is expected. Here both acetaldehyde, to produce adducts, and sodium cyanoborohydride, to stabilise them, were used.

This layout was used for every experiment. The results are therefore similar in format regardless of the subjects. The key feature is that native protein is present in lane one and the altered proteins of interest in lane four. It may be observed that some positive bands are present in all lanes. This indicates antibody activity against native proteins and is not an unexpected finding. Koskinas et al described similar bands in their experiments on acetaldehyde adducts in the liver and other workers using Western blotting with heart tissue also find these non-specific bands (Koskinas, 1992).

Thus the blots resulting from immunostaining have four lanes represented pictorially, from left to right, as:

LANE:	1	2	3	4
TREATMENT:	None	Acetaldehyde	Cyano-borohydride	Both
FUNCTION:	Native tissue	Control	Control	Result

Only bands present in lane 4 were considered positive. It was not uncommon to see bands in all four lanes in any of the groups of subjects studied.

Where bands are present in two lanes only this is usually lanes 3 and 4. Where they are of equal intensity they are counted as negative. Where measurement of band intensity by densitometry shows that the band in lane 4 is more than twice the intensity of another band it is counted as positive.

The following figures are set out according to this format, though each has a legend describing it in detail. The immunoblots are in order, starting with subjects from

the alcoholic cardiomyopathy group, followed by those of healthy volunteers, dilated cardiomyopathy, ischaemic heart disease and alcoholic liver disease. A table follows the last blot giving the overall results and then a table of the estimated molecular size of the positive bands.

Four of fourteen (28%) patients with ACM had detectable antibodies against acetaldehyde-modified cytosolic protein (figures 15-19 below), detected only against samples treated with acetaldehyde and cyanoborohydride, compared with none of 28 patients with cardiac failure of non-alcoholic aetiology ($p<0.05$, Chi-Square). Antibodies were of IgG class in 3 patients and two of these also had an IgA antibody. An IgM antibody was detected in the fourth patient. The proteins identified had molecular weights ranging from 63 to 82 kDa. No antibodies were detected in sera from any other subject. Patients with IgG antibodies also had antibodies of the same isotype against microsomal proteins of different size to the cytosolic target (figure 20). Antibodies against untreated heart were noted among all groups (figures 21-24).

8.1. Alcoholic Cardiomyopathy

The fourteen patients with alcoholic heart muscle disease were tested for IgA, IgG and IgM antibodies against normal human left ventricular cytosolic fractions treated with or without acetaldehyde in the presence or absence of sodium cyanoborohydride. Sera from patients with positive antibodies against cytosolic fractions were tested against microsomal fractions, again with IgA, IgG and IgM (where the amount of serum allowed). The resulting blots are illustrated below.

The series of blots illustrated serves to show the type of result obtained in performing Western immunoblotting with sera from these subjects. Many more blots were performed and blots were often repeated to confirm initial findings. The series is intended to show the important positive results in detail and then some illustrative negative results from control subjects.

Figure 15: Photograph of Western Immunoblot - Patient A tested against Human Cytosolic Protein.

The photograph shows a blot produced after development with chloro-napthol. The four lanes represent four fractions of human left ventricular cytosolic tissue treated with 1)nothing, 2)acetaldehyde alone, 3)sodium cyanoborohydride and 4)acetaldehyde and sodium cyanoborohydride. The blot was probed with serum from patient A, a subject with ACM. The secondary antibody was peroxidase conjugated anti-human IgG. The "X" marks a large band centred on 63kDa, which is present only in lane 4. This represents a positive result. The patient's serum reacts only with this antigen, produced by acetaldehyde and stabilised by sodium cyanoborohydride(lane 4), and not with the native protein (lane 1) or with either of the controls (lanes 2 and 3). Also seen are bands present in lanes 1-4 at approximately 20, 44 and 100 kDa. These bands are non-specific and are often observed in serum from control subjects.

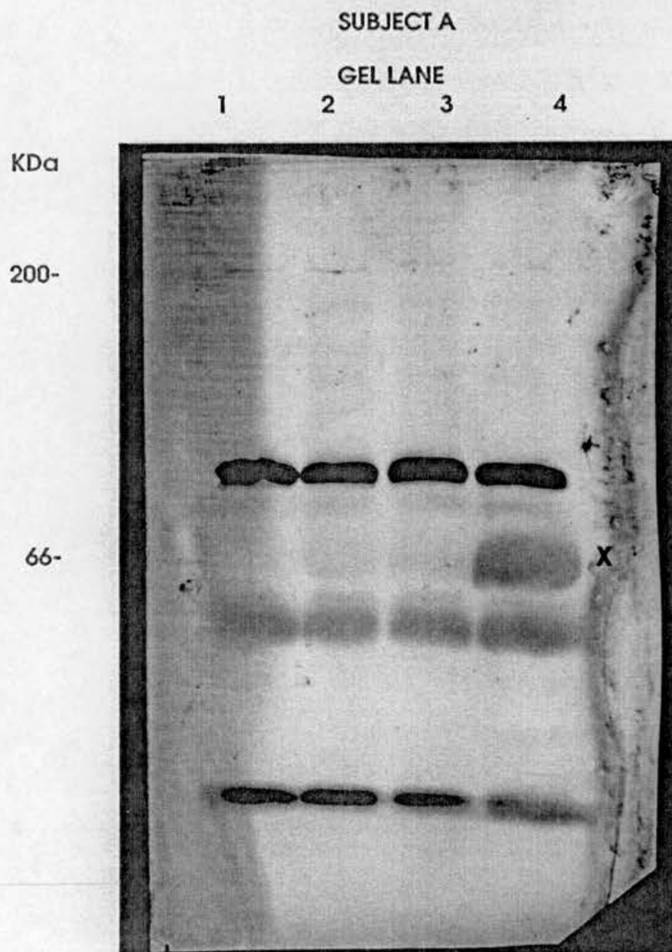


Figure 16: A Second Patient with ACM tested against Human Cytosolic Protein.

This blot is shown alongside one stained with Coomassie Blue R-250 for protein, for comparison. Subject D has ACM and the serum has reacted with a protein at approximately 69kDa, again present only in lane 4, marked "X". In this case the positive band is narrower than that in the previous figure. The secondary antibody was IgG. Once more, non-specific bands, present in lanes 1-4, can be seen. The coomassie-stained blot was an identical blot to that used for treatment with the patient's serum and was produced in the same experiment. The large number of protein bands on the blot is striking when compared with the small number with which the subjects serum has reacted.

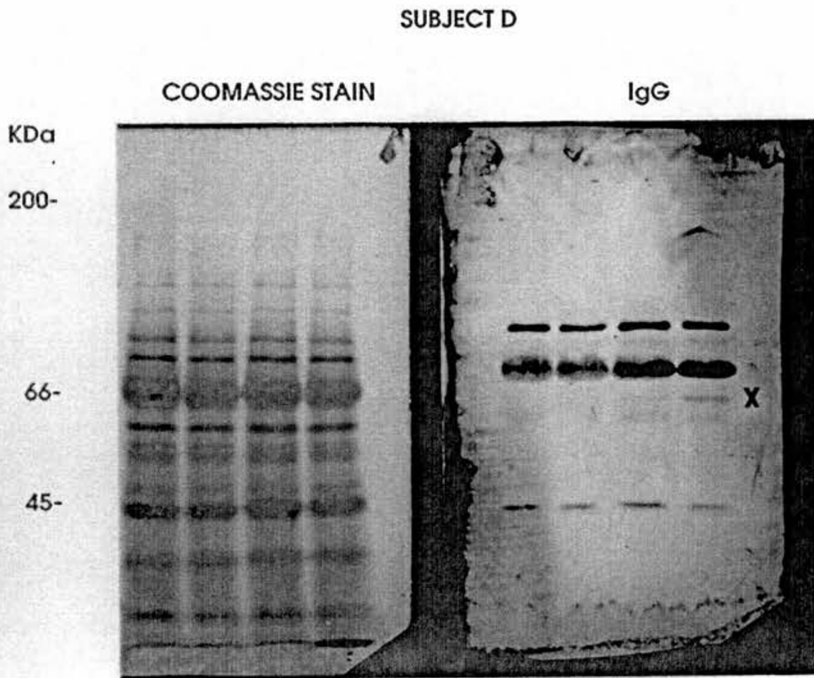


Figure 17: Western Immunoblot. Patient A tested for IgG, IgA and IgM against Human Cytosolic Protein.

Three blots reacted with serum from subject A are shown alongside another stained with coomassie for protein as above. The blots were developed with a secondary antibody to IgG, IgA and IgM respectively. Bands are present in lane 4 on the IgG (63kDa) and IgA (two bands - 74 and 82kDa) blots marked "X" and "Y" respectively. No specific IgM bands were detected for this subject. Bands are present which are seen in lanes three and four only.

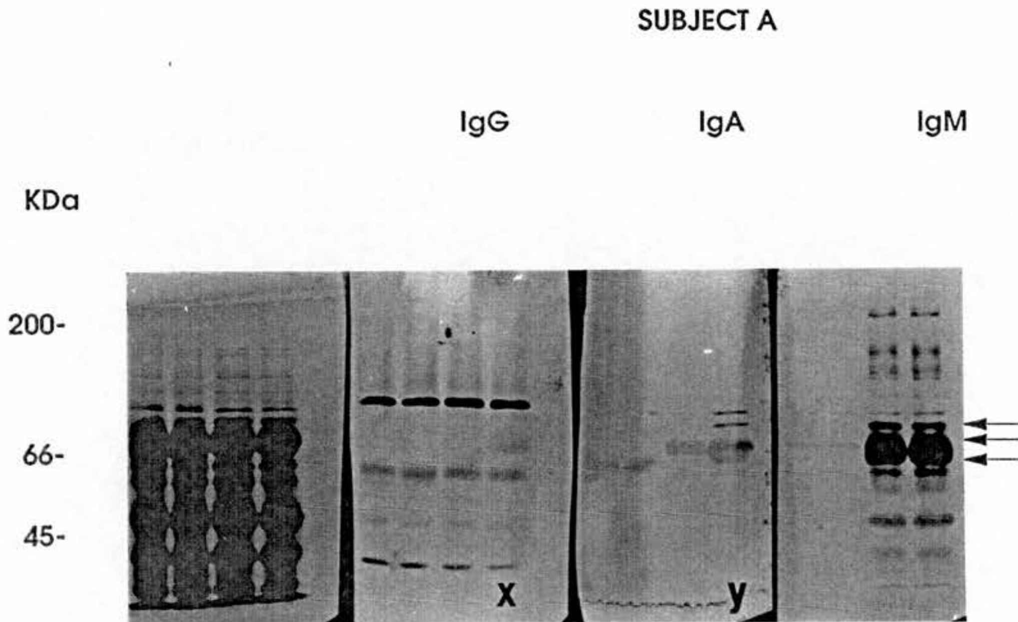


Figure 18: Western Immunoblot. Patient B tested for IgG, IgA and IgM against Human Cytosolic Protein.

Serum from subject B reacted with three blots of human left ventricular cytosol and compared with a protein stained blot as in the figure above. An IgG antibody is present at 63kDa in the lane marked "X". Faint IgA bands are present at 74 and 67kDa.

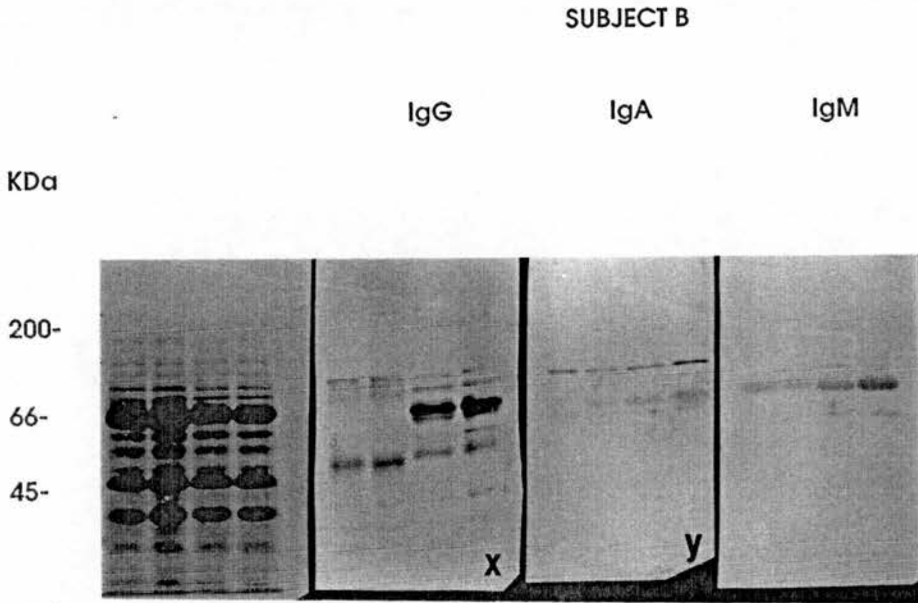


Figure 19: Western Immunoblot. Patient C tested for IgG, IgA and IgM against Human Cytosolic Protein.

This photograph shows blots produced when serum from subject C was reacted with three blots of human left ventricular cytosol. These are compared with a protein stained blot. Positive bands are present only for IgM. The main one is at 77kDa in the lane marked with an "X" but a band at a lower Mr is also visible. There are no specific IgG or IgA bands.

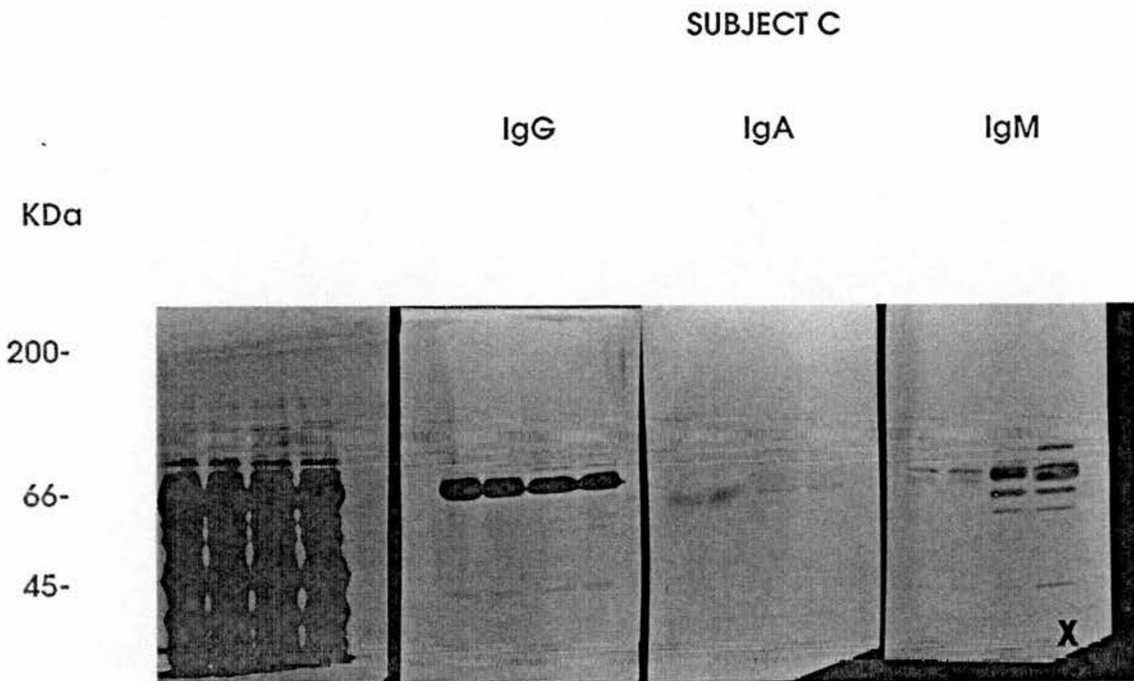
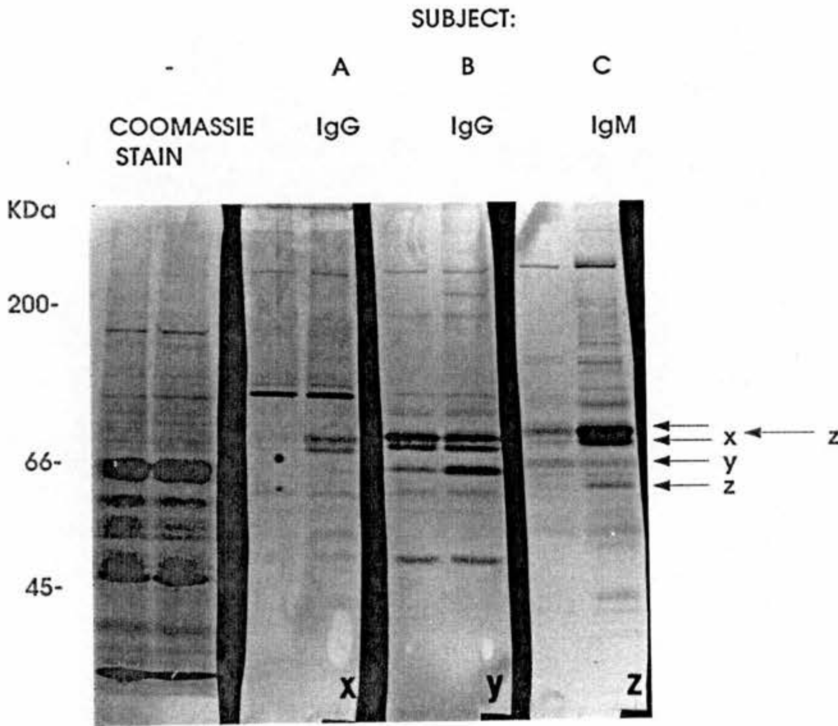


Figure 20: Patients A, B and C tested for IgG and IgM against Human Cardiac Microsomal Protein.

This photograph shows the results of an experiment where human cardiac microsomal fraction was treated in exactly the same way as the cytosolic fraction used in the experiments illustrated by the above blots. Lanes 3 and 4 of microsomal fraction blots are shown against lanes 3 and 4 stained with coomassie blue. Sera from subjects A, B and C have been reacted with the blots and secondary antibody was IgG, IgG and IgM respectively. For patient A bands are present as a doublet at about 70kDa, for patient B at approximately 66kDa (with a much weaker band in lane three which is more than three times less strong a reaction according to densitometry). Patient C's serum reacted with a protein at about 70kDa and also, less strongly, with a band at about 60kDa. Multiple other bands are present in lane four with patient C, particularly at higher molecular size. This experiment, conducted simultaneously with identical blots allows comparisons to be made directly between the bands reacting with each individual subjects serum.

IMMUNOBLOTS - HUMAN LEFT VENTRICULAR MICROSOMAL FRACTION



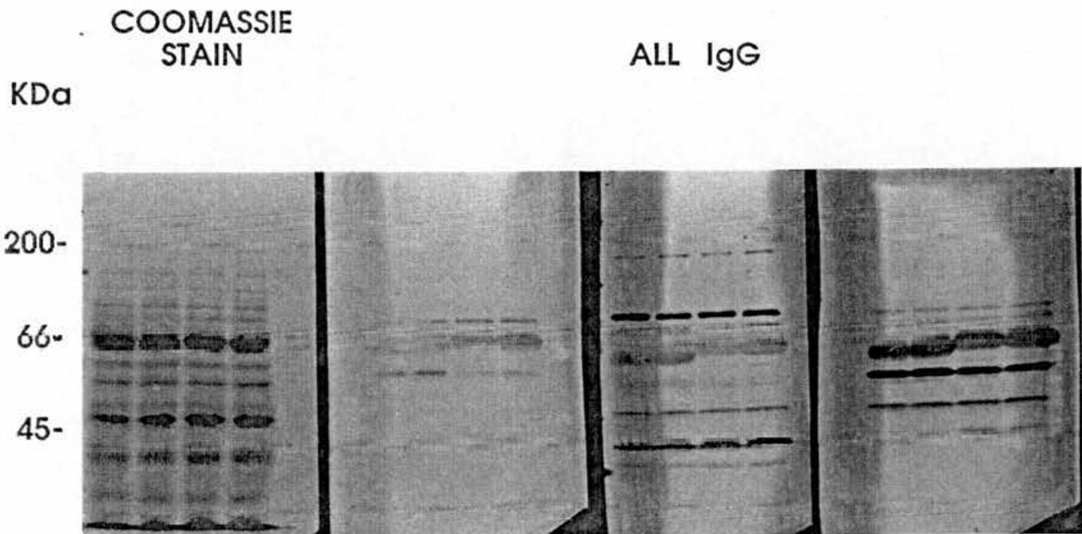
8.2 Healthy Volunteers

Sera from eleven healthy individuals (age range 24 to 56 years, one female) who drank from 0 to 16 units of alcohol per week, were examined. None of these sera contained antibodies to proteins present in lane four only, as illustrated below in figure 21. Blots developed with IgG are shown but all three antibody isotypes were tested and found to be negative.

Figure 21: Western Immunoblots. Healthy Volunteers tested for IgG against Human Cytosolic Protein.

This figure illustrates the results produced when sera from non-alcoholic volunteer subjects with no heart or liver disease were reacted with blots of human left ventricular cytosol. The secondary antibody was directed against human IgG. Many non-specific bands are present in the central blot. These results are similar to those produced with IgA and IgM except that there were fewer bands with IgA. When healthy volunteers' sera (not all were tested due to shortage of microsomal fraction) were reacted with microsomal blots similar results were obtained.

IMMUNOBLOTS - HUMAN LEFT VENTRICULAR CYTOSOL
CONTROL - THREE NORMAL SUBJECTS



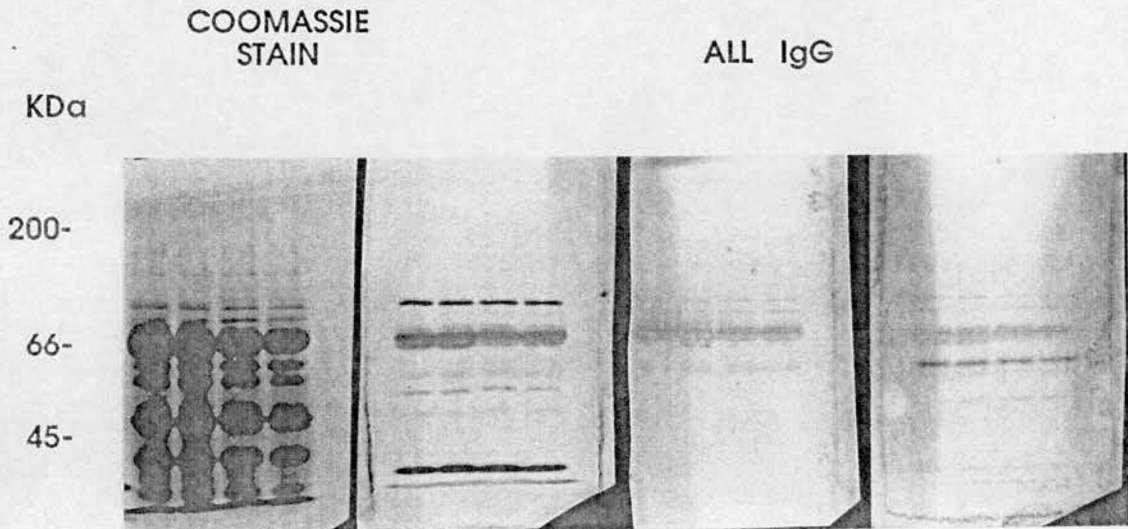
8.3. Idiopathic Dilated Cardiomyopathy

Patients with idiopathic dilated cardiomyopathy also had serum samples tested for antibodies to acetaldehyde adducts of human cardiac cytosolic tissue. Twenty of these subjects were studied (age range 20 to 65 years). None of these patients consumed more than 21 units of alcohol weekly.

Again only blots reacted with IgG are shown (fig. 22) but all isotypes were tested. No positive results were obtained, as no bands present in lane four only were observed.

Figure 22: Serum from subjects with Idiopathic Dilated Cardiomyopathy tested for IgG against blots of Human Cytosolic Protein.

Blots of human left ventricular cytosol reacted with serum from three different patients with dilated cardiomyopathy and shown alongside a blot stained for protein. Many bands are present but none solely in lane 4. These bands are non-specific auto-reactive antibodies often seen in such experiments. No IgA or IgM antibody activity was detected either (not shown).



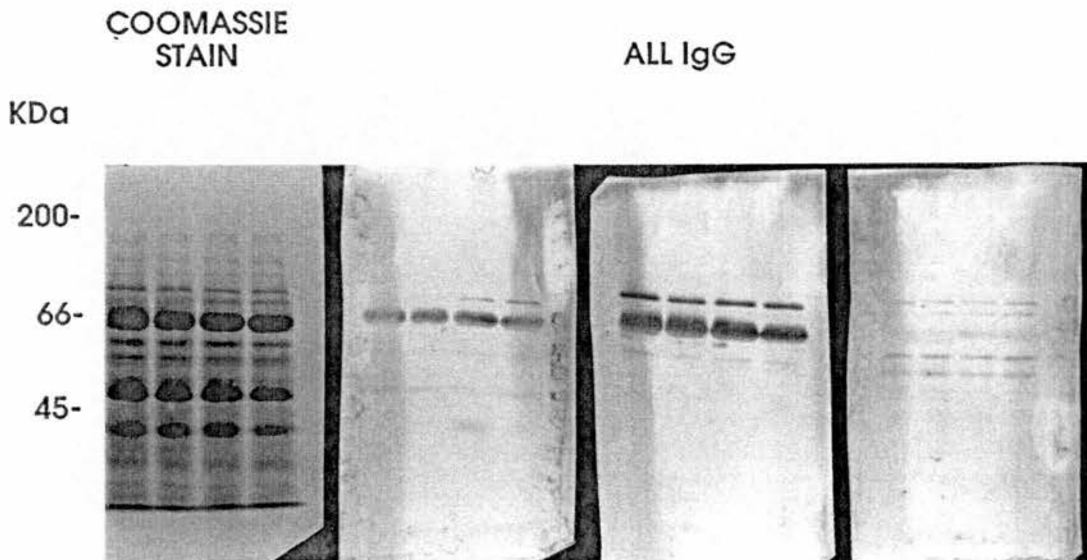
8.4. Ischaemic Heart Disease

Sera from eight patients with ischaemic heart disease (age range 49 to 86 years) and impaired left ventricular function were also studied. These patients were all consuming less than 21 units of alcohol weekly. Their sera were reacted with blots in the fashion described above. The secondary antibody used in the blots shown (fig.23) was IgG, but IgA and IgM were also examined. No positive results were observed (as defined above).

Figure 23: Subjects with Ischaemic Heart Disease tested for IgG against blots of Human Cytosolic Protein.

Blots of human cardiac cytosolic protein treated as described above reacted with sera from patients with IHD. A coomassie stained blot is shown for comparison. As above non-specific bands only are observed, frequently at 66kDa. No IgA or IgM antibody activity was detected (not shown).

IMMUNOBLOTS - HUMAN LEFT VENTRICULAR CYTOSOL
CONTROL - THREE SUBJECTS WITH ISCHAEMIC HEART DISEASE

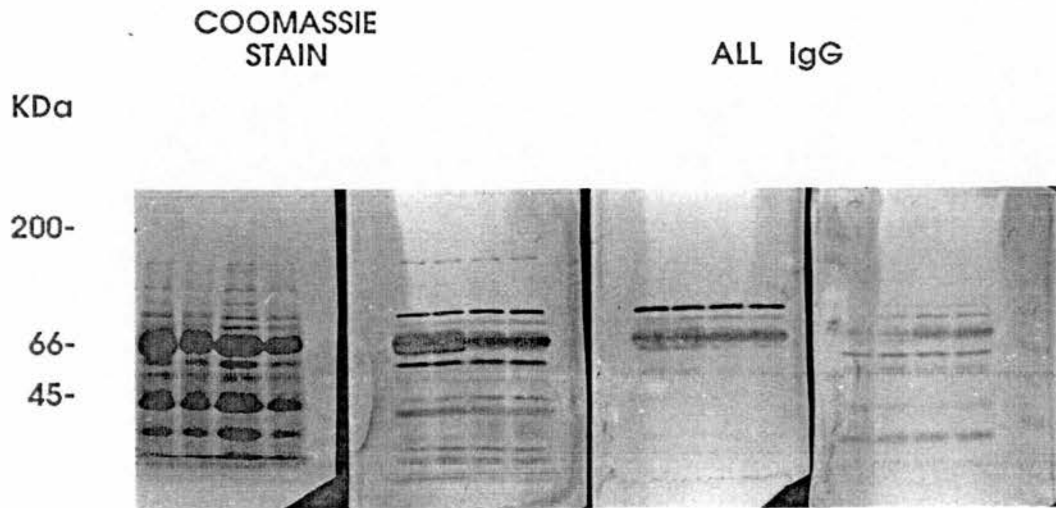


8.5. Alcoholic Liver Disease

Eight patients with alcoholic liver disease were studied (aged 31 to 68 years, two female) who were consuming 42 to 84 units of alcohol weekly. Sera were tested with IgG, IgA and IgM secondary antibodies. Only the IgG blots are illustrated (fig. 24) as the results were all broadly similar. No positive results were observed (as defined above).

Figure 24: Subjects with Alcoholic Liver Disease tested for IgG against blots of Human Cytosolic Protein

Blots of human left ventricular cytosol reacted with serum from three different patients with alcoholic liver disease and shown alongside a blot stained for protein. Again, multiple non-specific bands are present in each lane, in this case rather more than seen with subjects suffering from ischaemic heart disease.



8.6. Relative Molecular Mass of Positive Bands

The various estimated molecular masses of the positive reactions on the blots illustrated above are listed in table 22 below. It can be seen that no single molecular sized protein is common to all the patients, though the positive bands do seem to cluster between 60 and 80 kDa.

Table 22: Relative Molecular Mass of Positive Bands.

This table shows the relative molecular masses of bands judged as positive for each subject. The method used is described above in the methods section. These figures are estimates and, particularly for higher molecular masses, are not necessarily entirely accurate. There may be discrepancies between observed bands on individual blots and the figures listed in the table. There appear to be antigens at around 63 and 77 kDa which are detected by more than one subject's sera. LVC=left ventricular cytosolic fraction and LVM=left ventricular microsomal fraction. Subjects A to D, whose immunoblots are illustrated above, are identified in the table by their respective letters.

Molecular Weight Estimations of Positive Bands

Subject	Tissue	Antibody Isotype	Band Size
A	LVC	IgG	58 - 68
	LVC	IgA	82
	LVM	IgG	77
B	LVC	IgG	63
	LVC	IgA	62, 120
	LVM	IgG	69
C	LVC	IgM	77
	LVM	IgM	60, 87, 110
D	LVC	IgG	70
	LVM	IgG	72

9. Alcoholic Cardiomyopathy - Discussion

9.1 Main Findings

The major findings of this study are that a proportion of patients with alcoholic cardiomyopathy have circulating antibodies that recognise acetaldehyde-modified normal human cardiac protein. The antibodies identified were predominantly of IgG isotype, but IgA and IgM antibodies were also detected. The antigens recognised by these antibodies were in the range 58-120 kDa and were identified in both cytosolic and microsomal fractions. All the appropriate control populations, including those with heart disease not due to alcohol and those with alcohol-related illness not involving the heart, were always negative for antibody to acetaldehyde-modified cardiac protein.

In this study, the antibody was detected only in the presence of sodium cyanoborohydride, the reducing agent used to stabilise the acetaldehyde adducts. Acetaldehyde is a highly reactive species that yields stable and unstable adducts after binding to protein; the unstable adducts are probably Schiff bases formed with the epsilon amino group of lysine residues (Tuma, 1987). Because the antigen was detected in the presence only of both acetaldehyde and cyanoborohydride, it would appear that the generation of the antigen in-vivo involves the formation of stable adducts. Alternatively, unstable adducts might be continuously produced.

The results indicate that acetaldehyde modifies cardiac protein in some individuals consuming alcohol to excess and renders them immunogenic. No subjects from any of the four control groups were found to have specific antibodies of this nature. However, it is uncertain whether the generation of the antigen and the immune response to this antigen is the primary event in alcoholic cardiomyopathy or whether this is a non-specific response, but the presence of such antibody, exclusively in patients with alcoholic cardiomyopathy, suggests that this approach is worthy of further attention.

Experiments using immunofluorescence on sections of myocardium from patients with alcoholic cardiomyopathy might identify the cellular location of the antigens. In initial experiments, conducted by Dr N Cary and his assistants in the immunofluorescence laboratory at Papworth hospital, it was not possible to achieve low enough background staining to identify specific staining. Sufficient tissue for extensive experimentation was not available for this study. Future experiments may centre on the use of otherwise normal heart which has been treated in a similar way to the protein fractions used in western blotting experiments. The immunofluorescence techniques may then be refined until meaningful results are achieved.

It is not known what concentrations of acetaldehyde are achieved in cardiac tissues in man following alcohol consumption but it is known that the concentration in

rat ventricle exceeds that in plasma (Espinet, 1984). Neither the nature nor the location of the acetaldehyde-modified antigens in ventricular tissue are known, but the fact that these antigens have induced immune responses suggests that they are accessible to the immune system.

9.2. Sensitivity of Assay

In total, four of fourteen patients with alcoholic cardiomyopathy (sensitivity = 28%) possessed antibodies to acetaldehyde adducts of human left ventricle. One possible reason why the number of patients who were positive for antibody to acetaldehyde-modified cardiac protein was small is the nature of the assay seeking antibodies. Western blotting involves conformational changes and it may be that using an assay that does not involve such changes would give a higher yield of the relevant antibody. The small numbers may also relate to timing of the original blood sample. Patients labelled as having alcoholic cardiomyopathy may already have stopped drinking by the time of tertiary referral to a major centre (the source of the majority of these sera). A further explanation may be that the patients without the antibody response may have a true 'dilated cardiomyopathy' not related to alcohol consumption. Not all patients with alcoholic cardiomyopathy will recover if they stop drinking and these may represent cases of dilated cardiomyopathy. Alternatively it may be that more than one mechanism of myocardial damage is at work. The acetaldehyde adduct explanation fits the available evidence but many workers who have studied dilated cardiomyopathy specifically excluded subjects with alcoholic cardiomyopathy. This means that there is a paucity of literature on these patients. It is precisely because there is no diagnostic test and empirical diagnosis is required that it has been difficult to make more progress in studying the condition. It may be that the antibody response to acetaldehyde adducts is a way to identify patients with a true alcoholic cardiomyopathy.

9.3. Specificity of Assay

Antibodies were identified in the serum of subjects from each group studied to proteins not incubated with acetaldehyde. These antibodies are represented by the presence of bands in each lane, one to four, on the blots shown in the results section. These were clearly not specific to patients with alcoholic cardiomyopathy but this is not an unexpected finding. Two bodies of work suggest that this could have been predicted. Koskinas et al (Koskinas, 1992) also found that their subjects with alcoholic liver disease, non-alcoholic liver disease or healthy volunteers had antibodies to native proteins, in this case of liver origin. Some of these proteins will not be tissue specific and might be present in other organs, such as the heart. In studies using Western blotting of cardiac tissue and sera from subjects with dilated cardiomyopathy, acute

myocarditis and normal subjects, the presence of antibodies to native protein was observed (Neumann, 1990). Thus, non-specific antibody responses are not unusual and have been observed previously. Therefore, the only bands to which close attention has been paid were those present in lane four only - where stabilised acetaldehyde adducts were present.

In terms of the ability of the assay to detect patients with alcoholic cardiomyopathy in these studies the specificity was 100% since none of the control subjects had any antibodies to acetaldehyde modified human cardiac cytosolic proteins. The predictive value of a positive test is also 100% according to these figures.

9.4. Immunological Responses to the Heart

This thesis has demonstrated the presence of antibodies to acetaldehyde-adducts using experiments not previously performed for human cardiac proteins. However, there have been many previous studies looking for immunological responses directed against the heart. Only a few of these have involved patients with alcoholic cardiomyopathy. Early studies using direct immunofluorescence showed evidence of immunoglobulin binding to the heart in patients with cardiomyopathy (Sanders, 1965; Das, 1971; Das, 1972). Maisch et al used indirect immunofluorescence and found 'anti-interfibrillary antibodies' in 67% (of 30) patients with alcoholic cardiomyopathy versus 41% (of 79) with primary cardiomyopathy and 3% (of 200) in healthy controls (Maisch, 1983). In the current studies IgG, IgA and IgM antibody isotypes were present. Maisch found IgG in 77% and IgM in 19% of alcoholic cardiomyopathy subjects.

Another group used direct and indirect immunofluorescence to study 118 patients with 'congestive cardiomyopathy', of whom 38 were labelled as "definitely alcoholic" and 34 as "presumably alcoholic". They found antiheart antibodies were present in 25% of the "definitely alcoholic" group and 37% in the "presumably alcoholic" group (Hogye, 1989). It is noteworthy that the finding of positive immunofluorescence in 25% of the "definitely alcoholic" group compares with the figure of 28% of alcoholic cardiomyopathy subjects producing positive results in this thesis. It is unclear why there should be a higher proportion of positive results in the less well defined "presumably alcoholic" group.

The bands detected for healthy volunteers in this thesis may represent non-organ specific antibodies. These would be antibodies raised against substances present in many organs or tissues and would not represent a specific response against cardiac proteins. Whether or not these non-organ specific antibodies have significance is not known. This antibody activity recognises proteins regardless of whether or not they

have been treated with acetaldehyde or cyanoborohydride since the bands are present in all four lanes.

Caforio et al found that 17 of 65 (26%) patients with idiopathic dilated cardiomyopathy had organ-specific cardiac antibodies compared with only 7 of 200 (3.5%) normal subjects, using immunofluorescence (Caforio, 1990b). They found a relatively low incidence of anti-heart antibodies, whereas in the present study almost all subjects with heart disease had antibody activity against human cardiac proteins (the "non-specific bands" illustrated in figures 20 and 21). It may be that the antigens recognised as non-specific bands on these blots are not heart specific, but immunoblots using various other tissues were not used. Alternatively it may be that the different methods, that is immunofluorescence versus Western blotting, give very different results.

Neumann et al used Western blotting to detect IgG antibodies against an extract of normal human heart (Neumann, 1990). They studied 71 patients with idiopathic dilated cardiomyopathy, 17 patients with acute myocarditis and 15 healthy volunteers. There was no difference in the mean number of antigens recognized by serum from each group, which would fit with my results. However, in the same study, using indirect immunofluorescence, they showed high titre IgG antibodies to heart in 59% of patients with myocarditis, 20% with idiopathic cardiomyopathy and none in controls. The latter results are more akin to those of Caforio et al and the former are similar to my findings for non-specific antibodies. This is further evidence that the different methods produce different results. Since Neumann et al did not exclude (or did not state either way) alcoholics some of these results may relate to patients consuming excess alcohol.

Thus, other workers have detected antibodies directed against the heart in patients with alcoholic cardiomyopathy. They may not be organ specific and are detected differently with Western blotting and indirect immunofluorescence. The question of organ specificity of these antibodies was not addressed in this thesis. However, the presence of antibodies to other tissue preparations would merely show that this mechanism could function in these other tissues. This might be predicted from a knowledge of the wide range of tissues damaged in chronic alcohol abuse. These other studies support the finding of antibodies against native heart among control subjects. They also suggest additional antibody activity against the heart in these patients since no other studies were performed using acetaldehyde adducts. Although this might also indicate the presence of a stable acetaldehyde adduct in the tissues studied, or a protein sufficiently similar to an unstable adduct to allow cross-reactive antibody binding to occur.

9.5. Relationship to Antibodies in Alcoholic Hepatitis

A previous study has suggested that an IgA antibody circulating against a 200kDa protein is associated closely with alcoholic hepatitis. It is interesting in this context to note that none of the patients with alcoholic liver disease in this study were positive for antibody to the modified cardiac protein. Similarly, patients with alcoholic cardiomyopathy do not have IgA antibody to the 200kDa acetaldehyde-modified liver protein identified in alcoholic hepatitis. Other workers have shown that antibodies to acetaldehyde haptens are present in animals and humans exposed to ethanol long term in vivo. These investigations have used a variety of stable protein acetaldehyde adducts, often with the test antigen being produced in-vitro, for example with albumin or haemoglobin. These results are important in that they show that the antibody response to acetaldehyde-modified epitopes is not restricted necessarily to those with alcoholic liver disease and has been identified in several situations previously in heavy drinkers who do not have evidence of alcoholic liver damage (Hoerner, 1986; Niemela, 1987).

Patients with alcoholic liver disease, known to have an antibody to a 200 kilo-Dalton liver cytosolic protein, did not have an antibody to a similar sized protein in human heart (figure 22). This suggests that these proteins are organ specific, or at least have differential expression in these organs. This may explain the clinical impression that these two conditions do not co-exist - despite sufficient alcohol consumption for either to occur. It may be that the patients genetic constitution determines a susceptibility to the action of alcohol. This may be on the basis of production of a protein which has important cellular functions and which may react with acetaldehyde. This study did not examine alcoholic patients with neuropathy, pancreatitis, thrombocytopenia or other complications of chronic alcohol abuse for anti-acetaldhyde-adduct antibodies but it may be speculated that these different tissues may all be affected by this mechanism to a greater or lesser extent.

9.6. Pathogenesis and Identity of Antigens

The reason for the presence of specific antibodies to proteins in lane four has been discussed. Briefly, alcoholic subjects ingest ethanol which is metabolised in the liver to acetaldehyde. Circulating acetaldehyde is taken up by the heart where it reacts with various proteins, rendering them antigenic. When such subjects go on an alcoholic binge the direct toxic effects of the ethanol on the heart cause sufficient cellular damage to expose the altered proteins. These then raise an antibody response. The result of this antibody response then depends on the protein in question. It may be an enzyme important for energy use in the heart, for example ornithine decarboxylase (Behrens,

1988), or perhaps a structural protein, such as myosin, involved in myocardial contraction.

Evidence that this postulated mechanism can function in-vivo comes from a study by Yokoyama et al (Yokoyama, 1993). In these experiments hepatitis was produced in Guinea Pigs that were immunised with synthetic acetaldehyde adducts of human haemoglobin and bovine serum albumin. They were then fed on ethanol. The animals were divided into groups according to whether or not they were immunised and whether or not they went on to be fed with alcohol. The study showed that animals immunised with the adducts and then fed with alcohol developed hepatic necrosis with elevated serum liver enzyme levels, a condition analogous to alcoholic hepatitis in humans. Animals immunised with unmodified haemoglobin, fed on alcohol showed fatty change only. Animals immunised only had minimal inflammatory changes in the liver. This study is of great importance in showing that the mechanism proposed functions as postulated. It does not of course prove that the antibody response has a pathological function but it is highly suggestive.

The antibody response in the patients studied in this thesis may of course be secondary to any damage occurring in their hearts. It may simply signify the degree of myocardial damage caused by acetaldehyde which, incidentally, also leads to the raising of an antibody response. These antibodies may therefore be a marker of myocardial damage resulting from acetaldehyde production. As such they may offer a useful diagnostic tool. On the other hand they may contribute to myocardial dysfunction by interfering with the function of proteins against which they are raised. The clinical features of alcoholic cardiomyopathy suggest that whatever process is deranging myocardial contractility it is reversible, as a recent study has confirmed (Teragaki, 1993). However it does not reverse overnight. It takes weeks, or even months, to recover fully from this condition. It is necessary therefore to postulate a mechanism of reversible myocardial impairment which takes some time to diminish after the root cause is removed. An antibody response might fit this profile.

Determination of the identity of the antigen(s) modified by acetaldehyde could be critical in pinpointing the role of acetaldehyde-modified proteins in ACM. There is considerable evidence that acetaldehyde may alter the function of a large number of proteins and that protein synthesis is reduced in the presence of acetaldehyde (Schreiber, 1972; Schreiber, 1982; Schreiber, 1974; Siddiq, 1993). However, acetaldehyde can have positive inotropic and chronotropic effects due to secondary release of catecholamines (James, 1967; Gallis, 1971). Unlike ethanol (Gould, 1971) acetaldehyde has no demonstrable direct toxic effect on the myocardium and is therefore unlikely to be causing alcoholic cardiomyopathy directly. However, the generation of antibodies to unstable acetaldehyde adducts may explain the reversible nature of

alcoholic cardiomyopathy since persistence of the antigens would be dependent on chronic acetaldehyde generation. Abstinence from alcohol would be expected to result in disappearance of the antigen and its immune stimulus.

Whilst acetaldehyde may not play a role as a direct toxin in alcoholic cardiomyopathy, immune recognition of acetaldehyde-modified protein may be a secondary and irrelevant effect, leaving some as yet unknown mechanism responsible for the disorder. Alternatively, immune recognition of modified cardiac protein could be a primary event; however there is little evidence to indicate complement activation in alcoholic cardiomyopathy, nor do the histological findings support a role for immune insult. However, there is a paucity of reliable data in this field and an important role for immune involvement cannot be excluded on the published literature to date.

One intriguing possibility is that of cross-reactivity between the neo-antigens produced by acetaldehyde binding to cellular proteins and native proteins in the myocardium. In particular the concept of cross-reactivity between antibodies to acetaldehyde adducts and proteins expressed at a high level in the context of cellular injury is attractive. Sequence homology between hsp60, a ubiquitous heat shock protein, and several autoantigens has been demonstrated (Jones, 1993). Thus a theory might be proposed in which the heart is exposed to acute damage by excess alcohol. This induces hsp60 production and also the formation of acetaldehyde adducts. Further damage leads to antibody production which may then be targeted against both the adducts and the heat shock protein. It may be that tissues which have either adducts or heat shock protein expressed at high levels are not affected by the antibody response but that the combination is in some way synergistic. The genetic ability to form these two proteins may therefore determine susceptibility to disease.

This theory has interesting parallels in work examining anti-myosin responses in mice with acute myocarditis induced by coxsackievirus infection. Susceptible mice can be induced to develop acute myocarditis by immunising them with myosin (Neu, 1987). Furthermore, there is cross-reactivity between antibodies to certain coxsackievirus epitopes and cardiac myosin (Alvarez, 1987; Beisel , 1990). Thus, the evidence from carefully controlled animal studies suggests that the development of an antibody response to a neo-antigen may be deleterious, due to cross-reactivity with native cellular proteins.

9.7. Summary

Having shown in this work that some patients with alcoholic cardiomyopathy possess circulating antibodies to acetaldehyde adducts of human heart proteins it will now be necessary to demonstrate that these antigens are actually present in the subject's tissues. The pathogenetic relevance of having a positive antibody response will have to be determined in long term prospective studies. In particular it will be necessary to follow patients who stop drinking to see if the antibody response disappears, and perhaps if it reappears if patients begin drinking again. It remains to be seen whether possessing circulating antibodies to acetaldehyde modified human cardiac protein adducts is associated with a poorer outcome or not.

Experiments may also be conducted to demonstrate a pathological function for these antibodies, for example with myocardial cells in tissue culture, as previously (Gery, 1961). In addition it may be that a similar series of experiments to those of Yokoyama et al will be required to confirm the validity of the acetaldehyde hypothesis (Yokoyama, 1993).

The identification of the antigens recognised by the circulating antibodies will allow the development of an ELISA test. This would allow the rapid screening of large numbers of samples, allowing a study of all hospital admissions with heart failure to be performed for example. Whilst the rate of antibody detection in patients with alcoholic cardiomyopathy was low in this study, at around 30%, it is a highly specific test and as such may serve as a marker for detection of alcoholic cardiomyopathy in a population of patients with idiopathic dilated cardiomyopathy.

In addition to these experiments the T cell responses against the antigens recognised by these antibodies could be studied. It has been shown by two-dimensional electrophoresis that there are detectable abnormalities in cardiac proteins in patients with cardiomyopathy (Kovalyov, 1990). Two-dimensional electrophoresis has been used in association with electroelution to provide protein fractions for experiments of T cell responses to mycobacterial antigens (Gulle, 1990). Hence fractionation of human myocardium could be performed and peripheral blood lymphocytes from patients with and without alcoholic cardiomyopathy could be tested for proliferative responses against different fractions. Initial experiments were performed in healthy control subjects but consistent responses were not obtained and further experiments are planned. This approach may also be useful in cardiac transplant recipients, as discussed earlier. Thus a reliable method for obtaining fractions of human myocardium in solution is required, which led to the experiments in Part III.

Part III

Isolation and Identification of Unknown Myocardial Proteins

10. Isolation and Identification of Unknown Myocardial Proteins

10.1. The Problem

In the studies on lymphocyte culture from EMB it was observed that when attempts were made to sub-culture positive cultures they died if the biopsy was not present. Therefore the antigenic stimulus of the EMB itself was felt to be necessary. If the specific proteins responsible for this antigenic stimulation could be isolated, further experiments could be conducted to attempt to establish the cellular components most effective at promoting proliferation of cells cultured from EMB. In addition, in studies on patients with alcoholic cardiomyopathy, the T cell responses against the antigens recognised by circulating anti-acetaldehyde adduct antibodies could be studied if a similar technique for isolating the relevant proteins were available.

Human myocardium could be fractionated and the fractions tested for differential effects on growth of T cells. It would be possible to narrow down the identity of the molecules against which the T cell response is directed in these two groups of patients. As previously discussed, a similar approach has been used by Paque et al in their study of murine coxsackievirus myocarditis (Paque, 1979) and by Gulle et al in their study of T cell responses to mycobacteria (Gulle, 1990).

A given band on an immunoblot may represent a single protein or a number of proteins of similar molecular size. When antibody activity against a protein band is observed on a blot it may thus be directed against one or more proteins. It is very helpful to be able to isolate or purify the protein band in order that the protein identified by antibody binding may be further characterized. If experiments, such as those looking for circulating antibodies to acetaldehyde adducts of human heart, repeatedly identify a protein band of a specific molecular size, or if it is thought that a protein band is particularly significant, then it may be felt appropriate to attempt to identify the protein represented by the band. This may be because the band is recognised by many patient's sera, or because it is common to all blotting results in patients with particular clinical features. Furthermore isolated proteins may be important in looking for antigen specific T cell reactivity as considered in section 1, both to define specificity but also to provide a source of antigen for specific T cell expansion.

The identification of the protein recognised by the patient's antibodies would then be a necessary step in elucidating the nature of the disease process under study. In the case of alcoholic cardiomyopathy the cellular target for antibodies raised against acetaldehyde adducts would be of particular interest. A structural protein or a myocardial enzyme as a target for such antibody activity would be an intriguing result. Therefore the ability to isolate and identify positive bands observed on immunoblots would be extremely useful.

The subsequent isolation of proteins producing a positive band on Western immunoblots is a large technical problem. Identification of the protein may be achieved by N-terminal amino acid sequencing of the band in the solid phase using Edman degradation in automatic peptide sequencers (Choli, 1990). Two main difficulties may arise. As mentioned above, there may be other proteins co-migrating in the region of the positive band, or the N-terminus may be "blocked" by sugar moieties which prevent Edman degradation, the reaction central to microsequencing.

One solution to these problems is to perform limited proteolysis (Cleveland, 1977) producing peptide fragments which are not blocked (since their n-termini are freshly formed). These are then run on a two-dimensional gel, separated by charge and molecular size. The gel is blotted on to a membrane and probed with the relevant serum. A positive spot on this membrane, if produced, is highly likely to be a non-blocked specific peptide whose sequence might be successfully determined. Mass spectroscopy using fast atom bombardment would be an even better, though more expensive, approach for identification of these peptide residues.

Alternatively, if there is an available monoclonal antibody then high performance liquid chromatography (HPLC) may be the answer, using a column to which the antibody has been bound to extract the protein from solution in relatively pure form. This may produce manageable quantities of protein in microgram amounts but requires very large amounts of starting material (Kozuka, 1991). However, sometimes chromatographic separation may not be achievable (Cooperman, 1986; Kamp, 1988).

Therefore, where a positive band is found on a Western blot, there are a number of strategies for attempting to identify the protein producing the reaction. In doing this, a combination of the various approaches outlined above may be most fruitful, rather than adherence solely to one method. In the current investigations, examination of humoral and cellular responses to myocardial proteins was desired. The T-cell response to a range of myocardial protein fractions would be of special interest, in the area of rejection of cardiac transplants and in terms of responses to native myocardial protein in alcoholic cardiomyopathy. Bands detected in immunoblotting experiments may represent proteins whose amino-acid sequences may be capable of stimulating T cell responses as well as antigen production by B cells. In order to identify these proteins and to obtain them in manageable quantities for further experiments a method was required which would allow the isolation of soluble protein fractions containing protein that has been detected by antibody on Western blots.

One strategy would be to study EMB from patients with the relevant myocardial disease (for example acute rejection of cardiac transplants). The aim being to culture T cells from the biopsy and to detect humoral responses against blotted myocardial

protein using serum. Once this was achieved fractions of myocardium would be tested for ability to induce proliferation of T cells (both cultured and PBLs from the subject). This would identify which components of the myocardium were stimulating T cell responses. In parallel, the identification of positive bands detected on Western blotting would be performed by n-terminal amino-acid sequencing.

In order to facilitate this process an electroelution device was designed which would allow the isolation in soluble form of proteins that had been resolved by SDS-PAGE. The method required that the resolution of electrophoresis be largely preserved and that the protein fractions be in manageable quantities. A similar device has been reported by Gulle and co-workers who examined T-cell responses to mycobacterial proteins eluted from two-dimensional electrophoresis gels (Gulle, 1990). To test a device designed for one-dimensional gels, and to confirm the feasibility of the overall strategy, experiments were performed to isolate and partially purify a protein already known to be present in human myocardium, the Endothelin receptor (Davenport, 1991).

10.2. Electroelution Device

The device which will be described was designed for use with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), but it could be used with almost any form of electrophoresis gel. SDS-PAGE is a very widely used method of separating proteins by size. The quantitative recovery of proteins from such gels is an important analytical and preparative technique.

A number of elution devices and methods have been described for recovering protein from gels. These include electroelution into membrane traps from gel slices (Jacobs, 1986), continuous electroelution during the running of gels (Ofverstedt, 1983), and diffusion out of homogenised gel slices (Bernabeu, 1977). However there are important limits to the usefulness of these methods. Continuous electroelution yields large recovery volumes which may then have to be concentrated, diffusion methods apply to smaller proteins (less than 60kDa), and membrane trap devices are often time consuming and require gels to be sliced into pieces. Unless the size of the protein of interest is known, it is difficult to envisage using such devices to purify a number of proteins from a gel for screening purposes.

Gulle et al designed a device, mentioned above, which appears to overcome these problems. This device was reported to electroelute proteins simultaneously and rapidly from two-dimensional gels (Gulle, 1990). It consists of a plate with a square matrix of 480, 4mm diameter wells, which fits between the electrodes of a standard semi-dry blotter. For the experiments in this thesis a variant of this device was designed for one-dimensional gels by myself and Dr A.Persidis (Persidis, 1992).

The device consists of a plate with wells arranged in columns which correspond to the lanes of protein bands on a one dimensional gel which has been run on a BioRad IIXi 16cm gel apparatus. This plate is placed on to the positive electrode of a semi-dry blotter and the wells are filled with buffer. The plate is screwed down on to a polyacrylamide gel block lying on the electrode, which effectively seals the wells. The gel of interest is placed on top of the plate such that the protein lanes lie along the columns of wells. When the top negative electrode is placed on top of the gel to complete assembly of the device, the applied current is normal to the gel surface and forces the proteins to simultaneously migrate out of the gel and into the underlying wells. A multichannel pipette can be used to harvest the well contents. Two tips fit into each well across the columns and tips fit into alternate wells down the columns.

The recovery volume using this device is small (200μl per well), which facilitates subsequent handling of the protein solutions. Elution time is minimal because the proteins have only to migrate across the width of the gel (typically less than 1.5mm), instead of along a gel slice, before they are eluted.

In testing this device ^{14}C labelled molecular weight markers were used to demonstrate elution time course and recovery from an SDS-polyacrylamide gel. In addition β-galactosidase was run on a non-denaturing non-reducing gel and then eluted using the device. The recovered solution was tested for enzymatic activity following elution, to demonstrate that a biologically active enzyme could be obtained using this method.

10.3. Endothelin Receptor Isolation

The requirement to isolate myocardial proteins has been discussed above. Such proteins may be the antigens against which autoantibodies are raised in alcoholic cardiomyopathy, for example. Alternatively they may be proteins which stimulate T cell proliferation in acute rejection of cardiac transplants. The ability to isolate these proteins for further studies would be highly advantageous.

Once the device described above had been built and tested it was used to demonstrate that a protein whose location on a gel was initially unknown, in this case the endothelin receptor, could be isolated. The endothelin receptor was chosen because it is known to be present in human myocardium and because its ligands were easily available in radiolabelled form. In order to isolate the endothelin receptor, a peptide-protein "mobility shift assay" was developed using native polyacrylamide gel electrophoresis. This enabled the isolation of non-denatured receptor proteins from small amounts of human cardiac tissue. This work has been published (Persidis, 1993).

The endothelins are a related group of peptides which includes endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3). The snake venom sarafotoxin S6b is structurally related. All three ET-isoforms bind to cardiac tissue (Davenport, 1991) and have been found to increase cardiac contractility and cause vasoconstriction (Davenport, 1989). Indeed ET-1 is the most potent vasoconstrictor known. However, the nature of the cardiac endothelin(ET) receptor(s) is largely unknown. The study of the human ET receptor would therefore be greatly facilitated by a simple method for receptor isolation from small quantities (milligrams) of cardiac tissue.

Conventional biochemical methods require large amounts (grams) of starting material (Kozuka, 1991), whilst expression of cloned receptors in cell lines requires knowledge of the receptor sequence and produces receptors which have not undergone the same post-translational modifications as native receptors. Other techniques such as ligand blotting used in studies on the acetylcholine receptor (Gershoni, 1983) and on lipoprotein-binding proteins (Graham, 1987) do not permit recovery of solublised receptor in non-denatured form. Al-Hakim and Linhardt (Al-Hakim, 1990) reported the isolation of individual oligosaccharides following transfer from polyacrylamide gels onto a nylon membrane. However this method was not tested on proteins and required an additional step, namely the semi-dry transfer, before recovery could be effected by elution.

10.4. Aim

The aim of these experiments was therefore:

1. To design and test an efficient electroelution device.

2. To use this method to isolate and purify a known myocardial protein in a functional form. To illustrate this a protein known to be present in myocardium (the endothelin receptor) was selected.

11. Endothelin Receptor Isolation: Methods

11.1. Elution Device

The device used in these experiments was designed around a semi-dry blotter with a central plate screwed down onto the bottom or positive electrode of the blotter. The central plate was 6mm thick and had a grid of wells arranged in eight columns of thirty-two wells per column. Each well was shaped as a flattened ellipse which measured 4 x 15mm. There was a gap between the columns vertically of 0.5mm. The gap between the wells in each column horizontally was 0.2mm. The blotter electrodes measured 150 x 150mm. The rows were numbered, 1 to 32 and the columns designated with a letter, A to H, in order to facilitate identification of individual eluted fractions.

To assemble the device (fig.25) the bottom electrode was smeared with 20mm Tris pH 8.0 (elution buffer). Two pieces of Whatman 3MM filter paper (Whatman LabSales Ltd, Dorset,UK), wet in elution buffer and cut to the size of the electrode were placed on it.

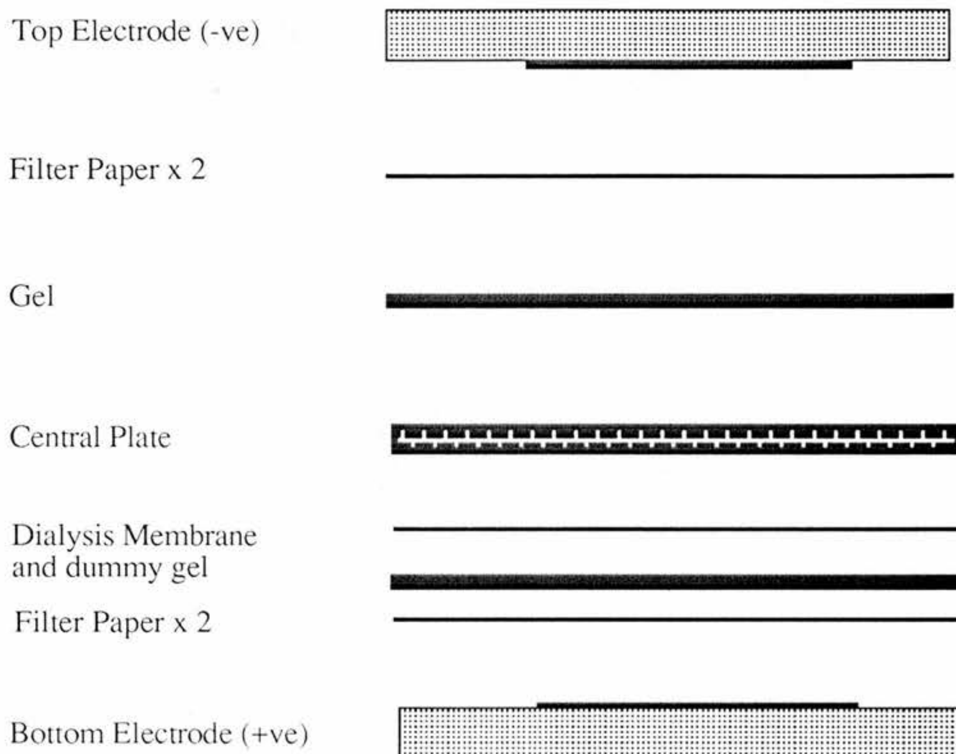
An identical sized 1mm thick polyacrylamide gel block (10%, in elution buffer with no SDS), was placed on the filter paper. An identical sized piece of dialysis membrane (Visking Dialysis tubing, Medicell International, Liverpool), equilibrated in elution buffer, was placed on top of the gel block. The central plate was then screwed down onto the membrane by means of threaded nuts on each side of the grid of wells. The wells were then filled with approximately 200µl per well elution buffer, filled by pouring from a beaker and then wiping off the excess. The gel of interest, previously equilibrated for 10min in elution buffer, was placed on top of the grid. The gel was placed such that each track, identified by the loading well ears, lay above a column of underlying wells. The dimensions of the grid of wells were chosen such that a whole gel prepared using a standard comb with one centimetre wide wells would fit almost perfectly aligned with the underlying wells.

On top of the gel were placed another two sheets of Whatman filter paper soaked in elution buffer. The top electrode was then sealed down onto this sandwich. The device was leak free since the underlying dialysis membrane and gel block effectively prevented cross-contamination between wells. Five to fifteen volts were applied across the electrodes, corresponding to approximately 50-400mA, depending on the area, thickness and density of the gel. After elution the top electrode and gel were removed and the contents of the wells aspirated using a multi-channel pipette (Flow Laboratories, Bucks, UK). The grid of wells was designed so that the tips of this pipette fit into alternate wells when sampled down the column and two tips into

each well when sampled across the columns. The contents of the wells were transferred to microtitre wells prior to scintillation counting.

Figure 25. Electroelution - Arrangement of Gel on Device

This figure is a diagrammatical representation of the assembly of the elution device. It shows the important elements and the order in which they are placed on the device. Each element is labelled on the left hand side and the text explains their function.



11.2. Native PAGE

β-galactosidase (Grade VI, from Escherichia Coli; Sigma Chemical Company Ltd, Dorset, UK) was prepared as a stock solution in 20mM Tris/HCl pH 7.3. Amounts ranging from 1 to 30μg were diluted in equal volumes of double strength native sample buffer (20% glycerol, 0.01% bromophenol blue, 0.1M Tris/HCl pH 7.3). Non-denaturing non-reducing PAGE was performed using 10% resolving gels. These were eluted for 30 minutes at 5V. A track containing 30μg of the enzyme was sliced prior to elution of the remaining gel, fixed and stained (50% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue) and then destained (10%methanol, 10% acetic acid) to locate the protein band so that the appropriate well contents could be assayed. The enzyme substrate (o-nitrophenyl-β-galactopyranoside, Sigma) was prepared at 4mg/ml in sodium phosphate buffer, pH 7.0 containing 10mmKCl, 1mM$MgSO_4$, 50mM 2-mercaptoethanol (assay buffer); 100μl of the substrate was

added to 200μl of the eluted enzyme and incubated at 37^{0}C for 1 hour prior to measurement of the absorbance at 414nm. Controls included the same enzyme amounts prepared in native sample buffer without dye.

11.3. SDS-PAGE

One-dimensional SDS-PAGE was performed as previously described (Laemmli, 1970), with 5% stacking gels and 10% resolving gels. ^{14}C-methylated molecular weight markers (Amersham, Bucks, UK) ranging from 200 to 14.3kDa, were loaded at 10nCi per track. The tracks were sliced and eluted at 5-15V for different time periods after which they were fixed in 50% methanol, 10% acetic acid, amplified (Amplify, Amersham), dried and exposed against Fuji X-Ray film for periods up to 3 days. The marker bands on the autoradiographs were quantified as described (Davenport , 1988), using a Cambridge Instruments Quantimet 970 Image Analyser.

11.4. Extract Production

Human atria (from the explanted hearts of cardiac transplant recipients, stored at -70^{0}C) were homogenised in ice cold buffer (50mM Tris/HCl, 5mM MgCl, 5mM EDTA, 1mM EGTA pH 7.5 supplemented with 250μl/100mls aprotinin). After filtering, washing and centrifugation at 30,000g, extract pellets were suspended in Hepes buffer (50mM Hepes, 5mM MgCl, pH 8.0) and stored at -70^{0}C. Thawed extract was solublised (150 μg per gel lane) with CHAPS in the presence of 1mg/ml BSA as described before (Nakajo, 1989) at 4^{0}C for thirty minutes. After centrifugation the pellet was discarded, the supernatant being the partially purified extract.

11.5. Electrophoresis

The ET and S6b peptides were labelled with ^{125}Iodine (specific activity 2,200 Ci/mmol; Amersham International plc, Amersham, Bucks, UK). Radiolabelled peptides (4 x10^{-10}M final concentration) and a structurally unrelated unlabelled 18-amino-acid peptide (100μg/ml final, kind gift from Dr S. Graham) were incubated with partially purified extract at room temperature for 30 minutes, in the presence or absence of the relevant unlabelled ET or S6b peptides (10 to 1000nM; Novabiochem, Nottingham, UK). The unrelated 18 amino-acid peptide was present to reduce non-specific interactions. The range of concentrations of unlabelled ET/S6b peptides was chosen to include up to a 1000-fold excess over the Kd, sufficient to block the receptor. In control samples extract was excluded from the incubation mixture. The solutions were run on native electrophoresis gels (5% polyacrylamide in TBE buffer: 0.09 M Tris, 0.09 M Boric acid, 10 mM EDTA, pH 8.0) for 4 hours at 25mA. Gels were fixed

(10% methanol, 10% acetic acid), dried and exposed to Fuji RX X-ray film (Genetic Research Instrumentation Ltd., Dunmow, Essex, UK) at -70°C for periods ranging from 16 hours to 7 days.

11.6. Antibody Production

A polyclonal antibody was raised in rabbits against a peptide derived from the predicted C-terminal sequence of the cloned human ET_B receptor (Sakamoto, 1991) (kind gift of Dr A.P.Davenport, Clinical Pharmacology Unit, Addenbrookes Hospital, Cambridge). The sequence chosen does not show sequence similarity with any other known peptide sequence on the Swiss-Prot database. An ELISA test (performed in the Clinical Pharmacology Unit, Addenbrookes Hospital, Cambridge, by Dr C.Plumpton) using the peptide antigen to which the antibody was raised showed the ET_B antibody to be sensitive and specific.

11.7. Electroelution

For electroelution of putative ^{125}I-ET/receptor complexes from native gels the method described above was used. The method has also been published previously (Persidis, 1992). The elution buffer (20 mM Tris/HCl, pH 8.0) used also contained 0.4% CHAPS. To dissociate the ET-1/receptor complex it was incubated in high salt (0.5M NaCl) for 30 minutes.

After dissociation the solution was subjected to four cycles of ultrafiltration by centrifugation through Centricon-10 ultrafiltration devices (Amicon Ltd., Stonehouse, Glos., UK) according to the manufacturer's instructions. These devices are small containers which contain a filter or membrane with a known molecular weight cut-off. They are designed for use in a centrifuge. On spinning the tubes, solutions placed in them can be filtered. The resulting solution, which has not passed through the filter is called the retentate. The solution which does pass through is the filtrate. It depends on the nature of the experiment whether the retentate or the filtrate or both are of interest.

In these experiments the membrane cut-off was 10kDa and hence it was the retentate, which did not pass through the membrane, which contained the putative endothelin receptors. This was designated receptor eluate (RE). No radioactivity was detected in this retentate, demonstrating that effective dissociation had occurred, the radiolabelled ET-1 having passed through the Centricon-10 membrane. Control eluate (CE) was obtained by elution from an irrelevant portion of the gel, producing a solution of concentration 32.8μg/ml. Estimations of protein concentration of atrial membrane preparation, enriched receptor preparation and control eluate were performed using the

BioRad protein assay kit according to the manufacturers instructions (BioRad UK, Hemel Hemspstead, Herts, UK).

11.8. Immunobinding

The retentates from the RE and CE preparations were applied to nitrocellulose filters (Schleiser and Schuell, Anderman & Co. Ltd, Kingston, Surrey), along with elution buffer (EB), which were then blocked with 5% dried milk (Marvel) in Tris buffered saline (TBS: 50 mM Tris/HCl, pH 7.4, 200 mM NaCl). Antisera (1:100) were added in blocking buffer with 0.1% Tween-20, the secondary antibody (1:500) was alkaline phosphatase conjugated goat anti-rabbit immunoglobulins (DAKO, High Wycombe, Bucks, UK) with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, SIGMA) as substrate.

11.9. Saturation Analysis

Fixed quantities of atrial extract (75μg) were solublised and incubated with ^{125}I-labelled ET-1 in concentrations ranging from 0.1 to 3nM as described above. Extracts were run on native gels and autoradiography was carried out as described above, with the addition of a calibrated radioactive scale to the cassette. Densitometry was performed using a Quantimet 970 image analyser (Cambridge Instruments, Cambridge, UK). Details relating to quantitative autoradiography have been described previously (Davenport, 1988). The specific band was measured using a sample box placed around each band. Values obtained fell within the linear range for quantitative radiography and the film was not saturated at the highest concentration of ET-1. Curves of optical density against final concentration were plotted and the equilibrium constant for ET-1 binding to the endothelin receptor in this system was determined using an iterative curve fitting program (Munson, 1980).

12. Results: Endothelin Receptor Isolation

12.1. Electroelution

The device described was used to demonstrate that a range of cardiac proteins could be electro-eluted. Various experiments were performed in testing the device. Only a few of the resulting gels are shown.

Figure 26. Human Right Ventricle - Before and After Elution

This figure shows the result of an experiment where human right ventricle was homogenised mechanically in SDS-PAGE sample buffer and then run on 10% SDS-PAGE. The gel was sliced at the left hand side. The right hand portion of the gel was eluted using the device and the left hand portion was not. After elution was finished both the portions of gel were stained with Coomassie blue. The lanes of the central plate from the device can be seen where protein has not been eluted. Some protein has remained on the gel in the middle of a lane. This is ascribed to air bubbles, an early problem with these experiments before their influence was abolished by more careful preparation.

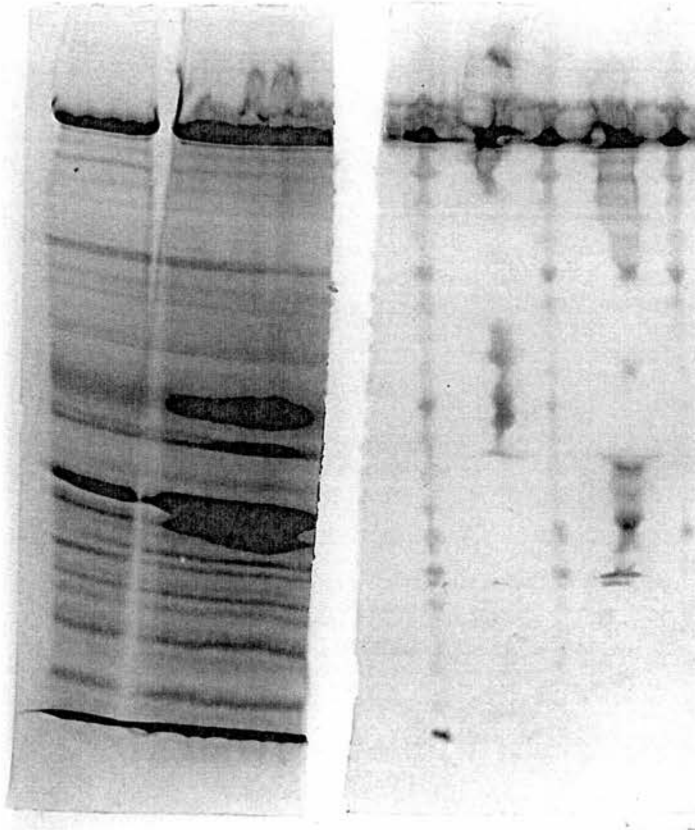


Figure 27. Eluted Human Right Ventricular Microsomal Fraction. *This figure shows two identical gels of human right ventricular microsomal fraction, prepared in an identical fashion to human left ventricular microsomal fraction as detailed in chapter 7. The fractions were treated with nothing, acetaldehyde alone, cyanoborohydride alone or both acetaldehyde and cyanoborohydride, producing samples 1 to 4, as detailed before. The preparations were run in two sets of four lanes on a 6% SDS-PAGE gel and then eluted using the device. The non-eluted gel, on the left, was prepared with a set of molecular weight standards for comparison (lane at far left). It can be seen that cleaner elution results have now been obtained than with the early elution in fig.26. Also note that the 200kDa band is eluted to a lesser degree than the other proteins on the gel. This is discussed in more detail later.*

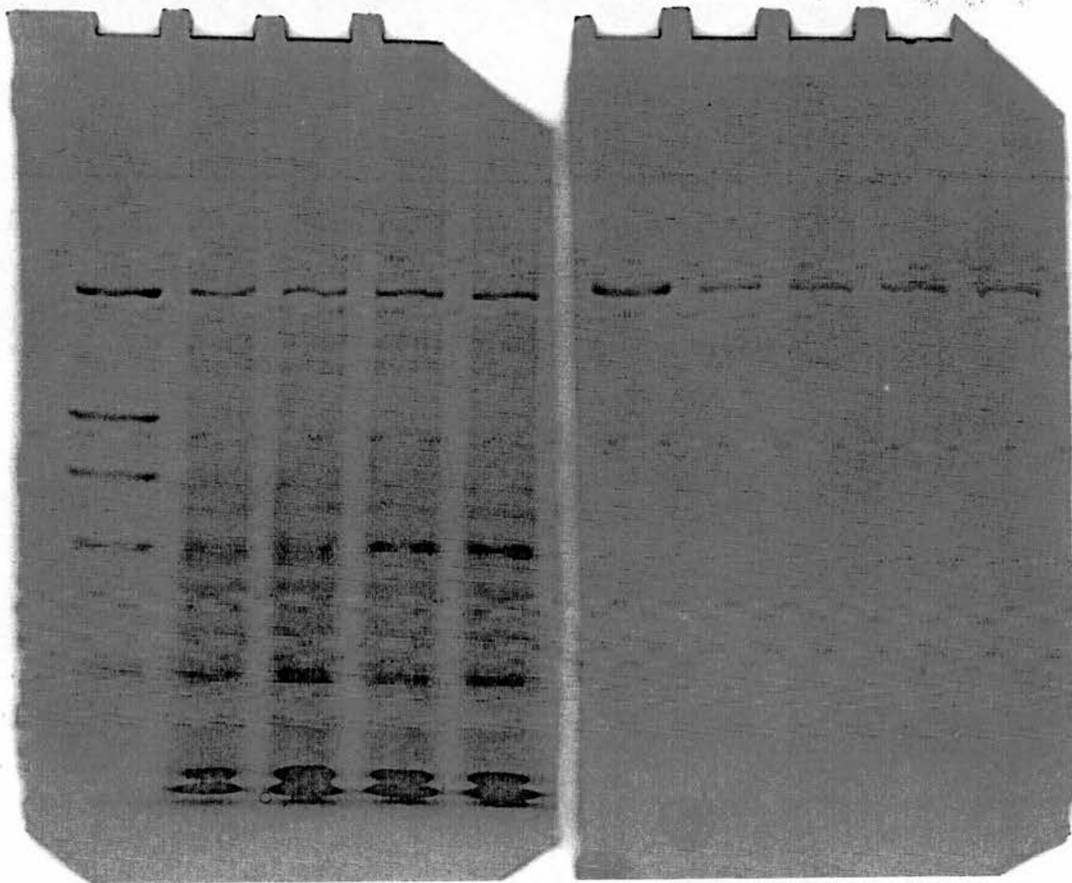
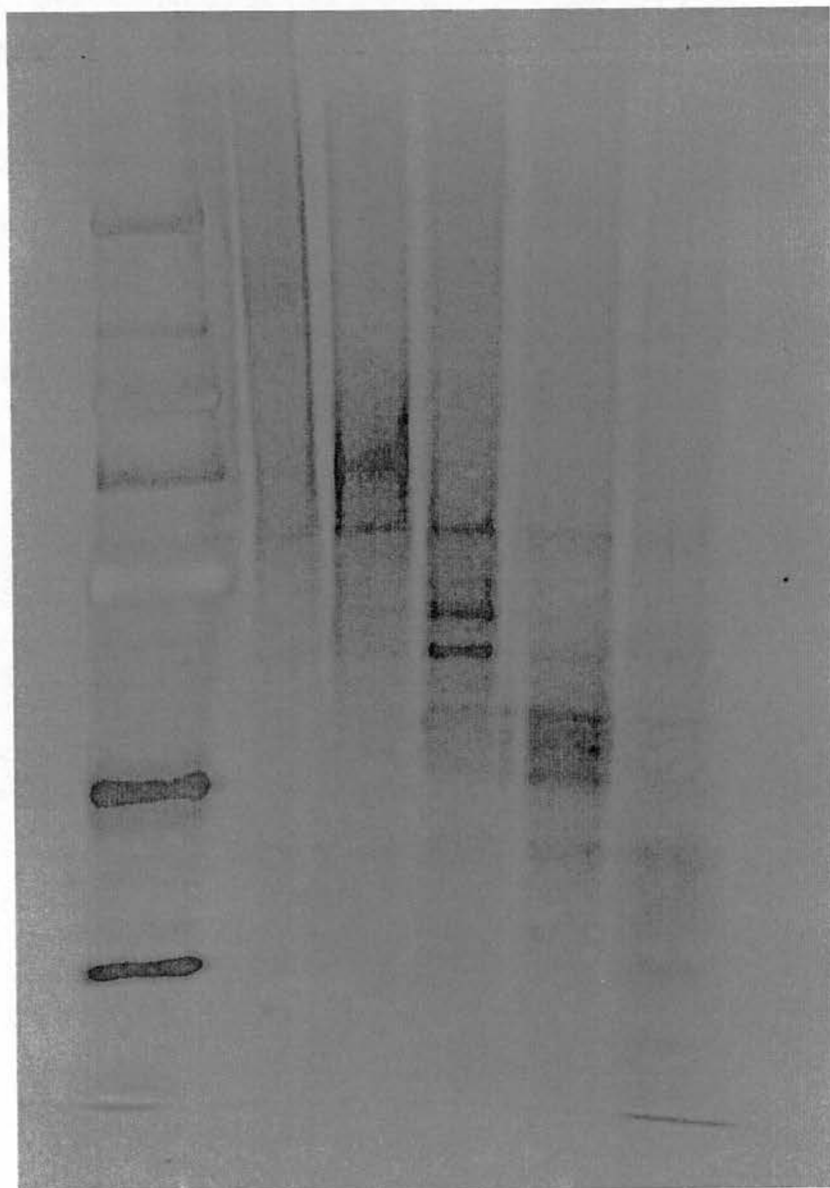


Figure 28. Silver Stain of Selected Eluted Fractions.

This is a photograph of a silver stained 10% SDS-PAGE gel. The gel was produced by taking different fractions eluted from the gel in fig. 27 and running them on another 10% SDS-PAGE gel. The concentration of protein in the individual wells does not allow Coomassie staining to be effective, suggesting that sub-microgram amounts are present. It can be seen that preferential elution of different fractions has been achieved. However, also visible are bands of some proteins which have cross contaminated the wells. The fractions selected were from wells numbered 1, 5, 10, 15 and 20. Molecular weight standards are seen in the lane on the far left.

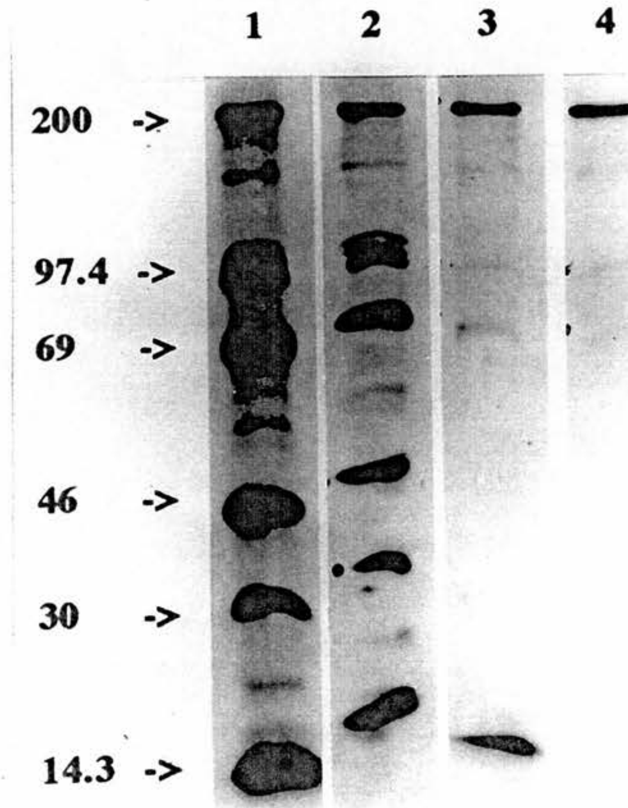


12.2. Elution of Radiolabelled Proteins

In order to gain some insight into the performance of the device with regard to the length of time different proteins would take to be eluted, radiolabelled proteins were resolved on 1mm thick gels and eluted for various time periods as shown below (fig. 29).

Figure 29. Elution Time Course - Radiolabelled Markers.

This is a photograph of an autoradiogram of eluted and non-eluted ^{14}C-labelled molecular weight marker proteins. The electrophoresis is described in the methods section. The applied voltage for elution was 5V, current 40mA. The molecular size in kDa is given at the left hand edge of the picture. The lanes are numbered 1 to 4 representing time of elution: 0, 15, 30 and 60 minutes respectively. At the end of each time point, an aliquot from each of the wells underlying a gel strip was measured for radioactivity, while the corresponding gel strip was removed from the device and processed for autoradiography. It can be seen that within 15min the intensity of some bands has diminished and by 30min extensive elution has occurred.



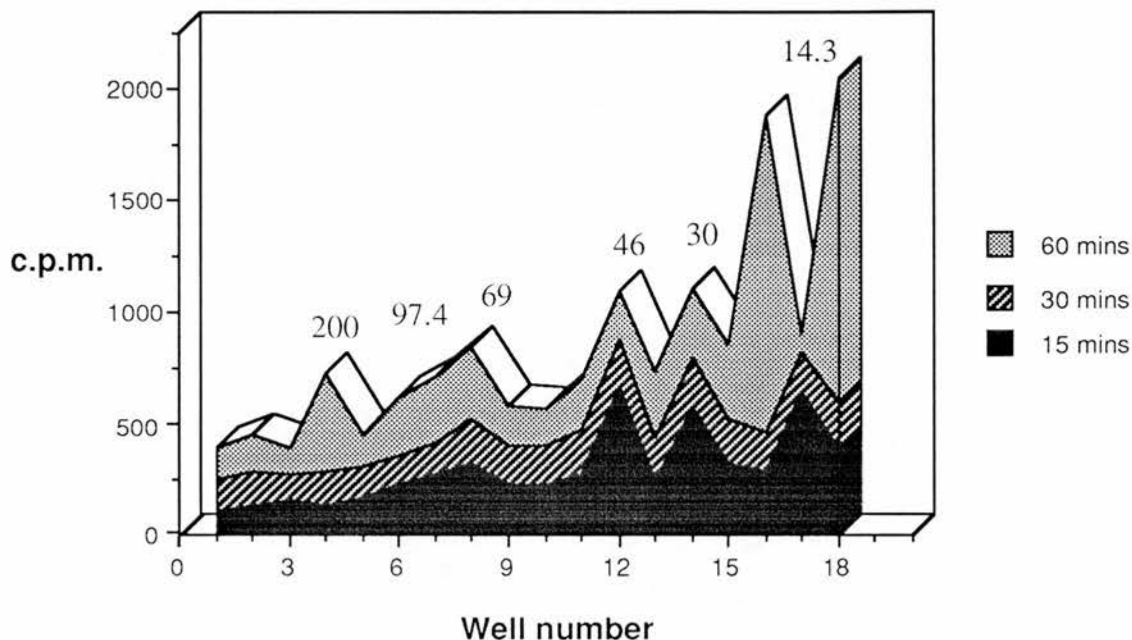
The time course of elution is illustrated in more detail (table 23), which shows the results of densitometric measurements made on serial autoradiograms of the six marker bands, at different time points. The table shows additional data to those represented in the photograph in figure 29.

Table 23. Elution Time Course of Radiolabelled Proteins
This table shows the measurements of optical density made on autoradiograms of the gel strips produced in the time course experiment using radiolabelled markers. The first column shows the actual optical density measurement but the other columns show a percentage of the measurement at time zero. It can be seen that after 15min the 97.4kDa protein was eluted by about 40% whereas none of the other proteins were eluted to any significant degree. By 30min elution was complete for the 30kDa protein and by 90% or better for the 46, 69 and 97.4 kDa proteins. The 200kDa protein took 120min to be eluted by 80%, this was reduced to 60min when elution was carried out at 15V. Increasing the voltage also caused the other proteins to be eluted by 90% or better within 15min.

^{14}C Markers (kDa)	Non-eluted OD_{abs}	Eluted markers ($\%OD_{abs}$ remaining on autoradiogram)		
(Gel Lane:)	0 min (1)	15 min (2)	30 min (3)	60 min (4)
Myosin (200)	0.655	100	95	93
Phosphorylase b (97.4)	0.711	62	11	10
Bovine serum albumin (69)	0.787	95	14	8
Ovalbumin (46)	0.818	0	6	0
Carbonic Anhydrase (30)	0.847	86	0	0
Lysozyme (14.3)	0.811	100	59	5

To confirm that the proteins had indeed been collected in the wells, 10μl aliquots were taken and the amount of radioactivity in each well was plotted against well number for the different time periods of elution. This is shown as an area graph in fig.30.

Figure 30. Area Graph of Protein Recovery after Elution
An aliquot was taken from each well (numbered 1 to 18) under each gel strip following elution for the time periods shown. Radioactivity for each well number, measured by liquid scintillation counting, is plotted for each of three time periods. The peaks correspond to protein bands eluted from the gel and are labelled accordingly (14.3 to 200 kDa, see fig.y). There are two peaks for the 14.3 kDa band at the 60min time period because the gel may have changed size due to absorbance of buffer solution. Radioactivity may therefore be collected from more than one well. (c.p.m.=counts per minute minus background count).



The autoradiograph of the non-eluted proteins (lane 1 fig.29) shows bands that directly correlate with the well numbers showing high counts, as shown in fig.30. Peaks are clearly identifiable, demonstrating the resolution of the wells. The peaks usually straddle one or more wells and the background count is also significant. However, examination of lane 1 in fig.29 shows that there are more bands than the six proteins of the cocktail, this being due to impurities and protein breakdown upon boiling in sample buffer. However, all bands are simultaneously eluted giving rise to a high background of radioactivity in all wells and reducing the sharpness of the peaks corresponding to the actual marker proteins. No radioactivity was detected on the dialysis membrane after elution and aspiration of the well contents, indicating that losses due to adsorption onto the membrane were minimal.

12.3. Elution of Active Enzyme

As additional evidence of the existence in the well eluate of the protein run on the gel, β-galactosidase was run on a native non-denaturing non-reducing gel (details in methods) and was subsequently eluted at 5V (40mA) for 45 min. The enzymatic activity of an aliquot of the eluate from the corresponding well was compared to that of an appropriate non-eluted standard. The standard (30μg) gave an OD_{414} of 0.482 and the eluate (30μg loaded on to the gel) OD_{414} was 0.505, indicating that active enzyme was obtained after elution.

In another experiment, 0.8mcg of β-galactosidase was run on a non-denaturing non-reducing gel and samples taken from around the row where the band was to determine the scatter of protein which had diffused sideways (table 24). Wells from further away from the target well did have detectable β-galactosidase according to the OD_{414} values but this did not usually extend beyond three wells from the target well.

Table 24. OD_{414} of Samples from Wells Around the Target Well of Eluted β-galactosidase

β-galactosidase was run on a non-denaturing gel in a concentration of 0.8mcg. The well directly overlying the band was tested for enzymatic activity as were the wells on either side. The results are shown both as the obtained OD value and as a percentage of the value obtained in the target well. From the table it can be seen that perhaps half or more of the electrophoresed protein diffuses into adjacent wells.

Well	OD_{414}	OD_{414} as % of Target Well OD
Above	0.247	47
Target Well	0.521	100
Below	0.251	48

12.4. Mobility Shift Assays of Endothelin Receptor and Peptides

These experiments involved running extracts of human atrium on SDS-PAGE after incubation with radiolabelled ligands for endothelin receptors. The gels were dried down and autoradiograms produced after exposure of the gels to X-Ray film for various time periods. The autoradiograms produced usually consisted of lanes where no extract was added as a control to show what pattern the radiolabelled ligand produced simply running in the gel alone. There were also various lanes where un-labelled (or "cold") ligand, corresponding to the radiolabelled-ligand in use, was added in increasing amounts to compete out the binding of the radiolabelled-ligand to the endothelin receptor. This competition by cold ligand is suggestive of a specific interaction.

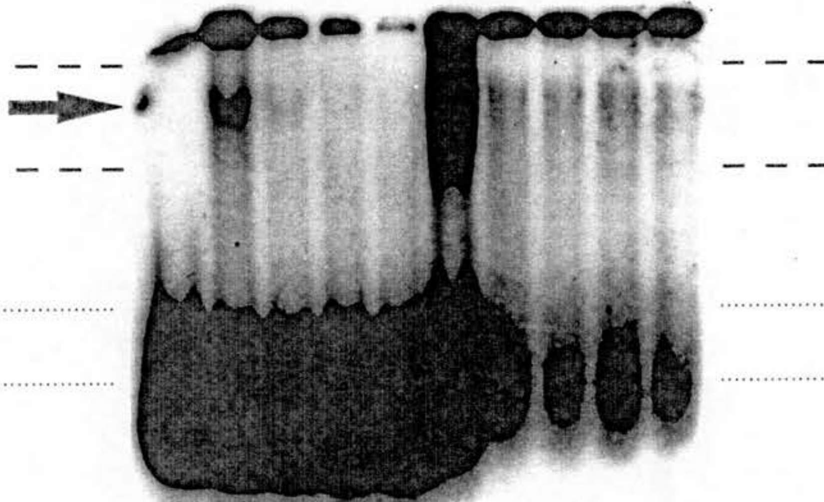
This pattern of lanes is followed for most of the autoradiograms shown below. Figures 31a to 31d show mobility shift assays with ET-1,-2,-3 and S6b. The autoradiograms show that radiolabelled ET-1, ET-2 and S6b all migrated to similar positions on different gels, but ET-3 was less mobile. Addition of membrane extract caused a retardation of the mobility of each radiolabelled peptide, suggesting a peptide-receptor interaction (Fig.31a-d,arrows). The addition of increasing quantities of the corresponding unlabelled peptide resulted in a reduction in intensity of this band and its eventual abolition (Fig.31a-d).

Membrane extract was incubated with ^{125}I-labelled ET-1, ET-2, ET-3 and S6b in the presence or absence of 1000nM unlabelled peptide and run on the same non-denaturing gel for comparison. ET-1 and ET-2 behaved in a similar manner, with the ET-receptor complex band appearing in the same position on the gel for each. The complex band appeared in a similar position with S6b, although the interaction was weaker and to visualise this band gels required to be exposed to X-ray film for longer.

Figure 31a. Mobility Shift Assay with ET1

The autoradiogram was produced in a mobility shift assay using human atrial extract and ET1, both radiolabelled and unlabelled. The lanes represent the different substances added to the incubation mix, as listed below. The origin of the gel is at the top of the figure. Insoluble material which complexes with the radiolabelled ligand is seen at the top of the gel. In this case, this supposedly non-specific interaction is reduced by addition of cold competitor. Radiolabelled ET-1 is retarded in the presence of extract to a position representing the ET-1/receptor complex (arrow). Competition with unlabelled ET-1 eventually abolishes this specific band. The regions between the dashed and dotted lines were selectively electroeluted to provide receptor and control material respectively (see Fig. 35). The presence or absence of extract is indicated by the plus or minus signs (respectively) in the first row under the photograph. The concentration of cold ET1 in the incubation mixture is given in nanomoles (nM) in the second row. The effect of addition of a reducing agent (2-mercapto-ethanol, right half of figure) is shown and the presence or absence of this substance is indicated in the third row with a plus or minus sign. The reducing agent alters the migration of the ligand alone such that it extends from the origin into the gel as well as occupying its' previous position in the gel. There appear to be bands in the area of the specific complex band but these are little affected by increasing amounts of cold ET1 and are not interpretable.

a



Extract	−	+	+	+	+	−	+	+	+	+
ET-1 (nM)	0	0	10	100	1000	0	0	10	100	1000
BME	−	−	−	−	−	+	+	+	+	+

Figure 31b. Mobility Shift Assay with ET2

In this photograph the autoradiogram was produced by mobility shift experiments using ET-2 as the radiolabelled ligand. All other aspects of the experiment were the same as for fig.31a. Again insoluble material marks the origin of the gel at the top of the picture. The arrow indicates the position of a specific complex band, which is eventually abolished with increasing unlabelled ET2. The addition of the reducing agent produces non-specific bands which are uninterpretable.

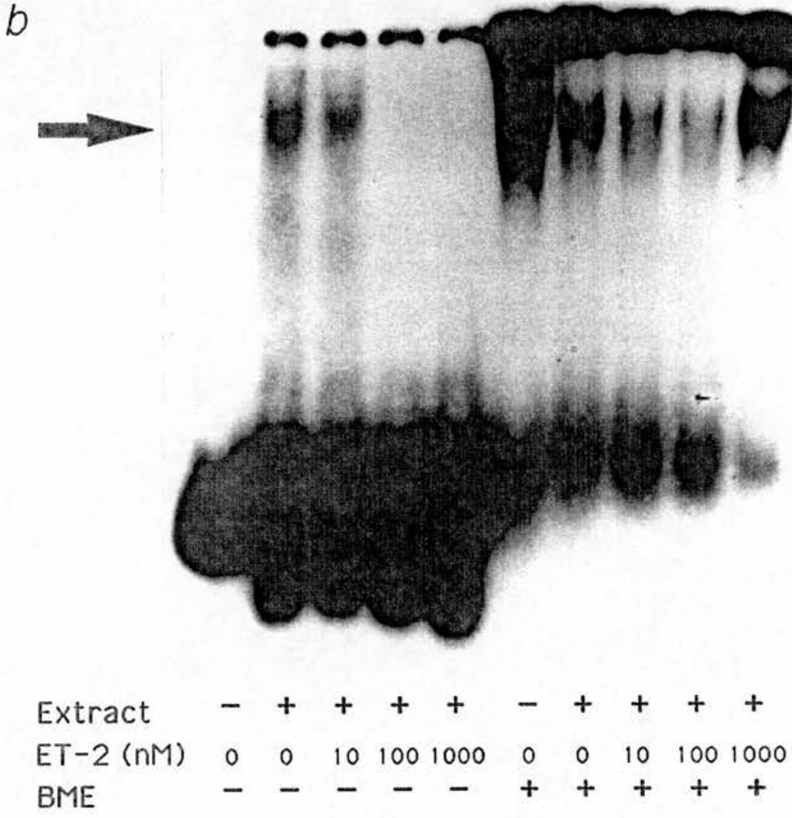
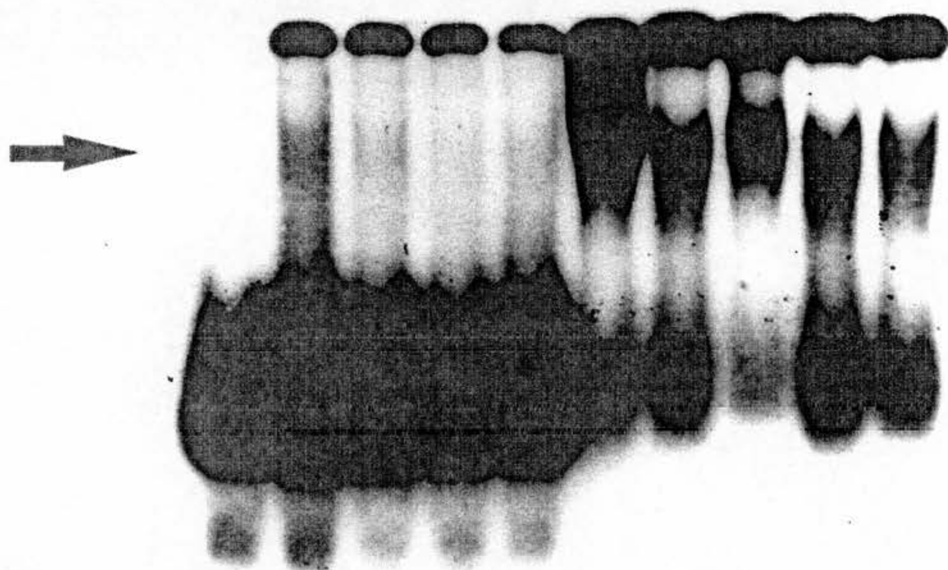


Figure 31c. Mobility Shift Assay with ET3

In this figure an autoradiogram produced by mobility shift assay with ET3 is shown. The experiment was identical in all important respects to that represented in fig.31a.The ET3 ligand does not migrate so far into the gel. Insoluble material in association with radiolabelled ligand is seen at the origin. The arrow indicates the position of the specific complex band, which is eventually abolished with increasing respective unlabelled ET3. Note that the insoluble materials' interaction with labelled ET3 is virtually unaffected by addition of cold ET3. The addition of reducing agent again produces non-specific bands.

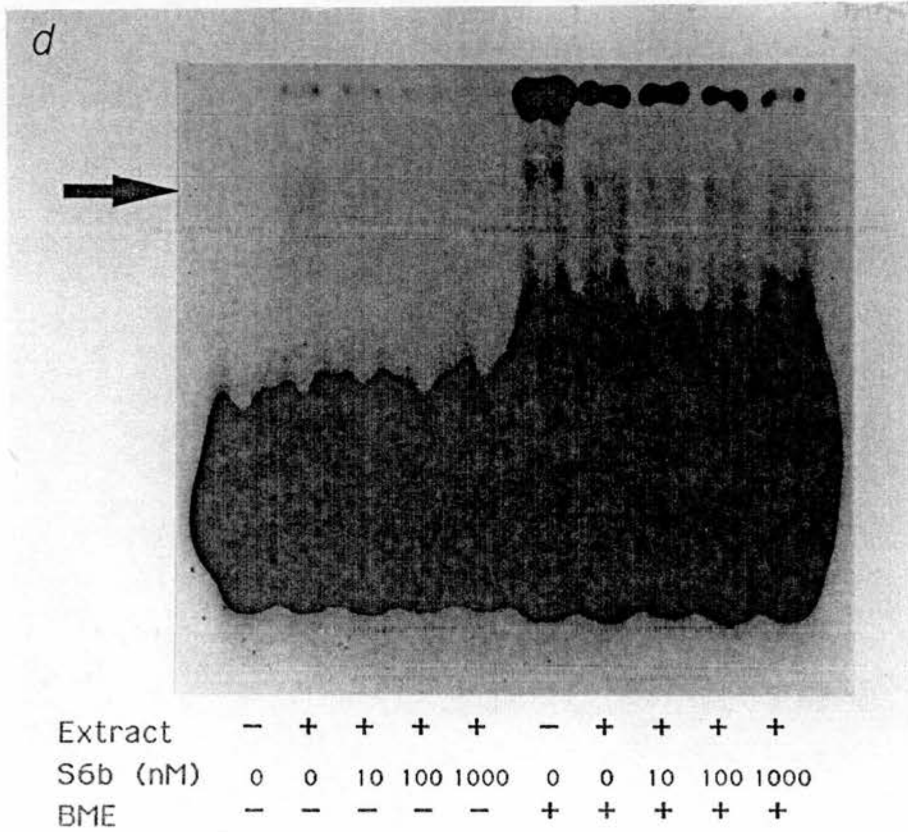
C



Extract	−	+	+	+	+	−	+	+	+	+
ET-3 (nM)	0	0	10	100	1000	0	0	10	100	1000
BME	−	−	−	−	−	+	+	+	+	+

Figure 31d. Mobility Shift Assay with S6b

In this figure an autoradiogram produced by mobility shift assay with S6b is shown. The experiment was identical in all important respects to that represented in fig. 31a. The arrow indicates the position of the specific complex band, which is much fainter in this autoradiogram. The autoradiogram was exposed for four days in order to show the band at all, illustrating the weaker interaction that S6b has with the endothelin receptor. The specific band is quickly abolished by the addition of unlabelled S6b. The addition of the reducing agent makes interpretation difficult, as before.

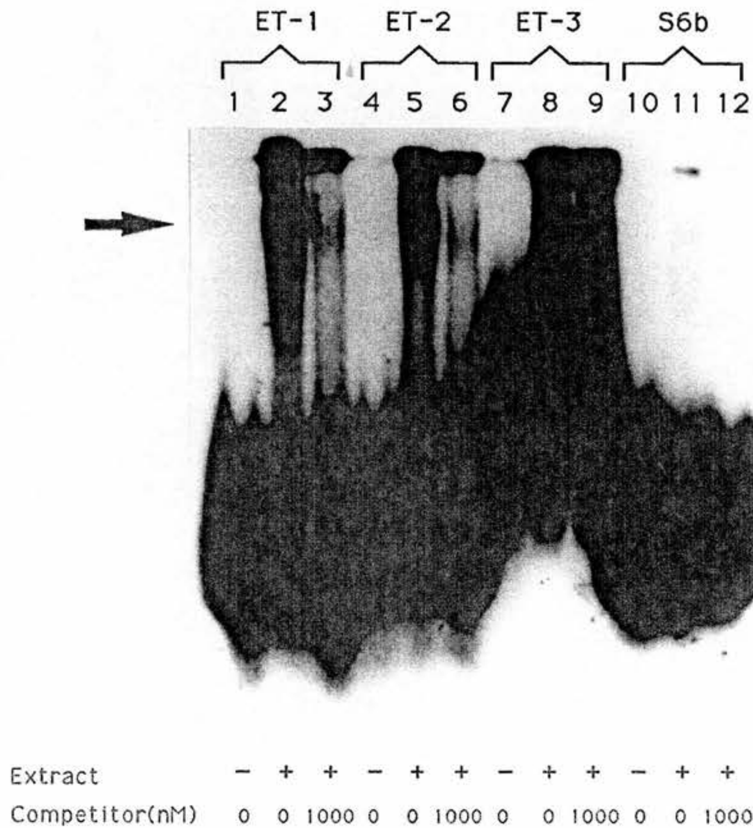


Extract	−	+	+	+	+	−	+	+	+	+
S6b (nM)	0	0	10	100	1000	0	0	10	100	1000
BME	−	−	−	−	−	+	+	+	+	+

The above figures represent the results of individual experiments using the ^{125}I-lablelled ET-1, 2 and 3 and S6b ligands. In order to demonstrate the relationship between these ligands and to show the interactions together for comparison, an experiment using all four ligands together on the same gel was performed (fig. 32, below).

Figure 32. Mobility shift of ET-1, ET-2, ET-3 and S6b

The autoradiogram shows all four peptides incubated in the presence or absence of extract and in the presence or absence of 1000nM unlabelled peptide run on the same gel. Lanes 1,4,7 and 10 are labelled ET-1,-2,-3 and S6b alone respectively; lanes 2,5,8 and 11 are the respective labelled peptides with partially purified extract and lanes 3,6,9 and 12 are the relevant labelled peptides with extract and the corresponding unlabelled peptide in excess. The appearances of the other peptides on this gel are different from those shown in fig.31 because the gel here was exposed for much longer with the X-Ray film in order to try to show the complex band for S6b.



12.5. Saturation Analysis

To determine the binding characteristics of the putative receptor, increasing amounts of labelled ET-1 were incubated with a fixed quantity of extract in mobility shift assays (fig.33). Specific binding was saturable with an equilibrium dissociation constant (K_d) of 2nM calculated by an iterative curve fitting program (Munson, 1980). Since the protein concentration in individual bands was not known, B_{max} could not be calculated.

Figure 33. Saturation Binding of ^{125}I-ET1 to Human Atrium
This experiment was conducted in a similar fashion to that illustrated in fig.31a. The autoradiogram shown in the photograph has six lanes which are each the equivalent of lane 2 in fig31a (and fig.32) except that increasing concentrations of labelled ET1 were added to the incubation mixture. A serial increase in density and eventual saturation of the specific complex band occurs. Human atrial extract (75µg/lane) was incubated with increasing concentrations of ET-1 from 0.1 to 3nM (lanes 1-6) prior to electrophoresis as for fig.31a. Optical density of the complex band was measured using a Cambridge Instruments Quantimet 970 image analyser on autoradiograms exposed for periods from three to ten days. Radioactive standards were exposed simultaneously for calibration purposes.

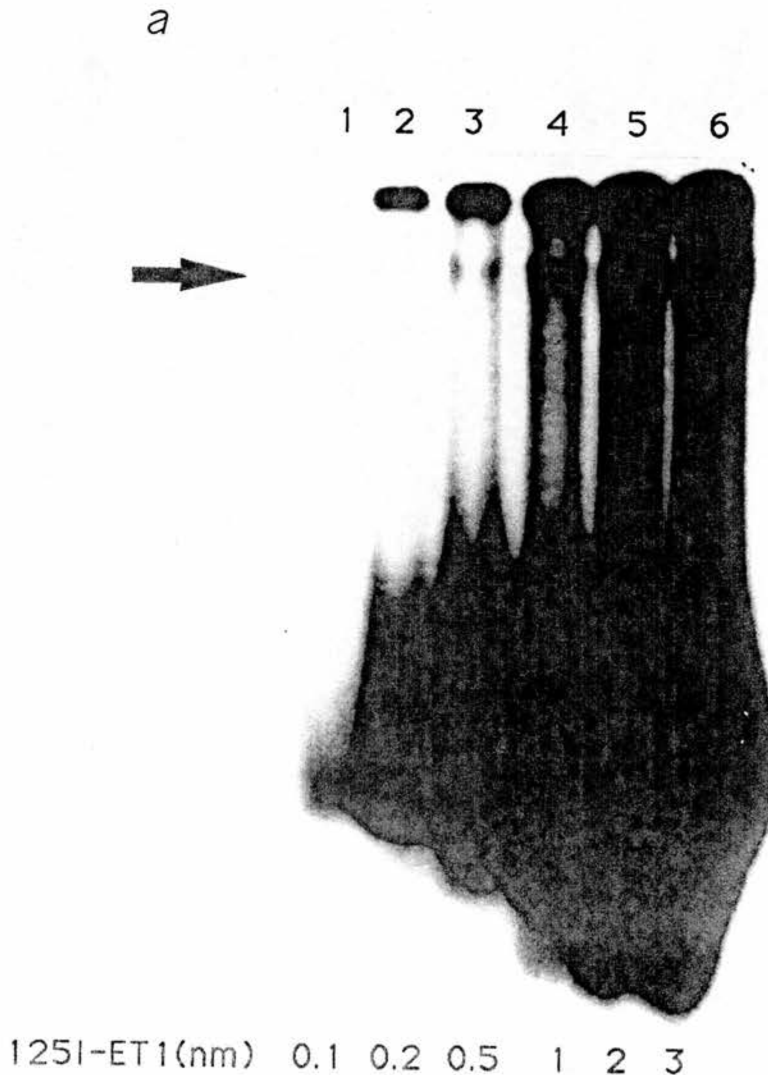
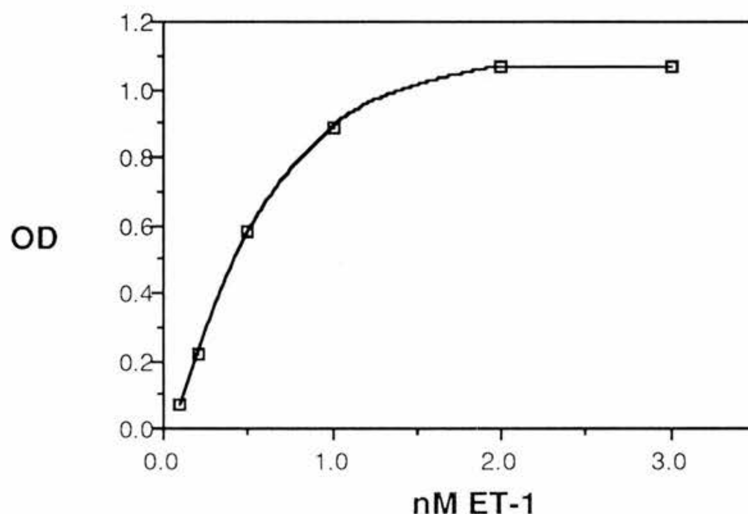


Figure 34. Graph of Saturation Binding

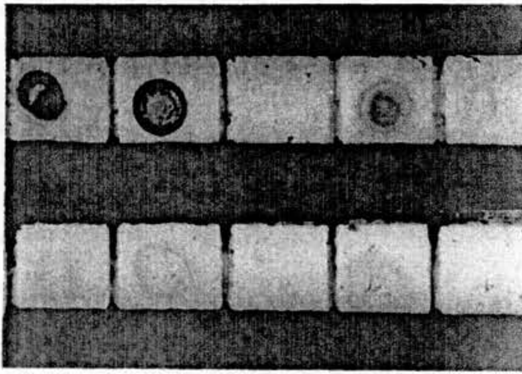
This graph is a plot of absolute optical density (OD) of the specific complex band, shown in fig.33, against concentration of ^{125}I-labelled ET-1. Data shown are single measurements from an experiment typical of the results obtained. A rectangular hyperbola is produced suggesting a one-to-one receptor-ligand correspondence. The equilibrium dissociation constant (Kd) was calculated as 2 nM using an iterative curve fitting program. Note that the calculated value is markedly different to that which would be ascertained from direct observation of the curve. This reflects the well recognised difficulties of obtaining these figures using graphical methods.



12.6. Receptor Elution

Receptor-enriched material was prepared by electroelution of the complex band. The components of the complexes were dissociated and the receptor fraction separated and concentrated. The radiolabelled peptide, being of low molecular weight, passes through the membrane chosen whereas the larger receptor protein is retained. Antibody raised against the ET_B receptor was used to identify the eluted, dissociated and concentrated receptor preparation. Dot blots of the concentrated receptor eluate probed with the antibody revealed the presence of immunoreactive protein (fig.35). The initial amount of starting material was 3mg and from this approximately 30μg of receptor-enriched eluate was obtained.

Figure 35. Dot-immunobinding of electroeluted ET receptor
This photograph shows blots produced after dot-immunobinding analysis of electroeluted and dissociated ET receptor with anti-ET_B receptor antibody. Receptor eluate (RE) was obtained from between the dashed lines and control eluate (CE) from between the dotted lines on the gel in Fig.31a by electroelution as described under methods. Both immune and pre-immune sera were tested as shown, with RE, CE and also elution buffer (EB). Only immune sera gave a positive result with RE material but not with CE or EB. The three RE preparations shown were each made in separate experiments.



13. Endothelin Receptor Isolation - Discussion

These studies demonstrated that efficient electroelution of a range of proteins was possible. Experiments confirmed that the use of non-reducing gels would allow the recovery of enzymes which retained their function. In addition a specific functional myocardial protein was eluted and shown to be present in the eluate by dot immunobinding assays.

13.1. Electroelution

The device described is effective at eluting a range of proteins initially resolved by SDS-PAGE. Most proteins can be eluted rapidly from a gel, but higher molecular weight proteins take longer. This might be predicted from first principles and is also the case in Western blotting. The time course experiment demonstrated that there is differential elution of proteins but that recovery of proteins from the gel is quantitative. Therefore the ability of the device to function as planned was confirmed.

This device is not confined to use with proteins alone however and the range of substances that could be eluted is large. Among the other macromolecules that can be electroeluted are nucleic acids (Symons, 1988) and oligosaccharides (Al-Hakim, 1990). Importantly, biologically active protein can be recovered, as demonstrated for β-galactosidase.

One of the potential problems encountered with electroelution following SDS-PAGE is the presence of SDS and denatured protein in the eluate. It is possible to remove SDS efficiently, together with low protein losses, by ice-cold acetone precipitation (Hager, 1990). During renaturation the presence of detergents can improve yields of active proteins (Tandon, 1986; Tandon, 1988) although renaturation must be adapted to each protein in question (Schultes, 1990). In these experiments this was not addressed.

13.2. Endothelin Receptor Isolation

In the experiments described above, radiolabelled endothelin-1 and related peptides were used to identify and isolate endothelin receptors from partially purified membrane extracts of human atrial tissue. Binding analysis using radiolabelled endothelin-1 gave a K_d of 2nM, similar to results from binding experiments conducted directly on tissue. Peptide-receptor complexes were electroeluted from native gels and dissociated. Receptor material was characterised by dot-immunobinding analysis of eluates using an antibody raised against a predicted human endothelin receptor sequence. This method therefore allows simple isolation of peptide receptors from small quantities of human material. The technique could be applied to receptors which have not been cloned. It could be applied to any protein for which a suitable ligand,

including an antibody, is available. All that is required is a means of locating the protein on the gel and it can be eluted on a preparative scale, limited only by the loading characteristics of the SDS PAGE gel used. In the experiments described it was possible to obtain milligram quantities of total eluate from 20 wells of crude extract.

The figures show that the use of native gels leads to more diffuse banding patterns on the autoradiograms than are usually seen on gels run under denaturing conditions, but specific bands are clearly identifiable. In these experiments, labelled peptide was retarded in the presence of extract, suggesting the formation of a peptide/receptor complex, for each of the four peptides studied. These experiments were repeated with partially purified extract from several patients to be sure that the findings were not peculiar to any one subject.

Differences in primary structure among the ET peptides were reflected in their different electrophoretic mobilities when compared on the same gel (Fig.32). In particular ET-3 was less mobile than the other peptides in these gels. This was not unexpected since the amino-acid sequence for ET-3 is significantly different from the other ET peptides (Inoue, 1989). However, variations in mobility might also result from the labelling of each peptide with ^{125}Iodine.

Saturation binding experiments suggested the presence of a specific ET-receptor in the position of the band arrowed in Figures 1 and 2. The K_d of 2nM obtained in these experiments compares favourably with a Kd of 0.787nM calculated by Dr AP Davenport and Dr P Molenaar (Clinical Pharmacology Unit, Addenbrookes Hospital, Cambridge) from conventional saturation binding experiments using ^{125}I-labelled ET-1 and adult human right atrium. It also compares with 0.64 nM, the concentration of unlabelled ET-1 required to reduce the binding of labelled ET-1 by 50%, obtained from radioligand competition binding studies on human fetal right atrium (Wharton, 1991). In another study, the concentration required to produce 50% of the maximal positive inotropic action of ET-1 on human right atrium was 2 nM (Davenport, 1989). Equilibrium data obtained in this study are therefore comparable with data obtained by saturation and competition studies and are closely similar to those estimated from physiological responses.

Immunoblotting also confirmed the presence of ET receptors in the complex band. Therefore this technique provides a simple and quick way of isolating receptors. The quantities of receptor isolated would enable the raising of antibodies, the partial sequencing of the protein and potentially X-ray crystallography. However, since the receptors are not present in pure form, further purification procedures may be required prior to other studies.

These studies demonstrate that gel electrophoresis followed by electroelution can be used to obtain target proteins from human myocardium. These may be receptors

or functional enzymes. Where more information is available, for example the relative molecular mass, such experiments would be more straightforward. Therefore, where a particular myocardial protein is targeted for study in the future, this approach could aid in its isolation and identification. When dealing with a band on an immunoblot for example, it would not be difficult to elute the equivalent protein from a preparative gel in order to facilitate further studies.

One potential problem involves the diffusion of target proteins into adjacent wells on the elutor. This may not be a problem where only a few proteins, well separated by SDS-PAGE, are present on the gel. However, in human myocardium there is a large number of proteins which all lie very closely. A number of solutions to this problem are possible. A larger gel could be run, allowing the dye front to migrate further and producing better resolution. A gradient gel could be employed or alternatively two-dimensional electrophoresis, again to better separate individual proteins. A technical solution to the problem would be to produce an elution plate with a bonded membrane rather than a free dialysis membrane cut to size. This would have the advantage of being watertight and would allow no leakage of the contents of the wells and less cross-over from one well to another. Presumably this would not completely eliminate diffusion since some of the sideways movement may be taking place within the gel itself if the electric field is not uniformly applied.

13.3. Protein Isolation, Alcoholic Cardiomyopathy and Acute Rejection of Cardiac Transplants

The relationship between the above studies and those concerning acute rejection of human cardiac transplants and the presence of circulating antibodies against acetaldehdye adducts in alcoholic cardiomyopathy lies in the ability to separate manageable quantities of protein. In order to identify the components of human myocardium against which T cell responses are directed in rejection it is necessary to have some means of isolating the relative proteins. Similarly B cell responses, in the form of circulating antibodies in patients with alcoholic cardiomyopathy, are directed against proteins whose isolation is of crucial importance in defining the role of antibody responses in this disease. Separation by molecular size is an obvious choice for initial resolution and the use of electroelution could readily provide protein fractions for testing with subjects T cells, whether they be cultured from EMB or from peripheral blood.

The use of electroelution could therefore be applied to either situation. The study of patients with alcoholic cardiomyopathy would be greatly aided by the ability to rapidly isolate proteins recognised by antibodies in patients sera. It is not known what components of the myocardium are dysfunctional in this condition and the ability to

identify proteins targeted by antibody responses would be a significant advance. It is assumed that since systolic dysfunction is a key clinical feature of this condition that structural proteins involved in myocardial contractility are damaged by alcohol, or its metabolites. However, it may be possible to show, using these methods, that a myocardial cytosolic enzyme or other protein, is the main target of the damage caused by alcohol, and that this damage occurs indirectly.

Hence, in either area of study, the ability to isolate proteins, known or unknown, from protein mixtures resolved on SDS-PAGE, would be of great assistance in further studies.

14. Final Conclusions and The Future

These studies demonstrated efficient culture of T lymphocytes from EMB from cardiac transplant recipients. The ability to culture lymphocytes relates to clinically significant rejection when medium with IL-2 alone, or IL-2 supplemented with PHA, was used, but not medium supplemented with anti-CD3. It was not possible to predict subsequent rejection. More cultures were obtained in the presence of 2 HLA DR mis-matches compared with one or no mis-matches. Positive cultures would most easily be obtained from EMB with acute rejection where 2 HLA DR mis-matches between donor and recipient were present. Biopsies should be cultured in Il-2 alone. PHA supplements could be used to produce larger cell numbers due to its polyclonal activating effect, whilst retaining a correlation with rejection. The ability to culture T cells from EMB more efficiently might be enhanced if specific myocardial proteins involved in stimulating proliferation could be isolated. The electroelution studies demonstrated that efficient electroelution of a range of proteins was possible. The recovery of functionally intact enzymes was shown and the ET_BR was successfully eluted. This suggests that this approach may be feasible and there are previous studies which have employed similar methods.

The study of patients with alcoholic cardiomyopathy demonstrated circulating antibodies that recognise acetaldehyde-modified normal human cardiac protein in 4 of 14 (28%) patients. Antibodies were mainly IgG, but IgA and IgM antibodies were also found. The antigens recognised by these antibodies were in the range 58-120 kDa. All the appropriate control populations were negative for these antibodies and thus this is not a sensitive but a highly specific test for alcoholic cardiomyopathy. The mechanism by which the myocardium might be damaged in this disorder is not clear but possibilities include antibody mediated damage or soluble factors secreted by activated T cells. The antigens against which the immune response is directed are not known. Identification of these using fractionated human myocardium may be possible in a similar way to that proposed for the study of lymphocyte culture from EMB.

The use of electroelution could therefore be applied to either situation. The study of patients with alcoholic cardiomyopathy would be greatly aided by the ability to rapidly isolate proteins recognised by antibodies in patients sera. It is not known what components of the myocardium are dysfunctional in this condition and the ability to identify proteins targeted by antibody responses would be a significant advance. It is assumed that since systolic dysfunction is a key clinical feature of this condition that structural proteins involved in myocardial contractility are damaged by alcohol, or its metabolites. However, it may be possible to show, using these methods, that a

myocardial cytosolic enzyme or other protein, is the main target of the damage caused by alcohol, and that this damage occurs indirectly.

Hence, in either area of study, the ability to isolate proteins, known or unknown, from protein mixtures resolved on SDS-PAGE, would be of great assistance in further studies.

14.1. Endomyocardial Biopsy

The initial experiments re-iterated previous findings, namely that it is possible to obtain proliferating lymphocytes from small biopsy fragments of the transplanted heart. It was not found that the ability to grow lymphocytes correlates very closely with cellular rejection in contrast with previous studies. This technique therefore has more to offer in terms of providing cells with which to perform functional studies than for diagnostic purposes. In the future such a technique will prove highly useful in the study of inflammatory conditions of the heart such as acute myocarditis. In addition cultured lymphocytes obtained in this way could be tested for their proliferative response when a target protein for cellular rejection is identified, which may help to predict patients at high risk of developing rejection.

14.2. Alcoholic Cardiomyopathy

These studies are the first reported which are able to propose a mechanism whereby alcoholic cardiomyopathy may be produced. They are based on similar studies of alcoholic liver disease but produced different findings. In alcoholic cardiomyopathy there is no single protein antigen detected but a pattern peculiar to each patient. This will make it harder to elucidate the nature of the processes involved.

In the future these studies will be extended to include a prospective study, which is in fact now in progress.

The identification of the antigens involved is crucial and this will be done using two-dimensional electrophoresis and n-terminal amino-acid sequencing, with the help of staff at the Microchemical Facility, AFRC Institute for Animal Physiology, Babraham, Cambridge. These studies are also in progress.

Part of the study now underway is directed at identifying the presence of the putative antigens in the tissues of subjects with alcoholic cardiomyopathy. This is possible using endomyocardial biopsy tissue from the subjects which is then treated in a similar fashion to the stock heart used in the above experiments. It is then blotted and the blots reacted with both the subjects serum and a polyclonal antibody to acetaldehyde adducts, provided by Dr JG Kenna, St Mary's Hospital, London. The polyclonal antibody is more sensitive than the patients serum and so far we have been able to

clearly demonstrate the presence of acetaldehyde adducts in the tissues of patients with both alcoholic liver disease and alcoholic cardiomyopathy.

The prospect of being able to explain the mechanism by which this particular non-ischaemic heart disease is produced is very enticing. However, it is important to state that so far all that has been shown is the presence of a circulating antibody. In only a few conditions, such as myasthenia gravis for example, are the antibodies truly functional. Many previous studies have demonstrated such antibodies which are then shown to have no pathological function.

These studies raise the prospect of an adjunctive treatment for use in these patients, particularly where they prove unable to give up alcohol. There are agents which can reduce the levels of circulating acetaldehyde which might reduce the production of tissue adducts and therefore abrogate the immune response. For example, human blood acetaldehyde concentrations can be lowered in vitro by doses of pantethine (Watanabe, 1985). Furthermore, a study in rats showed protection against lethal doses of acetaldehyde by combinations of thiamin, L-cysteine and L-2-methylthiazolidine-4-carboxylic acid (Sprince, 1974). These studies suggest that if the acetaldehyde adduct theory is correct there may well be clinical benefits to be gained by appropriate treatment.

14.3. Protein Isolation

This study showed that it is possible to isolate and partially purify a target protein from the myocardium without knowledge of its molecular size, provided a known ligand is available. The technique would be even more useful where this critical information was available. Hence, when a particular band on immunoblotting is to be identified this will be greatly aided by the ability to selectively elute relatively large quantities. In the study of antibody responses to acetaldehyde adducts this will be of great value in helping to isolate the protein targets involved.

In the experiments shown the endothelin type B receptor was obtained. This work has been published and may allow partially purified receptor preparations to be obtained in a simple series of experiments.

14.4. Summary

This thesis has shown that polyclonal stimulation offers little in the study of lymphocyte culture from EMB, that prediction of subsequent rejection is not feasible and that ELI are related to acute rejection. In addition the first assay of peripheral blood which is able to distinguish alcoholic cardiomyopathy from idiopathic cardiomyopathy has been developed, though its sensitivity is low it is very specific. Both these areas of

study may be furthered through the use of fractionated myocardial proteins and a technique to allow this has been developed.

The ability to detect, isolate and purify proteins of interest will be of great assistance in the further studies which are planned. It has therefore provided the opportunity to develop appropriate technical skills and knowledge to investigate cardiac disease in the future. As such it represents the work necessary to begin to answer questions about cardiac disease, rather than providing those answers in itself.

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Addendum

Alcoholic Cardiomyopathy: Diagnostic Criteria

The diagnosis of alcoholic cardiomyopathy (or alcoholic heart muscle disease) is one of exclusion. Therefore, for accurate research studies and precise interpretation of data, certain conditions must be satisfied before the diagnosis can be accepted. The features of a dilated cardiomyopathy must be present and patients must have been drinking a defined amount of alcohol for a sufficient length of time. The period of time taken as sufficient to accept a diagnosis of alcoholic cardiomyopathy has varied. Teragaki et al selected ten years (Teragaki, 1993), but most other authors have used five years (for example: Levi, 1977 and Cerqueira, 1991). To confirm the diagnosis, cardiac catheter studies must rule out significant coronary artery disease and endomyocardial biopsy is necessary to exclude other forms of heart muscle disease.

In this study, patients described as having alcoholic cardiomyopathy all had impaired left ventricular function, as defined by moderate or severely impaired left ventricular function on left ventricular angiography, 2D-echocardiography and/or on nuclear imaging (MUGA). At least two imaging modalities were used in each patient. Consumption of alcohol in these patients exceeded 80g per day (as in Richardson, 1986) for a five year period (or >250kg lifetime intake). All patients with alcoholic cardiomyopathy underwent coronary angiography and no significant coronary artery disease was detected (that is, no lesions causing greater than 50% reduction in luminal diameter in an epicardial artery). In addition, no regional wall motion abnormalities consistent with previous myocardial infarction were observed. All fourteen patients also underwent endomyocardial biopsy, with at least three biopsy fragments being obtained for histological examination.

Alcohol consumption, detailed in the methods section, was typical of that exhibited by patients at the time of hospital admission. However, information was not available on precisely when (or if) consumption ceased in these patients. From the records that were available, it appears that patients were drinking alcohol to within two weeks of invasive studies, including biopsy, being performed. The features observed at histology were non-specific, being those consistent with dilated cardiomyopathy. Typical findings were of myocyte hypertrophy, some fibrosis and occasional inflammatory cells.

The patients in this study were therefore well characterised according to accepted criteria. Other forms of heart disease were excluded by endomyocardial biopsy and ischaemic heart disease was ruled out. The specificity of the findings is therefore not in doubt.