ENDOMYOCARDIAL BIOPSY DIAGNOSIS OF ACUTE CARDIAC ALLOGRAFT REJECTION

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TO

BELINDA

DECLARATION

I, JAMES BARRIE HUNT, hereby declare that the work on which this thesis is based is original, (except where acknowledgements indicate otherwise), and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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CONTENTS

1. INTRODUCTION

Subsequent to the first clinical human cardiac transplantation which was performed by Christiaan Barnard in December 1967 at Groote Schuur Hospital, Cape Town, South Africa,[1] cardiac transplantation has now become an accepted therapeutic modality for terminal cardiac failure. Between December 1967 and November 1990 at Groote Schuur Hospital, 216 heart and heart-lung transplants have been performed. Table 1 lists the types of operations performed

TABLE 1:

PROCEDURE	NUMBER
ORTHOTOPIC HEART TRANSPLANTS	117
*HETEROTOPIC HEART TRANSPLANTS	69
RETRANSPLANTS	13
**HEART-LUNG TRANSPLANTS	17
TOTAL	216

*Includes two Xenogenic-transplants
**Includes three Retransplants

Dramatic increases in patient survival rates have occurred, particularly in the last decade due to the introduction of cyclosporine-based immunosuppression regimens, (at Groote Schuur from 1983 onwards), improved donor - recipient selection and more sophisticated rejection monitoring, with 12-month actuarial survival rates after heart transplantation of 94% being achieved. Despite these improvements, infection and graft rejection remain the most important problems limiting survival, and since the treatment of rejection requires an intensification of immunosuppressant therapy which predisposes the patient to opportunistic infections, the two factors are inter-linked.[2]

The prompt and accurate diagnosis of acute allograft rejection is essential in the post-operative period to avoid immunological damage to the graft, and cannot await the development of clinical signs.

2. OBJECTIVES OF THE STUDY

The aims of the present investigation are fourfold:

(i) to review the range of non-invasive methods that may be used to diagnose acute cardiac allograft rejection;
(ii) to review the use of the bioptome in sampling the donor heart endomyocardium;
(iii) to review the light microscopic and histological grading of acute cardiac rejection;
(iv) to characterise the mononuclear populations in endomyocardial biopsy samples and correlate the findings with the light microscopic appearances of the same biopsy specimens.

2.1. NON-INVASIVE MEANS OF DIAGNOSING ACUTE CARDIAC ALLOGRAFT REJECTION

Although there are many non-invasive methods being employed and tested for the diagnosis and surveillance of acute rejection, some of which are listed in Table 2.1, percutaneous transvenous endomyocardial biopsy (EMB) remains the most reliable method.[3, 4]

TABLE 2.1: NON-INVASIVE ADJUNCTS TO EMB IN DIAGNOSIS AND MONITORING OF ACUTE CARDIAC ALLOGRAFT REJECTION

1. ELECTROCARDIOGRAPHIC VOLTAGE CHANGES
2. RADIOLOGICAL MONITORING OF HEART SIZE
3. RADIONUCLIDE VENTRICULOGRAPHY
4. MYOCARDIAL IMAGING AGENTS: THALLIUM-201, TECHNETIUM-99M, GALLIUM-67, INDIUM-111 ANTIMYOSIN (FAB)
5. MAGNETIC RESONANCE IMAGING
6. CYTO-IMMUNOLOGICAL MONITORING
7. DOPPLER ECHOCARDIOGRAPHY
8. BIOCHEMICAL PARAMETERS: SERUM ALPHA 1- ANTITRYPSIN, ALPHA 2-MACROGLOBULIN, C- REACTIVE PROTEIN, C3, C4; URINARY NEOPTERIN, POLYAMINES.

A reduction in the electro-cardiographic voltage was used together with EMB in the 'pre-cyclosporine' era to screen for allograft rejection; however, with the introduction of cyclosporine as a primary immunosuppressant agent this close correlation no longer exists and the

electro-cardiogram appears to be an unreliable predictor of acute rejection. [5] Assessment of parameters such as cardio-thoracic ratio and cardiac volumes on plain radiographs show some correlation to acute rejection but are not sensitive enough to replace EMB. [6]

Radionuclide ventriculography was found to be predictive of a positive biopsy when changes in parameters of left ventricular volumes were measured, particularly reductions in stroke volume and end-diastolic volume.[7, 8] Studies utilising Doppler electrocardiography have shown that acute cardiac rejection is accompanied by alteration in left ventricular filling dynamics, as measured by isovolumetric relaxation times.

Various biochemical parameters such as alpha 1-antitrypsin, alpha 2-macroglobulin, C-reactive protein, C3, C4, neopterin and polyamines are metabolic by-products of lymphocyte activation. Their measurement in serum and/or urine has been investigated as a means to diagnose rejection. However their potential use is limited to screening procedures due to the inability to discriminate between infection and rejection states. [11,12,13] Cyto-immunologic monitoring (CIM) which combines morphological quantitation of activated lymphocytes and immunoblasts with immuno-phenotyping of peripheral blood has been shown to be neither sensitive nor specific enough to replace EMB in screening for acute rejection. [14] However, Garner *et al*[15], have shown that the application of flow cytometry to peripheral blood lymphocytes to develop an immune monitoring profile, may be useful in distinguishing between infection and rejection. Using similar applications Hammer *et al,*[16] have demonstrated that CIM has a sensitivity of 85% and a specificity of 90% for the diagnosis of acute rejection and can be used to provide information on viral or bacterial infections.

Of the techniques utilising myocardial imaging agents, a study of thallium -201, technetium -99m and gallium -67 showed that all three of these agents had unacceptably poor sensitivity (0 - 30%), [17] whereas in a separate study with indium -III antimyosin (FAB) imaging, sensitivity and specificity were both 80% and this technique may be of value in the identification of rejection.[18] Recently, magnetic resonance imaging has been employed to measure myocardial thickness and this was increased significantly in biopsy-confirmed rejection episodes in 8 patients.[19]

In summary, no single non-invasive test has been validated to replace EMB for the diagnosis of acute cardiac allograft rejection and, in addition, surveillance of acute rejection to guide immunosuppressant therapy still relies upon repetitive serial EMB. In a situation where repeated EMB is not feasible such as transplantation in neonates, a technique which measures left ventricular function, for example Doppler echocardiography, may be the only acceptable suitable method of diagnosing and monitoring acute allograft rejection.[20]

2.2 OBTAINING AN ENDOMYOCARDIAL BIOPSY USING THE CARDIAC BIOPTOME

The histological assessment of acute rejection requires biopsy material from the endomyocardium. The histopathological features of acute rejection in myocardial biopsies are the same as those seen in donor hearts examined at autopsy or when surgically removed.[21]

The non-surgical techniques to obtain cardiac biopsy material such as needle biopsy via a trans-thoracic approach were developed experimentally in the 1960's and subsequently applied to humans.[22, 23] The morbidity and mortality associated with this procedure was unacceptably high for routine clinical usage, but using a flexible cardiac catheter and a Konno-Sakakibara bioptome, a transvenous approach was shown to be safer and more dependable in obtaining biopsy material from the endocardium and myocardium.[24]

Caves,[25] in Stanford developed a new cardiac biopsy forceps and utilising an internal jugular transvenous approach this method was 100% successful in obtaining biopsy material from 19 patients during 85 biopsy procedures, without significant clinical complications. More importantly, this method could be performed with minimal patient discomfort under local anaesthetic on an out-patient basis. Thus, by the early 1970's a safe technique and instrument for serial biopsy of the transplanted heart was available and experience of this method was reported on in 1974 by the Stanford group.[26] Their results showed that serial percutaneous transvenous endomyocardial biopsy, (EMB), provided an objective index of cardiac allograft rejection and improved their ability to manage acute rejection episodes. In addition EMB enabled an accurate histological grading of the severity of acute rejection to be performed and an important observation was that the histological changes of acute rejection preceded clinical signs, and electrocardiographic changes by at least 2 days, allowing for earlier treatment of rejection episodes.

Certain modifications of the technique and instruments of the EMB have evolved. As an alternative to the internal jugular vein, venous access may be achieved via cannulation of the subclavian or femoral veins, and various bioptomes are in use (e.g. size 7 French, size 9 French, FBIC Olympus and Olympus 19 C). At our institution, (Groote Schuur Hospital), the preferred approach is the percutaneous supraclavicular route to the left or right subclavian vein and an Olympus 19 C bioptome with fenestrated forceps is used to obtain biopsy specimens.

Problems and Pitfalls of Endomyocardial Biopsy

Although technically safe and generally well tolerated by patients, serial EMB is not the perfect 'gold standard' for the diagnosis and surveillance of acute cardiac allograft rejection and certain limitations are associated with this procedure. (Table 2.2)

TABLE 2.2:	LIMITATIONS OF ENDOMYOCARDIAL BIOPSY
1.	INVASIVE NATURE OF THE PROCEDURE
2.	SAMPLING ERROR
3.	SAMPLING OF A PREVIOUS BIOPSY SITE
4.	BIOPSY INDUCED ARTEFACT

It is an invasive, uncomfortable investigation for the patient and is associated with a small but significant morbidity. In the Stanford

experience of over 10 000 endomyocardial biopsies there have been no deaths and the morbidity has been less than 0.3%.[4] Complications are usually confined to the venous site and include localised infections, pneumothorax and rarely, reversible nerve pareses related to the infiltration of the local anaesthetic. Occasionally the tricuspid valve or branches of the epicardial coronary arteries may be biopsied.

Therapeutic decisions regarding immunosuppressant therapy are made based on the pathologist's histopathological assessment of rejection in the tiny biopsy samples submitted. Do these samples taken from the apex of the right ventricle adequately represent the changes occurring in the entire myocardium? A study at our institution[27] addressed this question by comparing "biopsy" samples taken with a bioptome from formalin-fixed explanted human donor hearts with standard histological sections taken from the same hearts. Using a scoring system to grade severity of acute rejection, agreement of results between bioptome samples and routine sections was found in 86% of cases and rejection involved both ventricles equally. More important was the fact that in 285 biopsy samples, only two false-negative results were obtained. Zerbe *et al*[28] using autopsy acquired cardiac allograft specimens confirmed that for diagnostic purposes, rejection is evenly distributed throughout the right ventricular endomyocardium. In addition, by applying statistical models to different diagnostic approaches (myocyte injury and inflammatory pattern), they showed that no significant increase in sensitivity or specificity occurs beyond 3 fragments per biopsy based on the pattern of inflammation, although incremental increases in sensitivity

occurred with increasing the numbers of fragments per biopsy (up to 6) when utilising myocyte injury alone.[28]

Fenoglio *et al* [29] have stated that a minimum of three samples should be submitted for monitoring transplant rejection, whilst others[30] consider that adequate sampling requires at least 5 biopsy fragments for examination. At our institution between 3 and 4 fragments are obtained at each biopsy procedure and an additional 1 or 2 fragments are submitted separately in liquid nitrogen for frozen section and determination of lymphocyte subsets. If less than 3 fragments are received the pathologist should express his doubts as to the adequacy of the sampling.

The endomyocardial bioptome randomly samples a limited portion of the right ventricle, usually the apex or septum. However the bioptome tends to follow the trabecular pattern of the right ventricle to a similar anatomical position with each pass and sampling of a previous biopsy site is common with repeated procedures and may approach 16%.[28] An infiltrate of mononuclear cells may be present together with myocyte necrosis which can mimic acute rejection; however the presence of organising thrombus on the endocardial surface should alert the pathologist to the possibility of a previous biopsy site. (Figure 1) The progressive sequential changes which occur have been described by Rose[3], based on experimental work performed on baboons and include haemorrhage and myocyte necrosis followed by a mononuclear cellular infiltrate with eventual collagen deposition and the formation of a fibrous scar.

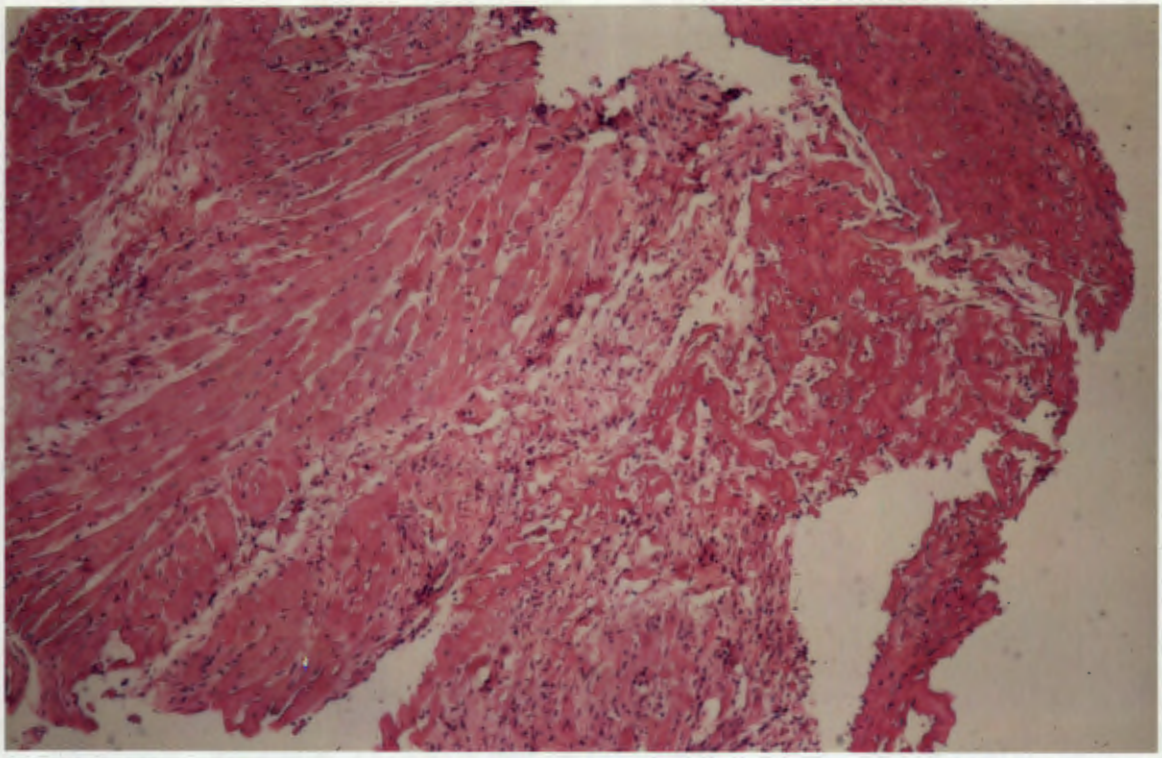


Figure 1: Previous biopsy site. Organising fibrin thrombus (top right) with deeper granulation tissue fills the endocardial defect. Scanty lymphocytes are present beneath the thrombus, but the myocardium is devoid of inflammatory cells (haematoxylin and eosin, x 200).

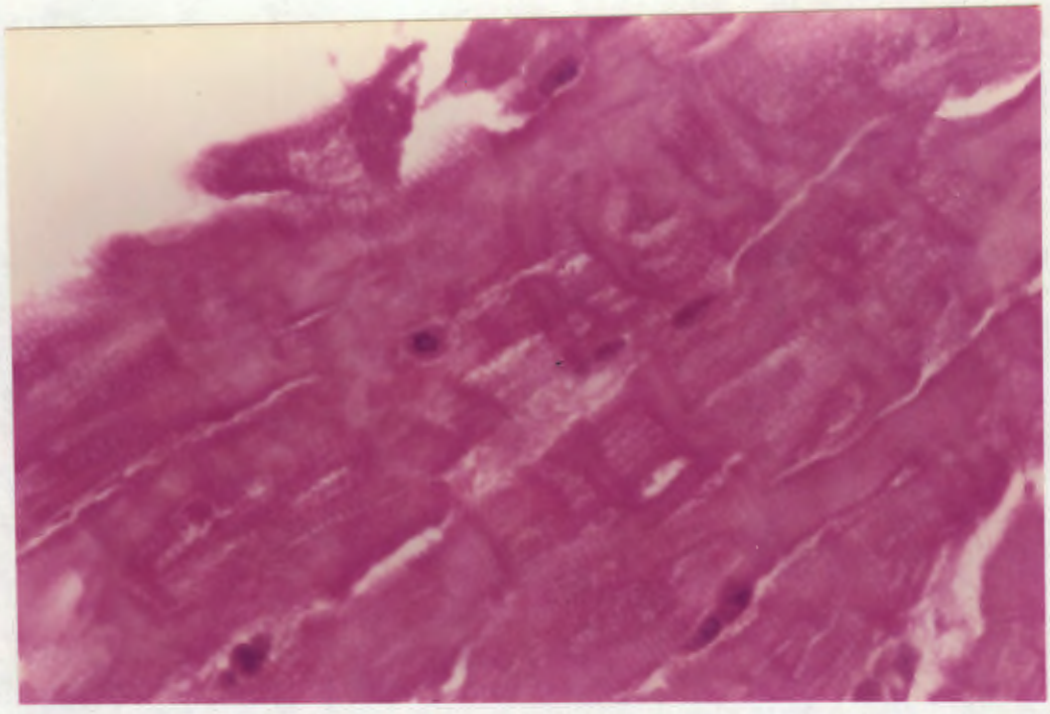
A commonly occurring biopsy-induced artefact is the presence of contraction band necrosis, usually at the edges of the biopsy fragments. (Figure 2a) Kemnitz *et al*[31] hold that contraction bands are a feature of mild acute rejection but their interpretation is more difficult in the early post-operative period (less than 1 week), when similar changes may result from the effect of donor brain death. Donor brain death may result in an over-production of catecholamines and this can cause myocyte damage represented by increased eosinophilia, contraction banding and an infiltrate of mononuclear cells adjacent to damaged myocytes. (Figure 2b).

Thus, although there are limitations and diagnostic pitfalls associated with EMB the procedure can be used safely and effectively to monitor acute rejection of the cardiac allograft and the overall efficiency (no safety problem; vascular access; and adequate sample), has been reported to be as high as 99%.[32]

2.3 LIGHT MICROSCOPIC APPEARANCES OF ACUTE CARDIAC REJECTION IN ENDOMYOCARDIAL BIOPSIES

The sequential pathological changes occurring in the myocardium during acute cardiac rejection as seen in serial endomyocardial biopsies of cardiac recipients have been well described. [3, 4, 26] Acute rejection in cardiac allografts is a special form of inflammatory heart disease (myocarditis) in which the immunological damage is mediated by the host-derived mononuclear cells which first come into contact with the graft via the microvasculature. In the earliest stage of acute rejection,

2a



2b

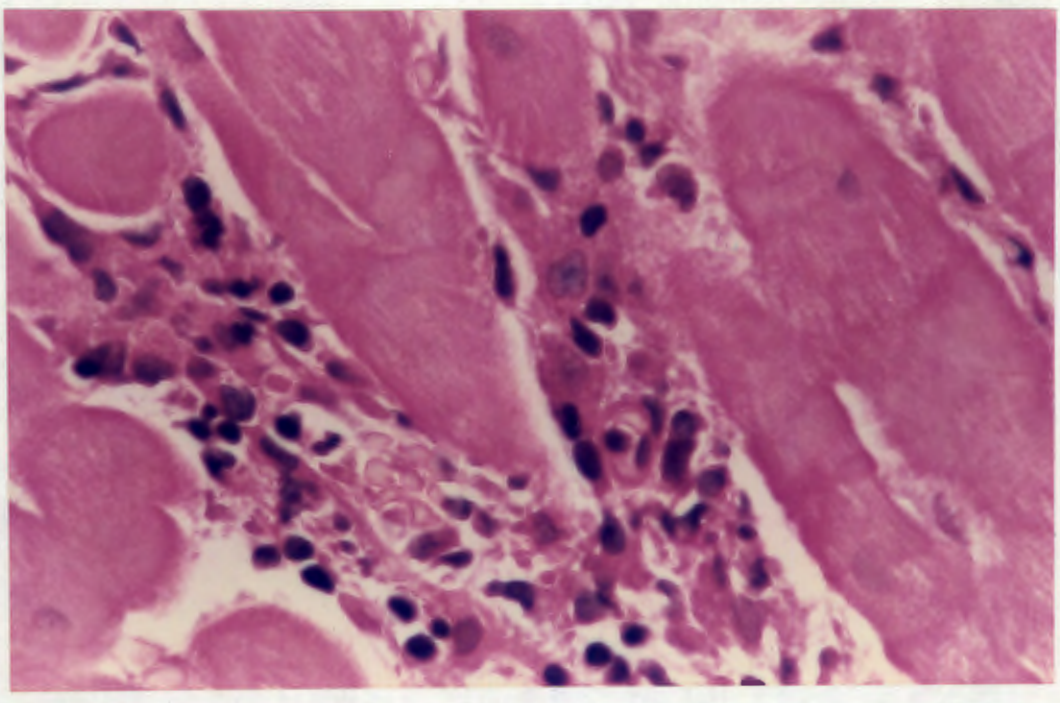


Figure 2: Example of contraction banding artefact induced by the biopsy procedure (a). Sympathetic nervous system over-activity resulting from donor brain damage may produce contraction band necrosis of donor myocytes which in time evokes a mononuclear cellular response (b) {(a) and (b) haematoxylin and eosin, x 400}.

increased numbers of mononuclear cells are present within the small vessels accompanied by interstitial oedema of the myocardium and occasionally small perivascular haemorrhages. These features were commonly associated with the usage of steroid-azathioprine based immunosuppressive regimens, but are now infrequently seen in the cyclosporine (cyclosporin-A) treated patients.

Inflammatory cells may be seen coursing through the vessel walls where they accumulate as perivascular aggregates. Initially, the infiltrate is composed chiefly of small lymphocytes with occasional histiocytes and granulocytes, but with the progression of the rejection process the lymphocytes show the features of immunoblastic activation: their nuclei enlarge, nucleoli appear and they acquire plump pyroninophilic cytoplasm due to the accumulation of RNA. This can be demonstrated by the Unna-Pappenheim (methyl green-pyronin) method (Figure 3).

With increasing severity of rejection the changes become more prominent and the mononuclear inflammatory infiltrates spread into the interstitium and become perimyocytic. The plump mononuclear cells appear to adhere to the myocytes, may indent or overlap them, and focal injury to myocytes may become evident. Injured myocytes may show a range of changes. Initially, there is increasing eosinophilia with loss of nuclear detail, and cytoplasmic cross-striations become indistinct. Eventually focal myocyte necrosis can develop which when present, indicates significant severe acute rejection, particularly in a patient on cyclosporine therapy. It is invariably coagulative necrosis which is seen, although colliquative myocytolysis and contraction band necrosis have

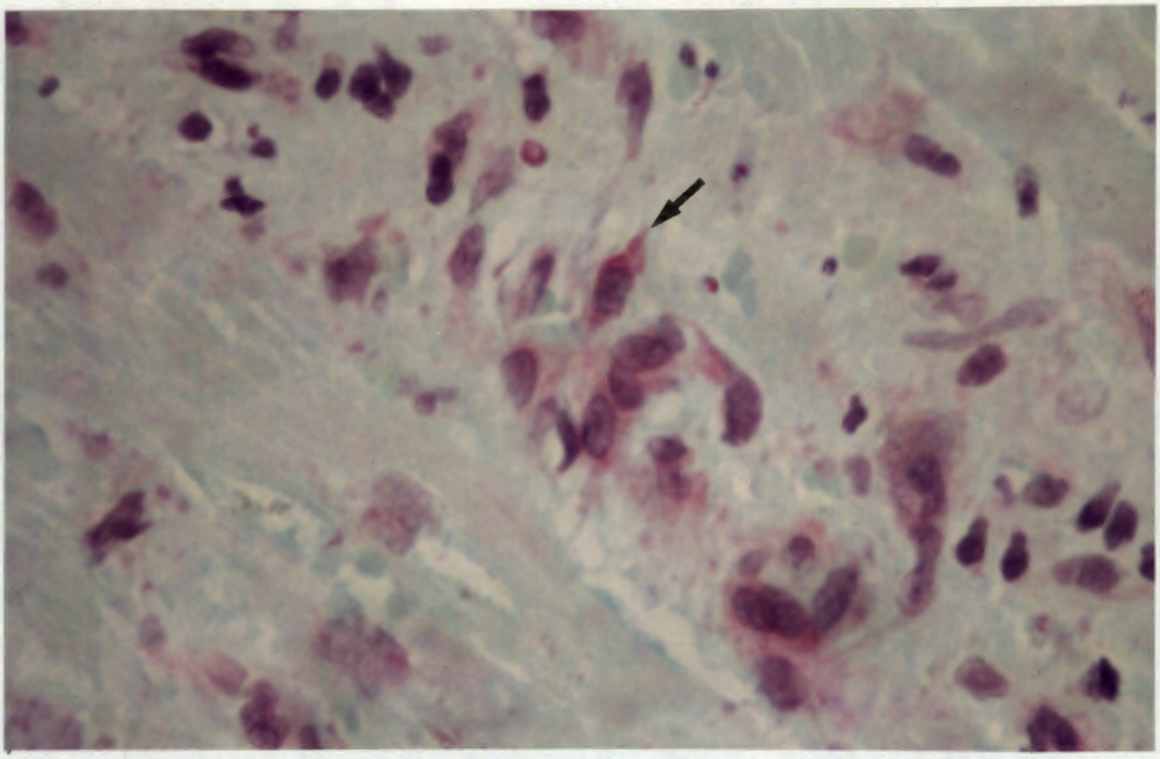


Figure 3: Immunoblastic activation of lymphocytes (arrow). The accumulation of abundant cytoplasmic RNA (magenta hue) is demonstrated by the methyl green-pyronin method (Unna-Pappenheim, x 400).

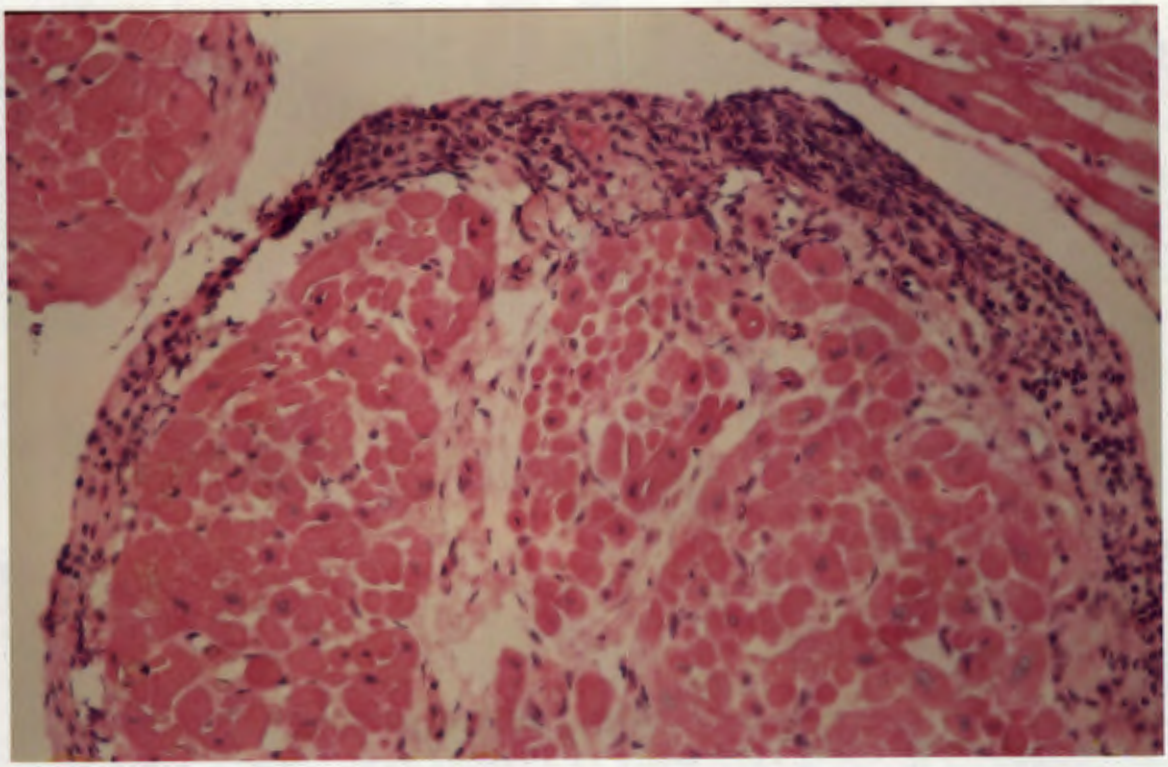
been noted. Myocytolysis, when prominent is usually a consequence of ischaemic damage to the graft due to chronic vascular rejection, whilst contraction band necrosis must be interpreted with caution as this is commonly a biopsy-induced artefact, or is related to catecholamine-induced myocyte damage resulting from donor brain death (discussed earlier above).

Immune-mediated vascular damage can be seen in the intramyocardial vessels. There is endothelial swelling and intimal oedema followed by disruption and loss of endothelial cells. Activation of the coagulation cascade may result in fibrin thrombi being formed in small vessels. In severe rejection a fibrinoid vasculitis can develop which may result in interstitial haemorrhages and thrombotic occlusion of the vessels and this process may involve the smaller branches of the epicardial coronary arteries.

There appears to be a progression of histological changes with increasing severity of acute rejection in patients treated with conventional (steroid and azathioprine) immunosuppressant therapy. However with cyclosporine-based regimens, acute rejection episodes develop more slowly and take longer to resolve, and the microscopic features of acute rejection in such patients show subtle but important differences from those previously described. Rose,[3] tabulated the comparative differences in the microscopic appearances of acute rejection as seen in endomycardial biopsies from patients on different immunosuppressant therapy: azathioprine and steroid versus cyclosporine-based regimens. In biopsies from patients being treated with cyclosporine there is often a

sparse infiltrate of inactive-looking mononuclear cells which occasionally form large endocardial aggregates (Figure 4a & 4b). If these are unaccompanied by myocardial inflammatory infiltrates they are not regarded as being indicative of significant rejection and the true significance of these endocardial infiltrates 'Quilty effect' remains to be elucidated. Myocyte necrosis when present is a sensitive sign of acute rejection and may be associated with only a mild or moderate degree of rejection. Some authors [29] hold that focal myocardial necrosis must be identified before a diagnosis of acute cardiac rejection can be established, but the Cape Town experience is that myocyte necrosis is seldom observed even when other features of severe acute rejection are evident and anti-rejection therapy should not be withheld until the development of myocyte damage. The Cape Town experience regarding the rarity of myocyte necrosis in moderate or severe acute rejection is not unique and similar observations have been made at the Cleveland Clinic.[33] Herskowitz *et al*[34] examined specific histological abnormalities that could predict early cardiac rejection and found that interstitial oedema, perivascular karyorrhexis and perivascular inflammation with intermyocyte extension were histological variables that probably represented early cardiac rejection before the development of myocyte necrosis, and when present in biopsy samples can be considered as an indication for the institution of immunosuppressive therapy.

4a



4b

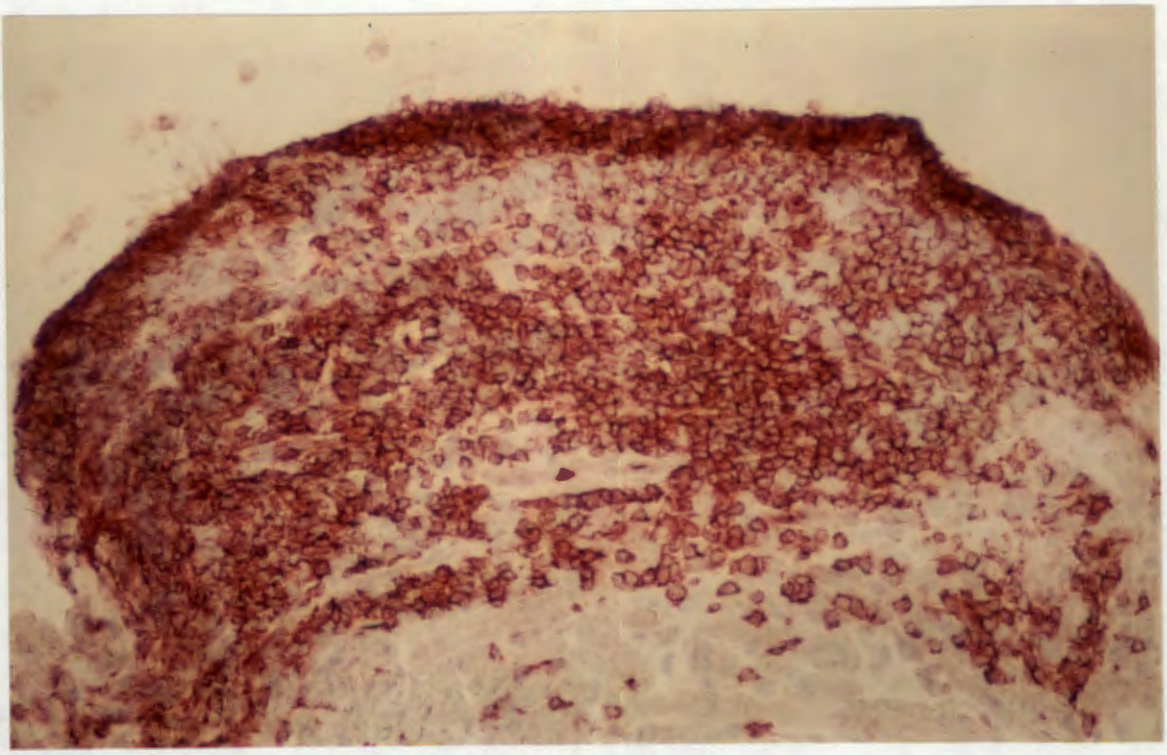
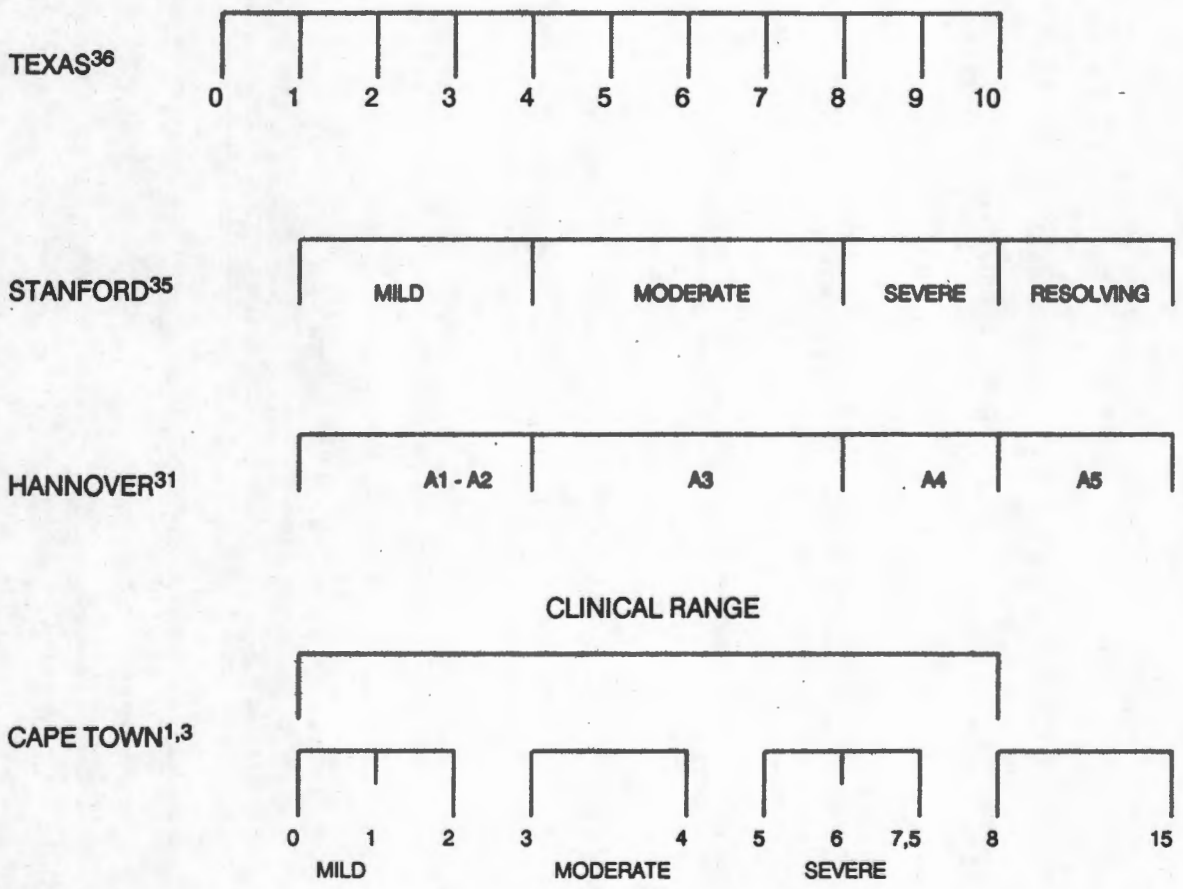


Figure 4(a): Large endocardial lymphoytic infiltrate ('Quilty effect'). The adjacent myocardium is devoid of lymphocytes (haematolyin and eosin, x 150). (b) Immunocytochemistry reveals the majority of the endocardial infiltrate to consist of T lymphocytes (see 2.4.2) (CD8+ antibody, peroxidase anti-peroxidase, x 150).

Histological Grading of Acute Cardiac Allograft Rejection

The Stanford (Billingham) grading system [35] of acute allograft rejection divides rejection episodes into mild, moderate, severe and resolving acute rejection. Several new grading systems are in use [31, 36] and the Stanford and comparative grading systems are demonstrated in Table 2.3.

TABLE 2.3: GRADING OF ACUTE REJECTION BY ENDOMYOCARDIAL BIOPSY



At Groote Schuur, Cape Town, a semi-quantitative scoring system has been used for many years to assess the degree of acute rejection in the biopsy material[1, 3]. Five histological criteria are individually assessed and assigned a score from zero to a maximum of three. The sum of these scores represents the total score for the biopsy. The five histological criteria assessed are:

1. Interstitial oedema.

2. Mononuclear inflammatory cell infiltration.

3. Pyroninophilia of the cytoplasm of the mononuclear cells.

4. Myocyte damage.

5. Abnormalities in the microvasculature, (vasculitis).

Theoretically a maximum score of 15 is possible but in clinical practice a score of greater than 6 is rarely encountered and would indicate irreversible damage to the graft. A score of 0 signifies that there is no evidence of rejection; 0,5 - 2,0 denotes mild rejection; 2,5 - 4,0 indicates moderate rejection and a score of greater than 4,5 is compatible with severe rejection. Examples of mild, moderate and severe acute rejection are given in Figures 5, 6 and 7 respectively. The main advantage of this semi-quantitative scoring system is that a numerical score is arrived at which is easily understood by the clinician, and scores attained on subsequent biopsies monitor the patient's progress and response to anti-rejection therapy during the acute rejection episode. In addition, reproducibility is improved since the same histological criteria are assessed on each biopsy.

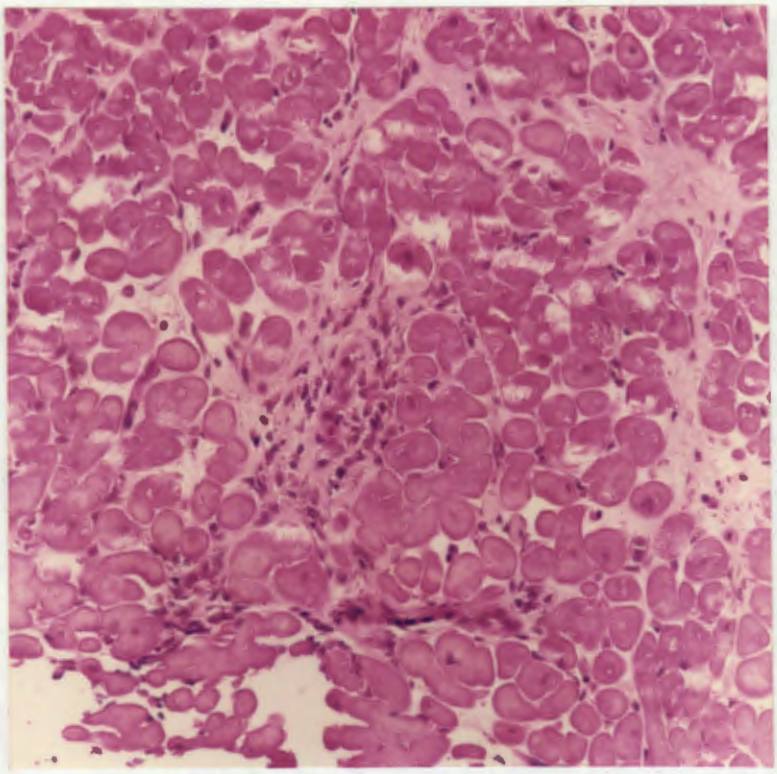


Figure 5: Mild acute rejection. Scanty perivascular lymphocytes are present (haematoxylin and eosin, x 200).

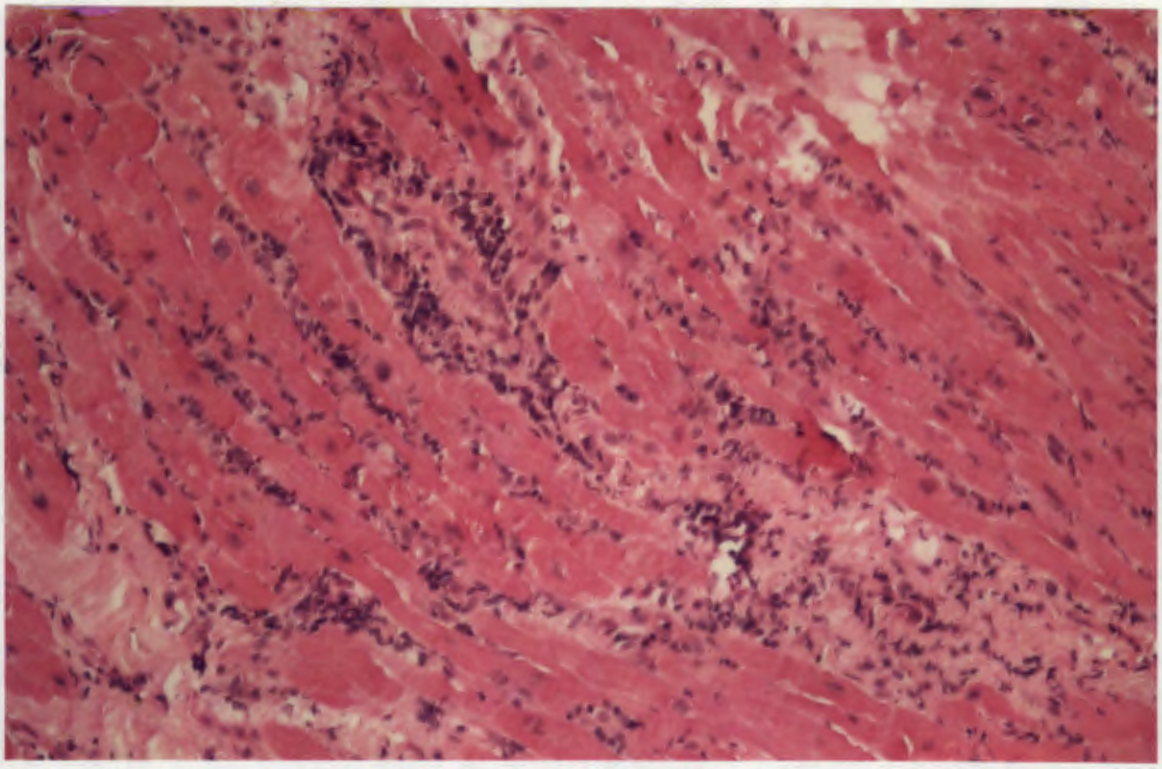


Figure 6: Moderate acute rejection is characterised by a more intense inflammatory infiltrate which extends from the perivascular into the perimyocytic interstitium (haematoxylin and eosin, x 200).

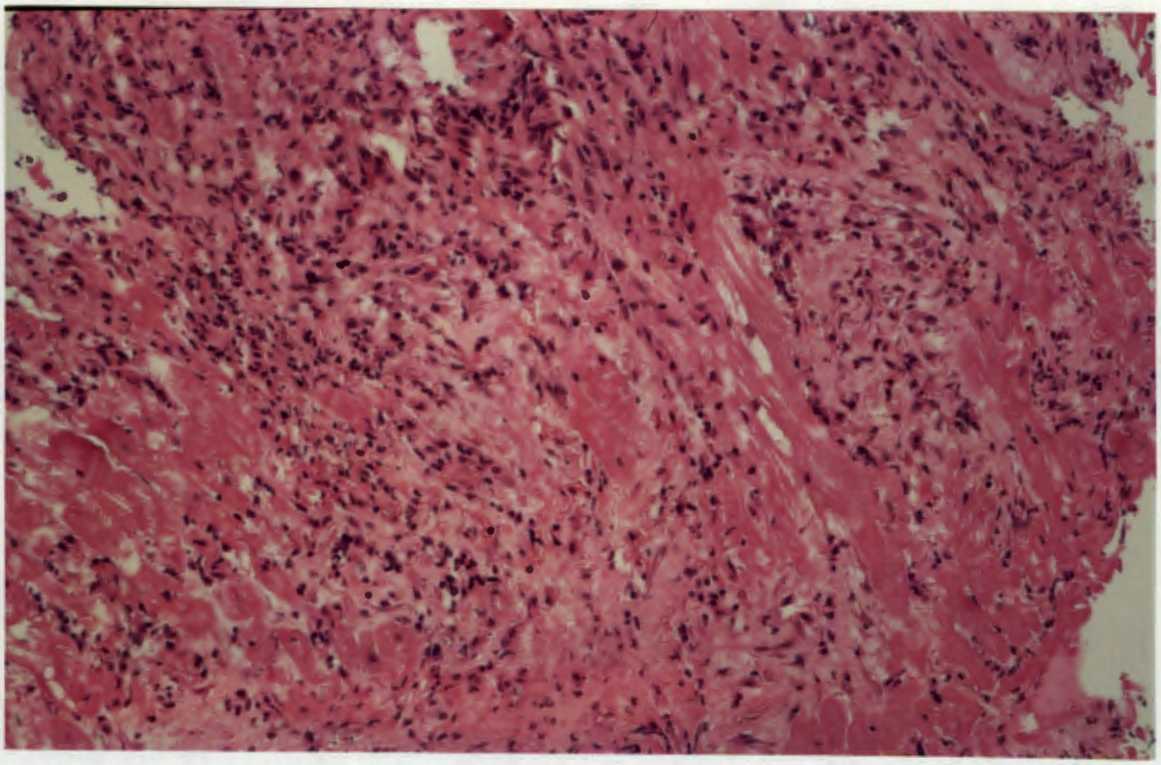


Figure 7: Resolving severe acute rejection showing prominent lymphocytic infiltration, focal myocyte necrosis, loss of myocytes and early sclerosis (haematoxylin and eosin, x 200).

Once a patient has been treated, follow-up biopsies may reveal evidence of repair. The features of resolving acute rejection include fibroblast infiltration with stromal fibrosis and the presence of macrophages. Residual lymphocytes may be present. These are small, non-pyroninophilic and are more common in patients being treated with cyclosporine.

2.4 IMMUNOHISTOCHEMICAL ANALYSIS OF MONONUCLEAR SUBPOPULATIONS

Although cyclosporine and steroids remain the cornerstone of immunosuppressant therapy, the problem of acute rejection refractory to repeated pulse therapy with methylprednisolone remains, and there is concern over the immediate and chronic renal toxicity associated with the use of high dosages of cyclosporine. Histologically acute rejection is characterised by an infiltration of inflammatory mononuclear cells into the graft (as discussed above). Monoclonal antibody immunoperoxidase studies performed on biopsy material obtained from acutely rejecting cardiac allografts have identified these cells as being predominantly T-lymphocytes-[37 33]

Thus, in an effort to find an optimum immunosuppressant protocol many institutions, including our own, are utilising some form of T-lymphocyte cytolytic therapy. Lymphocytolytics include antithymocyte globulin (ATG) and OKT3, a newly developed monoclonal antibody specifically developed against the CD-3 surface antigen of peripheral T-lymphocytes. These agents differ with respect to the host animal in which they are

made and also in their potency and specificity for cells that recognise antigens. Aspects of their development, mechanisms of action, and efficacy in treatment of acute cardiac rejection are discussed in detail elsewhere.[20, 39, 40]

At our institution an immunohistochemical study using monoclonal antibodies was performed on cardiac biopsy material to investigate the mononuclear subpopulations. Broadly, the aims of the study were twofold. Firstly, to characterise the nature of the mononuclear infiltrates in endomyocardial biopsies; secondly, to correlate the findings with the light microscopic appearances in the same specimens to establish whether immunoperoxidase markers could be useful in the assessment of acute cardiac rejection.

2.4.1 Patients and Methods

Patients

Following transplantation, donor hearts are biopsied once a week for the first month and then with decreasing frequency in subsequent months. The transvenous endomyocardial biopsy technique utilising the supraclavicular approach to the subclavian vein is used at Groote Schuur Hospital and is described in detail elsewhere by Cooper et al. [41]. All patients since September 1984 have received immunosuppression regimens based on low dose cyclosporine therapy (Cyclosporin A), steroids (methyl-prednisolone), and azathioprine. More recently,

modifications to this standard regime have been made. Patients have been randomly divided into sub-groups in order to determine the influence of adjuvant immunosuppression on infection-related morbidity and mortality, and in addition may receive either Rabbit antithymocyte globulin (RATG), or Murine monoclonal CD-3 antibody (OKT3) therapy.

During the study period from January 1988 until September 1990, 129 endomyocardial biopsies were examined for their lymphocyte subpopulations from a total of 636 specimens submitted. From the light microscopic examination the cases were divided into diagnostic groups. Table 2.4

TABLE 2.4: DIAGNOSTIC GROUPS

	NUMBERS	% OF CASES
NEGATIVE FOR REJECTION	31	24
MILD ACUTE REJECTION	67	51,9
MODERATE ACUTE REJECTION	13	10,1
SEVERE ACUTE REJECTION	2	1,6
INADEQUATE FOR ACCURATE ASSESSMENT	4	3,1
EVIDENCE OF PREVIOUS BIOPSY SITE	12	9,3
TOTAL	129	100

When the specimen shows evidence of a previous biopsy site (described earlier above), an accurate grading of rejection can be difficult to perform but the presence or absence of significant rejection can be commented on, if the biopsy site is represented in only one out of the several endomyocardial samples examined. Of the 12 cases showing the features of a previous biopsy site, 5 cases showed evidence of mild

rejection the remainder being negative for rejection. For statistical purposes these 12 cases were examined as a separate group. Within the diagnostic sub-group "negative for rejection", the semi-quantitative rejection score is consistently nil and further statistical analysis is therefore limited.

Histology

We routinely receive 3 - 4 endomyocardial specimens per biopsy procedure. They are transported in 5% buffered glutaraldehyde to facilitate subsequent ultrastructural examination of one of the fragments and the remaining fragments are post-fixed in 10% formol saline prior to routine processing in a vacuum infiltration processor. The paraffin embedded sections are stained by the haematoxylin-eosin, elastic van Gieson and Unna-Pappenheim methods for assessment of rejection based on light microscopy. In addition, a further one or two fragments are submitted fresh in liquid nitrogen and an immediate assessment of rejection can be made on the haematoxylin-eosin stained frozen sections. If significant numbers of lymphocytes are present (i.e. more than 10 lymphocytes per myocardial sample on frozen section), then cryostat sections are prepared for immunoperoxidase mononuclear subset determination; (to be discussed in detail below). The assessment of rejection is based on light microscopy using the semi-quantitative scoring system developed at this institution (discussed earlier above); and a numerical score indicating the grade of rejection is relayed to the clinicians as soon as possible giving them a guide as to the severity of the rejection process and the efficacy of treatment. Ultrastructural and immunofluorescent studies play an insignificant role in the diagnosis of acute rejection.

Monoclonal Antibodies and Immunoperoxidase Staining

Ten cryostat sections, each 4 microns thick, are cut and air dried then stored in a sealed container at -20 °C in a deep freeze for later immunoperoxidase staining. The air dried sections are fixed in cold acetone at 4 °C for 10 minutes, air dried again, then incubated for 10 minutes with non-immune rabbit serum at 1/20 dilution to block endogenous peroxidase. After a phosphate buffer saline (PBS) rinse at pH 7,4 the slides are incubated for 40 minutes with the first stage antibodies at a 1/10 dilution. The first stage monoclonal antibodies used in the study are listed in Table 2.4.

TABLE 2.4 **MONOCLONAL ANTIBODIES**

SOURCE	DESIGNATION	ANTIGEN DISTRIBUTION
*DAKO	T1 **(CD5)	ALL T CELLS
DAKO	T2 (CD7)	PRE-THYMOCYTIC T CELLS
+ORTHO	OKT4 (CD4)	HELPER/INDUCER T CELLS
DAKO	T8 (CD8)	CYTOTOXIC/SUPPRESSOR T CELLS
ORTHO	OKM1	MACROPHAGES/MONOCYTES
DAKO	PAN B (CD 22)	ALL B CELLS
DAKO	HLA - Dr	ALL IMMUNOCYTES/DENDRITIC CELLS EPITHELIUM UNDER IMMUNOLOGIC ATTACK

ABBREVIATIONS USED: *DAKO: DAKOPATTS; **CD: CLUSTER DESIGNATION; +ORTHO: ORTHO-MUNE

Plasma cells could be identified on routine haematoxylin-eosin stains and were encountered infrequently and thus specific immunoperoxidase stains for them were not performed.

After a rapid PBS rinse the slides are incubated with the second stage antibody, rabbit-anti-mouse IgG coupled with horse radish peroxidase (Dakopatts) for 30 minutes at a 1/25 dilution. To enhance the marker, a further 30 minute incubation with added human serum at room temperature is performed and the slides are given a final rinse with PBS.

The peroxidase reaction is induced by adding freshly prepared chromogen substrate 3-amino-9 ethylcarbozole in N,N- dimethylformamide. The slides are flooded for 20 minutes at room temperature and the reaction is stopped by adding distilled water. The sites of peroxidase fixation are identified as a red colour.

Finally the slides are counterstained with Mayer's haematoxylin and mounted in glycergel (an aqueous mountant). A negative control is prepared for each biopsy: the same process is performed but the first stage antibody is excluded.

To analyse the composition of the infiltrates a semi-quantitative method is used, based on the method described by Weintraub *et al,*[37] and described in Table 2.5

TABLE 2.5 SEMI-QUANTITATIVE MEASURE OF ENDOMYOCARDIAL INFILTRATES

		SCORE
TRACE =	FEW CELLS WITH POSITIVE SURFACE STAINING	0
+ =	NUMBER OF CELLS HAVE POSITIVE SURFACE STAINING	1
++ =	MANY CELLS HAVE POSITIVE SURFACE STAINING	2
+++ =	MOST INFLAMMATORY CELLS HAVE POSITIVE SURFACE STAINING	3

A semi-quantitative score was obtained for each monoclonal antibody tested, and a total score for each biopsy was obtained from the sum of the scores of the antibody panel used; the 'semi-quantitative score'. The monoclonal antibody HLA-Dr was excluded from the semi-quantitative analysis as this antibody stains all immunocytes. From the semi-quantitative scores a separate index, the CD4+/CD8+ subtotal was calculated for each biopsy.

Examples of the semi-quantitative measures "trace", "+", "++" and "+++" are given in Figures 8, 9, 10, and 11 respectively.

The same semi-quantitative method was applied to cases containing predominantly endocardial inflammatory infiltrates. Sometimes other inflammatory patterns were seen to be associated with the endocardial infiltrates. These were found to be either intramyocardial perivascular aggregates alone, or intramyocardial perivascular aggregates with intermyocyte extension. These patterns were noted and the cases separated into sub-groups and analysed separately.

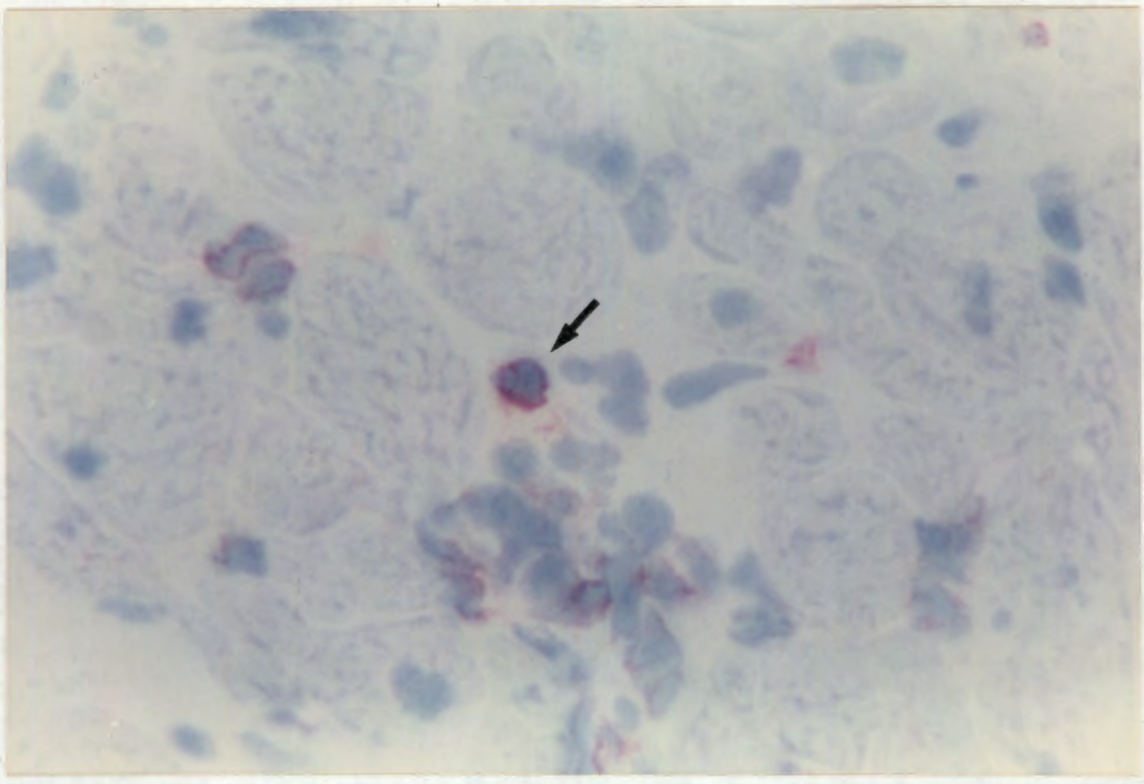


Figure 8: Section stained for T8(CD8+) cells with isolated lymphocytes (arrow) showing positive surface staining: semi-quantitative lymphocyte score, trace (0) (peroxidase anti-peroxidase, x 400).

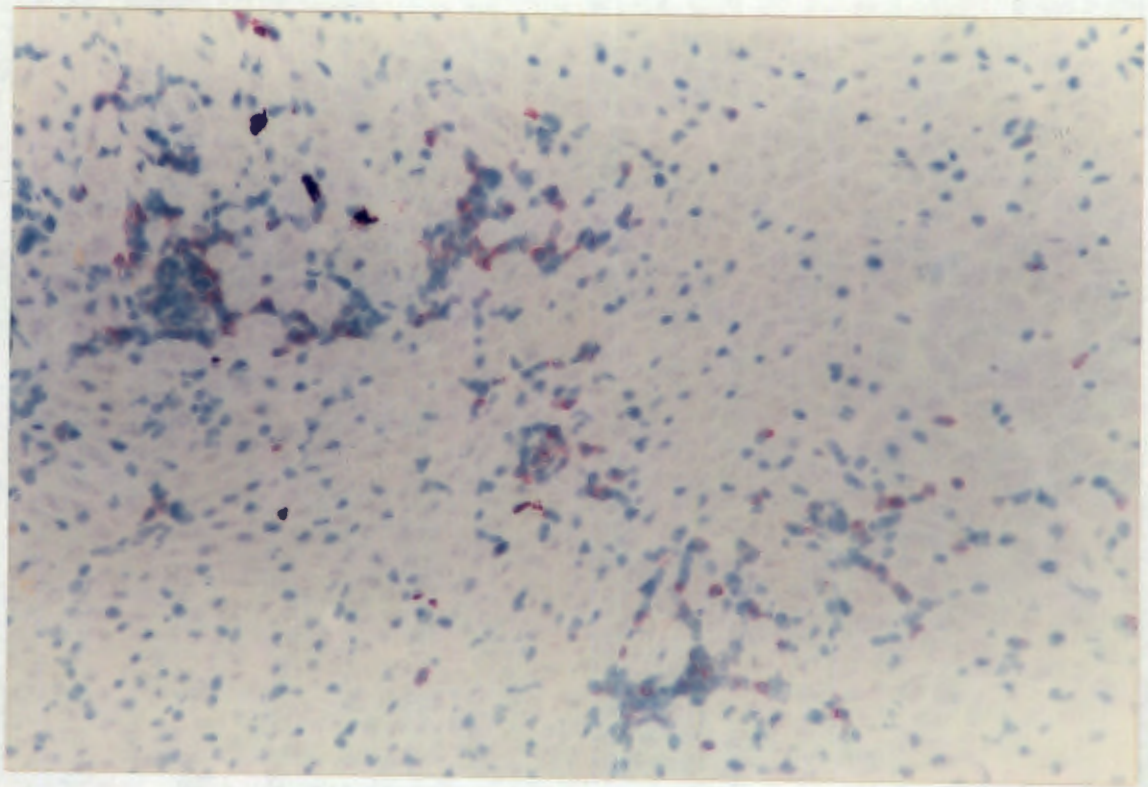


Figure 9: A number of lymphocytes showing positive surface staining for T8(CD8+) antibody; semi-quantitative lymphocyte score, (1) (peroxidase anti-perioxidase, x 200).

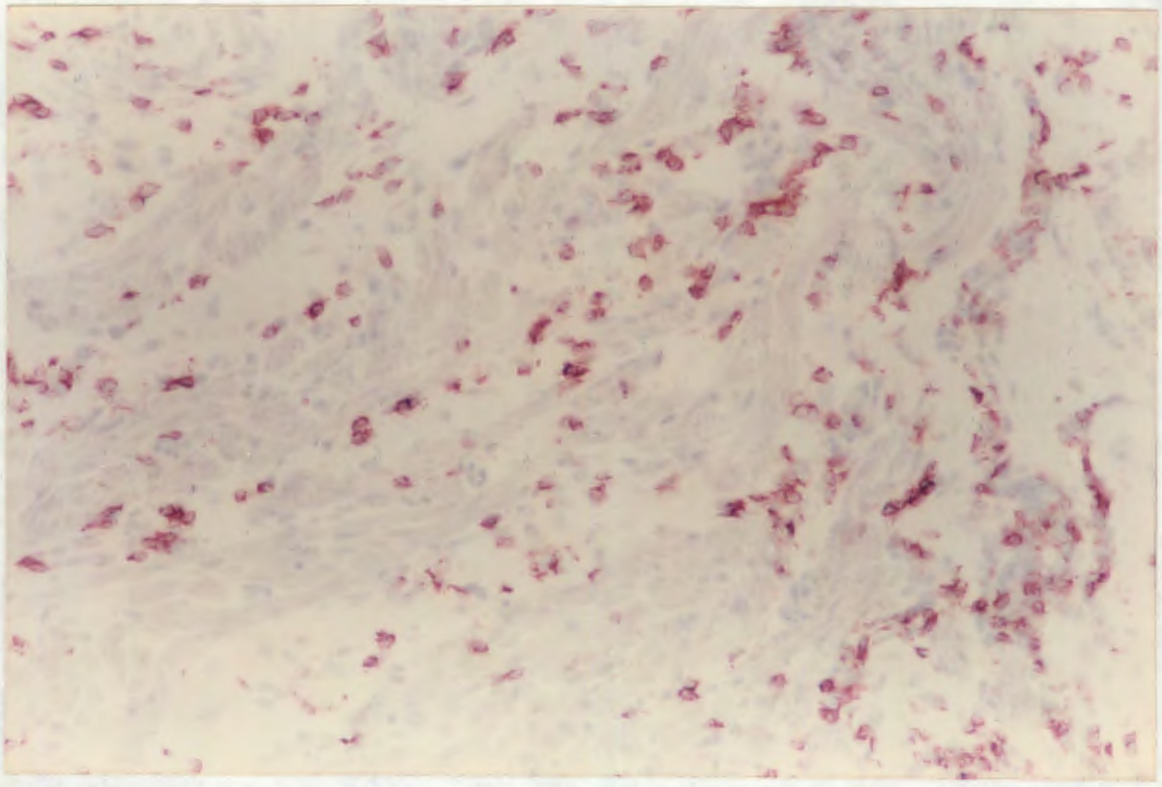


Figure 10: Semi-quantitative lymphocyte score (2). Many lymphocytes show positive surface staining for T8 (CD8+) antibody (peroxidase anti-peroxidase, x 200).

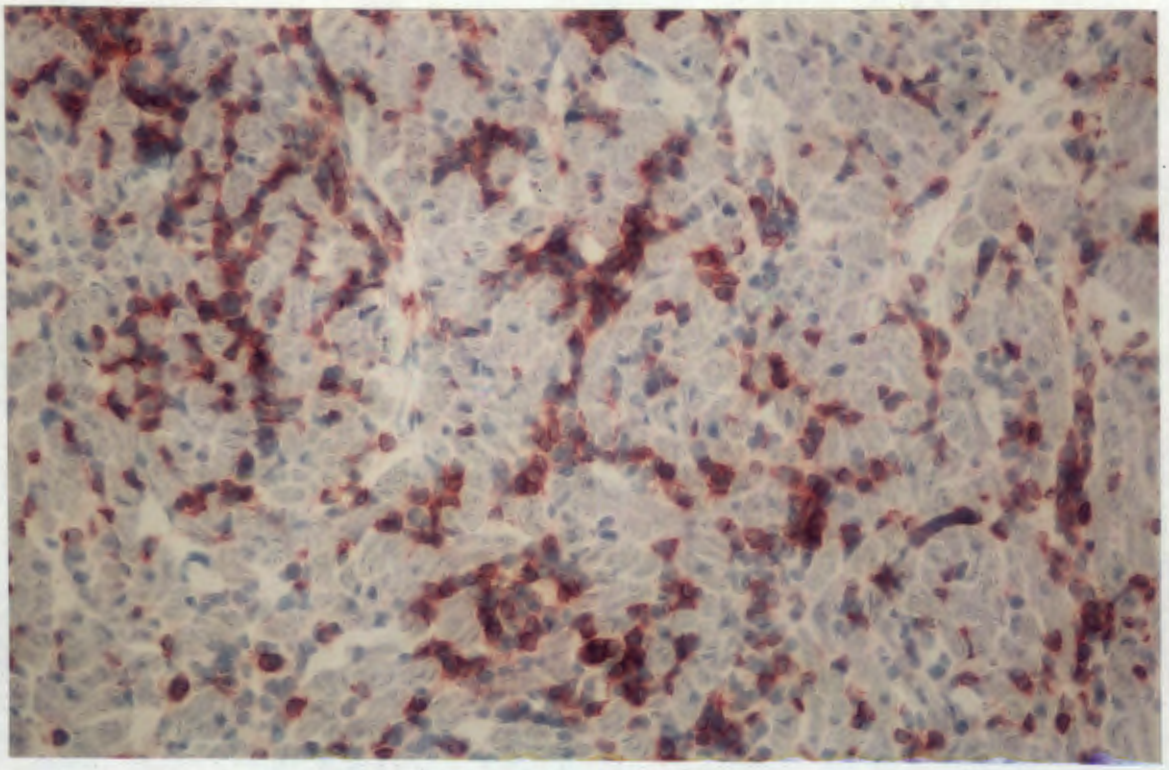


Figure 11: Numerous inflammatory cells in perivascular aggregates with intermyocyte extension stain positively for T8 (CD8+) antibody. Semi-quantitative lymphocyte score (3) (peroxidase anti-peroxidase, x 150).

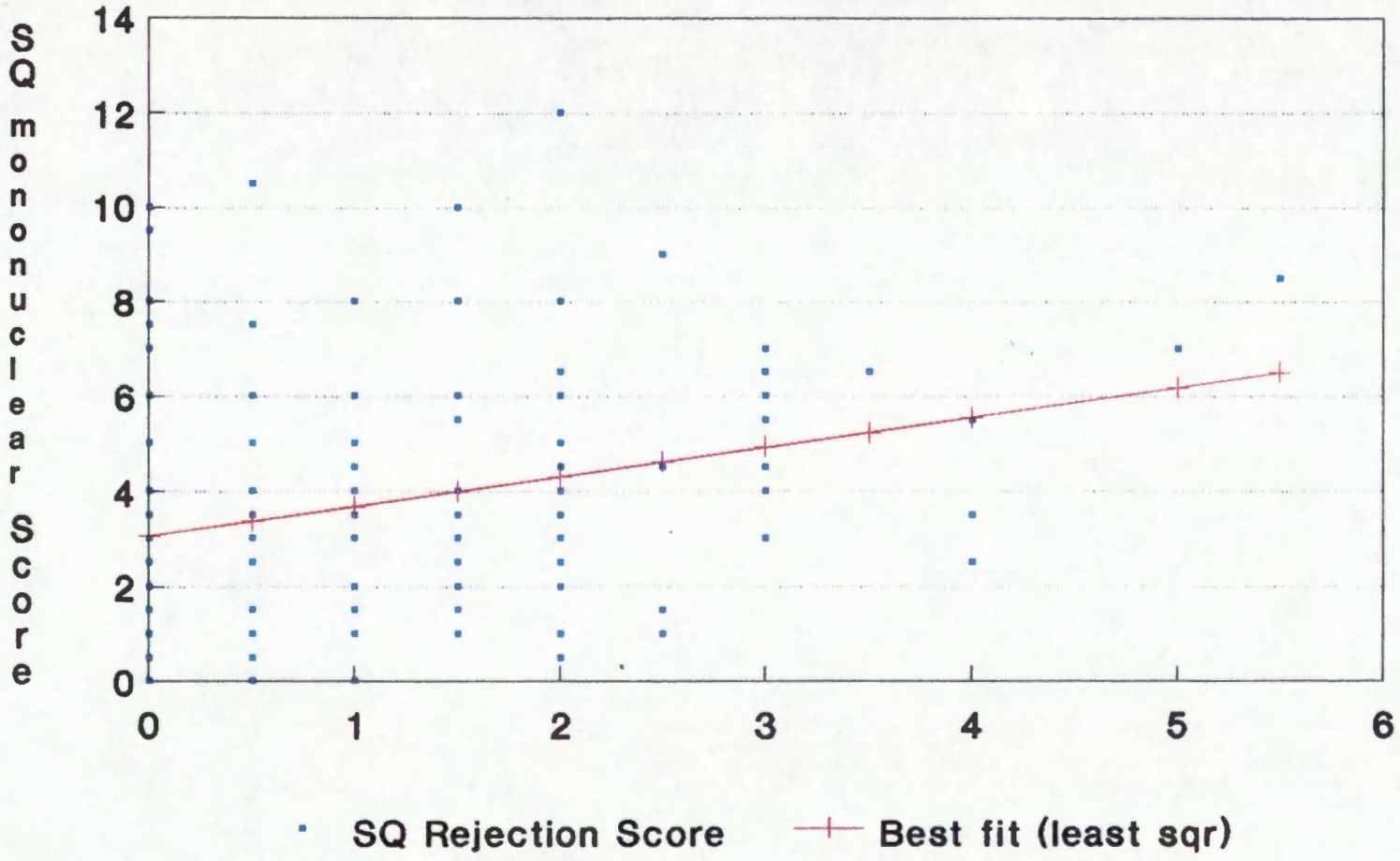
Statistical Methods

The total score obtained for each biopsy was weighted towards the contribution from the T-cell subpopulations. This was felt to be justified in view of the fact that previous studies had identified the infiltrates in acutely rejecting grafts as being composed predominantly of T-cells as discussed earlier above.

The database obtained for each biopsy included the semi-quantitative rejection score for acute rejection, a semi-quantitative mononuclear subpopulation score, and the CD4+/CD8+ subset totals. The relationship of the semi-quantitative scores, and the CD4+/CD8+ scores was tested by Pearson's Simple Correlation method. The method of multiple linear regression was used to predict the semi-quantitative rejection score using the variables; semi-quantitative mononuclear score and the CD4+/CD8+ subset totals in a step-wise manner. An analysis of variance was performed on the sub-groups of endocardial infiltrates to test whether an accompanying pattern of inflammatory infiltration was associated with variation within the semi-quantitative rejection score.

The statistical methods of analysis of variance used are described by Snedecor and Cochran,[42] and a statistical package, Programme S.P.S.S. was used for the multiple linear regression method.

Figure 12 Scatter plot of mononuclear score vs rejection score



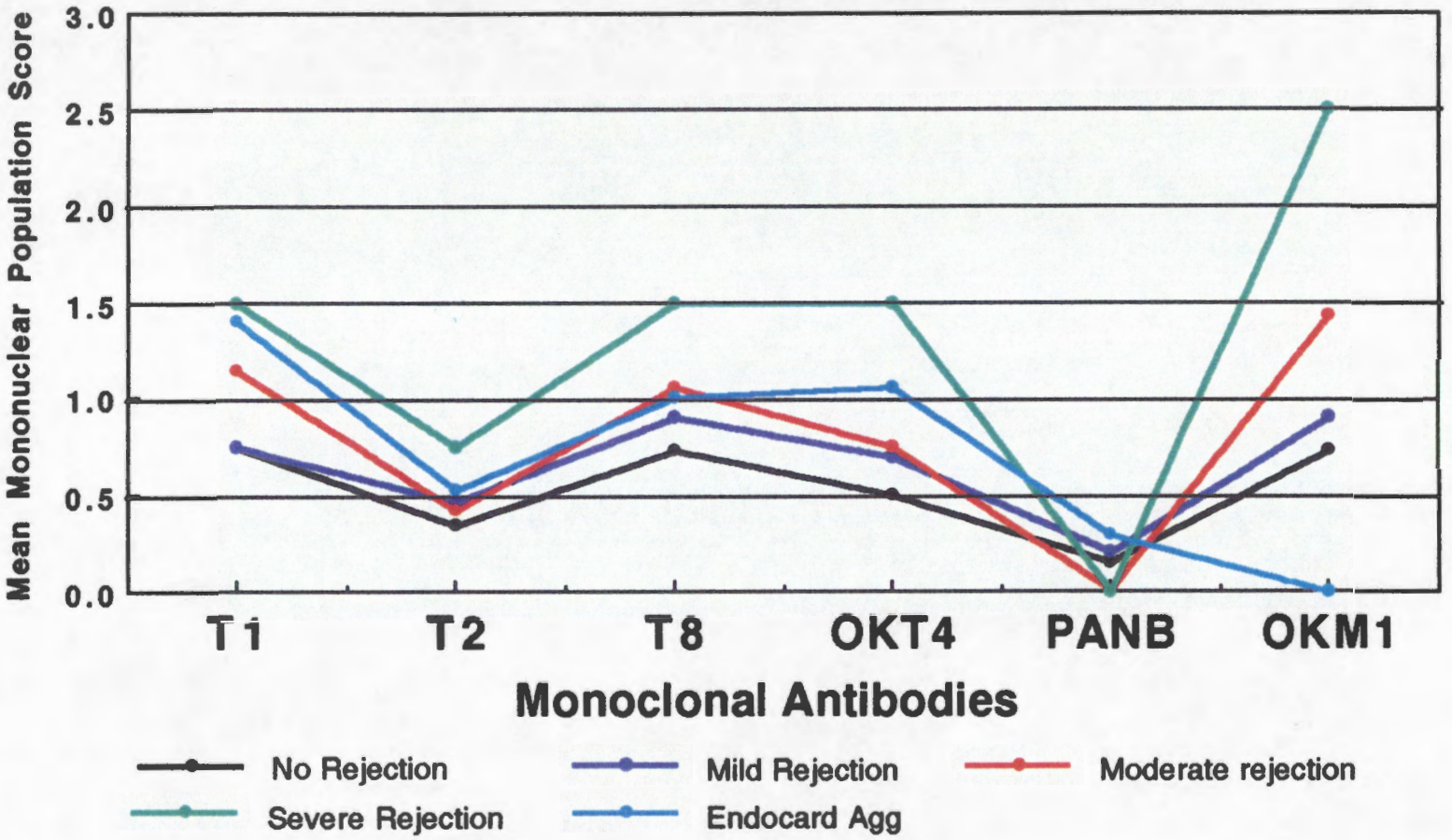
SQ:semi-quantitative

Figure 13 demonstrates the means of the mononuclear subpopulation scores for each antibody tested. An observation is that the majority of the cells are T lymphocytes with a mixture of T4(CD4+) and T8(CD8+) cells. For increasing grades of rejection the mean scores are higher. However, an analysis of variance shows that with each antibody studied no statistically significant difference occurs between each grade of rejection. The graph demonstrates a trend for OKMI. The mean score increases markedly for severe acute rejection, but there were only two cases and this must be interpreted with caution. Also shown in this graph are the corresponding figures for the mononuclear subpopulations present in cases with endocardial lymphocytic aggregates. It can be seen that a similar distribution is present.

Within the study population, 31 cases (24%), showed endocardial lymphocytic infiltrates (so-called 'Quilty effect'). The majority of cases were also associated with either endomyocardial perivascular aggregates or inter-myocyte extension of lymphocytes. The mean rejection scores associated with these patterns are shown in Appendix Table A-3, together with an analysis of variance. Although there is a trend towards a higher mean score when associated with another pattern, this trend is not statistically significant; (F value = 1.014247). This is probably due to the small sample sizes within each category.

Figure 13

Mean Mononuclear Sub-population Scores vs Antibodies Tested



3. DISCUSSION

The role of the EMB in the diagnosis and surveillance of acute cardiac allograft rejection has been discussed in the introduction. Although it is an invasive technique it is well tolerated by patients and morbidity and mortality associated with the procedure is low. In common with most diagnostic procedures where a tissue diagnosis is required, problems associated with the adequacy of sampling and representativeness of the sample obtained are inherent in the EMB procedure. This was discussed earlier above (see Problems and Pitfalls of Endomyocardial Biopsy), and it is generally accepted that adequate sampling has been achieved if 4 or more samples of endomydocardium are submitted with each biopsy procedure. Although several non-invasive adjuncts to EMB are available for the diagnosis of acute cardiac rejection, no single test is specific nor sensitive enough to replace EMB and it remains as the diagnostic "gold standard" for the diagnosis and surveillance of acute cardiac rejection.

In an attempt to grade the severity of the acute rejection process, at the University of Cape Town, a semi-quantitative scoring system has been developed. This method, together with other systems which have emerged from large transplant centres worldwide, were described and compared earlier above. Recently,[43] a Working Formulation has been proposed to standardise the nomenclature used in the diagnosis and grading of cardiac allograft rejection. Briefly, this recognises four grades of acute rejection; annotated as Grade 0 up to Grade 4. Roughly these correspond to "no rejection", "mild", "moderate" and "severe acute rejection" in the "old" nomenclature. Histological variables of major importance are the presence or absence of myocyte necrosis and the severity and nature of lymphocytic infiltrates. The pattern of

lymphocytlc infiltration is important i.e., focal perivascular or interstitial infiltrates versus diffuse infiltrates. Diffuse infiltrates are associated with increasing grades of severity of rejection. An additional feature to be noted in a report is the presence of an endocardial lymphocytic infiltrate 'Quilty effect'. It is believed that endocardial infiltrates are not significantly associated with acute rejection,[4] but the nature and significance of these infiltrates is not clear.

At the Cleveland Clinic[44] a large study was performed on endocardial lymphocytic infiltrates (ELI) in cardiac transplant patients to investigate their immunoreactivity and characterization. The authors distinguished a characteristic immunohistochemical pattern within ELIs; they were comprised predominantly of T cells associated with B cell clusters, with macrophages and plasma cells at the myocardial interface. In addition, although ELIs were not related to clinical rejection episodes, they could be associated with myocyte damage in the underlying subjacent myocardium. This they stated could make distinction from acute rejection difficult at histological examination, although the presence of macrophages and plasma cells at the endocardial-myocardial interface could help to exclude acute rejection.

From our study on mononuclear subpopulations, we failed to identify a distinctive immunohistochemical profile in ELIs when compared to those seen in myocardial infiltrates (see Figure 13). However, we observed that when ELIs were associated with lymphocyte infiltrations in the underlying myocardium there was a trend towards an association with higher rejection scores. This was not entirely unexpected, but does highlight the potential source of error that ELIs may present in the endomyocardial diagnosis of acute rejection.

Kottke-Marchant et al[44] discussed the likely aetiology of ELIs. They are restricted to cardiac transplant recipients on treatment with cyclosporine. The authors presented the evidence which suggests that they may represent Epstein-Barr Virus/cyclosporine-associated lymphoproliferative lesions. The reasons for their localisation to the endocardium are less clear and remain to be fully elucidated.

One of the major problems in cardiac transplantation limiting survival is the immune response of the recipient to the donor allograft. The immunological mechanisms of cardiac transplant rejection are complicated and are reviewed in detail elsewhere.[45 46] Of primary importance are antigens encoded by the Major Histocompatibility Complex (MHC), a closely linked group of genes mediating a variety of immunological functions including cell-to-cell interactions. The MHC complex encodes for transplantation antigens (HLA in humans) and is situated on the short arm of chromosome 6; HLA-A, HLA-B and HLA-C loci control expression of Class-I molecules and the HLA-D region encodes for the Class 11 molecules. The recipient immune response is directed towards these donor-specific MHC related transplantation antigens. Cell-mediated immunity is believed to be of major importance in acute cardiac rejection, mediated predominantly by T cells that have undergone activation and functional differentiation after exposure to transplantation antigens. Immunostaining has demonstrated that in biopsies from cyclosporine treated heart transplant recipients a mixture of CD4+ (T helper/inducer lymphocytes) and CD8+ (T cytotoxic/suppressor lymphocytes) are present in the myocardial infiltrate.[37] From our observations of mononuclear subpopulations we confirmed that the majority of cells in myocardial infiltrates are T cells predominantly of the CD4+ and CD8+ immunophenotype.

Cells expressing CD4 marker are reactive to Class II antigens and CD8+ cells are usually restricted to Class I antigen.[45] It has been reported[47] that during cell-mediated rejection CD4+ cells are the first to enter the heart followed by CD8+ lymphocytes. Assuming that mild rejection precedes grades of increasing severity of rejection, a similar phenomenon was identified by Schuurman *et al.*[48] From our study, we failed to identify significant changes in the CD4+ and CD8+ lymphocyte subpopulations within different grades of rejection, in keeping with the findings of Weintraub *et al.*[37]

In 1984 Hoshinaga *et al*[47] reported that the assessment of in-situ CD4+/CD8+ ratios in biopsies may be of benefit in monitoring rejection episodes. Subsequently other workers [49, 50] have investigated the CD4+/CD8+ ratios in peripheral blood and have found no associations between changes in T cell subsets and rejection episodes. When examining the lymphocyte status in endomyocardial biopsies and blood, Schuurman *et al*[48] found that myocardial tissue infiltrates have no direct correlations with lymphocyte characteristics in the circulation.

In our study, by assigning a numerical score based on a semi-quantitative analysis of lymphocyte subpopulations it precluded an accurate statistical analysis of CD4+/CD8+ ratios. However, as mentioned earlier above, our results demonstrated no significant changes in CD4+ and CD8+ populations within different grades of rejection and one could predict that no significant changes would be observed in CD4+/CD8+ ratios.

CONCLUSIONS

The endomyocardial biopsy remains the 'gold standard' for the diagnosis and surveillance of acute cardiac allograft rejection. From the tissue obtained by this procedure, the severity of the acute rejection process can be graded, and at our institution we have satisfactorily used a semi-quantitative histological scoring system. Aspects of myocardial lymphocytic infiltration comprise 2 of the 5 criteria assessed. In the present study the immunohistological characterization of myocardial lymphocytic infiltrates in donor heart biopsies was performed. A semi-quantitative scoring system of the mononuclear subpopulations was shown to have a limited predictive value to the variation within the semi-quantitative rejection score. A review of the literature reveals that the immunophenotypic characterization of lymphocytes in endomyocardial biopsies and peripheral blood has been of limited prognostic value in assessing cardiac transplant rejection.

Recent methodologies to propagate lymphocytes from cardiac transplant biopsies have enabled additional functional characteristics of T cell subsets to be studied during rejection.[51] [52] The growth of lymphocytes from histologically negative biopsies is associated with a risk of developing subsequent rejection.[53] When methods of lymphocyte culture and tests of functional alloreactivity become routine diagnostic tools they could replace the in-situ characterization of mononuclear subpopulations.

One concludes that the determination of mononuclear subsets is of little practical value in the routine diagnosis of acute cardiac rejection. Ordinary routine laboratory stains (haematoxylin-eosin, Unna-Pappenheim) yield sufficient information for the detection and grading of acute rejection changes in both frozen sections and paraffin embedded biopsies.

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APPENDIX

TABLE A-1 PEARSON'S SIMPLE CORRELATION METHOD

1. ALL CASES N = 129

CORRELATIONS	R SCORE	LYMPH	T4T8S
R SCORE	1.0000	.2785**	.2675*
LYMPH	.2785**	1.0000	.8858**
T4T8S	.2675*	.8858**	1.0000

2. NEGATIVE FOR REJECTION N = 31

CORRELATIONS	R SCORE	LYMPH	T4T8S
R SCORE	1.0000	-.2251	-.2047
LYMPH	-.2251	1.0000	.9150**
T4T8S	-.2047	.9150**	1.0000

3. MILD REJECTION N = 67

CORRELATIONS	R SCORE	LYMPH	T4T8S
R SCORE	1.0000	.2340	.2504
LYMPH	.2340	1.0000	.8838**
T4T8S	.2504	.8838**	I.0000

4. MODERATE REJECTION N = 13

CORRELATIONS	R SCORE	LYMPH	T4T8S
R SCORE	1.000	-.2324	-.1391
LYMPH	-.2324	1.0000	.7387*
T4T8S	-.1391	.7387*	1.0000

5. SEVERE REJECTION N = 2 INSUFFICIENT NUMBERS FOR STATISTICAL EVALUATION

6. PREVIOUS BIOPSY N = 12

CORRELATIONS	R SCORE	LYMPH	T4T8S
R SCORE	1.0000	.5013	.6255
LYMPH	.5013	1.0000	.8571**
T4T8S	.6255	.8571**	1.0000

SIGNIFICANCE: * - .01, ** - .001

ABBREVIATIONS:

R SCORE:	SEMI-QUANTITATIVE REJECTION SCORE;
LYMPH:	SEMI-QUANTITATIVE LYMPHOCYTE SCORE;
T4T8S:	CD4+/CD8+ SUBSETS TOTAL

TABLE A-2 MULTIPLE LINEAR REGRESSION

DEPENDENT VARIABLE : SEMI-QUANTITATIVE REJECTION SCORE
VARIABLES: : SEMI-QUANTIATIVE LYMPHOCYTE SCORE
: CD4+/CD8+ SUBSET TOTALS

ANALYSIS OF VARIANCE

DIAGNOSTIC GROUPS	R SQUARE	F	SIGNIF F
ALL CASES	.07957	5.44618	.0054
NEGATIVE FOR REJECTION	.05068	.74745	.4828
MILD REJECTION	.06345	2.16783	.1228
MODERATE REJECTION	.05635	.29859	.7483
SEVERE REJECTION	~	~	~
INADEQUATE BIOPSY	~	~	~
PREVIOUS BIOPSY SITE	.39588	2.29360	.1714

~ INSUFF. DATA FOR STATISTICAL ANALYSIS

TABLE A - 3 **ENDOCARDIAL LYMPHOCYTIC INFILTRATES**

ANALYSIS OF VARIANCE

	N	%	MEAN REJECTION SCORE	STANDARD ERRORS
GROUP A	8	25.8	.6875	.35527756
GROUP B	18	58.1	1.2778	.300379
GROUP C	5	16.1	1.5	.2738736
TOTAL	31	100		

GROUP A: ENDOCARDIAL INFILTRATES
GROUP B: ENDOCARDIAL INFILTRATES WITH ENDOMYOCARDIAL PERIVASCULAR AGGREGATES AND INTERMYOCYTE EXTENSION
GROUP C: ENDOCARDIAL INFILTRATES WITH ENDOMYOCARDIAL PERIVASCULAR AGGREGATES