AN ASSESSMENT OF THE MATRIX 96 DIRECT BETA COUNTER FOR CYTOTOXIC T LYMPHOCYTE PRECURSOR FREQUENCY ANALYSIS IN HUMAN CARDIAC TRANSPLANT RECIPIENTS

by

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
ATG	anti-thymocyte globulin
^{51}Cr	^{51}Chromium
CAD	coronary artery disease
CCM	complete culture medium
CI	confidence interval
CML	cell-mediated lympholysis
cpm	counts per minute
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T lymphocyte precursor
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
EBV	Epstein Barr virus
EMB	endomyocardial biopsy
FasL	fas-ligand
FBS	foetal bovine serum
HBSS	Hanks' balanced salt solution
HLA	human leukocyte antigen
HTL	helper T lymphocyte
HTLp	helper T lymphocyte precursor

LIST OF ABBREVIATIONS (CONTD.)

IFN-γ	interferon-gamma
IL-2	interleukin-2
IL-4	interleukin-4
ITIM	immunoreceptor tyrosine-based inhibitory motif
iv	intra venous
KIR	killer cell inhibitory receptor
LAK	lymphokine activated killer
LDA	limiting dilution analysis
LFA	lymphocyte function-associated antigen
MHC	major histocompatibility complex
MLC	mixed lymphocyte culture
MNC	mononuclear cell
mRNA	messenger ribonucleic acid
NF-AT	nuclear factor of activated T cells
NK	natural killer
PHA	phytohaemagglutinin
TCGF	T cell growth factor
TCR	T cell receptor
TNF	tumour necrosis factor
+ve	positive
-ve	negative

SUMMARY

This study was undertaken to assess the Matrix 96 Direct Beta Counter for the measurement of ^{51}Chromium (^{51}Cr)-release from cytotoxic T lymphocyte precursor (CTLp) frequency assays of human cardiac transplant recipients. The Matrix 96 was compared with a gamma counter for the measurement of maximal ^{51}Cr-release from graded numbers of labelled target cells. Repeated counts by both counters were consistent. Variation between the Matrix 96 and the gamma counter was similar to the variation between repeated counts using the gamma counter, indicating that the counters were comparable for the measurement of ^{51}Cr. The contribution of natural killer-like activity to target cell killing in the CTLp assay was investigated by the use of the K562 cell line in cold target competition assays. Ten responder-stimulator combinations were tested in the CTLp assay for cross-reactivity between K562 and allogeneic targets. Cytotoxicity was reduced in only one assay, suggesting that the cells which mediated lysis of allogeneic lymphocytes and K562 cells recognised distinct targets. Donor- and 3rd party-reactive CTLp frequencies in 15 human cardiac allograft recipients were then measured in samples taken before transplantation, and at two intervals after transplantation. In the group of patients, donor-reactive CTLp frequencies decreased by 3 to 7 months after transplantation, but increased to pre-transplant levels by 9 to 15 months after transplantation. Donor-specific decreases in CTLp frequencies relative to the pre-transplant frequencies were found in three patients by 9 to 15 months after transplantation. Pre-transplant donor-reactive CTLp frequencies were not predictive of acute rejection in the first 3 months or in the first year after transplantation. Donor-reactive CTLp frequencies were lower at 9 to 15 months after transplantation in patients

with concurrent endomyocardial biopsy (EMB) grade 2 or 3 rejection than in patients without rejection (EMB grade 0 or 1). Similarly, a negative correlation was found between donor-reactive CTLp frequencies at 9 to 15 months after transplantation and the number of EMB graded ≥2 in this period. Donor-reactive CTLp frequencies were not significantly different in patients who received induction immunosuppression with anti-thymocyte globulin (ATG) and in patients who received standard triple immunosuppression. The conclusion of all these experiments was that the Matrix 96 was comparable to the gamma counter for the measurement of ^{51}Cr-release and was a rapid and convenient method for counting large numbers of limiting dilution assays.

INTRODUCTION

1. The measurement of ^{51}Chromium-release in cell-culture supernatants using the Matrix 96 Direct Beta Counter.

Cytotoxic lymphocytes are cells of the immune system which can kill certain pathogen-infected cells, tumour cells and transplanted tissues (1). The cytotoxic activity of such cells is measured *in vitro* using cytolytic assays. Cytotoxic lymphocytes are cultured with target cells which have been labelled internally with a radioisotope or non-radioactive label. After four hours or longer incubation the amount of label released to the supernatant from lysed target cells is measured, giving an indication of the cytotoxic activity (2, 3).

The standard method of measuring cell-mediated cytotoxicity is by labelling target cells with the radioisotope ^{51}Cr in the form of sodium chromate ($Na_2{}^{51}CrO_4$). The radioactivity released from lysed cells is usually counted using a gamma counter (2). Gamma rays released from a sample cause the release of scintillations of light from a crystal and a detector system sorts and counts the emissions according to their energies (4). ^{51}Cr is counted in the 240 - 400 nm wavelength band and the result is expressed as counts per minute (cpm). Depending on the design of the gamma counter, samples may be counted sequentially, with the disadvantage that when the assay is large it can take several hours to count all samples. Alternative methods have been described, including the detection of the non-radioactive label europium (3, 5) or the enzyme lactate dehydrogenase (6) after target cell lysis. Non-radioactive methods are not widely used,

however, and the measurement of ^{51}Cr-release remains the most common detection method in assays of cell-mediated cytotoxicity.

^{51}Cr decays by electron capture, emitting beta particles as well as gamma radiation (7). Beta particles emitted by ^{51}Cr have been measured by scintillation counting, in which the sample is added to a liquid or solid scintillant and is counted in a beta counter (8 - 10). The Matrix 96 Direct Beta Counter can be used to count beta particles released from ^{51}Cr directly, without the need for scintillants. The Matrix 96 is a computer controlled beta counter which can simultaneously count 96 samples in the format of a 96 well microplate. It uses ninety-six uniform and independent avalanche gas ionisation detectors operating in the Geiger-Muller and proportional counting modes to maximise counting efficiency for weak beta and gamma isotopes (11). Samples to be counted are transferred and dried onto a "spotting" plate, which consists of a flat metal plate on which the pattern of a 96 well plate has been printed with hydrophobic ink. Sample preparation is thus more readily achieved than with a standard gamma counter, for which samples need to be transferred to individual tubes, which are then sealed. As samples from all the wells of a culture plate are counted simultaneously, the entire assay can be counted in a shorter time than if samples were counted sequentially.

The Matrix 96 correlated well with a gamma counter for the measurement of ^{51}Cr-release from cell-mediated lympholysis assays of lymphokine activated killer cells with tumour cell line targets (12). Similar percentage cytotoxicity was also found when a gamma counter was compared to the Matrix 96 in a ^{51}Cr retention assay, in which unlysed cells were harvested onto a fibre filter before counting (13). However, the assessment of the Matrix 96 for the measurement of ^{51}Cr-release in the above study involved the use of

correlation co-efficients (12). Correlation co-efficients measure strength of linear association, which is not the same as agreement, so this is not the most appropriate approach to method comparison studies (14). Methods of assessing agreement which give a useful indication of the differences between two methods are more informative (14).

Frequencies of cytotoxic T lymphocyte precursors (CTLp) in mixed cell populations can be estimated by limiting dilution assays (15). In each CTLp assay cytotoxicity is typically measured in at least 192 culture wells (16, 17) and studies often involve the testing of many samples (18, 19), so many culture supernatants need to be counted for ^{51}Cr. The 96-detector design of the Matrix 96 is ideally suited for this purpose.

The use of the Matrix 96 for measuring ^{51}Cr-release in CTLp assays has not been reported. To determine its suitability for such assays, the Matrix 96 was compared with a gamma counter by measuring ^{51}Cr-release from graded numbers of detergent-lysed phytohaemagglutinin (PHA)-stimulated lymphoid blast cells which gave a range of values that could be expected in CTLp assays. The method of analysis assessed the agreement and repeatability of the counting methods to give a useful indication of the differences between the two methods. ^{51}Cr-release from CTLp assays of cardiac transplant recipients was then measured to assess the suitability of the Matrix 96 for counting large numbers of limiting dilution assays in a clinical setting.

2. CTLp frequency analysis in human cardiac transplant recipients.

2.1. Cytotoxic lymphocytes and cardiac transplantation.

2.1.1. Histocompatibility genes and molecules.

Experiments in tumour and skin grafting between inbred mouse strains led to the identification of a single genetic region mainly responsible for the rejection of tissues from a genetically different, or allogeneic, individual of the same species (20, 21). This region was named the major histocompatibility complex (MHC), and in the mouse was called H-2 (22). The human equivalent is the human leukocyte antigen (HLA) complex, which is located on the short arm of chromosome six (23). An antigen is defined as a molecule which can be specifically bound by an antibody molecule (24).

MHC genes are classified in two groups, class I and class II, based on structural similarities between the members of each group (25). MHC class I and class II genes encode cell-surface glycoproteins which are involved in immune recognition (26, 27). HLA-A, -B and -C class I genes, and certain loci of the HLA-DR, -DQ and -DP class II genes encode functional HLA molecules which are expressed on cell surfaces (28). The expression of HLA genes is codominant, that is, an individual expresses two HLA alleles at each locus, one inherited from each parent (29). HLA genes are highly polymorphic, numbering several hundred class I and II alleles (28).

Class I HLA molecules consist of a polymorphic, HLA-encoded α polypeptide chain of about 44kD which is non-covalently associated with beta$_2$-microglobulin, an invariant

12 kD polypeptide encoded on chromosome 15 (30, 31). Class II HLA molecules consist of two non-covalently associated, polymorphic, HLA-encoded polypeptide chains: an α chain of about 34 kD and a β chain of about 29 kD (32, 33). Both class I and class II HLA molecules contain immunoglobulin-like loops or domains formed by disulphide bonds (34, 35).

The three-dimensional structures of HLA molecules were determined using X-ray crystallography, which confirmed similar structures for class I and class II molecules. Notable was a cleft or groove formed by polypeptide in the form of β-pleated sheet and α-helices, in which was found a short peptide (36, 37). Most of the polymorphism in HLA molecules occurs in the peptide-binding regions which are formed by the α1 and α2 domains of class I molecules and the α1 and β1 domains of class II molecules (38, 39). The peptides bound by MHC molecules are usually derived from normal cellular proteins, but during infections the bound peptides may be derived from pathogens (40). Such foreign peptides bound by MHC molecules are recognised by T lymphocytes during an immune response to the pathogen (41, 42).

Class I HLA molecules are constitutionally expressed on many cells and tissues, but the expression of class II HLA molecules is normally restricted to a limited number of cell types, including macrophages, dendritic cells, monocytes, B cells, activated T cells and also human endothelial and epithelial cells (43). HLA class I and II molecule expression can be upregulated by cytokines, soluble mediators produced by cells of the immune system (44, 45).

Matching of HLA antigens between recipients and donors is beneficial in cadaveric renal transplantation (46, 47). The selection of HLA matched recipients for cardiac transplantation has been limited by the short preservation time of donor hearts, but retrospective studies have demonstrated an effect of HLA matching on graft survival and cellular rejection (48 - 50).

Certain genes outwith the MHC encode antigens which give rise to weak or slow rejection responses. Such antigens have been termed minor histocompatibility antigens, but these can nevertheless stimulate rejection of transplanted organs (51). Minor histocompatibility antigens are peptides derived from polymorphic cellular proteins which are naturally processed and presented by MHC molecules (52).

2.1.2. Lymphocytes.

Lymphocytes are cells of the immune system, present in the blood and lymphoid tissues, which can specifically recognise foreign antigens (53). Lymphocytes and mononuclear phagocytes form the mononuclear cell component of white blood cells, or leukocytes. The three major classes of lymphocytes are T cells, B cells and natural killer cells.

T cells express T cell receptors (TCR) which recognise foreign peptides presented in association with self MHC molecules on cell surfaces (54, 55). The receptor specific for antigenic peptide-MHC complexes which is expressed by the majority of T lymphocytes is a heterodimer consisting of α and β polypeptide chains. The $\alpha\beta$ TCR is associated with CD3 proteins and other invariant proteins to form the T cell receptor complex (56).

A minority of T cells express the γδ receptor, the function of which is not fully understood (57).

Each T cell clone expresses T cell receptors of a unique specificity (54). The repertoire of specificities of αβ T cells is formed during T cell development in the thymus. A large repertoire of immature T cells is generated by somatic rearrangement of TCR gene segments which can then be combined in different ways, with further diversity arising when the rearranged segments are joined (58). The mature T cell repertoire is then shaped by a process involving positive selection of self MHC-restricted T cells and clonal deletion of autoreactive T cells (59).

In addition to the antigen receptors and MHC molecules already referred to, membrane proteins expressed by lymphocyte populations can be identified by specific monoclonal antibodies which define "clusters of differentiation" or CD markers (60).

αβ T lymphocytes can be categorised as being of two major groups, depending on mutually exclusive expression on the cell surface of either CD4 or CD8 molecules. CD4 +ve T cells recognise antigen presented by class II MHC molecules and CD8 +ve T cells recognise antigen presented by class I MHC molecules (61, 62). Classically, CD4 +ve T cells are regarded as cytokine-secreting "helper" T cells, whereas CD8 +ve T cells are seen as cytotoxic cells (63). These distinctions are not absolute, however, as CD4 +ve T cells can also be cytotoxic (64, 65), and CD8 +ve T cells secrete certain cytokines, notably interferon-γ (IFN-γ) (66, 67). T cells can also suppress immune responses in certain circumstances, but this activity may be mediated by particular

functions of subsets of both CD4 +ve and CD8 +ve T cells rather than by a distinct lineage of suppressor T cells (68).

In contrast to the TCR, the B lymphocyte antigen receptor recognises soluble or cell-surface antigens without the requirement for processing and presentation by MHC molecules. The proliferation and differentiation of B lymphocytes in response to most protein antigens is, however, dependent on help from T lymphocytes (69). B lymphocyte antigen receptors consist of membrane-bound forms of immunoglobulin molecules (70). Plasma cells, the terminally differentiated form of B lymphocytes, secrete immunoglobulin molecules of identical specificity to the membrane-bound form that was the initial trigger by which the B lymphocyte was activated (71). Secreted immunoglobulin molecules, also known as antibodies, have a variety of effector functions, including neutralisation of antigen, activation of serum proteins of the complement system, and coating of pathogens for enhanced removal by macrophages (72).

Natural killer (NK) cells, the third major class of lymphocytes, are large granular lymphocytes which do not express the T cell receptor, CD3 or membrane-bound immunoglobulin but commonly express CD16 (the low affinity receptor for IgG) and CD56 molecules (73). NK cells can kill certain tumour cells and other normal cells and cultured cell lines without prior sensitisation and in a non-MHC restricted manner (74). NK cells can thus kill targets which do not express MHC molecules, such as the human haematopoietic tumour cell line K562 (75). The function of NK cells is not fully understood, but they may provide a defence against virus-infected or tumour cells with reduced or absent MHC class I expression (76).

2.1.3. T lymphocyte activation and differentiation.

Resting T cells which have not encountered antigen are known as naïve or precursor T cells, and need to be activated by foreign antigen to develop effector functions (77). Activation occurs following the presentation of antigenic peptide-MHC complexes to T cells on the surfaces of antigen-presenting cells (APC). Two kinds of signal are required from APC to activate naive T cells (78). One is the result of the engagement of the TCR and its CD4 or CD8 co-receptor with an immunogenic peptide-MHC complex. The second signal is provided by non-polymorphic molecules on the surface of the APC which deliver essential costimulation to T cells. The best characterised costimulators are CD80 and CD86, members of the B7 ligand family, which interact with CD28 on T cells (79). Interactions of other adhesion molecules such as lymphocyte function-associated antigen (LFA)-1 with intercellular adhesion molecule (ICAM)-1 and ICAM-3, and CD2 with LFA-3 also enhance activation, but appear to be of lesser importance than the CD28 - B7 interactions (77). Naïve CD4 +ve T cells can only be activated by APC which provide the necessary costimulatory signals, but once activated T cells seem not to require costimulation and are able to respond to some extent to any APC bearing the appropriate peptide-MHC complex (77, 80). T cells which have been activated *in vivo* are sometimes described as "committed". Mature dendritic cells express high levels of MHC molecules as well as adhesion and costimulatory molecules, and are particularly potent activators of naive T cells (81).

T cell receptor binding to the antigenic peptide-MHC complex together with costimulatory signals activates intracellular signal transduction pathways (82). Intracellular portions of the CD3-γ, -δ, -ε and -ξ chains contain immunoreceptor

tyrosine-based activation motifs which are phosphorylated by the protein tyrosine kinases Fyn and Lck of the Src family. Ligation of CD4 or CD8 co-receptors by their MHC molecule ligands activates associated Lck protein tyrosine kinases. The phosphorylated TCR receptor complex ξ chain binds and activates zeta-associated protein (ZAP)-70, a Syk-family member protein tyrosine kinase which is required for T cell activation (83). These processes lead to the formation of at least two second messengers, inositol triphosphate and diacylglycerol, which bring about an increase in the concentration of intracellular Ca^{2+} and activate protein kinase C (82). Ca^{2+} ions bind to the protein calmodulin, forming a calmodulin-Ca^{2+} complex which activates calcineurin, a protein phosphatase. Calcineurin dephosphorylates the cytoplasmic subunit of the nuclear factor of activated T cells (NF-AT), which moves to the nucleus and combines with nuclear NF-AT to form a functional transcription factor (84). Functional NF-AT promotes the expression of interleukin-2 (IL-2) (84), a key regulatory cytokine in T cell activation. Protein kinase C plays a role in pathways leading to the activation of transcription factors (85). TCR stimulation also activates the Ras protein, which initiates a pathway leading to the induction of a calcium-independent nuclear component of NF-AT (86). Other transcription factors expressed rapidly after T cell activation include NF-κB, c-Fos and c-Myc. These early-immediate events are followed within hours of stimulation by the expression of genes encoding several cytokines, including IL-2 and IFN-γ, the alpha chain of the IL-2 receptor, and various other receptors and adhesion molecules (87).

The expression of IL-2 and the high-affinity IL-2 receptor is required for T cell proliferation. The IL-2 receptor (IL-2R) consists of three subunits, the α, β and γ polypeptide chains (88). The high affinity form of the receptor consists of the α and β

subunits (89), together with the more recently characterised γ chain. The γ chain does not bind IL-2 directly but appears to be involved in signalling and forms the intermediate-affinity IL-2R together with the β chain (90). The IL-2R β and γ chains are associated with protein tyrosine kinases of the Jak, Src and Syk families which, when activated, initiate multiple signal transduction pathways (91). Signalling mediated by the binding of IL-2 to the high affinity IL-2 R promotes the transition of the T cell from the G_1 to the S phase of the cell cycle and so to cell division (92).

Differentiation to effector cells occurs several days after stimulation and is accompanied by the expression of late activation antigens such as MHC class II molecules (93) and members of the β1 integrin family (94). During this period cytotoxic T lymphocytes synthesise the lytic proteins which are later used in target cell killing (95).

The development of cytotoxicity by CD8 +ve T cells is usually dependent on help from CD4 +ve T cells (96, 97). This may be in the form of cytokines, of which IL-2 and IFN-γ are considered to have significant roles (98, 99). Other cytokines, including IL-4, IL-6, IL-7 and IL-12 have also been reported to provide help for the generation of CTL (100 - 103). It has also been suggested that the role of the helper T cell may be to deliver an activating signal to the APC, permitting the APC to stimulate the CD8+ve T cell directly (104). The interaction between CD40 ligand on helper T cells and CD40 on the APC is thought to provide such a signal (105 - 107). Certain subsets of CD8 +ve T cells can also be activated directly by dendritic cells in the absence of CD4 +ve T cells (108, 109). Costimulation by CD80 and CD86 can provide the second signal in this model (110). Such helper T cell-independent activation of CD8 +ve T cells may

result from the ability of the CD8 +ve T cell to produce and respond to IL-2 in an autocrine manner (111). The contribution of CD4 +ve-T cell-independent CD8 +ve T cells to *in vivo* alloreactivity in humans is not known, but HLA class II differences are usually considered necessary for reactivity in mixed lymphocyte culture (MLC) *in vitro* (112, 113).

After encountering antigen some T cells differentiate into memory cells. These are resting cells which develop effector functions on re-stimulation with specific antigen, producing a faster, stronger response than occurs with primary stimulation of naïve T cells (114). Memory T cells can be differentiated from naïve T cells by positivity or negativity for the different isoforms of the CD45 molecule. In humans memory T cells are chiefly characterised as CD45RO positive and CD45RA negative, whereas naïve T cells are CD45RA positive and CD45RO negative (115). Effector cells share some surface markers with memory cells, however, so effector and memory cells cannot be clearly distinguished from each other by phenotype alone (116). Memory T cells are present at higher frequencies than naïve T cells and have higher affinity for antigen (117). Memory T cells are also more readily activated than naïve T cells and require less co-stimulation: resting B cells and macrophages can activate memory T cells whereas naïve T cells require dendritic cells or activated B cells for activation (118, 119).

When resting T cells are stimulated through the TCR in the absence of costimulatory signals they become unresponsive to subsequent stimulation by APC which do express the required costimulator molecules. This state has been described as T cell clonal anergy, and is characterised by an inability of the T cell to produce IL-2 (120). T cell

anergy may contribute to peripheral tolerance towards self or allogeneic antigens (121). The anergic state can be reversed by stimulation with high concentrations of IL-2 (122).

2.1.4. T lymphocyte-mediated cytotoxicity.

T lymphocyte-mediated cytotoxicity is antigen-specific. Effector CTL recognise peptide-MHC complexes on target cells by their TCR complexes. The formation of a CTL-target cell conjugate is initiated by weak interactions between adhesion molecules such as CD2 and LFA-1 and their respective ligands LFA-3 and ICAM-1. TCR binding activates CD8, LFA-1 and very late activation integrins which bind their receptors, strengthening the conjugate and providing additional costimulatory signals (95, 123). Specifically recognised target cells are then killed by the CTL.

There are two main mechanisms by which lymphocytes kill target cells. The first is mediated by perforin and granzymes, soluble proteins which are released from cytoplasmic lytic granules in the CTL. TCR-mediated recognition and conjugate formation triggers the movement of the lytic granules towards the point of contact with the target cell, followed by exocytosis of the granules' contents (124). Perforin polymerises and forms pores in the target cell membrane which can lead to osmotic lysis and necrotic cell death (125). In the presence of perforin, granzyme proteases enter the target cell and activate proteolytic caspases (cysteine aspartases) which cleave cellular proteins leading to death by apoptosis (126). Apoptic cell death is characterised by chromatin condensation, DNA fragmentation and blebbing of the cell membrane (127).

The second main killing mechanism is mediated by the interaction between Fas-ligand (FasL) on the membrane of the CTL and the Fas receptor (CD95) on the target cell surface. Cross-linking of Fas by FasL initiates a cascade of protein interactions leading to the activation of intracellular caspases and the induction of apoptic cell death (128). Other related molecules, such as tumour necrosis factor (TNF) and TNF-related apoptosis-inducing ligand can induce apoptosis in a similar way to Fas after binding to a corresponding "death receptor" (129).

The effector CTL is not itself lysed in the killing process. After lysis, the CTL disengages the target cell and is able to kill additional targets (95). Effector CTL function is short-lived, however, and most effector CTL die within one to five days or become memory cells (130).

2.1.5. Natural killer cell-mediated cytotoxicity.

In contrast to T cells, which kill target cells recognised through the TCR, NK cells express inhibitory receptors which recognise polymorphic class I MHC molecules and prevent the lysis of recognised cells (131). In this way, NK cells are prevented from killing the individual's own normal cells. In humans, the inhibitory receptors are the CD94/NKG2 receptors, which belong to the C-type lectin superfamily, and the killer cell inhibitory receptors (KIR), which are members of the immunoglobulin superfamily (132). CD94/NKG2 receptors bind to HLA-E, a non-classical HLA class I molecule (133), whereas KIR recognise HLA-B and -C molecules (132). These receptors possess

immunoreceptor tyrosine-based inhibitory motif (ITIM) sequences in their cytoplasmic domains which mediate the inhibitory signals (134, 135).

The interactions through which NK cells are stimulated to kill target cells are not well understood. The ligation of membrane receptors such as CD2, CD69 and CD44 may provide activation signals (136 - 138). Other proteins which may function as activating receptors include NKR-P1 (139), certain members of the CD94/NKG2 family of receptor complexes (140, 141) and KIR with short cytoplasmic domains that lack the ITIM motif (142, 143). Receptors associated with such ITIM-lacking KIR may mediate activation of the NK cell (144, 145). The ability to lyse targets is thought to depend on the balance between the activating and inhibitory signals received by the NK cell (131, 146).

The mechanisms used by NK cells to kill target cells are the same as those used by CTL, namely granule exocytosis and Fas-L-mediated apoptosis (95, 147). NK cells can also be activated by ligation of their CD16 receptors by IgG antibodies coating a target cell. NK cells activated in this way mediate antibody-dependent, cell-mediated cytotoxicity (148, 149).

Peripheral blood or spleen lymphocytes stimulated with high concentrations of IL-2 acquire the ability to lyse a variety of target cells, including NK-sensitive and NK-resistant tumour cells, in a non-MHC restricted fashion (150, 151). Such cells have been called lymphokine-activated killer (LAK) cells and are NK-like cells or non-MHC restricted T cells (152).

The activity of NK cells can be regulated by cytokines. IL-2 enhances the cytotoxic activity of NK cells, and induces them to proliferate and produce several cytokines, including IFN-γ (153 - 155). The IL-2R β chain is expressed on most NK cells and mediates signal transduction when bound by IL-2 (156). The IL-2R α chain can be induced on most NK cells by IL-2 (157) and forms the high affinity IL-2R in conjunction with the β and γ chains. Enhanced cytotoxicity and LAK activity, and the induction of proliferation are mediated by the intermediate-affinity IL-2R, but the α chain and high-affinity IL-2R binding is required for maintenance of proliferation and cytokine secretion (158).

IL-6 enhances NK cell cytotoxic activity in mononuclear cell cultures by inducing IL-2 production (159). LAK activity is induced in peripheral blood mononuclear cell cultures by IL-7 (102, 160). IL-12 enhances NK activity in peripheral blood lymphocytes and stimulates IFN-γ production (161, 162), but has little effect on proliferation (163).

The interferons (IFN-α, -β, and -γ) can have varying effects on NK cell cytotoxicity. IFN-α and -γ can enhance NK cell cytotoxic activity alone or in combination with IL-2, but prolonged co-culture of IFN-γ and IL-2 for more than six days inhibits cytotoxicity (164, 165). TNF induces IL-2Rα expression, proliferation and LAK activity in NK cells in combination with IL-2 (166, 167). IL-4 inhibits the enhancement of NK cell cytotoxic activity and proliferation by IL-2 (168), but induces proliferation of NK cells in combination with IL-12 (169).

Cytokines mediate their effects by binding to specific cytokine receptors. Most cytokine receptors lack intrinsic protein tyrosine kinase domains, but induce intracellular signal transduction by the recruitment or activation of cytoplasmic protein tyrosine kinases (91).

CTL cultured in the presence of IL-2 can also develop non-MHC restricted cytotoxicity (170). Cells cultured in IL-2 that have NK activity have therefore been called cells with NK-like activity to distinguish them from freshly-isolated NK cells (171). This terminology has been adopted here to describe cells mediating the lysis of the NK cell target K562 after *in vitro* culture.

2.1.6. Allorecognition.

T cells recognise allogeneic MHC molecules in two main ways, termed direct and indirect allorecognition. In direct allorecognition, T cells recognise intact antigenic peptide-MHC complexes on the surface of allogeneic antigen presenting cells. The antigen-specific and allo-specific T cell repertoires overlap, suggesting that direct allorecognition is a result of cross-reactivity of self-restricted T cells (172, 173). About 1 - 10 % of T cells can respond to an alloantigen, a much higher frequency than is found for conventional antigens (174). This high frequency of allorecognition could be explained by two models of the interaction between T cells and allogeneic MHC: the multiple determinant model and the high ligand density model (175). In the multiple determinant model, the peptide bound by an allogeneic MHC molecule forms part of the antigenic determinant recognised by the T cell receptor. Different MHC molecules bind

peptides with distinct sequence motifs (176, 177), and each MHC molecule can bind in the region of 2000 to 10000 different peptides (178). Allogeneic MHC molecules therefore present a different set of bound peptides from self-MHC molecules. Thus a single allogeneic MHC molecule could give rise to many different antigenic peptide-MHC complexes and stimulate many different T cells. In the high ligand density model the T cell receptor interacts with antigenic determinants on the allogeneic MHC molecule itself, irrespective of the bound peptide. This could result from TCR specific for antigenic peptides presented by self-MHC cross-reacting with determinants on the TCR-contacting parts of an allogeneic MHC molecule (179). MHC molecules are present on APC at high density and so could be recognised by large numbers of T cells, including those with low-affinity receptors which would not normally be stimulated by lower density ligands (180). Peptide-specific and MHC-specific allorecognition could operate in different circumstances, depending on the structural similarity of the responder and stimulator MHC molecules. When responder and stimulator MHC molecules are similar, the bound peptide may be the major recognition element, but when the MHC molecules are very different, determinants on the allogeneic MHC molecule itself may contribute more (179).

In the second allorecognition pathway, T cells recognise alloantigen-derived peptides presented by host APC in the context of self-MHC in a process known as "indirect" presentation (181). This is analogous to conventional self-MHC restricted antigen recognition.

CD8 +ve cytotoxic T lymphocytes recognise allogeneic target cells directly, but indirect presentation of alloantigens may contribute to CD4 +ve T cell helper or regulatory

responses (182, 183). Helper T lymphocytes activated by the indirect presentation pathway can induce delayed-type hypersensitivity (DTH)-type responses, CD8 +ve T cell-mediated cytotoxicity and antibody production by B cells in animal models (184).

2.1.7. Cardiac allograft rejection.

Allograft rejection can take different forms which are defined on the basis of the time after transplantation at which they occur and the histopathology of biopsy specimens or of the rejected organ (185).

Hyperacute rejection occurs within hours of transplantation and is mediated by pre-existing antibodies that bind to graft vascular endothelium (186, 187). The binding of antibody activates complement and effects changes in vascular endothelial cells, leading to increased vascular permeability, the attraction of phagocytes, platelet aggregation and the attraction of inflammatory cells such as neutrophils and platelets (188). In cardiac allografts hyperacute rejection is characterised histologically by disruption of the microcirculation with interstitial haemorrhage and oedema (189, 190) and results in heart failure. Hyperacute rejection of transplanted hearts is rare (191 - 193), but has been reported after the transplantation of blood group ABO incompatible hearts (194), in some but not all patients with a positive lymphocytotoxic crossmatch (190, 193, 195, 196), and in some patients with a negative lymphocytotoxic crossmatch but with antibody to vascular endothelial cells or other myocardial proteins (187, 197, 198).

Acute cardiac allograft rejection occurs most frequently in the first few months after transplantation, and is infrequent after the first year (199, 200). Acute cellular rejection is monitored by endomyocardial biopsy (EMB) (201) and is characterised by infiltration of the heart by inflammatory leukocytes, accompanied by myocyte damage in the moderate and severe forms. The cellular infiltrate can comprise lymphocytes, including CD4 +ve and CD8 +ve T lymphocytes, B lymphocytes and NK cells; monocytes and macrophages; and granulocytes (202 - 204).

CD3 +ve T cells increase in number in biopsies showing histological rejection, but the relative proportions of CD4 +ve and CD8 +ve T cells observed is influenced by the type of immunosuppression received by the patients (205). Before the introduction of cyclosporin A (CsA), CD8 +ve T cells predominated during rejection, but a mixed infiltrate of CD4 +ve and CD8 +ve T cells is seen in CsA-treated patients (202, 206, 207). The activation status of graft infiltrating cells has also been studied. An immunohistological analysis of CD45 isoforms in EMB showed that during mild rejection naïve CD45RA, CD4 +ve T cells predominated, whereas during moderate rejection there was a shift towards activated CD45RO T cells, with the greatest increase in CD45RO, CD8 +ve cells (208). Activated CTL express perforin and granzymes and both mRNA encoding these proteins, and the proteins themselves have been found in association with histological rejection (209, 210).

Increased expression of HLA class I and class II molecules is found in EMB, often but not always corresponding with histological rejection (205). HLA class I is upregulated on the myocardial plasma membrane and intercalating discs, and HLA class II is upregulated on vascular endothelium and on the endocardium (205). Increased HLA

expression is presumably induced by cytokines released from infiltrating cells. The expression of adhesion molecules ICAM-1 and vascular cell adhesion molecule (VCAM)-1 are also upregulated in EMB during rejection and are involved in migration of lymphocytes through the endothelium and into the graft (211).

Anti-heart and donor cell-reactive antibodies have been found in association with cellular rejection and may contribute to acute rejection (198, 212, 213). Antibody-mediated acute ("vascular") rejection has also been described in the absence of cell-mediated rejection (214).

Chronic rejection of cardiac allografts takes the form of accelerated coronary artery disease (CAD) (215). CAD is visible by angiography in 10 - 15 % of patients after one year and 40 - 50 % of patients after five years (216, 217) but as many as 75 % of patients show intimal thickening at 1 year by intravascular ultrasonography (218). Both humoral (219, 220) and cell-mediated (221, 222) mechanisms may be involved in the pathogenesis of CAD, although non-immunological factors also contribute (215).

Rejection in recipients previously unsensitised to donor alloantigens is initiated by host T cells which have been activated by alloantigens of the graft (223). The cells thought to be primarily responsible for the initiation of rejection by the direct allorecognition pathway are MHC class II-positive "passenger" leukocytes of donor origin which are present within the transplanted graft (224, 225). Dendritic-like cells have been found in normal rat and mouse hearts (226, 227), and in normal human hearts, although in the latter they form a minority of class II-positive cells (228). In mice, dendritic leukocytes have been shown to migrate from transplanted hearts to the spleen, where central

activation of T cells could occur (227). It is also possible that T cells could be activated by donor passenger leukocytes within the graft itself (229).

Graft endothelial cells may be able to directly activate alloreactive memory, but not naive T cells (230), and although the response is relatively weak it may increase the number of activated T cells in the graft (231). Approximately 20 % of the primary CTL precursor response to class I alloantigen can be activated by antigen alone, without costimulation, and so may represent cross-reactive memory CTL primed by environmental antigens (119).

Activated helper T cells of the host release cytokines which to act the recipient's lymphoid cells and on graft tissue to promote the effector mechanisms that result in rejection (232 - 234). T cell-derived cytokines provide help for the activation of CTL and antibody production, and bring about DTH-type responses through inflammation and macrophage activation (185). CD4 +ve T cells may also be directly cytotoxic to the graft (235).

If prior sensitisation to donor alloantigens has occurred, donor antigen-reactive memory T cells may also be present, and as discussed above, memory T cells are more readily activated than naïve T cells. Memory CD8 +ve T cells are also less dependent on CD4 +ve T cells for help and both memory CD4 +ve and CD8 -ve T cells are more potent mediators of rejection than naïve T cells (233, 236, 237).

2.1.8. The role of cytotoxic T lymphocytes in cardiac allograft rejection.

Whether allograft rejection is mediated by specific CTL or by CD4 +ve T cell-mediated DTH-type mechanisms has been the subject of debate between workers studying animal models of transplantation and rejection (238, 239). The rejection of organ allografts can be delayed or abrogated in animals depleted of CD4 +ve T cells but not in animals depleted of CD8 +ve T cells (240 - 243). However, there is evidence from adoptive transfer studies of collaboration between CD4 +ve and CD8 +ve T cell subsets in rejection (244 - 246), and for antigen-specific as well as non-specific tissue damage (247 - 249). It is now generally accepted that both CD4 +ve and CD8 +ve T cells contribute to allograft rejection by a combination of CTL-mediated and DTH-type mechanisms (250, 251). The relative contributions of CD4 +ve and CD8 +ve T cells to rejection in different models may depend on the degree of dependence of CD8 +ve T cells on CD4 +ve T cells for help (250), the type of graft, the nature of the differences between donor and recipient MHC and the sensitisation state of the recipient (251). A further possible rejection mechanism identified in rodents involves the presentation of processed allogeneic class I MHC molecules by host APC to CD4 +ve T cells, which then provide help to B cells for alloantibody production (252).

Graft-infiltrating lymphocytes cultured from endomyocardial biopsies have provided evidence for a role for CTL in the acute rejection of human cardiac allografts (253). Donor-specific CTL can be present in rejection-free allografts (254, 255). However, higher frequencies of donor-specific CTL were found in graft-infiltrating lymphocytes from patients who had rejection episodes compared to those without rejection (256,

257). Moreover, graft-infiltrating committed CTL with high avidity for donor HLA class I and class II antigens were present during rejection episodes, whereas in the absence of rejection low avidity cells predominated (235, 258). The differences in avidity of CTL may thus explain why alloreactive CTL can be present in grafts without histological rejection (258). Graft-infiltrating lymphocytes have also been shown to lyse donor heart endothelial cells (259), although the ability of CTL to directly injure myocytes is not proven (260, 261). The expression of perforin and granzymes in graft-infiltrating lymphocytes is also associated with rejection and provides further evidence for a direct role of CTL (209, 210).

2.1.9. Immunosuppression.

In clinical transplantation the administration of drugs which suppress the recipient's immune system is necessary to prevent allograft rejection. Different combinations of drugs have been used at different centres, but triple therapy with CsA, azathioprine and corticosteroids has been common in the 1980s and 1990s (262). These drugs formed the basis of the immunosuppression given to patients in the present study.

CsA, azathioprine and steroids act in complementary ways to suppress allograft rejection. CsA inhibits T cell activation by blocking signal transduction pathways involved in the expression of the IL-2 gene. It does this by blocking the action of calcineurin (263). CsA binds to the cytoplasmic protein cyclophilin, an isomerase involved in protein folding. The CsA-cyclophilin complex then binds active calcineurin

and inhibits its activity, preventing the dephosphorylation of cytoplasmic NF-AT and its translocation to the nucleus, where it is required for gene transcription.

An alternative mechanism of CsA action has been proposed, based on the observation that CsA stimulates the production of transforming growth factor (TGF)-β, a cytokine with immunosuppressive activity (264). This effect is mediated by TGF-β-dependent induction of the cell cycle inhibitor p21 (265).

Azathioprine is metabolised into the purine analogue mercaptopurine which intercalates into a cell's DNA. It inhibits de novo purine synthesis, the synthesis of DNA and RNA, and can create breaks in chromosomal DNA (266). Azathioprine is more active on metabolically active cells, and particularly on replicating cells, whose proliferation is thereby inhibited (267)

Steroids used in transplantation include methylprednisolone, which is given intravenously, and the oral preparations of prednisone and prednisolone. Their immunosuppressive action involves several mechanisms, including a potent anti-inflammatory effect, and the inhibition of macrophage function and T lymphocyte activation (266). Steroids are bound by receptor molecules in the cytoplasm and transported to the nucleus (268). There, the steroid-receptor complexes bind to glucocorticoid response element sites in the promoter regions of specific genes and regulate gene transcription (269). Steroids inhibit the secretion of lymphokines and inflammatory mediators by macrophages (266), and also directly inhibit T cell activation by blocking IL-2 gene expression and signal transduction through the IL-2 receptor (270, 271).

Antibodies directed towards lymphocytes or to specific lymphocyte antigens have also been used for immunosuppression after transplantation. Polyclonal antisera are produced by immunising animals such as rabbits, goats or horses with human lymphocytes derived from lymph nodes, yielding anti-lymphocyte globulin (ALG), or with thymus gland lymphocytes, yielding anti-thymocyte globulin (ATG) (266). Monoclonal antibodies to specific T cell markers such as CD3 (known as OKT3) have also been used in attempts to selectively manipulate the immune response to the allograft (272).

Polyclonal anti-thymocyte globulin contains antibodies specific for numerous T cell-specific and non-T cell specific cell-surface molecules (273). Anti-lymphocyte serum was historically thought to selectively deplete circulating lymphocytes by complement-mediated lysis and opsonisation and phagocytosis in the liver (274 - 276). More recently, it has been proposed that the mechanism of ATG-mediated suppression could also involve the engagement of multiple T cell surface receptors resulting in partial activation and anergy (273, 277, 278). The immune response to an allograft regenerates after treatment with anti-lymphocyte serum (279, 280), but lymphocyte subsets can be altered for extended periods. Decreased CD4 : CD8 ratios were seen for up to five years in anti-thymocyte globulin treated renal transplant recipients as a result of depletion of CD4 +ve T cells and increased repopulation of CD8 +ve T cells (281).

Anti-lymphocyte antibodies have been used as induction therapy given for a short period immediately after transplantation to prevent early rejection (282), and for the treatment of acute rejection episodes (283). Some patients in the present study received induction immunosuppression with ATG as part of a study of the effectiveness of this agent.

2.1.10. Transplantation tolerance.

Transplantation tolerance has been defined as "the indefinite survival of a graft in the absence of ongoing immunosuppression" (284). This state has been achieved experimentally in animals (285, 286) and has been demonstrated in a small number of human transplant recipients (287, 288).

Several different mechanisms may contribute to the induction and maintenance of transplantation tolerance. Potential mechanisms include the deletion of developing T cells by the introduction of allogeneic cells or peptides to the thymus; the deletion of T cells in the periphery; the induction of immunological ignorance by blocking costimulation; T cell anergy; and T cell-mediated suppression (289).

The survival of solid organ allografts with continued immunosuppression has sometimes been called "graft acceptance" or "operational tolerance" (290, 291), although the distinction between tolerance and acceptance is not always made.

Human transplant recipients initially require intense immunosuppression to prevent rejection, but doses are reduced after the first few weeks and, in the longer term, individual components such as steroids are sometimes stopped completely (292). Many studies have shown reduced cellular immune responses of patients towards their donors after transplantation (293). It is apparent, therefore, that some form of adaptation of the recipient's immune system to the graft can occur in immunosuppressed patients.

Reduced immune reactivity to the donor is also sometimes referred to as tolerance (294, 295). It is perhaps more accurate, however, to refer to this as hyporeactivity or unresponsiveness of the particular cell or process studied, since in most cases immunosuppression is not withdrawn to determine if "true" transplantation tolerance has developed.

2.2. Cellular assays of direct allorecognition.

2.2.1. Mixed lymphocyte culture.

When lymphocytes from two unrelated individuals are cultured together *in vitro* some of the cells are transformed into proliferating lymphoblasts (296, 297). Proliferation in MLC is stimulated mainly by differences in the HLA class II region (112, 298). The degree of proliferation is related to the degree of mismatching for HLA class II antigens between the individuals tested (299 - 301).

The MLC was first used as a cellular compatibility test before living related renal transplantation (302, 303), and has since been used to monitor immunological reactivity between patient and donor after solid organ transplantation. In several studies reduced donor-specific responses ("hyporesponsiveness") in MLC were found in some organ allograft recipients (292, 304 - 308) and in some studies this was associated with the absence of rejection or a reduced incidence of rejection (292, 305 - 308).

2.2.2. Cell-mediated lympholysis.

Some of the lymphocytes which proliferate in MLC are cytotoxic to cells of the stimulator (309). These cytotoxic lymphocytes are predominantly directed towards class I MHC molecules, although class II-specific CTL can also occur, but class II differences are necessary for T cell proliferation and the generation of cytotoxicity (113, 310). Cell-mediated lympholysis (CML) can be assayed quantitatively by measuring the cytotoxicity of MLC-generated effector cells at defined effector to target cell ratios (311).

CML assays have also been used for immunological monitoring after transplantation, and were found by some investigators to correspond better to clinical events than MLC (312 - 314). More transplant recipients are hyporesponsive to donor antigens by the CML assay than by the MLC assay (313, 315, 316) and hyporesponsiveness is found more consistently by CML than by MLC, in which responses appear to vary (317 - 319). Overall, donor-specific hyporeactivity is found in about 70 % of recipients by the CML assay, and in about 30 % of recipients by the MLC assay (293).

Although CML hyporesponsiveness is frequently observed after transplantation, the clinical value of the CML assay is uncertain. In a large study CML non-responsiveness had only limited predictive value for graft survival, moreover rejection occurred in some patients who were non-responsive in the CML assay (320).

CML hyporesponsiveness may theoretically occur as a result of hyporesponsiveness of either CD8 +ve or CD4 +ve T cells, which could explain the higher prevalence of hyporesponsiveness by CML compared to MLC. However, CML reactivity has been

reported despite reduced MLC responses (313), so there may be other reasons for the different prevalences of hyporesponsiveness. It has been suggested that MLC reactivity could be stimulated by indirect as well as by direct presentation pathways, whereas cytotoxic T cells mainly recognise targets directly (293), but there is conflicting evidence regarding MLC stimulation by the indirect pathway after transplantation (321, 322). Different regulatory mechanisms may also function in MLC and CML responses. The involvement of suppressor cells in CML hyporesponsiveness has been proposed in several reports (317, 319, 323).

2.2.3. Limiting dilution analysis of T lymphocytes.

Frequencies of T lymphocyte precursors in various mixed cell populations can be estimated by the technique of limiting dilution analysis (LDA) (15). Limiting dilution assays are quantitative, and are thus more informative than semi-quantitative assays such as MLC and CML. LDA has, to a large extent, replaced MLC and CML assays in post-transplant monitoring, and has provided a more detailed analysis of the relationship between helper and cytotoxic T cell subsets after transplantation.

Limiting dilution assays have been applied to the study of CTLp in bone marrow and solid organ transplantation (18, 19, 324), and allospecific T cell repertoires (325, 326). Limiting dilution analysis has also been used to quantify helper T lymphocyte sub-populations, including precursors of IL-2 producing cells (referred to as the helper T lymphocyte precursor assay, HTLp) (327) and precursors of cells which produce other cytokines, such as IL-4 (328).

In limiting dilution assays cells from the test population are cultured in replicate in a series of dilutions together with defined stimulator cells. Culture conditions are optimal for the proliferation of the precursor cell being studied. After a period of incubation to allow the precursor cells to proliferate, the proportion of cultures at each dilution which received the precursor cell of interest are determined by assaying effector function in each replicate culture (329).

Poisson probability statistics are applied to these data to provide an estimate of the frequency of the precursor cell in the original population (330). When cells from a homogeneous cell suspension are distributed between culture wells in identical volumes, the number of precursor cells in individual replicate cultures varies due to random distribution with a probability described by the Poisson distribution. Interpretation of limiting dilution assays is based on the fraction of replicate wells at each dilution which are negative for effector function, because these are known to have received no precursor cells, whereas the number of precursors received by a positive well is not known. A minimum of 20 replicates are required at each dilution to give reliable frequency estimates (330).

The proportion of negative wells at each dilution is represented by the zero term of the Poisson distribution, which predicts that 37 % of culture wells will be negative when there is an average of one precursor cell per well (329). Each dilution of the original population yields a different fraction of negative cultures. Different statistical treatments of the data to give the frequency estimate have been used, including least squares fitting (329) and minimum chi square methods (331), but the maximum likelihood method is

widely accepted as the procedure of choice (330, 332). Maximum likelihood estimation is a complex, iterative procedure, which is usually performed by calculator or computer.

For the frequency estimate to be valid, the assay conditions should be such that the precursor cell type whose frequency is being estimated is the only limiting factor in the culture system, and a single precursor cell should be able to generate a detectable response. This is known as the single hit model (330). Conformity to single-hit kinetics is tested for each assay by generating a chi^2 goodness-of-fit statistic and determining the corresponding p value.

The frequency of allospecific CTLp is determined by culturing the test population with allogeneic stimulator cells in the presence of interleukin-2. Lysis of stimulator-type target cells after 7 to 10 days of culture indicates that one or more CTL precursors were present in a culture well (17).

The ability of every human T cell to undergo clonal expansion under appropriate culture conditions was demonstrated using a limiting dilution assay in which human peripheral blood T cells were cultured in medium containing T cell growth factor (TCGF) and PHA, which activates most T cells (333). Most of the CTLp in peripheral blood belong to the CD8 +ve T cell subset and are HLA class I restricted (16, 334). However, high frequencies of class II-specific CTL are found in CD4 +ve enriched populations (335). Alloreactive CTLp can be specific for mismatches at the HLA-A, -B and -Cw loci (336 - 339) and can also recognise subtypes of HLA class I antigens (340, 341). CTL specific for the public HLA class I determinants Bw4 and Bw6 are not detected (337, 342). CTLp specific for minor histocompatibility antigens are present at very low frequencies

in unsensitised individuals (343, 344), but can be detected at high frequencies after *in vivo* sensitisation (345, 346). CTLp frequencies between HLA-identical siblings are very low (less than 5 per million peripheral blood mononuclear cells (MNC)) or undetectable (347, 344). Those few precursors that do react in HLA-identical combinations may be specific for mismatched minor histocompatibility antigens.

2.2.4. CTLp assay methods.

Different methods have been used for limiting dilution analysis of CTLp (15). The addition of a source of TCGF was shown to be essential in all methods (16, 17, 325). In earlier studies supernatant from PHA-stimulated cells or MLC was used for this purpose (16, 325, 333), but it was later shown that IL-2 is the only lymphokine necessary for the expansion of alloreactive CTLp (17). Interleukin-4 (IL-4) did not increase the CTLp frequency and interleukin-1 was inhibitory. Moretta *et al.* found the highest clonal efficiency when TCGF was added after two days of culture, and Kaminski *et al.* found that the addition of low concentrations (5 units/ml) of IL-2 on days three and six gave optimal CTLp frequency without non-specificity (17, 333). The use of filler cells was shown to be unnecessary (17).

CTLp frequencies have been determined in populations of peripheral blood MNC and T cells purified by positive (16, 333) and negative (335) selection methods. Cells used as stimulators in the CTLp assay have included peripheral blood MNC, spleen MNC and Epstein Barr virus (EBV)-transformed B cell lines. B cell lines are stronger stimulator

cells than peripheral blood MNC, but also induce high levels of non-specific reactivity (348).

Mitogen-stimulated T cell blasts are commonly used targets for cytotoxic activity in the CTLp assay, although some researchers have preferred EBV-transformed B cell lines or unstimulated peripheral blood MNC (337, 348). Higher frequencies of specific alloreactive CTLp are detected after ten days of culture than after six or seven days of culture, especially in combinations which give rise to low CTLp frequencies (16, 17).

The CTLp assay methods referred to above do not distinguish between *in vivo* activated CTL, naïve CTL precursors or memory cells. CTL of all these activation states which can proliferate and develop effector function *in vitro* are detected. *In vivo* activated, committed CTL can be distinguished from naïve CTLp by their ability to grow in culture with IL-2 in the absence of allogeneic stimulator cells (349). Other modifications to the CTLp assay permit the identification of CTL with differing avidity for target cells, based on the susceptibility to inhibition by anti-CD4 or anti-CD8 antibodies (258, 350), or the identification of CTL with differing susceptibility to immunosuppressive drugs (351, 352). In this context, avidity is defined as the overall strength of binding between a T cell and an APC or target cell, and reflects the interactions between T cell receptors and antigenic peptide-MHC complexes, and between CD4, CD8 and other accessory molecules and their ligands (235, 353).

2.2.5. The contribution of natural killer cell-like activity to estimates of CTLp frequency.

For the CTLp frequency estimate to be accurate, the cytotoxicity measured in the assay should be mediated by CD8 +ve or CD4 +ve CTL which specifically recognise HLA class I or class II molecules on the surface of the target cells. Other forms of cytotoxicity, such as NK cell-like activity and LAK activity, which are not MHC restricted, have been found after MLC (354, 355) and could influence CTLp frequency estimates if cytotoxic to allogeneic targets.

LAK activity can be induced *in vitro* by high concentrations of IL-2 (greater than 1 nM or 30 International Units) (356). An IL-2 concentration of 5 units/ml was found to give minimal non-specific reactivity against autologous targets in the CTLp assay (17). The absence of lymphokine-induced non-specificity in the CTLp assay at low IL-2 concentrations has been further demonstrated by the absence of lysis of 3rd party or autologous targets in split-well assays (16, 325).

It has been suggested in several studies that NK-like activity is distinct from alloreactive cytotoxicity in MLC and in CTLp assay cultures. Allogeneic lymphocytes and K562 cells did not cross-compete as targets in cold-target competition assays with MLC-generated cytotoxicity (355, 357). In an HLA class II-specific CTLp assay, the depletion of CD56 +ve cells before culture did not affect the frequencies found, or specific lysis of allogeneic B lymphoblastoid cell line targets (335). Furthermore, cell-sorter purified CD8 +ve, CD16 -ve T cells and CD16 +ve, CD3 -ve NK cells cultured in limiting dilution with exogenous IL-2 were specific for allogeneic lymphoblasts or K562 cells respectively, with very little cross-reactivity (358). There is, however, at least one report

in which the depletion of CD56 +ve cells resulted in lower CTLp frequencies in two of three cases (359).

NK cells appear to have a repertoire of killing for allogeneic specificities (360, 361). Susceptibility of the target to lysis depends upon both the nature of the HLA mismatch between the responder and stimulator cells and the repertoire of inhibitory receptors expressed by the responder's NK cells (362, 363). Populations of NK cells generated after the culture of certain combinations of allogeneic lymphocytes can specifically lyse stimulator-derived T cell blasts (364, 365).

The involvement of NK-like activity in the CTLp assay was investigated in the present study using the K562 cell line. K562 is a haematopoietic tumour cell line which does not express HLA class I molecules and is a highly sensitive target for NK cell activity (75). Firstly, the K562 cell line was used as a target in split-well assays to assess the extent of NK-like activity in CTLp assay cultures. Then cold target competition assays were used to determine if K562 cells could inhibit lysis of specific allogeneic targets, and conversely, if allogeneic cells could inhibit lysis of K562 targets. Five different responder-stimulator combinations were tested in each case, using splenic stimulator cells and peripheral blood mononuclear responder cells, as used in the subsequent assays of cardiac transplant patients. Inhibition of cytotoxicity would suggest the involvement of NK-like activity in the lysis of allogeneic target cells and a possible contribution to CTLp frequency estimates.

2.3. The use of cellular assays of direct allorecognition for immunological monitoring after cardiac transplantation.

There are many reports of the use of cellular assays of peripheral blood lymphocytes for immunological monitoring after renal transplantation (293, 366), but fewer reports have been published relating to cardiac transplantation. Reinsmoen *et al.* found donor antigen-specific hyporesponsiveness in 6 of 27 cardiac transplant recipients using MLC. Hyporesponsiveness was associated with the absence of chronic rejection in these patients (308). However, van Besouw *et al.* found no differences in MLC or in frequencies of helper T lymphocytes between patients with and without chronic rejection, but found higher reactivity to a non-specific stimulus in patients with chronic rejection (367). Different stimulator cells were used in the two studies: Reinsmoen *et al.* used homozygous typing cells which shared HLA antigens with the donor, whereas Van Besouw *et al.* used transformed B cell lines from the donor. Van Besouw *et al.* also found comparable MLC responses before and after transplantation, independent of acute rejection (321). Wijngaard *et al.* reported CML nonreactivity to donor cells in 7 of 10 cardiac recipients in the first three years after transplantation, coincident with the absence of acute rejection (294).

Some investigators used limiting dilution assays of CTLp to examine donor-specific responses. Reader *et al.* found higher CTLp frequencies in samples taken during biopsy-diagnosed rejection than in samples taken in the absence of rejection, and some correspondence of CTLp frequencies with clinical events after transplantation (368). Hu *et al.* reported an overall donor-specific decrease in CTLp frequency within one year of transplantation, with individual patients showing different variations in frequency with

time (19). Whereas Hu *et al.* found that pre-transplant donor-specific CTLp frequencies correlated with rejection, Reader *et al.* did not. In contrast to the study of Hu *et al.*, Loonen *et al.* found no differences between CTLp frequencies in samples taken before and up to three years after transplantation (369). However, the same group found that CTLp with high avidity for donor cells were associated with rejection (370).

Peripheral blood HTLp frequencies have also been studied after cardiac transplantation. Increases in donor-reactive HTLp frequencies have been reported during rejection episodes (370, 371), and decreased HTLp frequencies were found one year after transplantation in patients who tolerated steroid withdrawal (372). Cattell *et al.* found decreased HTL frequencies in three of four cardiac recipients, and an increased frequency in one patient after rejection (373). Hornick *et al.* reported donor-specific hyporesponsiveness of helper T lymphocytes in five of ten cardiac recipients with chronic rejection, four of whom also had low frequencies of donor-specific cytotoxic T lymphocytes (374).

The limiting dilution assay methods used by these investigators varied. Differences in the concentrations of IL-2 added in the CTLp assays, in the timings of the addition of IL-2, and in the duration of culture make direct comparisons difficult. Different patient groups, immunosuppression regimens and the use of other treatments such as pre-transplant blood transfusions may also have contributed to the different results. The frequency of sampling and length of follow-up after transplantation also varied between the studies. Some studies attempted to correlate CTLp frequencies with acute rejection, which is most common in the first months after transplantation, and results from these studies might be expected to differ from those which looked for evidence of longer-term

graft acceptance. With the exception of the study of Hu *et al.*, serial changes in CTLp frequency were not reported for most patients. There is wide variation in the CTLp repertoire of different individuals towards mismatched HLA antigens (325), so it may be more informative to study serial changes in individual patients than to compare groups of frequencies at various time points.

The value of assays of peripheral blood CTLp in clinical cardiac transplantation is, therefore, uncertain. One reason for a lack of correspondence with rejection could be that lymphocytes in the peripheral blood may not reflect those within the graft (370). Frequencies of donor-specific CTLp (375) and committed CTL (376) are higher in graft infiltrating cells than from peripheral blood. Another possible explanation is that most studies have measured direct recognition of donor antigen presenting cells, which ignores the recent suggestion that that indirect allorecognition may also be involved in acute and chronic rejection of cardiac grafts (377, 378).

However, as already indicated, donor-specific hyporesponsiveness of peripheral blood lymphocytes was reported in some studies (19, 294, 308, 372 - 374) and is an established phenomenon in some recipients of other solid organ transplants (18, 293, 379, 380). Limiting dilution analysis of lymphocyte populations in cardiac transplant recipients could therefore be useful in the identification of patients who develop immunological hyporesponsiveness towards their graft (293), and assist in the elucidation of mechanisms of immunological acceptance of allografts (288, 381). Patients so identified as hyporesponsive to donor antigens could be candidates for the reduction of immunosuppression, thus minimising the increased risks of malignancy, infections and

other complications of immunosuppressive therapy which are found in transplant recipients (382).

The development of donor-specific hyporesponsiveness after cardiac transplantation was investigated in the present study by measuring donor- and 3rd party-reactive CTLp frequencies in peripheral blood samples from 15 patients. The sampling was designed to allow the comparison of pre-transplant CTLp frequencies with CTLp frequencies at two time points in the first year after transplantation. CTLp frequencies were also analysed for associations with EMB-diagnosed rejection, induction immunosuppression with ATG, and HLA mismatching between recipient and donor.

3. Aims.

The aims of this thesis were as follows. Firstly, to compare the Matrix 96 Direct Beta Counter with a gamma counter for measuring ^{51}Cr-release in cell culture supernatants, with regard to ease of use and rapidity for counting large assays, and to define a protocol for the application of the Matrix 96 to assays of clinical samples. Secondly, to apply the derived protocol to the clinically relevant question of cardiac allograft rejection and its monitoring by means of CTLp frequency analysis. Subsidiary objectives were, firstly, to identify and quantify the contribution of NK-like activity to CTLp frequency analysis in order to determine the specificity of the assay. Secondly, to use the Matrix 96 to measure ^{51}Cr-release from CTLp assays of samples taken from human cardiac transplant recipients before transplantation and on two occasions after transplantation. And finally, to analyse the CTLp frequencies obtained from cardiac transplant recipients for evidence of donor-specific immunological hyporesponsiveness and associations with acute cellular rejection.

METHODS

1. Reagents.

AB serum (heat inactivated, group AB serum from untransfused male donors; Quest Biomedical, Knowle, England).

Acetic acid (BDH Laboratory Supplies, Poole, England).

^{51}Cr (sodium chromate in sodium chloride soln., 7.4-18.5 GBq/mg Cr, 0.37-1.3 GBq/ml, order no. CJS4; Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England).

Crystal violet dye (Sigma-Aldrich Co. Ltd., Poole, England).

Dimethyl sulphoxide (DMSO; BDH Laboratory Supplies, Poole, England).

Eosin-Y (Sigma-Aldrich Co. Ltd., Poole, England).

Foetal bovine serum (FBS; Life Technologies Ltd., Paisley, Scotland).

L-glutamine (Life Technologies Ltd., Paisley, Scotland).

Hanks' balanced salt solution (HBSS; Sigma-Aldrich Co. Ltd., Poole, England).

Hydrochloric acid (HCl; BDH Laboratory Supplies, Poole, England).

IL-2 (human, recombinant (*E.coli*); Boehringer Mannheim UK, Lewes, England).

Medium 199 (with Earle's salts,100 mg/l L-glutamine and 5.96g/l HEPES; Life Technologies Ltd., Paisley, Scotland).

Benzylpenicillin sodium BP (Britannia Pharmaceuticals Ltd., Redhill, England).

Phytohaemagglutinin (PHA; Cat. no. HA16, Murex Biotech Ltd., Dartford, England).

Polysucrose/sodium diatrizoate solution (density 1.077; Histopaque-1077, Sigma-Aldrich Co. Ltd., Poole, England).

RPMI 1640 medium with sodium bicarbonate buffering (2 g/l $NaHCO_3$, pH 7.0-7.4, Life Technologies Ltd., Paisley, Scotland).

Sodium chloride, 0.9 % (Baxter Healthcare Ltd., Thetford, England).

Sodium heparin (Leo Laboratories, Princes Risborough, England).

Streptomycin sulphate BP (Evans Medical Ltd., Leatherhead, England).

Triton X-100 (BDH Laboratory Supplies, Poole, England).

Zapoglobin (Coulter Electronics Ltd., Luton, England).

The K562 cell line was a kind gift from Ms. A. Galvin, Dept. of Immunology, Queen's Medical Centre, Nottingham.

2. Media used for cell culture and washing.

Cell washing medium consisted of HBSS containing 100 units/ml penicillin and 100 μg/ml streptomycin.

Complete culture medium (CCM) consisted of RPMI 1640 medium, supplemented with 10 % vol/vol AB serum, 292 mg/l L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

3. Equipment and disposables.

Caesium 137 source (Gammacell irradiator, Molsgaard Medical).

Coulter DN counter (Coulter Electronics Ltd., Luton, England).

Cell culture plates, 24 well, flat bottom (Corning, Bibby-Sterilin, Stone, England).

Cell culture plates, 96 well, U-bottom (Costar UK Ltd., High Wycombe, England).

Cryogenic vials, sterile (LIP Ltd., Shipley, England).

Gamma counter (Packard Auto-Gamma 5650 gamma counter, Packard Instrument Co., Meriden, CT, USA).

Humidified incubator (RS Biotech, Finedon, England).

Matrix 96 Direct Beta Counter (Packard Instrument Co., Meriden, CT, USA).

Nalgene Cryo 1°C freezing container (McQuilkin & Co., Glasgow, Scotland).

Nylon fibre (3 denier, 3.81 cm, type 200; Fenwall Laboratories, Deerfield, Illinois, USA).

SpotWell-96 metal plate (Canberra Packard, Pangbourne, England).

25 cm^2 tissue culture flasks (Corning, New York, USA).

0.75 ml tubes (Luckham LP2, Life Sciences International, Basingstoke, England).

4. Cell preparation and cryopreservation.

Mononuclear cells from human peripheral blood and spleen were required in the subsequently described assays and were prepared as follows. Peripheral blood MNC were aseptically separated by density gradient centrifugation over polysucrose/sodium diatrizoate solution, then were washed once at 250 g for 15 min, then twice at 400 g for 5 min in washing medium. Spleen cells were aseptically recovered into washing medium by teasing apart the spleen tissue using forceps. Stromal elements were separated from the cell suspension by passing the mixture through a syringe containing loosely packed nylon fibre, which had previously been primed with 0.5 ml each of FBS and washing medium. The cells were collected and washed at 400 g for 5 min, then were resuspended in washing medium. MNC then were separated by density gradient centrifugation over polysucrose/sodium diatrizoate solution as described above.

Peripheral blood and spleen MNC were frozen in sterile cryogenic vials at a concentration of 10 to 20 x 10^6 cells/ml, in a mixture consisting of equal proportions of FBS and 20 % vol/vol DMSO in 0.9 % sodium chloride. The vials were frozen in a Nalgene Cryo 1°C freezing container overnight in a -70°C freezer, then were stored in the vapour phase of liquid nitrogen until required.

5. Determination of cell concentration and viability.

Cell concentrations were determined either by an automated method (Coulter DN counter) or manually. For manual counting the cell suspension was diluted with an equal volume of 2 % vol/vol aqueous acetic acid, coloured pale violet with crystal violet dye, and counted in an Improved Neubauer counting chamber. All cell concentrations for a specific patient's CTLp assays were quantified in the same way to eliminate inter-method variability. Cell viability was assessed by exclusion of the vital dye, Eosin-Y, and cells were used in the CTLp assay only if their viability was 90 % or greater. ^{51}Cr-labelled PHA stimulated blast cells were counted manually as described above, but using undiluted cell suspensions.

6. Preparation of ^{51}Cr-labelled PHA blasts.

Frozen cells were removed from liquid nitrogen and thawed rapidly in a water bath at 37°C, then were added to 15 ml tubes containing 1 ml each of RPMI 1640 and AB serum. RPMI 1640 was slowly added to each tube to a volume of 10 ml. The cells were pelleted by centrifuging at 300 g for 5 min, then were washed twice in RPMI 1640 and resuspended in CCM.

1 x 10^6 cells in 1 ml of CCM were added to duplicate wells of 24 well, flat bottomed culture plates. PHA was added to each well at 2 μg/ml. The plates were placed in a humidified incubator at 37°C with 5 % CO_2 in air for 10 days. On days three and seven

the PHA blast cultures were split and supplemented with CCM containing 10 % vol/vol IL-2 to maintain a concentration range of 10^5 to 10^6 cells/ml.

The PHA blast cultures were harvested and washed twice in RPMI 1640. 1 x 10^7 cells of each type were resuspended in 200 µl of CCM and labelled with 7.4 MBq of ^{51}Cr at 37°C for 90 min. The ^{51}Cr labelled cells were washed three times with RPMI 1640, then were resuspended in CCM and were counted manually.

7. Assessment of the Matrix 96 Direct Beta Counter.

7.1. Determination of counting time for the Matrix 96 Direct Beta Counter.

^{51}Cr-labelled PHA blast cells were prepared from spleen mononuclear cells from a single donor as described above. A series of dilutions of the labelled cells (10000, 8000, 6000, 4000, 2000 and 1000 cells per well; 16 wells per dilution) were added to a 96 well, U-bottom cell culture plate and the cells were lysed by the addition of 100 µl of 1 % vol/vol Triton X-100 in water followed by incubation at 37°C for 4 hours. The plate was centrifuged at 250 g for 5 min. 100 µl of the supernatant from each well was transferred using a multi-channel pipette to 0.75 ml tubes for counting with a gamma counter, then 50 µl was transferred to the corresponding positions of a SpotWell-96 metal plate for counting with the Matrix 96 Direct Beta Counter. The 0.75 ml tubes were sealed with wax and stored at room temperature overnight. The SpotWell-96 plate was placed in a fume cupboard overnight at room temperature to allow drying to completion.

The 0.75 ml tubes were counted once, to obtain counts per min (cpm). Samples were counted in sequence to allow direct comparison with the counts obtained from the same wells by the Matrix 96. Observation of real-time counts of the SpotWell-96 plate indicated that counts of 17 to 19 min gave results closest to the gamma counter cpm. The SpotWell-96 plate was therefore counted for 17, 18 and 19 min and these counts were used for detailed analysis.

The experiment was repeated, varying the labelled cell concentration to 1000, 800, 600, 400, 200 and 100 cells per well to assess responsiveness at a lower range of counts. The 0.75 ml tubes were counted once, to obtain cpm. The Spotwell-96 plate was counted for 18 and 19 min, the times which were found in the first experiment to give counts closest to the gamma counter cpm.

7.2. Comparison of the Matrix 96 with a gamma counter.

Lysates from ^{51}Cr-labelled PHA blasts from a single donor were prepared as above, using blast cell dilutions of 1000, 800, 600, 400, 200 and 100 cells per well, with 16 replicates at each dilution. The 0.75 ml tubes were counted three times, in sequence, using a gamma counter, to obtain cpm. The SpotWell-96 plate was counted three times using the Matrix 96, each time for 19 min.

The counting methods were compared using an analysis of differences between the values obtained by each method (14). Limits of agreement between two sets of counts were given by the mean of the differences between the counting methods plus and minus

two standard deviations. The 95 % confidence intervals for the upper and lower limits of agreement were calculated as described. The limits of agreement of the Matrix 96 and gamma counter were calculated using two repeated sets of measurements for each counter. Variation in repeated measurements ("repeatability") was assessed by the co-efficient of repeatability, calculated as twice the standard deviation of differences between pairs of repeated measurements.

The counters were not compared directly for counting CTLp assays because the volume of supernatant required (150 μl) could not be taken without also taking up unlysed cells. This was avoided by the use of detergent-lysed preparations in the experiments described above.

8. The Cytotoxic T lymphocyte Precursor Assay.

The method of Kaminski *et al.* was used for all CTLp assays (17). Aseptic technique was used for all stages except the ^{51}Cr-release assay.

8.1. Limiting dilution cultures.

Frozen cells were thawed as described above. Stimulator cells were irradiated with 30 Gy from a Caesium 137 source.

The limiting dilution cultures were set up as follows. Eight dilutions of the responder cell population (usually 4, 3, 2, 1, 0.5, 0.25, 0.125 and 0.0625 x 10^4 cells per well, 24 wells per dilution) were added in 50 µl volumes to 96 well, U-bottom cell culture plates. 5 x 10^4 irradiated stimulator cells were added to each well in a volume of 100 µl. Control wells consisted of 24 wells of stimulator cells in the absence of responders as a baseline control, 8 wells of stimulator cells with unrelated responder cells, 8 wells of unrelated stimulators with patient responder cells, 8 wells of stimulators with irradiated unrelated responder cells, and 8 wells containing culture medium alone for the measurement of spontaneous ^{51}Cr release.

The plates were placed in a humidified incubator at 37°C with 5 % CO_2 in air for 10 days. On days three and seven each well of the limiting dilution and control cultures was supplemented with IL-2 in 25 µl of CCM, to a final concentration of 5 units/ml IL-2 per well.

8.2. ^{51}Cr-release assay.

^{51}Cr-labelled PHA blast cells were prepared from each stimulator cell type as described above, and the concentration of each was adjusted to 1 x 10^5 cells/ml. 100 µl of supernatant was removed from each well of the assay plates using a multi-channel pipette, and the cell pellets were resuspended by pipette in the remaining 100 µl of culture medium. 100 µl of the labelled PHA blast cells was then added to each well of

the assay and control plates (1 x 10^4 cells/well), the target cell added being the same as the stimulator cell in the limiting dilution cultures. Total release of ^{51}Cr was determined by adding 100 µl of each labelled cell to 100 µl of 0.1M HCl. All plates were then incubated for four hours at 37°C with 5 % CO_2.

After the four hour incubation, the plates were centrifuged at 250 g for 5 min, then 50 µl of supernatant from each well was transferred by multi-channel pipette to a Spotwell-96 metal plate in the same pattern as in the 96-well culture plates. The Spotwell-96 plates were counted for 19 min using the Matrix 96, as previously determined to be optimal.

8.3. Calculation of results.

Positive wells were defined as those with counts greater than the mean count plus three standard deviations of the 24 wells containing only irradiated stimulators and labelled target cells (baseline control). In eight assays where the release from these wells had a high standard deviation (mean + 3 SD > 8 % of total release), wells containing labelled target cells alone (spontaneous release) were used to determine positiveness. Spontaneous release has been used by others to determine positiveness (19) and was considered valid here because the use of an erroneously high threshold results in underestimation of the CTLp frequency. CTLp frequencies with 95 % confidence intervals (95 % CI) were calculated from the numbers of negative wells at each dilution by the maximum likelihood method, using a general linear interactive modelling-based computer program (developed at Hammersmith Hospital, London). The chi^2 goodness-

of-fit value was used to test for single-hit kinetics, that is, that a single factor was limiting. Chi^2 values with $p \geq 0.05$ are usually considered acceptable. Frequency estimates were considered to be different if their 95 % confidence intervals did not overlap. The control wells were examined to ensure that stimulation and proliferation had occurred in the appropriate cell populations. Control wells containing stimulators with irradiated unrelated responder cells confirmed the absence of proliferation of irradiated cells. Results were displayed graphically by converting the fraction of negative cultures at each dilution to its natural logarithm and plotting these against responder cell number. If the data conform to single-hit kinetics then such a plot takes the form of a straight line through the origin.

9. Assessment of natural killer-like activity in the CTLp assay.

9.1. Culture of the K562 cell line.

The K562 cell line was cultured in medium consisting of RPMI 1640 supplemented with 10 % vol/vol FBS, 292 mg/l L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. K562 cells were stored in liquid nitrogen at a concentration of 4×10^6 cells/ml in the freezing mixture described above. Four days before needed as targets, frozen cells were removed from liquid nitrogen and thawed rapidly in a water bath at 37°C. Thawed cells were transferred to 15 ml tubes and 10 ml culture medium was slowly added to each tube. The cells were pelleted by centrifuging at 300 g for 5 min, then were resuspended in 10 ml culture medium and the contents of each tube were transferred to a 25 cm^2 tissue culture flask. The flasks were placed in a humidified

incubator at 37°C with 5 % CO_2 in air. On the third day, two volumes of fresh culture medium were added and the cultures were split between three 25 cm^2 flasks. On the day of the ^{51}Cr- release assay, K562 cells were washed and labelled as described above for PHA blast cells.

9.2. K562-directed cytotoxicity in CTLp assay.

Four CTLp assays were set up as described above. In two of the assays the cultures were split into two equal portions on day 10 by transferring 100 µl from each well to the corresponding well of a fresh 96 well, U-bottom plate. Cytotoxicity was then measured against both the stimulator-type target cell and against K562 (10^4 target cells per well in each case). In the other two assays the cultures were only tested against K562 targets because stimulator-type target cells were not available in sufficient numbers to carry out this assay. The responder and stimulator cells were from HLA-identical siblings in one assay and from unrelated individuals in the remaining assays. Peripheral blood MNC were from normal individuals and spleen MNC were from cadaver donors. Peripheral blood responder cells and splenic stimulator cells were used where possible to be consistent with the responder and stimulator cell types used in subsequent assays of patient samples.

Spontaneous release of ^{51}Cr from K562 targets was obtained by incubating 100 µl of labelled cells with 100 µl of culture medium (mean of 24 replicate wells). Total release of ^{51}Cr was obtained by incubating 100 µl of labelled cells with 100 µl of Zapoglobin

(two drops per ml saline; mean of four or eight replicate wells). Test release was the mean ^{51}Cr release of the 24 replicates at each dilution.

Specific lysis of the K562 targets was calculated using the formula:

$$\text{Specific lysis} = \frac{\text{test release - spontaneous release}}{\text{total release - spontaneous release}} \times 100$$

9.3. Determination of target cell ratios for cold target competition experiments.

Cold target competition assays were based on the method of Leclercq *et al.* (383).

Due to the large size of limiting dilution assays it was only practical to test cold target competition at a single unlabelled : labelled target cell ratio. A preliminary experiment was carried out to determine a suitable cell ratio. 96 replicates of 5×10^4 splenic stimulator cells (HLA-A2, -A25, -B35, -B57, -Cw4, -Cw6, -DR7, -DR8) were cultured with 5×10^4 peripheral blood mononuclear cell responder cells from an unrelated individual (HLA-A2, -A3, -B7, -B35, -Cw4, DR not tested) for 10 days with the addition of 5 units/ml IL-2 on days three and seven. On day 10, 50 µl of the supernatant was removed from each well, then the cells were resuspended the remaining volume and 100 µl of each culture was discarded to simulate the splitting of wells in subsequent assays. 50 µl of unlabelled cells were added to appropriate wells to give final

unlabelled : labelled target ratios of 0, 10:1, 20:1, and 40:1, chosen to cover a wide range of values. Eight replicates of each target ratio were set up for each of the following combinations:

1. unlabelled K562 : ^{51}Cr K562
2. unlabelled stimulator-type targets : ^{51}Cr stimulator-type targets
3. unlabelled stimulator-type targets : ^{51}Cr K562

The plate was centrifuged at 80 g for 5 min, then incubated at 37°C for 30 min. 100 µl of the appropriate labelled target cells (1000 cells per well) was then added to each well, the plate was centrifuged as before, and cytotoxicity was measured in a four hour ^{51}Cr release assay. Percentage inhibition of ^{51}Cr release was calculated for each target ratio, using the mean ^{51}Cr release from eight replicates.

9.4. Cold target competition by stimulator-type PHA blasts of killing of K562 targets.

Five assays were performed to determine if unlabelled stimulator-type target cells could inhibit the K562-directed cytotoxicity which developed in the CTLp assay cultures. The CTLp assays were carried out as described above. On day 10, 50 µl of the supernatant was removed from each well of the cultures, then the contents of each well were resuspended and 50 µl was transferred to the corresponding wells of each of two fresh sets of 96 well, U-bottom plates. 50 µl of medium was added to two sets of plates and to the third set was added 50 µl of unlabelled PHA blast cells (2×10^4 cells per well) from

the stimulator in the CTLp assay (cold competitors). The plates were centrifuged at 80 g for 5 min, then incubated at 37°C for 30 min. ^{51}Cr K562 targets were added to one set of plates without cold competitors (1000 cells per well), and ^{51}Cr stimulator targets were added to the other (1 x 10^4 cells per well). ^{51}Cr labelled K562 targets were added to the set of plates with cold competitors (1000 cells per well), giving a competitor : target ratio of 20:1. All plates were centrifuged as before, then cytotoxicity was measured in a four hour ^{51}Cr release assay. Specific lysis was calculated as before.

9.5. Cold target competition by K562 cells of killing of stimulator-type targets.

Five assays were performed to determine if unlabelled K562 cells could inhibit ^{51}Cr release from labelled stimulator-type target cells and reduce the frequency estimate in CTLp assays. The CTLp assays were carried out as described above. On day 10 the contents of each well were resuspended and 100 µl was transferred to the corresponding well of a fresh set of 96 well U-bottom plates. 50 µl of culture medium was added to each well of one set of plates, and 50 µl of unlabelled K562 cells was added to the other set (4 x 10^4 cells per well). The plates were centrifuged at 80 g for 5 min, then incubated at 37°C for 30 min. ^{51}Cr stimulator- type target cells were then added to both sets of plates (2000 cells per well), the plates were centrifuged as before, and cytotoxicity was measured in a four hour ^{51}Cr release assay. The competitor : target ratio was 20:1, as determined previously. The CTLp frequency was calculated for split-well cultures with and without the presence of unlabelled K562 competitors.

10. Reproducibility of the CTLp assay.

Identical assays were set up on three separate occasions to assess the reproducibility of the CTLp assay. Frozen splenic mononuclear cells from a single responder-stimulator pair (responder: HLA-A2, -B62, -B27, -Cw3, -Cw6, -DR4, -DQ8; stimulator: HLA-A2, -A29, -B35, -B44, Cw4, -DR1, -DR4, -DQ1, -DQ7) were thawed on each occasion and CTLp assays were carried out as described above. One of the assays was set up in duplicate from the same starting cell populations. All assays were counted using a gamma counter.

11. Patients.

15 patients who received orthotopic cardiac transplants between January 1994 and July 1995 were included in this study. 14 other patients transplanted during this period were excluded: 5 patients died in the follow-up period (3 of whom died before discharge), and samples were inadequate for 9 patients. Of the patients studied, 10 were male and 5 were female. Age at transplantation ranged from 19 to 59 years (median 54 years). Indications for transplantation were: ischaemic heart disease (eight patients), dilated cardiomyopathy (six) and valvular heart disease (one). Patient details are shown in table 1.

HLA class I antigens of patients, donors and control cells were typed using the complement-dependent lymphocytotoxicity test, based on the National Institutes of

Health method (384). Local, national (United Kingdom Transplant Support Services Authority), and exchanged HLA typing reagents were used. HLA-DRB1 and -DQB loci were typed using the polymerase chain reaction sequence-specific primer method (385, 386).

Patient	Sex	Age at transplant (years)	Indication for transplantation	ATG induction
IM	F	58	Ischaemic heart disease	No
PK	F	43	Dilated cardiomyopathy	No
SS	M	19	Dilated cardiomyopathy	Yes
GM	F	48	Ischaemic heart disease	Yes
RF	M	55	Ischaemic heart disease	No
JH	M	55	Ischaemic heart disease	No
GS	M	56	Valvular heart disease	Yes
WM	M	54	Dilated cardiomyopathy	Yes
JC	F	59	Dilated cardiomyopathy	No
TF	M	55	Ischaemic heart disease	No
GF	M	49	Ischaemic heart disease	Yes
AM	M	33	Dilated cardiomyopathy	Yes
TD	M	59	Ischaemic heart disease	No
WB	M	41	Dilated cardiomyopathy	Yes
JD	F	46	Ischaemic heart disease	No

Table 1. Patient characteristics. Patients are listed in chronological order of transplantation. ATG induction = patients who received induction immunosuppression with anti-thymocyte globulin.

Pre-transplant serum samples were retrospectively crossmatched against donor T and B lymphocytes for each patient, with the exception of patient IM, who was crossmatched prospectively. HLA typing and crossmatching were performed by staff of the Tissue Typing Laboratory at Glasgow Royal Infirmary.

12. Immunosuppression.

Standard maintenance immunosuppression consisted of CsA (two to three mg/kg pre-operatively, then titrated to achieve a whole blood trough level of 600 ng/ml (enzyme immuno-assay) by one week post transplant, reduced to a target level of about 250 ng/ml by one year post-transplant), azathioprine (three mg/kg pre-operatively, thereafter one to two mg/kg/day) and steroids (iv methyl prednisolone peri-operatively, then oral prednisolone at one mg/kg/day tapered to 0.2 mg/kg/day at four weeks). Seven of the fifteen patients were selected at random to receive cytolytic induction therapy with ATG. These patients received 500 mg equine ATG (Merieux) intravenously pre-operatively. Post-operatively, daily doses were given for seven days, titrated against the daily peripheral blood CD2 count to give a count of 100 cells/mm^3. The induction group received oral prednisolone at 0.5 mg/kg/day for the first two weeks post-transplant, reduced thereafter to 0.2 mg/kg/day. CsA was as for the standard group pre-operatively, then was titrated to achieve a whole blood trough level of 600 ng/ml by four days post-transplant. Azathioprine was as for the standard triple immunosuppression group.

Acute cardiac rejection was diagnosed by endomyocardial biopsy, according to the grading system of the International Society of Heart and Lung Transplantation (201). Rejection of grade two or above in the first six weeks after transplantation was treated with intravenous methylprednisolone (1 g daily for three days). After this period rejection episodes were treated by increasing oral prednisolone to 1 mg/kg/day for three days, then tapering to a maintenance dose over two weeks. One patient (GS) with rejection refractory to steroids (more than three pulses of steroid therapy) received OKT3. The same patient was further treated with methotrexate after continuing rejection. Patient WM also received cyclophosphamide.

13. Patient sampling.

Peripheral blood samples were taken from each patient immediately before the transplant operation (T1) and between 3 and 7 months (79 to 200 days, median 104; T2) and 9 and 15 months (257 to 455 days, median 301; T3) after transplantation. At each sampling point 50 to 80 ml of venous blood from each patient was collected into an equal volume of Medium 199 containing 20 units/ml sodium heparin, 100 units/ml penicillin and 100 µg/ml streptomycin. The diluted blood was kept in a 22°C incubator until processed (usually within 24 hours).

A portion of spleen was collected from the cardiac donor at the time of organ retrieval. The spleen was stored in Medium 199 at 4°C for no more than 48 hours before processing.

14. CTLp assays of patient samples.

CTLp assays were carried out on the peripheral blood samples received before transplantation and on two occasions after transplantation from each patient. The patient's mononuclear cells were used as the responder cell population in all of the assays. For each sample CTLp frequencies were determined against both the cardiac donor and a 3rd party which shared no HLA-A,-B or -DR antigens with the cardiac donor. Spleen mononuclear cells from the cardiac donor and the 3rd party were used as stimulator cells in each case. An additional unrelated cell was used as a control in each assay as described.

15. Statistical analysis of results.

CTLp assays not consistent with single hit kinetics (chi^2 values with $p<0.05$) were excluded from the statistical analysis. For tests which compared results in the different sample time groups, patients were included only if all three donor-reactive or 3^{rd} party-reactive CTLp frequencies were available.

Pre-transplant donor- and 3^{rd} party-reactive CTLp frequencies grouped by the number of HLA mismatches between recipient and donor were compared using the Mann-Whitney T statistic. CTLp frequencies were compared between patients with the following HLA mismatches: 0,1 and 2 HLA-A and -B mismatches compared with 3 and 4 HLA-A and -B mismatches; 0,1 and 2 HLA-DR and -DQ mismatches compared with 3 and 4

HLA-DR and -DQ mismatches; 0 to 4 HLA-A, -B, -DR and -DQ mismatches compared with 5 to 8 HLA-A, -B, -DR and -DQ mismatches. HLA mismatches were defined as the number of donor HLA antigens not present in the recipient.

The relation between pre-transplant donor- and 3rd party-reactive CTLp frequencies and EMB-diagnosed rejection (number of EMB graded ≥ 2 by 90 days and by 1 year after transplantation, summed grades of EMB graded ≥ 2 by 90 days and by 1 year after transplantation, number of days to first EMB graded ≥ 2) was investigated using the Spearman rank correlation coefficient. Summed EMB grades were used to reflect the severity and persistence of rejection.

CTLp frequencies in the different sample time groups (T1, T2 and T3) were compared using the sign test with paired frequencies.

Donor-reactive CTLp frequencies from samples taken at the time of grade 0 or 1 EMB were compared with donor-reactive CTLp frequencies from samples taken at the time of grade 2 or 3 EMB using the Mann-Whitney T statistic (there were no grade 4 EMB at any of the sampling times).

The relation between post-transplant CTLp frequencies and rejection was analysed by the Spearman rank correlation test. Spearman rank correlation co-efficients (r_s) were calculated for the T2 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period up to the sampling time; for the T3 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period

between the T2 and T3 samples; and for the T3 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period from transplantation to the T3 samples.

The relation between T3 CTLp frequencies and total doses of CsA and azathioprine received, and T3 CTLp frequencies and the number of additional courses of steroids received for acute rejection were analysed by the Spearman rank correlation test.

Donor- and 3rd party-reactive CTLp frequencies at each of the two post-transplant sample time groups (T2 and T3) were compared between patients who received induction therapy with ATG and patients who received standard triple immunosuppression using the Mann-Whitney T statistic.

Test statistics were calculated using the methods and tables described by Altman (387).

RESULTS

1. Assessment of the Matrix 96 Direct Beta Counter.

1.1. Determination of counting time for the Matrix 96 Direct Beta Counter.

A 19 min count using the Matrix 96 gave results which most closely matched the gamma counter cpm in both the range of counts observed (table 2), and the mean of 16 replicate wells at each dilution (figures 1 and 2).

At low values Matrix 96 counts were less than gamma cpm, because of the lower background counts of the Matrix 96. Regression lines extrapolated to zero labelled cells gave a background of about 30 counts for the 19 min Matrix 96 count compared to about 68 cpm for the gamma counter. For 19 min counts the mean calculated cpm of the Matrix 96 were about 5 - 6 % of gamma counter cpm over most of the counting range, falling to 4.8 % at 200 cells per well and 3.9 % at 100 cells per well.

Dilution range	Gamma counter	Matrix 96 counts		
	cpm	17 min	18 min	19 min
10000 to 1000 cells/well	364 to 2495	327 to 2355	327 to 2520	363 to 2634
1000 to 100 cells/well	100 to 601	NT	46 to 602	68 to 683

Table 2. Ranges of counts from samples counted in the gamma counter and the Matrix 96. Ranges shown are from 96 samples (16 replicates at each of 6 dilutions), counted once for each time interval. NT = not tested.

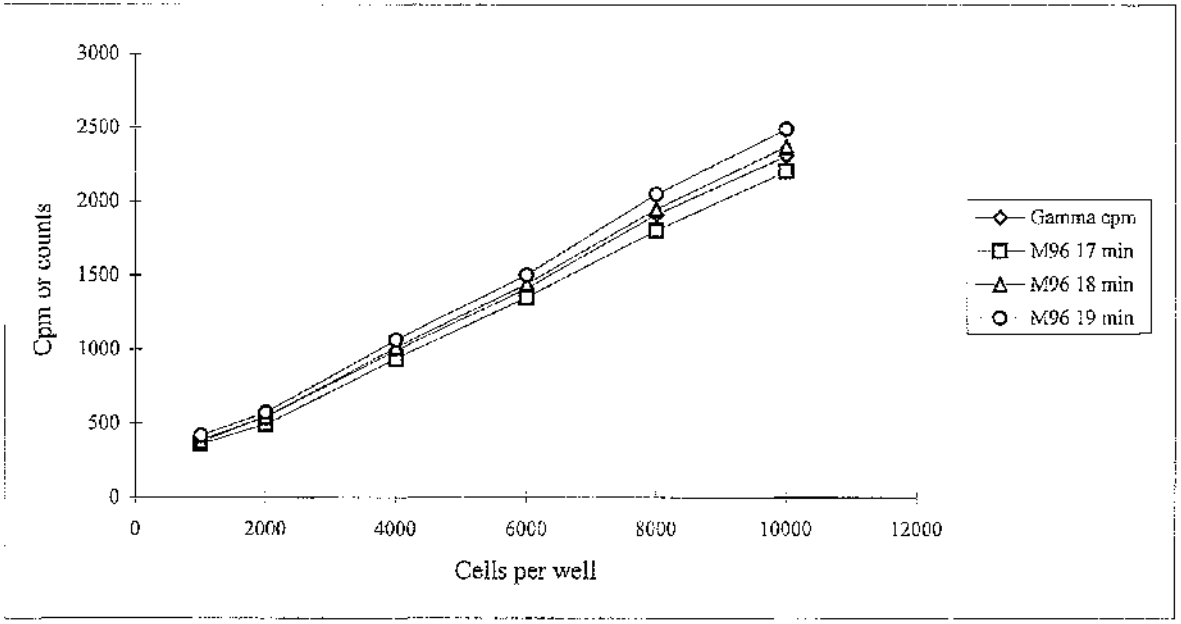

Figure 1. Comparison of 51Cr-release from 1000 to 10,000 cells counted using the Matrix 96 and gamma counter. 51Cr-labelled PHA blasts (1000 to 10,000 cells per well) were lysed by detergent and the supernatants were counted for cpm using the gamma counter (100 ul supernatant) and for 17, 18 and 19 min using the Matrix 96 (50 ul supernatant). Each point represents the mean cpm or counts measured from 16 replicate wells at each concentration of 51Cr-labelled PHA blasts.

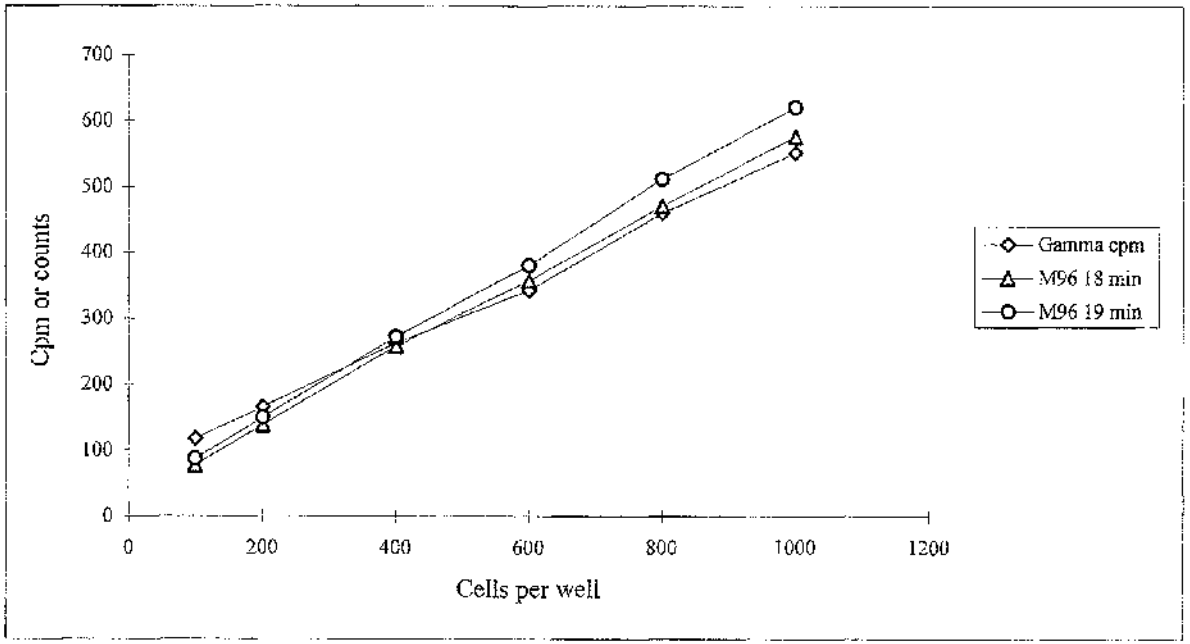

Figure 2. Comparison of 51Cr-release from 100 to 1000 cells counted using the Matrix 96 and gamma counter. 51Cr-labelled PHA blasts (100 to 1000 cells per well) were lysed by detergent and the supernatants were counted for cpm using the gamma counter (100 ul supernatant), and for 18 and 19 min using the Matrix 96 (50 ul supernatant). Each point represents the mean cpm or counts measured from 16 replicate wells at each concentration of 51Cr-labelled PHA blasts.

1.2. Comparison of the Matrix 96 with a gamma counter.

1.2.1. Agreement of the counters.

A plot of a 19 min Matrix 96 count against gamma counter cpm for the same samples is shown in figure 3. The differences between the counts by each method were plotted against their means (figure 4). The differences between the counters changed from positive at low values to negative at high values, that is, gamma cpm were higher than Matrix 96 counts at low values and lower than Matrix 96 counts at high values.

The limits of agreement (with 95 % CI for upper and lower limits) of the Matrix 96 and gamma counter were -69.2 (-82.3 to -56.1) to 79.6 (66.5 to 92.7).

1.2.2. Repeatability of the counters.

Plots of the differences between two sets of counts against their means for each method are shown in figures 5 and 6. The differences between repeated Matrix 96 counts increased with increasing ^{51}Cr-release, but were less than the differences between repeated gamma counter counts at low values. The gamma counter showed similar differences over the whole range of values. The co-efficients of repeatability, as defined in the methods, were 50.0, 48.2 and 54.3 between the three sets of gamma counter cpm and 57.6, 52.8 and 52.3 between the three Matrix 96 counts. The co-efficient of repeatability between the gamma counter and the Matrix 96 using two repeated sets of measurements for each counter was 74.4.

The mean differences for repeated measurements using the gamma counter were not significantly different from zero. For the Matrix 96, the mean difference for repeated measurements was less than zero for two of the three comparisons (counts 1 and 2, mean = -10.3 (95 % confidence interval = -16.1 to -4.5); counts 1 and 3, mean = -6.2 (95 % confidence interval = -11.6 to -0.8). 94.8 % of differences were within two SD of the mean for the gamma counter and 93.75 % of differences were within 2 SD of the mean for the Matrix 96. The Matrix 96 differences were related to the mean, however (figure 6), and when the data were $\log_{10}$ transformed 95.8 % of differences were within two standard deviations of the mean.

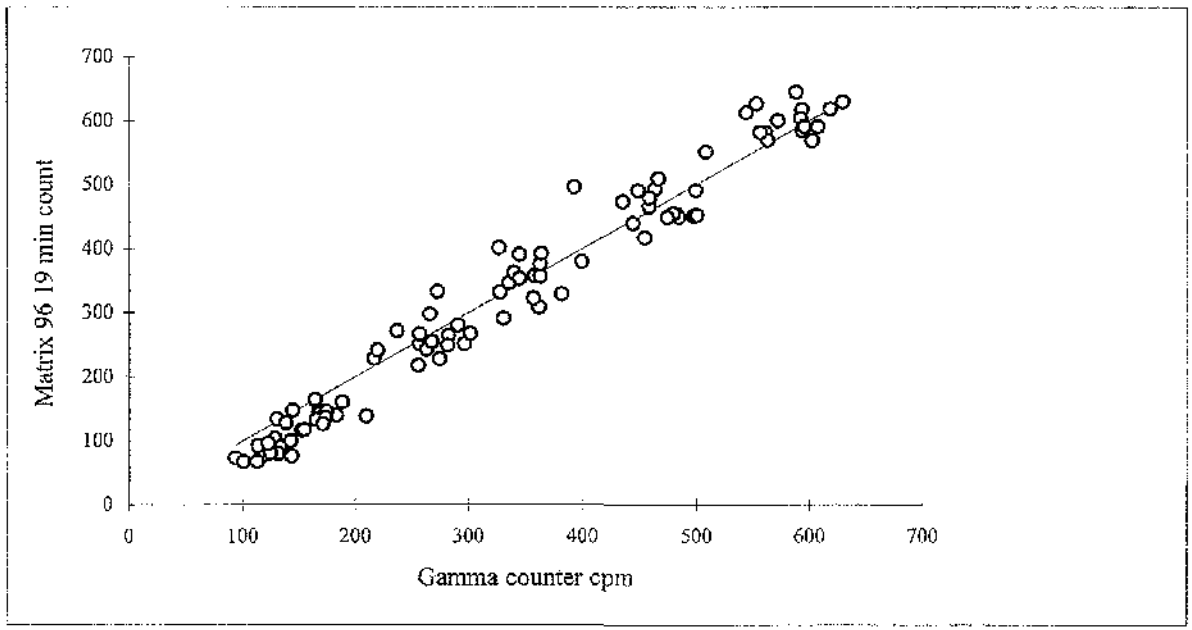

Figure 3. Comparison of 51Cr-release from 96 samples counted using the Matrix 96 and gamma counter. 51Cr-labelled PHA blasts (6 dilutions from 100 to 1000 cells per well, 16 replicates per dilution) were lysed by detergent and the supernatants were counted for cpm using the gamma counter (100 ul supernatant), and for 19 min using the Matrix 96 (50 ul supernatant). Each point represents the mean cpm or counts measured from an individual well The solid line is the line of equality.

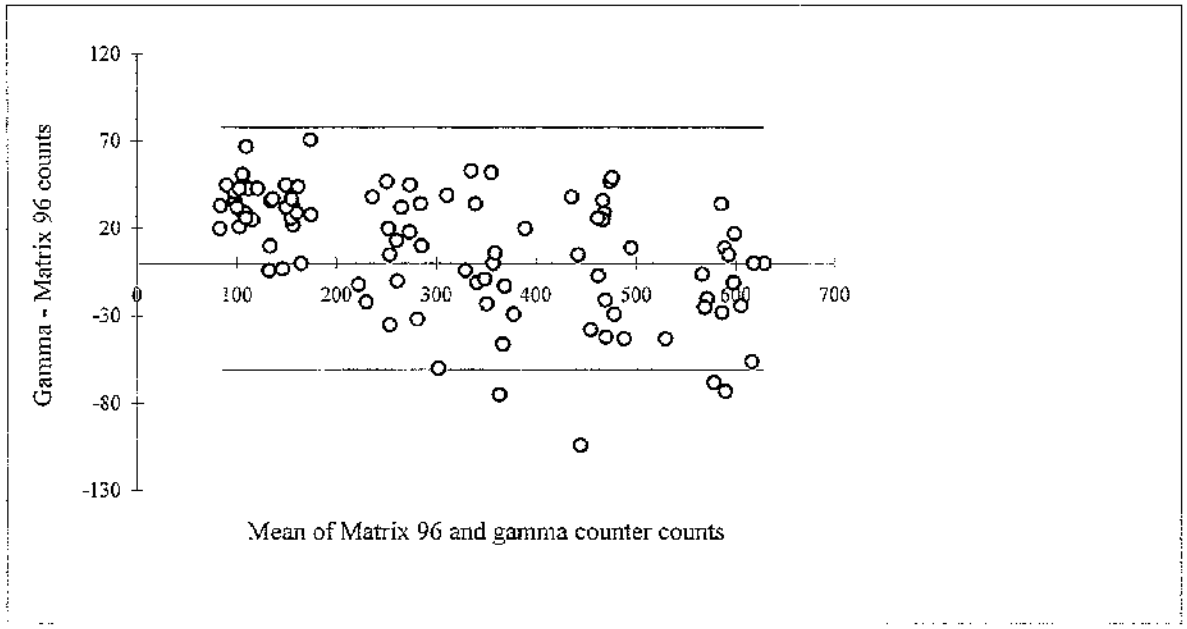

Figure 4. Differences plotted against means for 51Cr-release from 96 samples counted using the Matrix 96 and gamma counter. For each sample in Fig. 3 the difference between the counts from the two counters was plotted against the mean of the counts. The horizontal lines represent mean difference plus and minus 2SD.

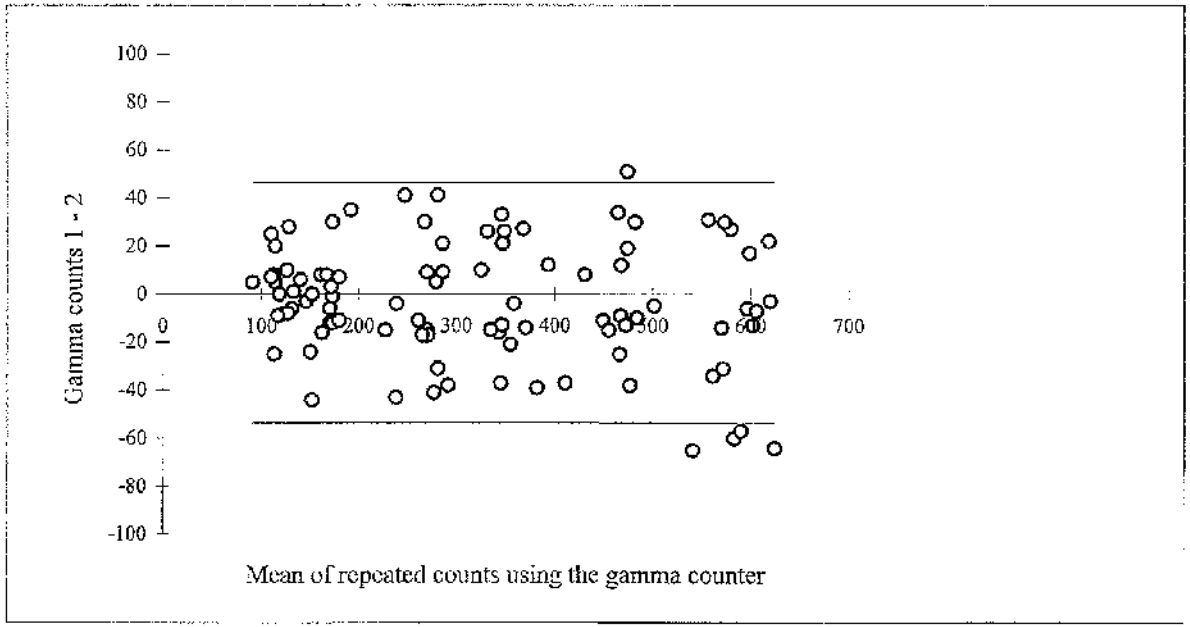

Figure 5. Differences plotted against means for 51Cr-release from 96 samples counted twice using the gamma counter. 51Cr-labelled PHA blasts (6 dilutions from 100 to 1000 cells per well, 16 replicates per dilution) were lysed by detergent and the supernatants were counted twice, in sequence, for cpm using the gamma counter (100 ul supernatant). The difference between the values from the repeated counts was plotted against the mean of the counts. The horizontal lines represent mean difference plus and minus 2SD.

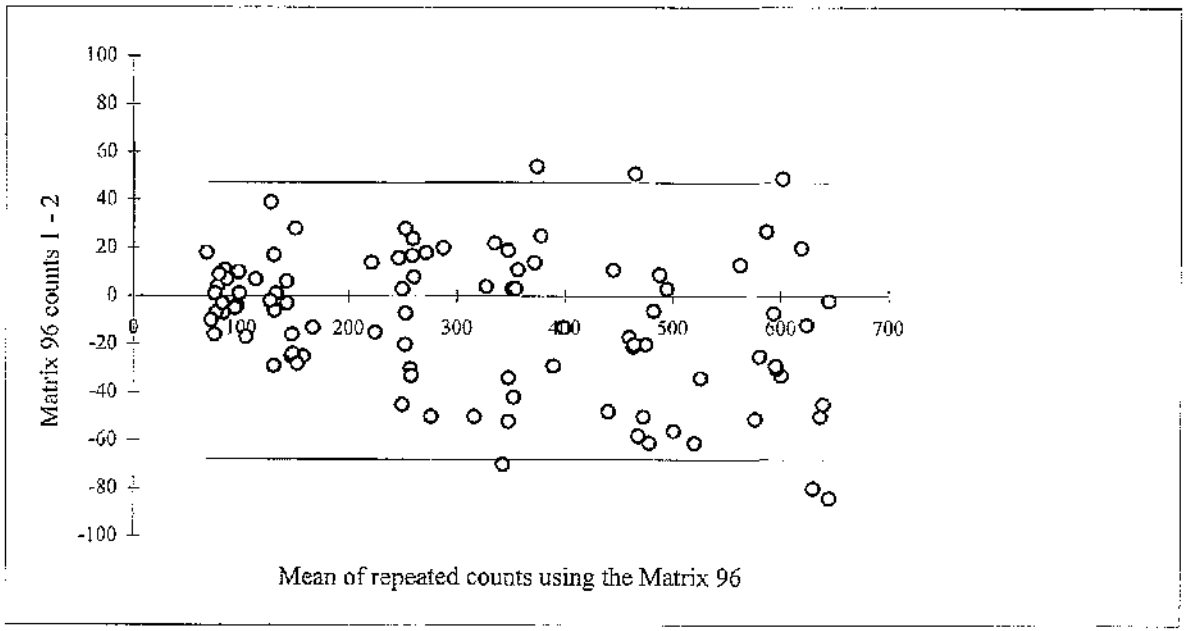

Figure 6. Differences plotted against means for 51Cr-release from 96 samples counted twice using the Matrix 96. 50 ul of supernatant from the same samples as were counted by the gamma counter was counted twice for 19 min each using the Matrix 96. The difference between the values from the repeated counts was plotted against the mean of the counts. The horizontal lines represent mean difference plus and minus 2SD.

2. Assessment of natural killer-like activity in the CTLp assay.

2.1. K562-directed cytotoxicity in CTLp assays.

The HLA types of the responder and stimulator cells used in the assays of K562-directed cytotoxicity are shown in table 3. K562-directed cytotoxicity was present in CTLp assay cultures in an assay between an HLA-identical sibling pair and in three assays between unrelated individuals with varying numbers of HLA mismatches. The maximum specific lysis was about 70 % in three of the assays (figures 7 to 10). In assay 2, different dilutions of responder cells were used due to a shortage of cells.

The CTLp frequency for assay 1 was 3 cells per million peripheral blood MNC (95 % confidence interval = 2 to 7 cells per million, $chi^2 = 3.15$, $p = 0.53$) and for assay 2 was 1190 cells per million peripheral blood MNC (95 % confidence interval = 829 to 1709 cells per million, $chi^2 = 0.19$, $p = 1.0$). Specific lysis of K562 cells was similar in each unrelated combination and appeared not to be influenced by the nature of the HLA mismatch.

K562 Assay	Cell	Cell type	HLA locus							
			A		B		Cw		DR	
1	R	PBMNC	3	29	39	44	-	-	NT	
	S	PBMNC	3	29	39	44	-	-	NT	
2	R	PBMNC	2	3	7	-	7	-	15	-
	S	SMNC	1	2	8	37	6	-	3	7
3	R	PBMNC	2	3	7	15	3	-	15	13
	S	SMNC	2	28	35	60	3	4	4	-
4	R	PBMNC	2	3	7	35	4	-	NT	
	S	SMNC	2	25	35	57	4	6	7	8

Table 3. HLA phenotypes of responder and stimulator cells used for the assessment of K562-directed cytotoxicity in CTLp assays. R = responder, S = stimulator, PBMNC = peripheral blood mononuclear cells, SMNC = spleen mononuclear cells, NT = not tested.

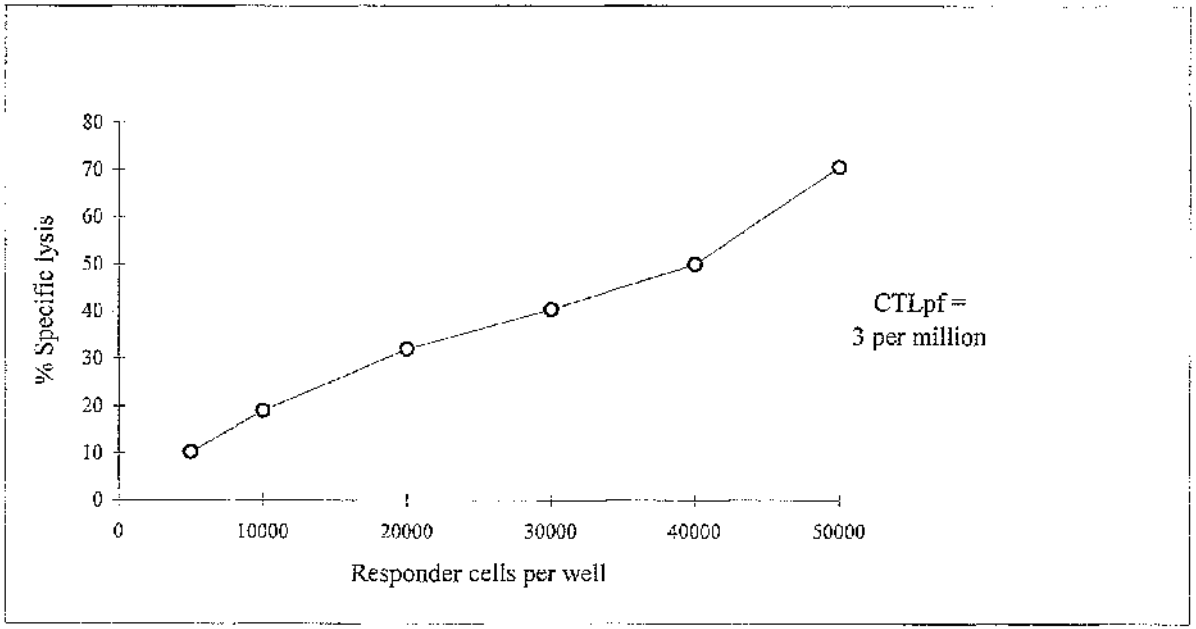

Figure 7. K562-directed cytotoxicity in assay 1. The contents of each half-well of day 10 CTLp cultures were tested for lysis of 51Cr-labelled K562 targets. Percent specific lysis was calculated from the mean of 24 replicate wells at each responder cell concentration and the spontaneous and total 51Cr-release. Responder and stimulator cells were from HLA-identical siblings.

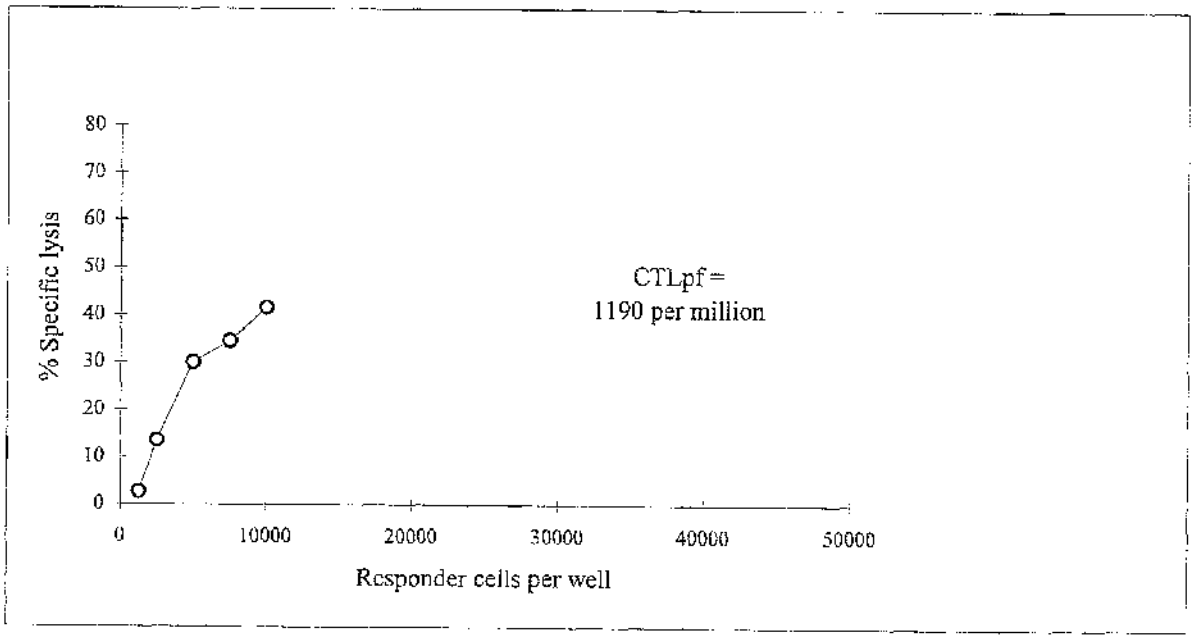

Figure 8. K562-directed cytotoxicity in assay 2. The contents of each half-well of day 10 CTLp cultures were tested for lysis of 51Cr-labelled K562 targets. Percent specific lysis was calculated from the mean of 24 replicate wells at each responder cell concentration and the spontaneous and total 51Cr- release. Responder and stimulator cells were from unrelated individuals.

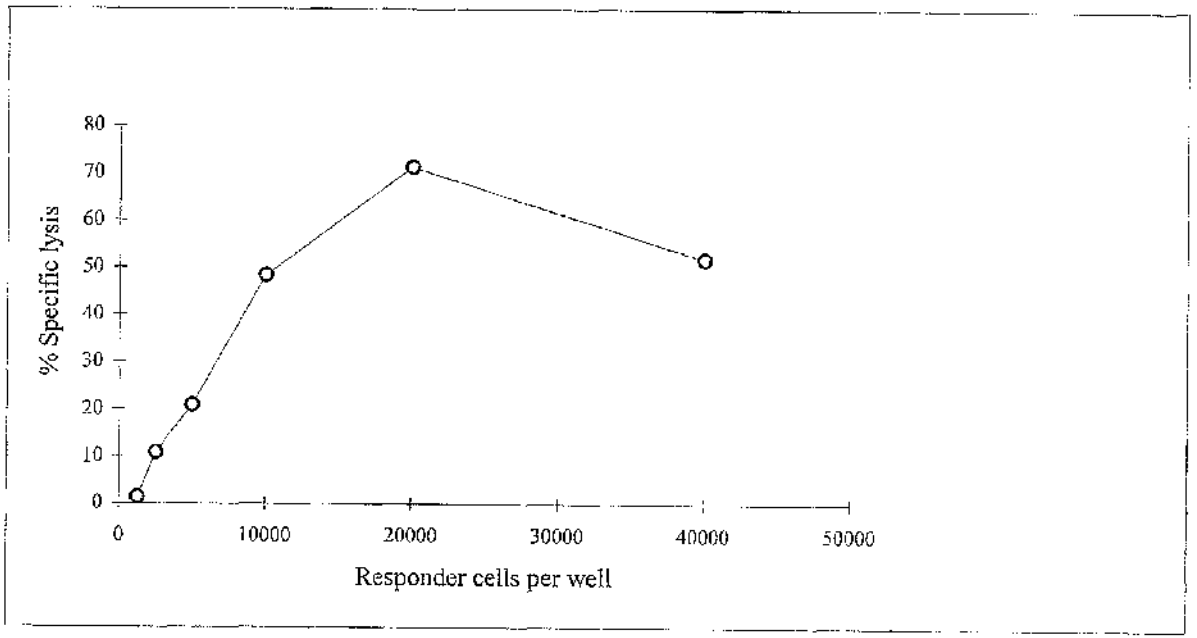

Figure 9. K562-directed cytotoxicity in assay 3. The contents of each full-well of day 10 CTLp cultures were tested for lysis of 51Cr-labelled K562 targets. Percent specific lysis was calculated from the mean of 24 replicate wells at each responder cell concentration and the spontaneous and total 51Cr- release. Responder and stimulator cells were from unrelated individuals.

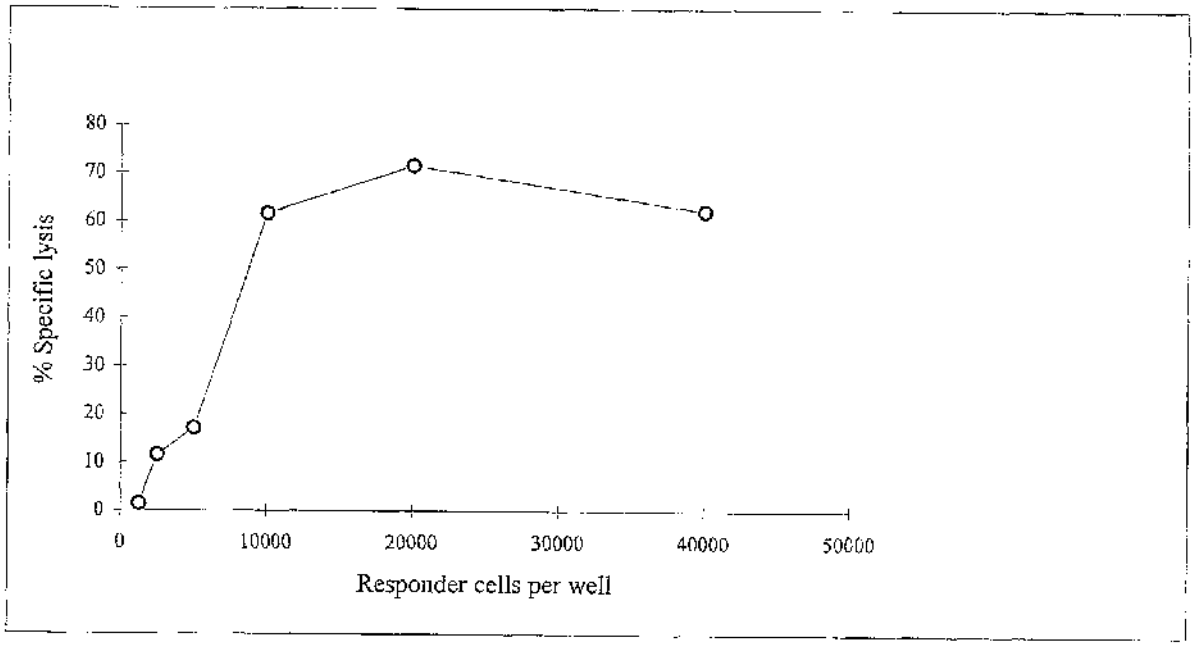

Figure 10. K562-directed cytotoxicity in assay 4. The contents of each full-well of day 10 CTLp cultures were tested for lysis of 51Cr-labelled K562 targets. Percent specific lysis was calculated from the mean of 24 replicate wells at each responder cell concentration and the spontaneous and total 51Cr- release. Responder and stimulator cells were from unrelated individuals.

2.2. Determination of target cell ratios for cold target competition experiments.

The results of the experiment to determine an appropriate cold : hot target cell ratio for competition experiments are shown in figure 11. As expected, unlabelled stimulator-type PHA blasts inhibited the release of ^{51}Cr from the same stimulator-type targets and unlabelled K562 inhibited release of ^{51}Cr from K562 targets. Unlabelled stimulator-type PHA blasts did not inhibit release of ^{51}Cr from K562, but a trend towards increased lysis of K562 was seen with the addition of increasing numbers of unlabelled PHA blast cells. However, the increase was small at the 20 : 1 ratio (2.8 %), and this ratio was chosen for subsequent competition experiments because it gave good homologous inhibition and was less demanding in numbers of cells.

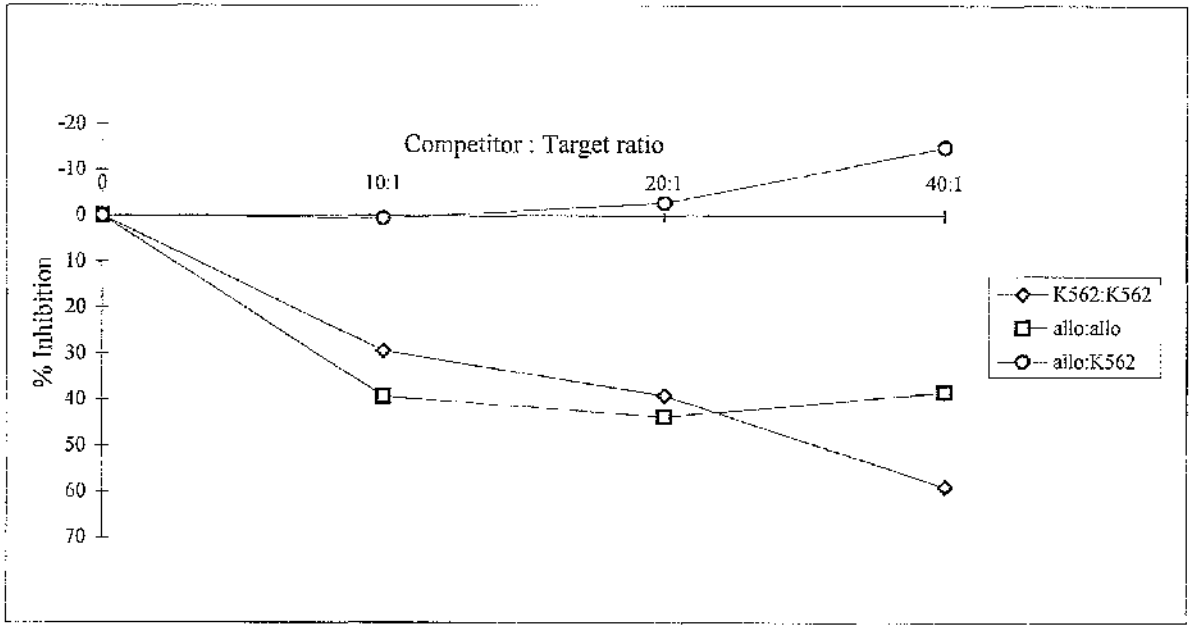

Figure 11. Determination of target cell ratios for cold target competition experiments. 50,000 responder cells were cultured with unrelated stimulator cells for 10 days under CTLp assay conditions. Unlabelled and 51Cr-labelled target cells were then added to give the competitor : target ratios shown. Unlabelled K562 cells were added to 51Cr K562 targets (K562:K562). Unlabelled stimulator-type targets were added to 51Cr stimulator-type targets (allo:allo) and to 51Cr K562 targets (allo:K562). Percent inhibition at each target cell ratio was calculated in comparison to wells containing no competitor cells. Values shown are the mean of 8 replicate wells at each target cell ratio.

2.3. Cold target competition by stimulator-type PHA blasts of killing of K562 targets.

The HLA types of the responder and stimulator cells used in competition assays 1 to 5 are shown in table 4. Specific lysis in the presence and absence of unlabelled competitors in each of the assays is shown in figures 12 to 16.

^{51}Cr release was augmented by the unlabelled stimulator-type PHA blast cells in the absence of responder cells and at low responder cell numbers in four of the assays. At higher responder cell numbers the ^{51}Cr release was similar in the presence and absence of cold competitors, with the exception of assay two, in which specific lysis of K562 targets was higher in the presence than in the absence of unlabelled competitor cells at each responder cell dilution.

The CTLp frequency of responder cells reactive with the stimulator cell type in each of the assays is shown in table 5. In assays one and two the numbers of positive wells at the 4×10^4 responder cell concentration were inconsistent with single-hit kinetics. This could have resulted from overgrowth of the cultures due to the high numbers of proliferating lymphocytes at these cell concentrations. It was considered valid to calculate adjusted CTLp frequencies with these values omitted, using values from the straight-line part of the limiting dilution curve.

Competition Assay	Cell	Cell type	HLA locus							
			A		B		Cw		DR	
1	R	PBMNC	2	3	7	15	3	-	15	13
	S	SMNC	2	28	35	60	3	4	4	-
2	R	PBMNC	2	3	7	-	7	-	103	15
	S	SMNC	2	28	35	60	3	4	4	-
3	R	PBMNC	1	3	8	60	3	7	17	13
	S	SMNC	30	31	13	18	5	6	7	14
4	R	PBMNC	1	26	22	37	6	-	10	14
	S	SMNC	30	31	13	18	5	6	7	14
5	R	PBMNC	2	3	7	-	7	-	8	13
	S	SMNC	1	2	8	44	-	-	17	4

Table 4. HLA phenotypes of responder and stimulator cells used in assays of cold target competition by stimulator-type PHA blasts of K562 killing. R = responder, S = stimulator, PBMNC = peripheral blood mononuclear cells, SMNC = spleen mononuclear cells. PBMNC were from normal individuals and SMNC were from cadaver donors.

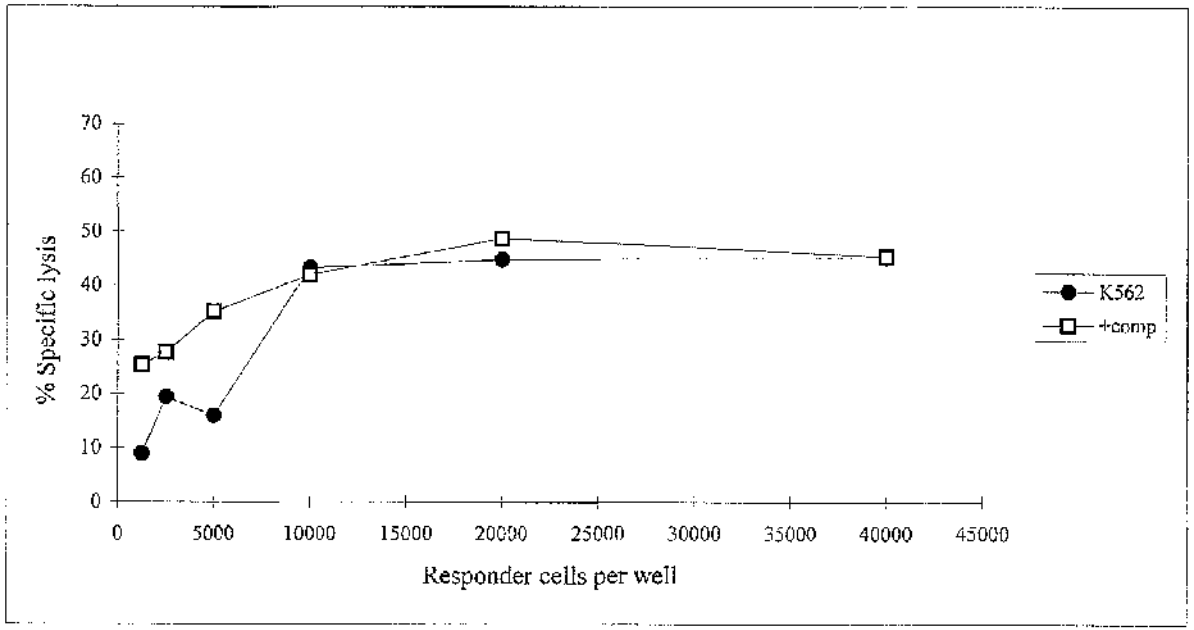

Figure 12. Cold target competition by stimulator-type PHA blasts of K562 killing: competition assay 1. A CTLp assay was set up for an unrelated responder-stimulator pair. After 10 days of culture each well was split into three portions and tested against stimulator-type targets, against K562 targets, and against 51Cr K562 targets in the presence of unlabelled stimulator-type competitor cells (+comp) at a 20 : 1 competitor : target ratio. Values shown are the mean percent specific lysis from 24 replicate wells at each responder cell concentration.

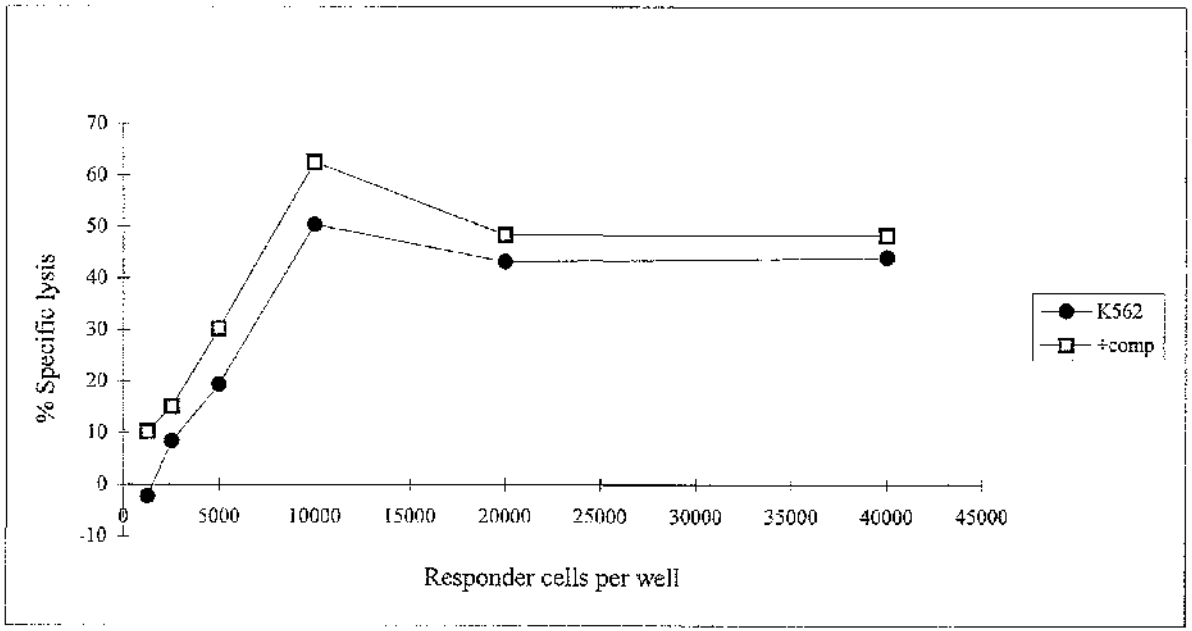

Figure 13. Cold target competition by stimulator-type PHA blasts of K562 killing: competition assay 2. A CTLp assay was set up for an unrelated responder-stimulator pair. After 10 days of culture each well was split into three portions and tested against stimulator-type targets, against K562 targets, and against 51Cr K562 targets in the presence of unlabelled stimulator-type competitor cells (+comp) at a 20 : 1 competitor : target ratio. Values shown are the mean percent specific lysis from 24 replicate wells at each responder cell concentration.

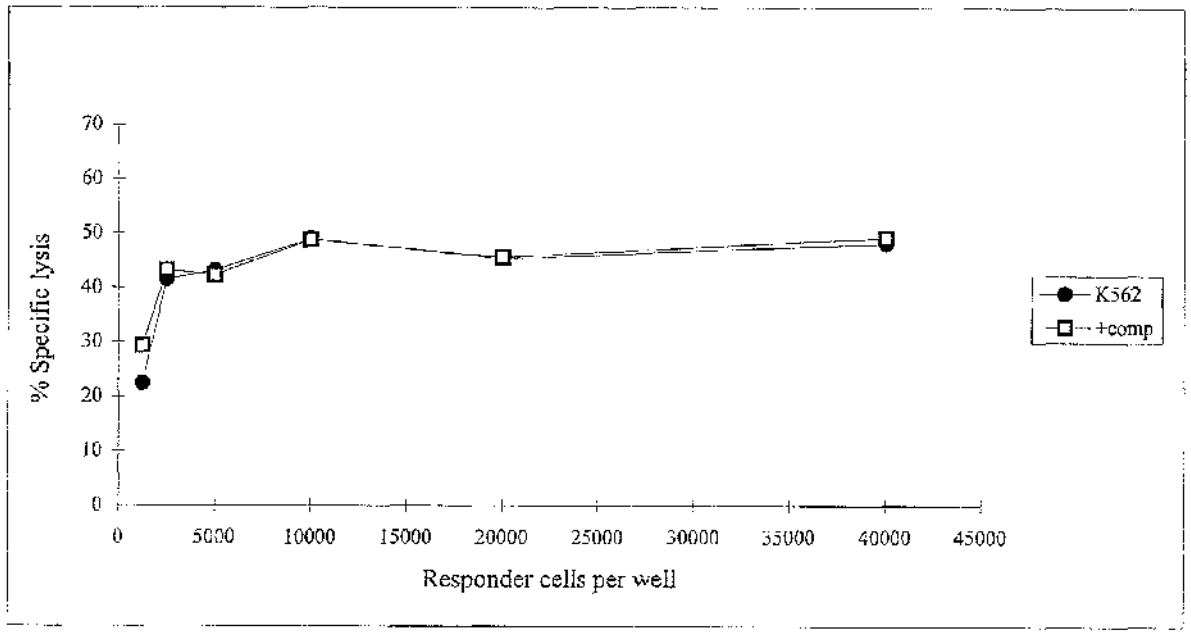

Figure 14. Cold target competition by stimulator-type PHA blasts of K562 killing: competition assay 3. A CTLp assay was set up for an unrelated responder-stimulator pair. After 10 days of culture each well was split into three portions and tested against stimulator-type targets, against K562 targets, and against 51Cr K562 targets in the presence of unlabelled stimulator-type competitor cells (+comp) at a 20 : 1 competitor : target ratio. Values shown are the mean percent specific lysis from 24 replicate wells at each responder cell concentration.

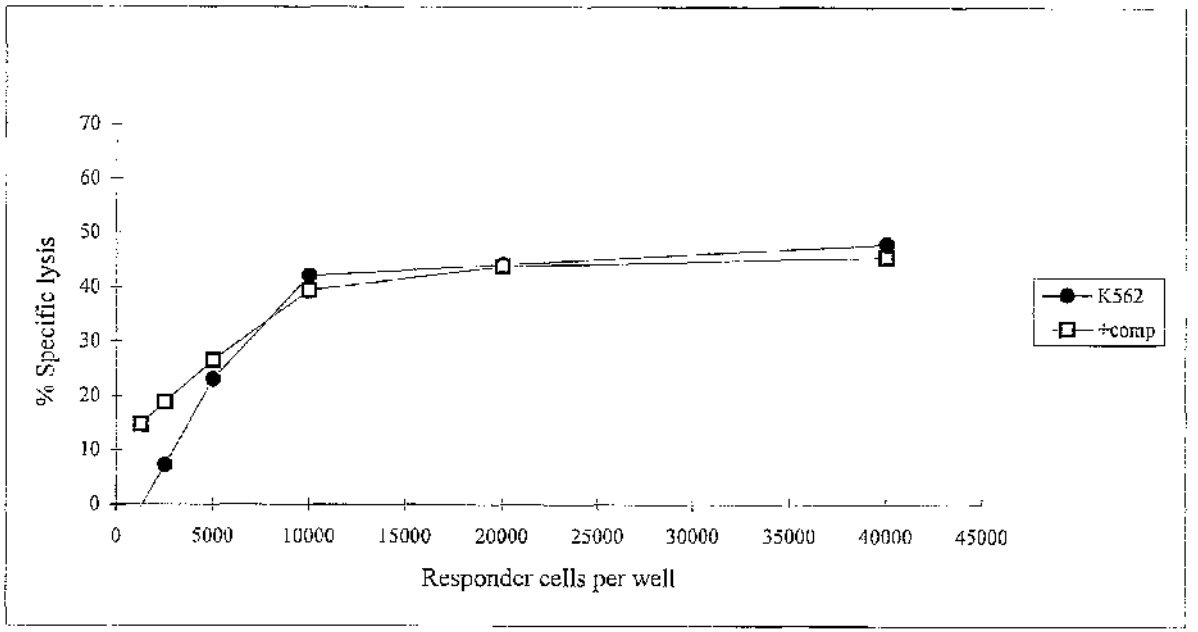

Figure 15. Cold target competition by stimulator-type PHA blasts of K562 killing: competition assay 4. A CTLp assay was set up for an unrelated responder-stimulator pair. After 10 days of culture each well was split into three portions and tested against stimulator-type targets, against K562 targets, and against 51Cr K562 targets in the presence of unlabelled stimulator-type competitor cells (+comp) at a 20 : 1 competitor : target ratio. Values shown are the mean percent specific lysis from 24 replicate wells at each responder cell concentration.

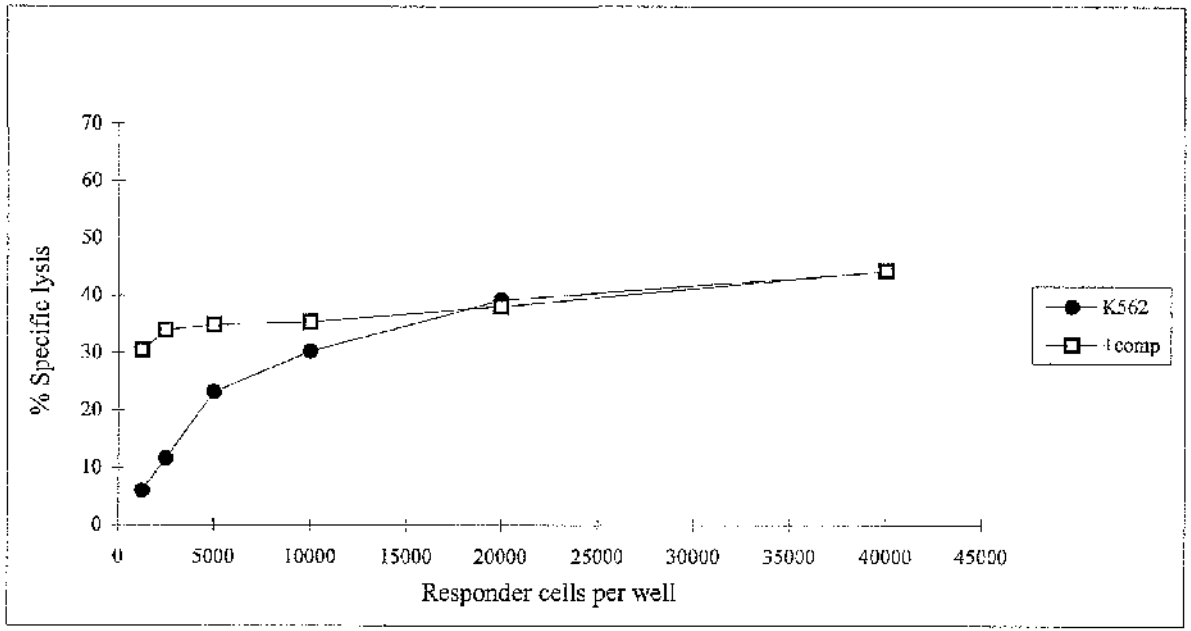

Figure 16. Cold target competition by stimulator-type PHA blasts of K562 killing: competition assay 5. A CTLp assay was set up for an unrelated responder-stimulator pair. After 10 days of culture each well was split into three portions and tested against stimulator-type targets, against K562 targets, and against 51Cr K562 targets in the presence of unlabelled stimulator-type competitor cells (+comp) at a 20 : 1 competitor : target ratio. Values shown are the mean percent specific lysis from 24 replicate wells at each responder cell concentration.

Competition Assay	CTLp	95% CI	Chi^2	p
1	36[a]	25 - 53	7.15	0.13
2	147[a]	111 - 193	2.70	0.61
3	1050	741 - 1488	0.97	0.97
4	71	54 - 94	1.44	0.92
5	177	135 - 232	8.08	0.15

Table 5. Stimulator cell-specific CTLp frequencies in assays used to investigate cold target competition by stimulator-type PHA blasts of K562 killing. CTLp = frequency per million peripheral blood MNC. a = adjusted CTLp frequency.

2.4. Cold target competition by K562 cells of killing of stimulator-type targets.

The HLA types of the responders and stimulators used in competition assays 6 to 10 are shown in table 6. The CTLp frequencies of responder cells reactive with the stimulator cell type in the presence and absence of unlabelled K562 competitors are shown in table 7.

While there appears to be a trend towards inhibition, with the exception of assay 9 the CTLp frequencies obtained in the presence and absence of unlabelled competitors had overlapping confidence intervals and were thus not significantly different from each other.

In assay 10, in the presence of unlabelled K562, the 3×10^4 and 4×10^4 responder cell concentrations contained fewer positive wells than higher dilutions and were inconsistent with single-hit kinetics. An adjusted CTLp frequency was calculated with these values omitted, using values from the straight-line part of the limiting dilution curve.

Competition Assay	Cell	Cell type	HLA locus							
			A		B		Cw		DR	
6	R	PBMNC	1	25	18	-	-	-	15	8
	S	SMNC	2	29	44	52	-	-	4	-
7	R	PBMNC	3	11	7	44	-	-	2	7
	S	SMNC	2	29	44	52	-	-	4	-
8	R	PBMNC	1	26	18	-	-	-	11	13
	S	SMNC	2	3	51	18	2	-	11	-
9	R	PBMNC	2	26	35	60	3	4	1	103
	S	SMNC	2	3	14	44	5	-	4	13
10	R	PBMNC	1	34	8	13	-	-	2	7
	S	SMNC	1	2	7	8	7	-	103	17

Table 6. HLA phenotypes of responder and stimulator cells used in assays of cold target competition by K562 cells of killing of stimulator-type targets. R = responder, S = stimulator, PBMNC = peripheral blood mononuclear cells, SMNC = spleen mononuclear cells. Responders in assays 6 and 8 were normal individuals and responders in assays 7, 9 and 10 were cardiac transplant recipients tested against their donors.

Competition Assay	Competitor	CTLp	95% CI	Chi^2	p
6	No competitor	409	311 - 538	16.19	0.02
	K562	289	221 - 379	14.27	0.05
7	No competitor	158	122 - 204	2.69	0.91
	K562	103	80 - 131	19.64	0.006
8	No competitor	485	368 - 640	6.25	0.51
	K562	393	299 - 517	10.01	0.19
9	No competitor	121	96 - 154	10.37	0.17
	K562	71	56 - 91	14.92	0.04
10	No competitor	87	69 - 111	11.50	0.12
	K562	80[a]	59 - 107	7.12	0.21

Table 7. Stimulator cell-specific CTLp frequencies in the presence and absence of unlabelled K562 competitors. CTLp = frequency per million peripheral blood MNC. a = adjusted CTLp frequency.

3. Reproducibility of the CTLp assay.

The CTLp frequencies obtained from the same responder-stimulator pair assayed on different dates had overlapping confidence intervals, and were thus not significantly different from each other (table 8). Intra-assay reproducibility was demonstrated in assay three.

Repeated assay	CTLp	95% CI	Chi^2	p
1	81	64 - 102	10.9	0.14
2	111	87 - 141	10.7	0.15
3*	97	76 - 123	7.4	0.39
3*	83	66 - 104	11.8	0.11

Table 8. CTLp frequencies for a responder-stimulator pair tested on different occasions. CTLp = frequency per million peripheral blood MNC. * Tested in duplicate from the same starting cell populations.

4. Analysis of CTLp frequency in cardiac transplant recipients.

4.1. HLA typing of patients, donors and 3rd party cells.

HLA typing results are shown in table 9. All patients had a negative crossmatch with donor T and B lymphocytes, except GS and TF who had negative T cell, but positive B cell crossmatches.

4.2. Donor- and 3rd party-reactive CTLp frequencies.

The donor-reactive and 3rd party-reactive CTLp frequencies for all patients before transplantation and at two time points after transplantation are shown in table 10. In four assays, the numbers of positive wells at one or two responder cell dilutions were inconsistent with single-hit kinetics. After examination of the results it was considered valid to omit these data points from the analysis. Adjusted CTLp frequencies were calculated with these values omitted, using values from the straight-line part of the limiting dilution curve.

Limiting dilution curves representative of different patterns of change after transplantation are shown in figures 17 and 18. Figure 17 shows assays of donor-reactive CTLp frequency for patient IM, in which CTLp frequency decreased at both sampling times after transplantation. In the assays for patient GS represented in figure 18, the donor-reactive CTLp frequency decreased in the T2 sample, then increased in the T3 sample.

Identification	HLA locus													
	A		B		Bw		Cw		DR				DQ	
IM	3	11	7	44	4	6	-	-	15	7	51	53	2	6
Donor	2	29	44	52	4		-	-	4	-	53	-	7	8
3rd party	1	26	8	-	-	6	7	-	17	-	52	-	2	-
PK	1	31	8	60	-	6	3	7	15	4	51	53	6	8
Donor	3	32	7	18	-	6	-	-	4	13	52	53	6	7
3rd party	1	2	8	37	4	6	6	7	17	7	52	53	2	-
SS	2	3	62	18	-	6	3	-	4	-	53	-	7	8
Donor	2	11	35	49	4	6	NT		103	4	53	-	5	8
3rd party	30	31	13	18	4	6	5	6	7	14	52	53	2	5
GM	2	23	44	62	4	6	3	4	17	4	52	53	2	8
Donor	24	28	7	44	4	6	-	-	15	7	51	53	2	6
3rd party	2	-	62	27	4	6	3	-	4	-	53	-	8	-
RF	3	32	22	35	-	6	3	4	1	4	53	-	5	7
Donor	1	-	7	60	-	6	3	7	4	13	52	53	6	8
3rd party	24	11	62	56	-	6	1	3	1	11	52	-	5	7
JH	1	3	7	-	-	6	-	-	17	11	52	-	2	7
Donor	1	-	8	44	4	6	-	-	17	7	52	53	2	-
3rd party	3	31	51	60	4	6	3	-	4	11	52	53	7	8
GS	2	26	35	60	-	6	3	4	1	103	-	-	5	-
Donor	2	3	44	14	4	6	5	-	4	13	52	53	6	7
3rd party	1	11	57	50	4	6	6	-	7	-	53	-	2	9
WM	1	34	8	13	4	6	-	-	15	7	51	53	2	6
Donor	1	2	7	8	-	6	7	-	103	17	52	-	2	5
3rd party	3	31	51	60	4	6	3	-	4	11	52	53	7	8
JC	2	3	35	44	4	6	4	5	1	13	52	-	5	6
Donor	2	3	51	18	4	6	2	-	11	-	52	-	7	-
3rd party	1	26	45	57	4	6	6	-	4	7	53	-	7	9
TF	2	3	35	51	4	6	4	-	4	11	52	53	7	8
Donor	2	-	44	-	-	-	5	-	4	-	53	-	7	-
3rd party	30	31	13	18	4	6	5	6	7	14	52	53	2	5

Table 9. HLA typing of patients 1 to 10, their cardiac donors and 3rd party cells used in the CTLp assays. NT = not tested. (Contd. over).

Identification	HLA locus													
	A		B		Bw		Cw		DR				DQ	
GF	1	2	8	44	4	6	5	-	15	4	51	53	6	7
Donor	1	2	8	62	-	6	3	7	17	4	52	53	2	8
3rd party	25	32	18	47	4	6	2	-	11	15	51	52	6	7
AM	2	11	7	27	4	6	2	-	1	4	53	-	5	7
Donor	1	-	8	39	-	6	-	-	4	14	52	53	5	8
3rd party	3	34	7	-	-	6	7	-	15	-	51	-	6	-
TD	2	11	7	50	-	6	6	7	7	14	52	53	2	5
Donor	1	31	8	-	-	6	7	-	17	-	52	-	2	-
3rd party	2	11	5	44	4	-	5	-	15	4	51	53	6	8
WB	1	-	7	8	-	6	NT		15	17	51	52	2	6
Donor	2	11	8	35	-	6	NT		103	11	52	-	5	7
3rd party	3	28	18	62	-	6	3	-	12	14	52	-	5	7
JD	2	3	8	49	4	6	7	-	17	4	52	53	2	8
Donor	2	11	35	60	-	6	3	4	4	-	53	-	7	8
3rd party	26	32	17	62	4	6	3	6	7	14	52	53	7	9

Table 9 (contd.). HLA typing of patients 11 to 15, their cardiac donors and 3rd party cells used in the CTLp assays. NT = not tested.

Patient	Specificity	Day	CTLp	95 % CI	Chi^2	p
IM	Donor	0	1479	1106-1976	0.80	0.85
		82	728	537-989	3.91	0.79
		356	304	237-389	8.75	0.27
	3rd Party	0	216	139-335	2.64	0.45
		82	410	316-532	2.06	0.96
		356	185	146-234	7.59	0.37
PK	Donor	0	590	443-786	3.21	0.86
		104	136	108-171	4.20	0.76
		272	429[a]	330-559	8.88	0.18
	3rd Party	0	7518	75-1x10[6]	0.00	1.0
		104	475	360-627	0.96	1.0
		272	1131	818-1562	0.35	1.0
SS	Donor	0	1122	830-1517	3.81	0.70
		91	623	476-816	6.61	0.47
		259	536	413-696	4.41	0.73
	3rd Party	0	758	572-1004	4.47	0.72
		91	594	455-775	6.04	0.54
		259	766	578-1014	5.84	0.56
GM	Donor	0	351	264-467	2.23	0.95
		80	41	31-54	7.85	0.35
		290	14	10-19	15.68	0.03
	3rd Party	0	399	299-533	11.75	0.11
		80	320	251-407	11.62	0.11
		290	135	107-170	11.76	0.11
RF	Donor	0	293	231-372	18.41	0.01
		200	36	28-47	7.38	0.39
		257	150	116-193	16.14	0.02
	3rd Party	0	375	293-479	6.21	0.52
		200	27	20-36	12.64	0.08
		257	216	166-282	14.57	0.02

Table 10. Frequencies of donor-reactive and 3rd party-reactive CTLp for patients 1 to 5. CTLp = frequency per million peripheral blood MNC. 95 % CI = 95 % confidence interval for the frequency estimate. a = adjusted CTLp frequency. (Contd. over).

Patient	Specificity	Day	CTLp	95 % CI	Chi^2	p
JH	Donor	0	117	91-151	13.08	0.04
		163	254	194-332	11.12	0.05
		289	19	11-31	5.62	0.35
	3rd Party	0	699	530-920	5.94	0.55
		163	628	473-833	4.50	0.72
		289	216	166-281	4.50	0.72
GS	Donor	0	109[a]	85-141	11.46	0.08
		112	28	21-37	7.16	0.41
		455	110	86-140	12.10	0.10
	3rd Party	0	334	262-426	9.72	0.21
		112	64	50-82	3.81	0.80
		455	136	106-174	6.27	0.51
WM	Donor	0	362[a]	277-471	6.23	0.18
		85	79	62-100	8.64	0.28
		295	82	64-105	11.22	0.13
	3rd Party	0	582[a]	446-759	7.43	0.19
		85	220	168-287	8.31	0.30
		295	2304	1545-3436	0.50	1.0
JC	Donor	0	342	260-449	3.07	0.88
		112	53	42-68	10.55	0.16
		373	28	21-37	16.32	0.02
	3rd Party	0	174	135-226	19.52	0.007
		112	72	57-92	12.30	0.09
		373	104	82-133	7.90	0.34
TF	Donor	0	102	79-132	4.75	0.58
		124	AF			
		306	255	195-333	12.13	0.06
	3rd party	0	198	153-258	12.18	0.06
		124	AF			
		306	256	196-335	10.64	0.16

Table 10 (contd.). Frequencies of donor-reactive and 3rd party-reactive CTLp for patients 6 to 10. CTLp = frequency per million peripheral blood MNC. 95 % CI = 95 % confidence interval for the frequency estimate. a = adjusted CTLp frequency. AF = assay failure. (Contd. over).

Patient	Specificity	Day	CTLp	95 % CI	Chi^2	p
GF	Donor	0	74	58-93	10.47	0.16
		121	26	19-37	21.41	0.002
		303	130	99-171	10.64	0.06
	3^{rd} Party	0	168	130-217	10.59	0.16
		121	259	198-339	24.30	0.001
		303	685	515-912	14.70	0.01
AM	Donor	0	256	196-335	14.43	0.04
		105	94	72-123	20.90	0.002
		303	125	97-161	7.83	0.35
	3^{rd} Party	0	512	388-677	3.93	0.79
		105	121	93-158	17.40	0.008
		303	139	106-182	2.61	0.76
TD	Donor	0	295	225-386	7.60	0.37
		79	146	112-190	6.60	0.36
		273	335	255-439	5.20	0.64
	3^{rd} party	0	281	214-367	3.93	0.79
		79	98	75-129	7.23	0.30
		273	156	121-202	16.15	0.02
WB	Donor	0	1149	848-1557	11.97	0.10
		85	155	120-201	6.69	0.46
		301	505	382-667	5.84	0.56
	3^{rd} party	0	410	311-539	10.91	0.09
		85	150	115-195	13.10	0.04
		301	188	145-244	14.41	0.04
JD	Donor	0	404	307-531	14.50	0.04
		102	165	128-213	5.67	0.58
		354	605	456-802	5.78	0.57
	3^{rd} party	0	610	460-809	9.92	0.19
		102	351	267-461	15.42	0.03
		354	746	560-995	3.51	0.83

Table 10 (contd.). Frequencies of donor-reactive and 3^{rd} party-reactive CTLp for patients 11 to 15. CTLp = frequency per million peripheral blood MNC.

95 % CI = 95 % confidence interval for the frequency estimate.

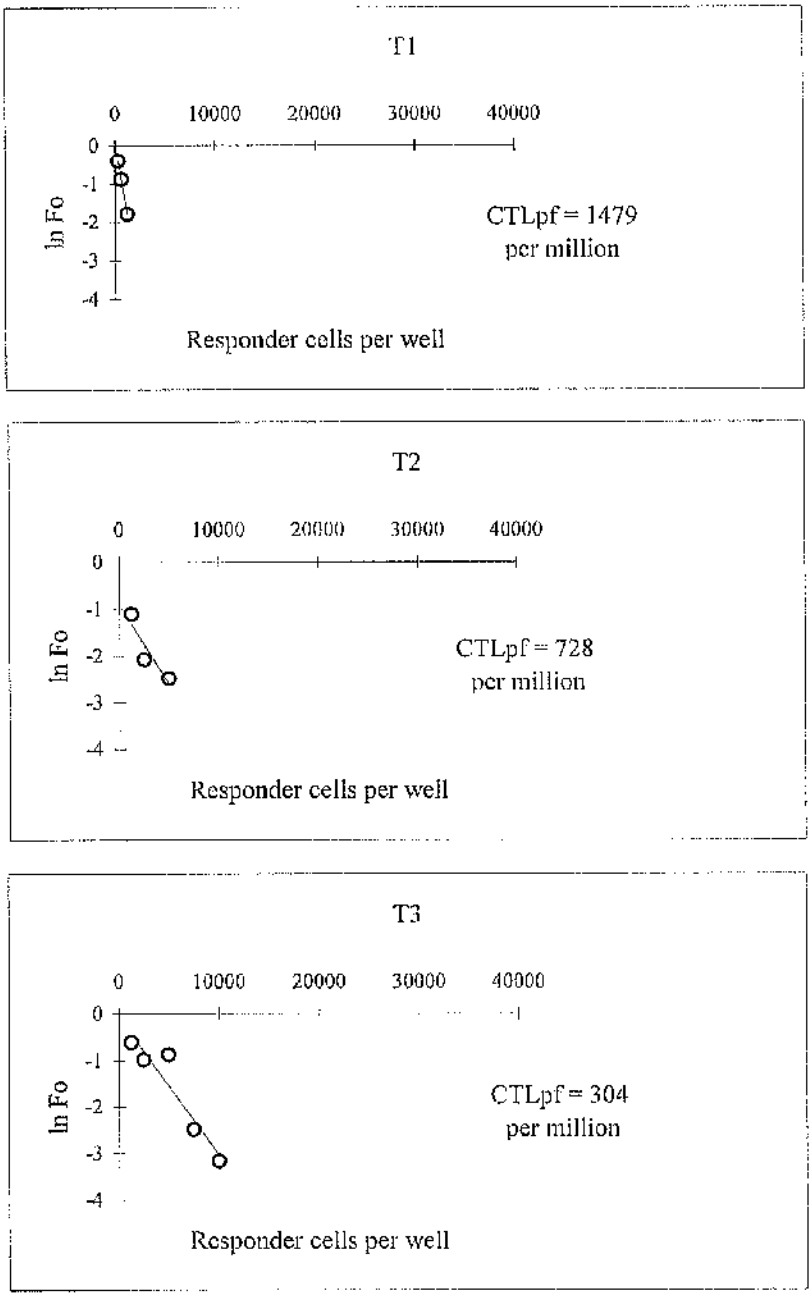

Figure 17. Limiting dilution curves of donor-reactive CTLp assays for patient IM pre-transplant (T1) and at three months (T2) and one year (T3) post transplant. Log to base e of the fraction of negative cultures (lnFo) was plotted at each responder cell concentration. Responder concentrations with all cultures positive were omitted. A straight line was fitted to the points by regression analysis.

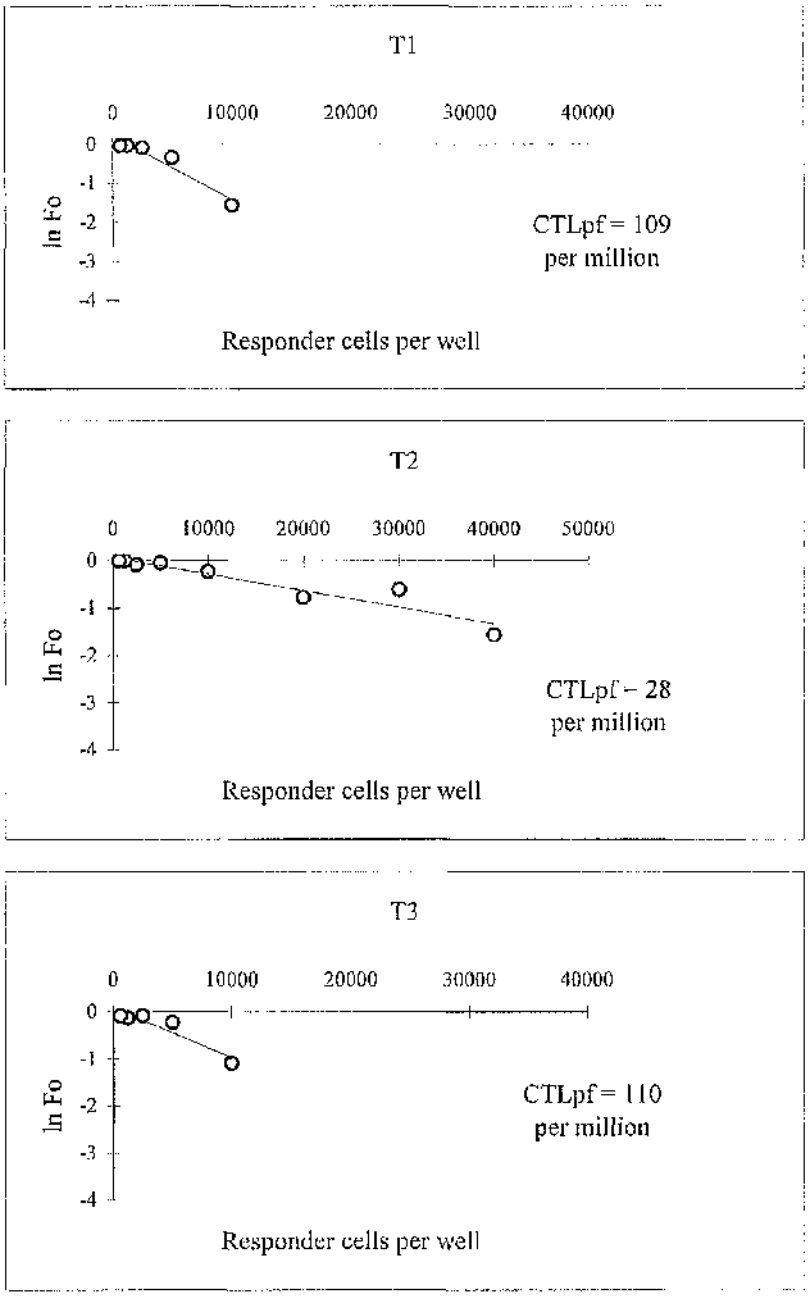

Figure 18. Limiting dilution curves of donor-reactive CTLp assays for patient GS pre-transplant (T1) and at 4 months (T2) and 15 months (T3) post transplant. Log to base e of the fraction of negative cultures (lnFo) was plotted at each responder cell concentration. Responder concentrations with all cultures positive were omitted. A straight line was fitted to the points by regression analysis.

4.3. Acute rejection and immunosuppression.

The number of EMB graded ≥ 2 in the first 90 days and in the first year after transplantation ranged from 0 to 5 (median 2), and 1 to 10 (median 3), respectively. The time from transplantation to the first EMB graded ≥ 2 ranged from 15 to 124 days (median 29 days). Details of EMB results are given in table 11.

Total doses of CsA and azathioprine received by patients ranged from 64,200 to 141,575 mg (median 111,025 mg), and 18,750 to 67,525 mg (median 45,520 mg), respectively. The number of additional courses of steroids given to treat acute rejection ranged from 0 to 6 (median 2). Details of immunosuppression are given in table 12.

4.4. Comparison of pre-transplant CTLp frequencies in patients grouped by the number of HLA mismatches with their donor.

Pre-transplant donor- and 3rd party-reactive CTLp frequencies grouped by the number of HLA mismatches between recipient and donor were compared using the Mann-Whitney T statistic. Pre-transplant donor-reactive CTLp frequencies were lower in the HLA-A, -B, -DR and -DQ 0 to 4 mismatch group than the HLA-A, -B, -DR and -DQ 5 to 8 mismatch group (figure 19, $p < 0.05$, $n = 10$). No differences were seen in donor-reactive CTLp frequencies grouped by HLA class I or class II mismatches independently, or in 3rd party-reactive CTLp frequencies in any of the mismatch groups.

Patient	T1 to T2[a]		T2 to T3		T1 + 90 days		T1 + 1 year		Days to first[d]
	No.[b]	Sum[c]	No.	Sum	No.	Sum	No.	Sum	
IM	3	8	2	5	3	8	5	13	34
PK	1	2	0	0	0	0	1	2	104
SS	3	6	1	2	3	6	4	8	16
GM	1	3	0	0	1	3	1	3	15
RF	2	6	0	0	0	0	3	9	124
JH	2	4	4	9	1	2	7	15	30
GS	5	15	0	0	5	15	5	15	27
WM	3	9	3	8	3	9	10	25	29
JC	2	5	1	3	2	5	3	8	21
TF	4	11	2	5	3	8	6	16	22
GF	1	3	2	5	1	3	4	12	46
AM	2	5	1	2	2	5	3	7	16
TD	1	2	1	3	2	5	2	5	48
WB	2	4	0	0	2	4	2	4	22
JD	2	6	1	2	1	3	3	8	32

Table 11. Summary of EMB results for the 15 cardiac transplant recipients. a = time interval during which EMB took place (T1 = pre-transplant, T2 = 79 to 200 days post-transplant, T3 = 257 to 455 days post-transplant), b = number of EMB graded ≥ 2, c = summed grades of EMB graded ≥ 2 (eg. one grade 2 EMB and two grade 3 EMD gives sum of 8), d = number of days from transplantation to first EMB graded ≥ 2.

Patient	Cyclosporin A (mg)[a]	Azathioprine (mg) [b]	Courses of steroids [c]
IM	82675	47925	3
PK	88750	25725	1
SS	125325	46900	3
GM	64200	32775	1
RF	92400	18750	2
JH	109950	27775	4
GS	122700	47800	6
WM	111025	61250	5
JC	140625	59375	3
TF	141575	51125	5
GF	102475	42250	2
AM	135950	67525	2
TD	90675	34075	2
WB	120750	36750	0
JD	118220	45520	2

Table 12. Summary of immunosuppression received by the 15 cardiac transplant patients. a and b = total dose of CsA and azathioprine, respectively, given to each patient in the period from transplantation to the T3 sample. c = total number of additional courses of steroids given for acute rejection in the period from transplantation to the T3 sample. Full details of immunosuppression protocols are given in the methods.

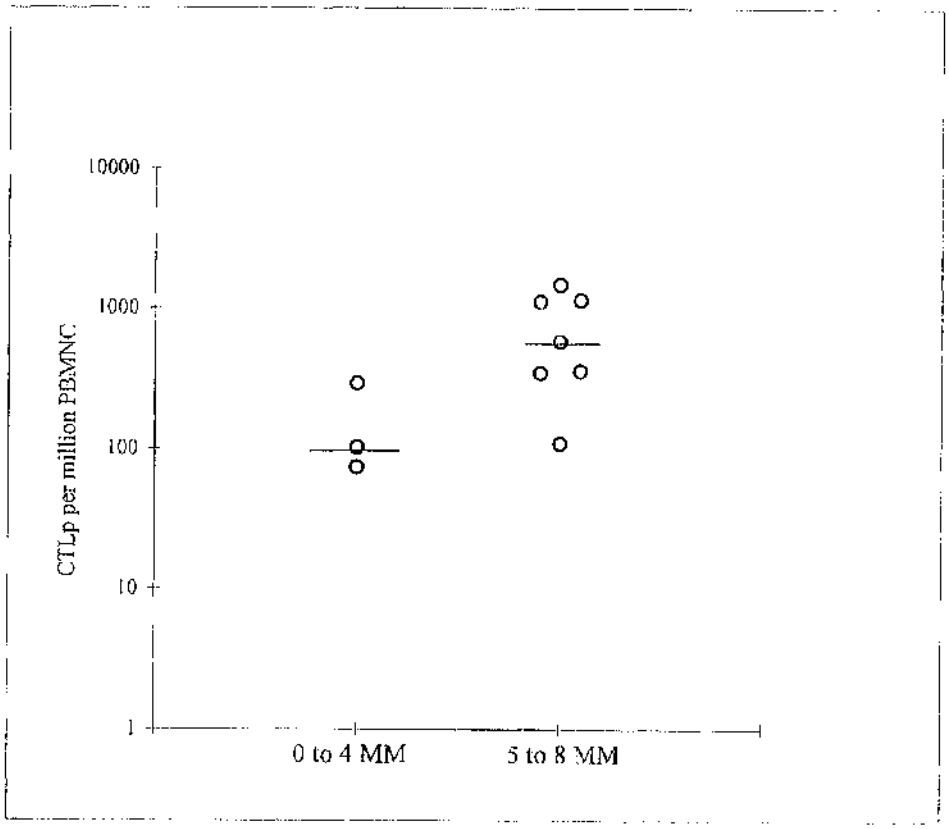

Figure 19. Pre-transplant donor-reactive CTLp frequencies (CTLp per million peripheral blood MNC (PBMNC)) grouped by number of HLA-A, -B, -DR and -DQ mismatches (MM) between recipient and donor. Frequencies were lower in the better matched group ($p < 0.05$). The medians are shown by horizontal lines.

4.5. Relation between pre-transplant CTLp frequency and acute rejection.

The relation between pre-transplant donor- and 3rd party-reactive CTLp frequencies and EMB-diagnosed rejection (number of EMB graded ≥ 2 by 90 days and by 1 year after transplantation, summed grades of EMB graded ≥ 2 by 90 days and by 1 year after transplantation, number of days to first EMB graded ≥ 2) was investigated using the Spearman rank correlation coefficient. No correlation was found with $p < 0.05$.

4.6. CTLp frequencies in cardiac transplant recipients.

Pre-transplant donor- and 3rd party-reactive CTLp frequencies were high before transplantation, ranging from 74 to 7518 CTLp per million peripheral blood MNC (median 375).

In assays with single-hit kinetics, by 3 to 7 months after transplantation donor-reactive CTLp frequencies were lower than pre-transplant frequencies in nine patients, but by 9 to 15 months this number had fallen to four (table 10). 3rd party-reactive CTLp frequencies after transplantation were lower than pre-transplant frequencies in four patients at both post-transplant time intervals, indicating that changes in CTLp frequency were not always donor-specific. Two patients had higher donor-reactive CTLp frequencies and one patient had a higher 3rd party-reactive CTLp frequency by 9 to 15 months after transplantation compared to pre-transplant frequencies. Changes in 3rd party-reactive CTLp frequencies were seen less frequently than changes in donor-reactive CTLp frequencies (table 10).

The largest decrease in donor-reactive CTLp frequency by 9 to 15 months after transplantation was by 4- to 5-fold in two patients, IM and WM, with T3 frequencies of 304 and 82 per million peripheral blood MNC, respectively. The frequency in one other patient (JH) was 19 CTLp per million peripheral blood MNC 9 months after transplantation, but the pre-transplant frequency estimate did not conform to single-hit kinetics so a comparison could not be made. Donor-reactive CTLp frequency decreased by 8-fold (to 41 cells per million) in patient GM, and 6-fold (to 53 cells per million) in patient JC by 2 to 4 months after transplantation. Donor-reactive cytotoxicity decreased further by 9 to 12 months in these patients, but these assays had non-single hit kinetics.

4.7. CTLp frequencies at different sampling times in the group of patients.

CTLp frequencies in the different sample time groups (T1, T2 and T3) were compared using the sign test with paired frequencies. Only the donor-reactive pre-transplant (T1) and 3 to 7 month (T2) CTLp frequency groups were different (figure 20, $p = 0.02$, $n = 7$). No significant differences were seen in 3rd party-reactive CTLp frequencies between any of the three time groups, or when donor- and 3rd party-reactive CTLp frequencies were compared at each of the time intervals.

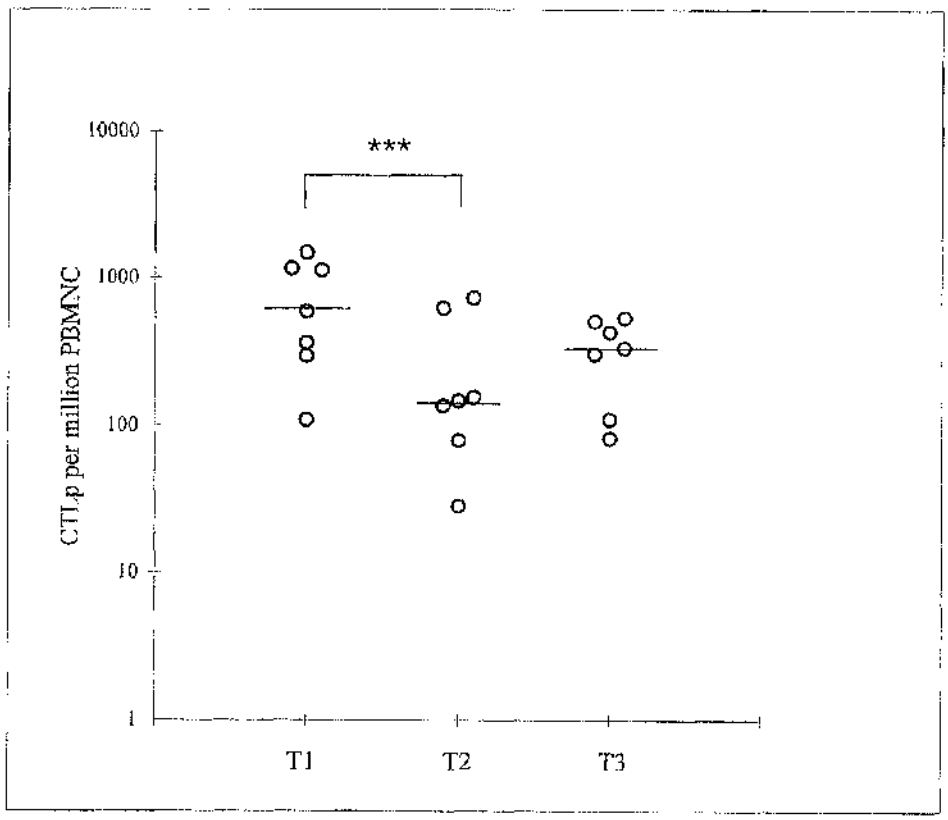

Figure 20. Donor-reactive CTLp frequencies (CTLp per million peripheral blood MNC (PBMNC)) pre-transplant (T1), and at 3-7 months (T2) and 9-15 months (T3) after transplantation.
*** Pre-transplant and T2 frequencies were different ($p = 0.02$). There were no other significant differences between groups. The medians are shown by horizontal lines.

4.8. Serial changes in CTLp frequency in individual patients.

Donor-reactive CTLp frequencies with single-hit kinetics were available at all three sampling intervals for seven patients. Donor-reactive CTLp frequencies decreased at the first post-transplant sample in all seven patients, but thereafter the patterns of change differed, with one patient showing a further decrease, two patients no further change and four patients showing an increase in CTLp frequency (figure 21). A concomitant decrease in 3rd party reactive CTLp frequency was seen in three samples (WM, TD and GS, T2 samples).

4.9. Comparison of post-transplant CTLp frequencies grouped by acute rejection grade.

Donor-reactive CTLp frequencies from samples taken at the time of grade 0 or 1 EMB were compared with donor-reactive CTLp frequencies from samples taken at the time of grade 2 or 3 EMB using the Mann-Whitney T statistic (there were no grade 4 EMB at any of the sampling points). No difference between the two groups was seen when the T2 and T3 samples were analysed together ($p > 0.1$, $n = 22$). Similarly, no difference was seen between the two groups in the T2 samples ($p > 0.1$, $n = 11$). In the T3 samples donor-reactive CTLp frequencies were lower in samples taken at the time of grade 2 or 3 EMB than grade 0 or 1 EMB (figure 22, $p < 0.05$, $n = 11$).

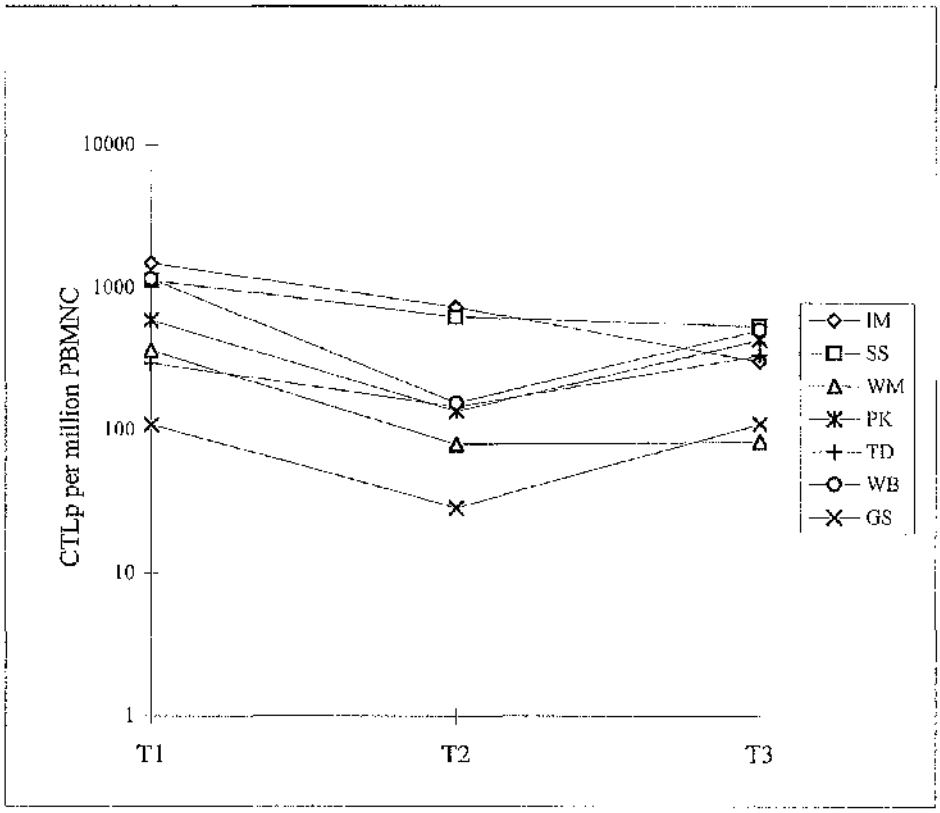

Figure 21. Serial changes in donor-reactive CTLp frequency (CTLp per million peripheral blood MNC (PBMNC)) for seven patients. T1 = pre-transplant, T2 = 3-7 months post- transplant, T3 = 9-15 months post-transplant.

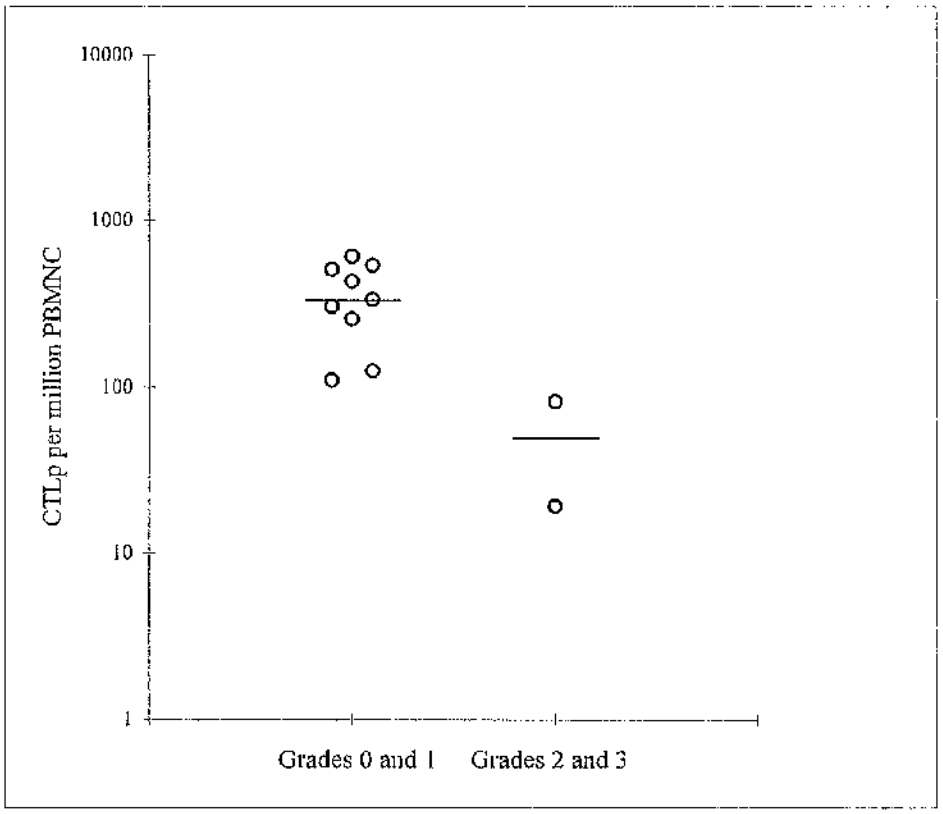

Figure 22. T3 (9-15 months post-transplant) donor-reactive CTLp frequencies (CTLp per million peripheral blood MNC (PBMNC)) from samples taken at the time of EMB graded 0 and 1 and EMB graded 2 and 3. Frequencies were lower at the time of grade 2 and 3 EMB ($p < 0.05$). The medians are shown by horizontal lines.

4.10. Relation between post-transplant donor-reactive CTLp frequencies and rejection in preceding periods.

Spearman rank correlation co-efficients (r_s) were calculated for the T2 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period up to the sampling time. Similar calculations were made for the T3 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period between the T2 and T3 samples, and the T3 sample CTLp frequencies and the number and summed grades of EMB graded ≥ 2 in the period from transplantation to the T3 samples. A significant negative correlation was found for the T3 sample donor-reactive CTLp frequencies and number of EMB graded ≥ 2 in the period from transplantation to the T3 samples (r_s = - 0.71, 0.05 > p > 0.02, n = 9, figure 23).

Donor-reactive CTLp frequencies in the T3 samples did not correlate with the total dose of CsA or azathioprine received in the period from transplantation to the T3 sample. There was a negative correlation of donor-reactive CTLp frequencies with the number of additional courses of steroids in the period from transplantation to the T3 samples, but this did not reach significance (r_s = -0.56, 0.1 > p > 0.05, n = 12, figure 24).

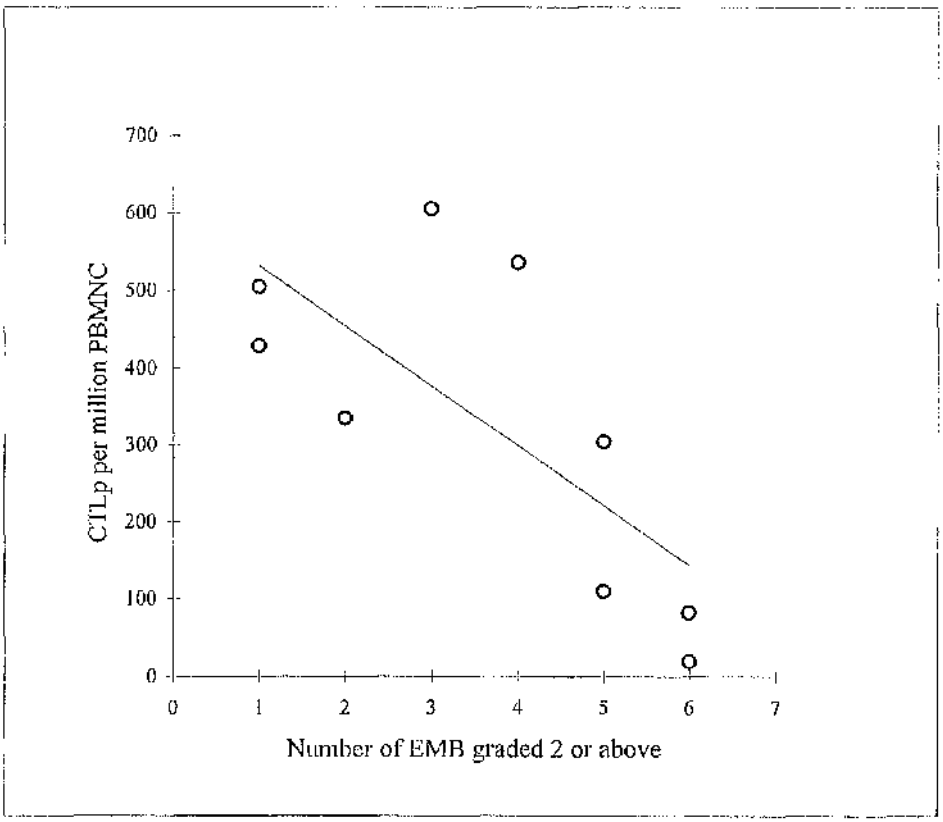

Figure 23. Donor-reactive CTLp frequencies (CTLp per million peripheral blood MNC (PBMNC)) at T3 (9-15 months post-transplant) plotted against number of EMB graded 2 and above from transplantation to T3. A significant negative rank correlation was found ($r_s = -0.71$, $0.05 > p > 0.02$).

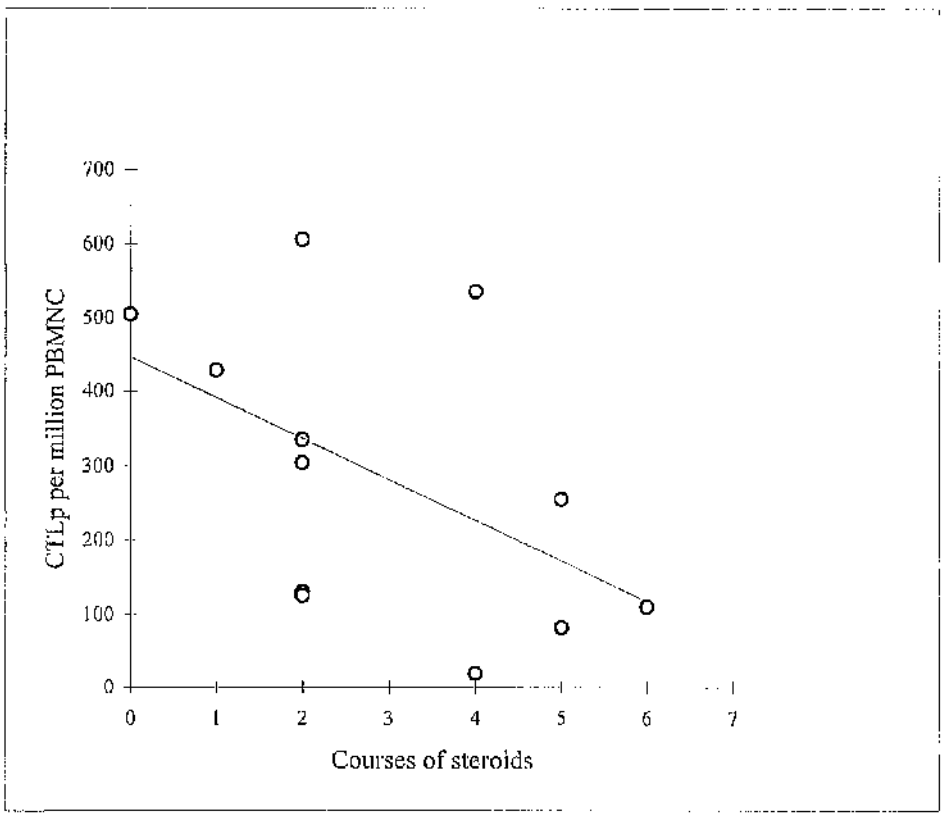

Figure 24. Donor-reactive CTLp frequencies (CTLp per million peripheral blood MNC (PBMNC)) at T3 (9-15 months post-transplant) plotted against number of courses of steroids from transplantation toT3. Rank correlation was not significant ($r_s = -0.56$, $0.1 > p > 0.05$).

3rd party-reactive CTLp frequencies with single-hit kinetics were available for the T2 and T3 samples for six of these patients, but no significant correlation was found. The negative correlation between the T3 sample donor-reactive CTLp frequencies and number of EMB graded ≥ 2 in the period from transplantation to the T3 samples was higher for these six patients than the initial nine (r_s = -0.87, 0.02 > p > 0.01), however the correlation with summed EMB grades did not reach significance (r_s = -0.73, 0.1 > p > 0.05).

4.11. Influence of induction immunosuppression with ATG on CTLp frequency.

Donor- and 3rd party-reactive CTLp frequencies at each of the two post-transplant time groups were compared between patients who received induction therapy with ATG and patients who received standard triple immunosuppression. The Mann-Whitney T statistic was used for comparisons. No differences between ATG-treated patients and patients given standard triple immunosuppression were seen in donor-reactive (n = 9) or 3rd party-reactive (n = 8) CTLp frequencies.

DISCUSSION

1. Assessment of the Matrix 96 Direct Beta Counter.

1.1. Determination of counting time for the Matrix 96 Direct Beta Counter.

A 19 min count using the Matrix 96 gave results which most closely matched the gamma counter cpm. Counts in the lower part of the range tested were given greater consideration than higher counts because the threshold value for defining positive wells in the CTLp assay is usually less than 500 counts. The divergence seen at higher counts was considered acceptable, as values in this region would be positive in a CTLp assay.

For the 19 min count the calculated mean cpm of the Matrix 96 were about 5 - 6 % of gamma cpm over most of the counting range, falling to 4.8 % at 200 cells and 3.9 % at 100 cells. The latter two values were lower because the Matrix 96 counts fell below the gamma counts at these cell numbers. Converting these values for equivalent sample volumes gives a Matrix 96 counting efficiency of 10 - 12 % of the gamma counter over most of the range. This compares to a value of 17 % reported previously (12). This difference could be explained by the different harvesting procedures used for the gamma counters. The value of 17 % efficiency was obtained by counting 150 µl supernatant, compared to 100 µl in the present study. A different model of gamma counter was used in the previous work and this may also have had a different counting efficiency from the model used in this study.

1.2. Comparison of the Matrix 96 with a gamma counter.

1.2.1. Agreement of the counters.

The limits of agreement of the Matrix 96 and gamma counter were -69.2 to 79.6. Matrix 96 counts may thus be 69.2 less or 79.6 more than cpm obtained by the gamma counter. While these differences appear large, with increasing counts the between counter bias changes from positive to negative with respect to the gamma counter (figure 4). The analysis may tend to overestimate the limits of agreement under such circumstances due to the complexity of the relationship (14).

1.2.2. Repeatability of the counters.

Differences between the counters were anticipated in view of the variation in repeated counts by each counter. Both counters had good repeatability. After log transformation, more than 95 % of differences between repeated counts using the Matrix 96 were within 2 SD of the mean. The co-efficient of repeatability for the Matrix 96 was similar to that for the gamma counter. Differences between repeated counts were less for the Matrix 96 than for the gamma counter at lower ^{51}Cr-release values (figures 5 and 6) which are critical for defining positive wells in the CTLp assay. The co-efficients of repeatability for repeated counts by either counter were lower than that found for the two counters compared. This could be expected given the changing bias between the counters with increasing counts. The conclusion of all these experiments was that the Matrix 96 was considered to be an acceptable alternative to the gamma counter for the measurement of ^{51}Cr in cell-culture supernatants.

A correlation coefficient of 0.984 between Matrix 96 and gamma counter counts was reported in the measurement of ^{51}Cr release from cell-mediated lympholysis assays (12). The present results give a correlation coefficient between the Matrix 96 and gamma counter of 0.985. As correlation co-efficients measure strength of linear association, which is not the same as agreement, and do not give a useful indication of the differences between two methods (14), the present approach can be considered more informative.

The Matrix 96 has quality control software which ensures that the detectors are operating at the optimal voltages (11). Optimal detector voltages were maintained throughout the period of the study. That the means of differences were below zero in two of three repeated Matrix 96 counts may suggest that there is some alteration of the samples during counting and would caution against using repeated measurements of the same samples for the analysis of clinical results.

Sample preparation was also found to be easier for the Matrix 96 than for a gamma counter. When this is considered with the ability to count 96 samples simultaneously, the use of the Matrix 96 gave faster, more easily obtained, yet comparable results.

After this initial assessment the Matrix 96 was used to count ^{51}Cr-release in CTLp assays of 45 samples from cardiac transplant recipients, and was found to be a rapid and convenient method for counting large numbers of limiting dilution assays.

2. Assessment of natural killer-like activity in the CTLp assay.

2.1. K562-directed cytotoxicity in CTLp assays.

K562-directed cytotoxicity was present in CTLp cultures in an assay between HLA-identical siblings and in three assays between unrelated individuals with varying numbers of HLA mismatches. Comparable lysis of K562 targets has previously been reported in CTLp cultures (50 % specific lysis at 10^4 responders with 5 units/ml IL-2), together with increased proportions of CD56 +ve cells (335).

As expected, a low CTLp frequency (3 CTLp per million peripheral blood MNC) was found in the assay of HLA-identical siblings. Specific lysis of K562 was lower in this assay than at equivalent responder cell numbers in the assays of unrelated individuals. The K562-directed cytotoxicity in the assay of HLA-identical siblings is probably the result of activation of NK-like activity by the IL-2 added to the cultures. The higher specific lysis of K562 at equivalent responder dilutions in the assays between unrelated individuals suggests that alloactivation also enhances the K562-directed activity in the CTLp cultures. This may be mediated by cytokines such as IL-2, IFN-γ, interleukin-6 and TNF-α, which increase the activity of NK cells (388) and are released during MLC (389). In future work, the cytokines released by HLA-identical and unrelated cells cultured in CTLp assay conditions could be measured to confirm this.

Cytotoxicity towards K562 is increased during primary MLC, but peaks at five to six days and returns to its initial level by day nine (354, 390). The time course of K562-directed cytotoxicity was not measured in the present study, so a comparison with, and

comment on the kinetics in the primary MLC cannot be made. However, it is possible that alloactivation-enhanced K562-directed activity is maintained over the ten day culture period by the IL-2 added to the CTLp assay.

2.2. Cold target competition by stimulator-type PHA blasts of killing of K562 targets.

The specificity of the NK-like activity was investigated by adding unlabelled target cells to the cytotoxicity assays. The increased lysis of K562 in the presence of unlabelled stimulator-type blast cells was higher than expected from the experiment in which target cell ratios were determined, and this complicated the interpretation of these assays. This increased lysis of K562 could have been mediated directly by the unlabelled PHA blasts. Stimulation of lymphocytes with PHA and IL-2 induces cytotoxicity towards tumour cell targets (391, 392) and such cytotoxicity could be responsible for the augmented killing of K562. The IL-2 added to the PHA blast cultures, as well as IL-2 and other cytokines released by the PHA-activated T cells would also be expected to enhance the activity of NK cells present in the PHA cultures (153, 388).

An alternative explanation considered was that the PHA blast cells, although washed before being added as competitors, continued to release cytokines in sufficient amounts to enhance the NK-like activity in the CTLp assay cultures. This could explain why augmentation of NK-like activity shows apparent dose dependency, with lysis of K562 not seen at higher responder cell numbers. The amount of cytokine available to each

effector would be reduced at high cell numbers, with less enhancement of NK-like activity. However, cytokine gene expression by activated T cells is rapid and short-lived. Increased expression of genes for IL-2 and IFN-γ is detected between 1 and 4 hours after *in vitro* stimulation of peripheral blood mononuclear cells with PHA or anti-CD3 monoclonal antibody (393). mRNA levels for IL-2 and IFN-γ peak at 5 to 8 hours, with a second peak of IFN-γ mRNA at 20 hours (393, 394). Other cytokines are expressed during T lymphocyte activation, and although the expression kinetics of individual cytokine genes varies, the rapid accumulation of mRNA followed by the cessation of transcription is a general feature (87, 395).

Levels of secreted IL-2 peak within 24 hours of stimulation, whereas IFN-γ can continue to increase for up to 40 hours (396, 397). IL-2 is removed from the culture medium by the stimulated lymphocytes and its activity declines to undetectable levels after 4 to 5 days (397, 398). The PHA blasts were washed and used as competitors after 10 days of culture by which time cytokine expression would have ceased, therefore it seems unlikely that NK-like activity could be enhanced by this mechanism.

If not a cytokine-mediated effect, the abrogation of augmented lysis of K562 at higher responder cell numbers could be accounted for by lysis of the unlabelled PHA blast cells by the more numerous alloreactive CTL likely at higher responder cell concentrations.

Treatment of the PHA blast cells to inhibit their cytotoxicity could prove difficult as both granule-mediated and Fas-mediated mechanisms would need to be addressed without affecting the cytotoxic activity of effectors generated in the CTLp cultures. Granule-

mediated cytotoxicity can be inhibited by agents such as concanamycin A (399), and Fas-mediated cytotoxicity can be inhibited by a soluble Fas-immunoglobulin fusion protein (400). The practicalities of the use of such agents would require further investigation. The use of a lower competitor to target ratio could permit the reduction of the augmented cytotoxicity, but could also reduce the ability to detect inhibition. The size of split-well CTLp assays makes difficult the use of different competitor : target ratios in the same assay. To resolve this it may be necessary to use effectors generated in limiting dilution cultures at defined effector : target ratios, but this would not fully reflect CTLp assay conditions, which was the intention in these experiments.

K562 cell lysis was not inhibited in any of the assays, although the possibility that inhibition may have been masked by the augmented cytotoxicity seen in the presence of unlabelled stimulator-type blasts cannot be excluded. Specific lysis of K562 was similar at higher responder cell numbers with and without competitors.

2.3. Cold target competition by K562 cells of killing of stimulator-type targets.

The addition of excess unlabelled K562 cells did not significantly change the CTLp frequency estimates in four out of five assays, including two assays of cardiac transplant patients tested against their donors. In one assay (number 9), also of cells from a cardiac transplant recipient, there was a reduction in CTLp frequency in the presence of unlabelled K562 cells, but this assay exhibited non-single hit kinetics.

Three of the ten CTLp assays were not consistent with single-hit kinetics. The increased number of manipulations required for the competitor assays could have introduced error, resulting in deviations from single-hit kinetics. The use of fewer ^{51}Cr-labelled target cells than in the standard CTLp assay was necessary to achieve high competitor : target ratios and may have decreased the sensitivity of the assay. In split-well assays, wells which are positive but have low cytotoxicity may sometimes only test positive in one of the split wells (401), and this could also have influenced the results. The technical difficulties of the CTLp assay were apparent in a recent multi-centre study in which non-single hit kinetics were reported in about 20 % of assays (402).

Five different responder-stimulator cell combinations were tested in each type of inhibitor assay. The results were broadly in agreement with previous studies which suggested that NK-like activity is distinct from alloreactive cytotoxicity in MLC and CTLp assays (335, 355, 357, 358). However, NK cells express inhibitory receptors which recognise different groups of HLA alleles and inhibit the lysis of recognised target cells (131), so it is possible that inhibition could occur in other combinations of responder and stimulator cells which express different inhibitory receptors and HLA alleles.

Despite a lack of unequivocal evidence for NK cell-mediated allocytotoxicity in the cold-target competition assays undertaken, the existence of a repertoire of lysis of allogeneic targets by NK cells has been established (360, 362, 363). Greater consideration may therefore have to be given to the possible influence of NK cell activity on assays of cytotoxic alloreactive cells in the future. This will become clearer as more is learned of

the nature and patterns of expression of the receptors and ligands involved in the regulation of NK cell- and CTL-mediated cytotoxicity.

3. Kinetics of the CTLp assays.

Several of the CTLp assays of samples from cardiac transplant patients were not consistent with single hit kinetics. When material was available such assays were repeated. Acceptable chi^2 values were found in 5 of 9 repeated assays, but invalid kinetics were still present in 9 of 44 donor assays and 9 of 44 3rd party assays. In most cases, repeated assays gave CTLp frequencies with overlapping confidence intervals, even when the chi^2 values indicated non-single hit kinetics.

The reasons for these invalid results are not clear. In assays with many reactive precursors growth was vigorous at high responder cell numbers and this could have led to nutrient or cytokine depletion or cell overcrowding. The responder cell dilutions were chosen to enable a broad range of frequencies to be estimated. However, with the retrospective knowledge that most frequencies remained high after transplantation, this problem may have been avoided by the use of a different dilution series.

Cellular interactions can produce non-single hit kinetics (329). The non-single hit kinetics observed here could not be clearly identified with previously described limiting dilution curves (329), so it is uncertain if cellular interactions were involved. Such interactions may be unlikely in pre-transplant samples and 3rd party-reactive CTLp.

Non-single hit kinetics consistent with T cell suppression have been described after renal transplantation (18, 381, 403). The number of positive wells increases with increasing responder cell number initially, then falls, giving a limiting dilution curve with a characteristic V-shaped appearance. This pattern of kinetics could not be clearly identified in the present study. The limited numbers of patient cells available from samples with invalid kinetics did not permit the use of different dilutions of responder cells or purified responder T cells which could have been informative in these cases.

4. Analysis of CTLp frequency in cardiac transplant recipients.

4.1. Comparison of pre-transplant CTLp frequencies in patients grouped by the number of HLA mismatches with their donor.

The high donor-reactive CTLp frequencies found in pre-transplant samples reflect the high degree of mismatching for donor antigens in the patients studied. Donor hearts are not matched for the recipient's HLA type at this centre. The median number of HLA-A, -B, -DR and -DQ mismatches was 5 (range 1 to 7). The range of CTLp frequencies found between normal individuals varies broadly with the number of HLA mismatches (344, 347, 404). High frequencies are also sometimes found between unrelated individuals who are matched for HLA phenotype, with presumed specificity for differences in HLA antigen subtypes or HLA loci often excluded from matching, such as HLA-Cw (405).

The two patients with positive B cell crossmatches (GS and TF) did not have higher pre-transplant donor-reactive CTLp frequencies than the others. This is consistent with the finding that sensitisation is associated with a change in the activation state, rather than the frequency of CTLp (406).

In the patients studied here, although lower donor-reactive CTLp frequencies were found in pre-transplant samples in the group with fewer HLA mismatches, no differences were found in 3rd party-reactive CTLp frequencies. The degree of mismatching with the 3rd party cells (median 7 HLA mismatches, range 1 to 8) was similar to that with donors. The absence of a consistent relationship between CTLp frequency and HLA mismatches

with donors and 3rd party cells is probably due to the scarcity of well matched combinations and the small numbers studied.

4.2. Relation between pre-transplant CTLp frequency and acute rejection.

No correlation was found between pre-transplant donor-reactive CTLp frequency and acute rejection. This agrees with previous reports in cardiac (368) and renal transplantation (18, 407 - 409), however Hu *et al.* did find a positive correlation between pre-transplant CTLp frequency and summed rejection score in the first eighteen months after cardiac transplantation (19).

Other features of T lymphocytes, such as their activation state, avidity for donor cells or susceptibility to inhibition by immunosuppressive drugs, may be more relevant to rejection than the total frequency of alloreactive T cells, especially when prior sensitisation has occurred (410 - 412).

Patients who died during the follow-up period were not tested because the main aim of the clinical part of the study was to follow changes in CTLp frequency over a 9 to 12 month period. It is possible, however, that the exclusion of these patients may have influenced this particular analysis. Testing of pre-transplant CTLp frequencies in such patients may be of interest in the future.

4.3. Post-transplant CTLp frequencies in cardiac transplant recipients.

Group analysis of paired CTLp frequencies showed that donor-reactive CTLp frequencies decreased significantly by 3 to 7 months after transplantation, but by 9 to 15 months were not different from pre-transplant frequencies. Within the group, however, individual variation was seen. In the seven patients analysed sequentially, donor-reactive CTLp frequencies decreased by 3 to 7 months, but thereafter, donor-reactive CTLp frequencies decreased further in one patient, remained unchanged in two patients and increased in four patients. In three of the seven patients the decrease by 3 to 7 months was not donor-specific, as decreases were also seen in 3rd party-reactive frequencies. No consistent patterns of change were seen in 3rd party-reactive CTLp frequencies, and these were above 100 per million peripheral blood MNC by 9 to 15 months in all patients.

Donor-specific CTLp frequencies decreased in three patients (IM, SS and WM) by 9 to 15 months compared to the pre-transplant frequency. Patient WM developed a B cell lymphoma and CsA was discontinued two weeks before the T3 sample was taken. These three patients did not receive higher total doses of CsA and azathioprine than most other patients, although the number of courses of steroids was to the higher end of the range (table 12). Closer examination of the period before the T3 sampling times showed that patient SS had a biopsy with grade 2 rejection one week before sampling which was treated by increasing CsA, azathioprine and steroids. Patients IM and WM had no rejection within 200 and 100 days of their T3 samples, respectively, although WM had rejection immediately afterwards. The doses of immunosuppression given to these three patients in the weeks before the T3 samples were comparable to those given to other patients. It is possible that the decreased CTLp frequencies in these patients were the

result of immunosuppressive therapy over the post-transplant period, and in the case of SS, following late rejection. However, given that 3rd party-reactive CTLp frequencies were not significantly different from pre-transplant frequencies for IM and SS, and increased over the pre-transplant frequency for WM, donor-specific hyporesponsiveness of CTLp cannot be excluded. Greater than 10-fold decreases in donor-reactive CTLp frequencies were seen in a further two patients (GM and JC), but the T3 sample CTLp assays did not conform to single hit kinetics and there was also a decrease in 3rd party-reactive CTLp frequencies, although of a much smaller magnitude. All except one of these five patients (GM) had three or more EMB indicating rejection in the follow-up period (table 11), so with the possible exception of GM could not be held to exhibit graft acceptance.

Increases in donor-reactive CTLp frequency over the pre-transplant value were seen in two patients. Such increases may be explained by *in vivo* expansion of donor-specific lymphocytes. In one instance, the patient (GF) developed a B cell lymphoma and CsA therapy was stopped. The sample in which the increase in CTLp frequency was found was taken four weeks after cessation of CsA. CsA has a median half-life of 6.4 to 8.7 hours (413), so should have been eliminated before this time. Two weeks later the patient had a grade 4 rejection episode and died. In the second instance (patient TF) the increased CTLp frequency was in a sample taken at the time of a grade 1A EMB with unchanged immunosuppression. If an *in vivo* expansion of donor-specific lymphocytes had occurred in the peripheral blood of this patient it did not appear to be reflected in an adverse reaction in the graft.

Hu *et al.* also found varying patterns of change in CTLp frequency after cardiac transplantation, with donor-reactive CTLp frequencies fluctuating more in some patients than in others. Patients were sampled more frequently than in the present study. The CTLp frequencies in the ten patients studied by Hu *et al.* were less than about 30 per million peripheral blood MNC by 9 to 10 months after transplantation, and remained low for 1 to 5 years (19). CTLp frequencies of such low magnitude were rare in the present study, but the pre-transplant frequencies were also higher. The report by Hu *et al.* does not give details of the type of heart disease the patients presented with, so it is not known if this could account for some of the differences. There were differences in CTLp assay methods, however (7 days versus 10 days incubation, 20 U/ml versus 5 U/ml IL-2). The immunosuppression given and the degree of matching for HLA antigens was similar to the present study. Hornick *et al.* also found low CTLp frequencies in 4 of 10 cardiac transplant recipients but not in samples taken less than 4 years after transplantation (374). The follow-up period in the present study may have been too short to demonstrate decreases of this magnitude. The present findings are similar to those of Loonen *et al.* who found that donor-specific CTLp frequencies were not different before cardiac transplantation and between 3 months and 3 years after transplantation (369).

In renal transplantation, substantial reductions in donor-reactive CTLp and HTLp frequencies have also been reported in only a minority of patients (379 - 381, 414). As in cardiac transplantation, the proportion of patients hyporesponsive to donor antigens may increase with time (409). A transient decrease in donor-reactive CTLp frequencies similar to that seen in the group analysis and four individual patients in the present study was also found by Mestre *et al.* in a subset of renal graft recipients (18). The decrease was more marked, however, in the study of Mestre *et al.* Of possible relevance to this

was that the renal patients in the study of Mestre *et al.* were better matched with their donors for HLA antigens than in the cardiac patients in the present study. Such decreases in donor-reactive CTLp frequencies may reflect the initial high immunosuppression administered to transplant recipients, or could be evidence of a short-lived tolerisation of direct allorecognition. If the latter explanation is true further investigation of this process would be valuable in understanding donor-specific responses after organ transplantation.

The reasons why some patients develop donor-specific hyporesponsiveness after transplantation while others do not are not understood. It has been suggested that donor passenger leukocytes could be involved in this process, as hyporesponsiveness is found more often after the transplantation of livers, which contain large numbers of haematopoietic cells, than after renal or cardiac transplants (380, 415). However, different mechanisms of graft acceptance may operate in cardiac and renal transplantation, compared to liver transplantation. Clonal anergy, immune deviation or active suppression may operate in cardiac and renal transplantation (291), whereas high-dose or activation induced tolerance and mechanisms dependent on lymphoid cell microchimerism have been proposed for liver transplantation (290, 416).

If the reduced T3 CTLp frequencies found in three patients were a result of donor-specific hyporesponsiveness, the clinical relevance of this is not clear. In some studies of renal transplant recipients, decreases in donor-reactive CTLp and HTLp were more common in patients with stable graft function than in those who experienced rejection (18, 409, 414). However, stable graft function can occur in the absence of hyporesponsiveness (369, 379, 380), and graft loss can occur after hyporesponsiveness

has developed. This was shown when low frequencies of donor-directed, primed HTLp were found after late failure of renal grafts (417) and hyporesponsiveness of donor-specific cytotoxic and helper lymphocytes was found in 5 of 10 cardiac transplant patients with chronic rejection (374). These findings have been interpreted as evidence of the involvement of indirect presentation pathways in chronic rejection. It has been suggested in recent reports that indirect presentation may contribute to both acute and chronic rejection of human cardiac allografts (377, 378, 418).

4.4. Relation between post-transplant CTLp frequencies and acute rejection.

No differences were seen in post-transplant donor-reactive CTLp frequencies grouped by EMB grade, except in the T3 samples in which CTLp frequencies were lower in the group with rejection. These patients had the most biopsies indicating rejection (6 each) up to their T3 sample point among the patients studied, so the decreased CTLp frequencies may have been the result of increased immunosuppression. T3 donor-reactive CTLp frequencies were negatively correlated with the number of additional courses of steroids since transplantation, although this was not statistically significant.

Consistent with this was the finding of a significant negative correlation for the T3 sample donor-reactive CTLp frequencies and the number of biopsies indicating rejection in the period from transplantation to the T3 sample. However, no correlation was seen with 3rd party-reactive CTLp frequencies in six of these patients for whom results were available, which suggests that the mechanism could be donor-specific.

In a previous study a decrease in donor-specific reactivity in mixed lymphocyte cultures of cardiac transplant patients with one or more acute rejection episodes was attributed to "the occurrence of rejection and rejection therapy" (419). CML responses were not reduced in these patients.

An alternative explanation could be that CTLp in these patients had migrated from the peripheral blood to the graft. Evidence of sequestration of lymphocytes in the allograft during rejection has come from canine renal transplantation (420). MLC reactivity decreased across the allograft during unmodified rejection, and lymphocytes recovered from rejecting kidneys showed accelerated responses in MLC. Consistent with this was the finding of reduced MLC reactivity in the peripheral blood of human renal transplant recipients, which preceded rejection and changes in immunosuppression (421).

That decreased CTLp frequencies were associated with rejection in only the T3 samples in the present study suggests, however, that entrapment within the graft is not the main explanation and that repeated anti-rejection therapy may have had a cumulative effect on peripheral blood lymphocytes.

Although Reader *et al.* found significant differences between CTL frequencies in the peripheral blood of cardiac transplant patients with different rejection grades (368) this was not confirmed by Vaessen *et al.* (370), who found no relationship between total donor-specific CTL and rejection. The latter group found higher percentages of CTL resistant to inhibition by anti-CD8 monoclonal antibody in the blood during rejection, however, suggesting that the avidity rather than total frequency of CTL is of relevance.

Increased frequencies of donor-specific IL-2 producing cells have also been found during rejection (370, 371).

The lack of a positive association between donor-reactive CTLp frequency and rejection in this and other studies may be explained if cytotoxic lymphocytes in the periphery do not reflect those in the graft in terms of their frequency, activation state or avidity. Donor-specific CTL frequencies are higher in lymphocytes derived from EMB than from peripheral blood (375) and committed CTL are found more often in EMB-derived cell lines than in peripheral blood during rejection (376). Frequencies of high avidity CTL resistant to inhibition by anti-CD8 monoclonal antibody are higher in patients with rejection than in rejection-free patients in EMB-derived cultures, but not in MLC-stimulated peripheral blood MNC (256). Furthermore, preferential expression of TCR Vβ gene families seen in EMB-derived T-cell lines was not seen in peripheral blood T cells, suggesting clonal expansion of activated donor-specific CTL in the graft (422). These findings are consistent with histological evidence for an infiltration of activated lymphocytes during rejection (208). Donor-reactive cells in the periphery also differ in activation state and frequency from those in the central lymphoid organs (423), which are presumably also directly relevant to the host response.

Van Emmerik *et al.* have proposed that committed CTL with high avidity for donor cells are required for rejection (235, 258). Such CTL are found in EMB cultures from rejecting hearts and may also be detected in peripheral blood by modified CTLp assays, but are not reflected in total numbers of alloreactive CTLp (370). This may explain why the persistence of donor-specific CTLp in peripheral blood appears not to be harmful to the graft, as in the present study no positive correlation between donor-specific CTLp

frequencies and rejection was found, and Loonen *et al.* also found no differences in CTLp frequencies between patients with and without rejection (369). In the absence of rejection, peripheral blood CTLp may be mainly naïve or low affinity cells susceptible to suppression by CsA and steroids (350, 352).

It is also possible that peripheral blood CTLp are not harmful because of the absence of activating factors such as IL-2 from helper T cells. Wijngaard *et al.* found unresponsiveness to donor cells in 7 of 10 cardiac transplant patients 3 to 27 months after transplantation using a cell-mediated lympholysis assay, but unresponsiveness was reversed in one patient when IL-2 was added to the cultures (294). Reduced numbers of donor-reactive IL-2-producing cells were reported in cardiac transplant patients with long-term acceptance of their grafts (372). Decreased frequencies of IL-2-producing cells do not appear to be essential to good graft function, however (379, 381), so this is unlikely to be the only explanation for the lack of association of peripheral CTLp frequencies with rejection.

CTLp reactive with donor spleen cells may not be the only CTL which contribute to rejection. Cytotoxicity to more relevant targets such as heart endothelial cells (259) may differ from that directed towards donor cells of haematopoietic origin. CTL which recognise tissue-specific peptides may fail to lyse lymphoid cells, as has been demonstrated with kidney cell lines (424, 425), and would thus not be detected in assays which use splenic target cells.

The lack of association of peripheral CTLp with acute rejection could also reflect the multifactorial nature of graft rejection, which can include DTH-type and antibody-

mediated mechanisms (260). Finally, *in vitro* assays of cellular hyporesponsiveness may not fully reflect the situation *in vivo*, due to cell separation and culture resulting in the loss of regulatory mechanisms (426).

4.5. Influence of induction immunosuppression with ATG on CTLp frequency.

No differences were seen in donor-reactive or 3rd party-reactive CTLp frequencies between patients who received induction immunosuppression with ATG and those who did not. Induction immunosuppression with anti-lymphocyte globulin has been reported to decrease the HLA-DR-directed cytotoxicity of graft-infiltrating cytotoxic T cells after cardiac transplantation (427). Such an effect is unlikely to be detected by the CTLp method used in the present study because PHA blasts are inefficient targets for HLA class II-directed cytotoxicity (235, 335). Treatment of cardiac transplant patients with ATG for rejection has also been reported to reduce the frequencies of donor-reactive committed CTL, which were replaced with CTL with lower avidity for donor antigen (428). Thus, although the T cells depleted by ATG treatment are replaced when treatment is discontinued, the replacement cells may differ from the depleted cells in activation state or avidity for donor antigen. That no effect of ATG induction on CTLp frequencies was found in the present study indicates that donor-reactive CTLp were replaced after ATG treatment ended. The CTLp assay method used does not distinguish activation status or avidity of CTL, however, so no comment can be made on qualitative differences between the different treatment groups.

5. Future work.

Further testing of most of the samples collected in this study would be limited by the numbers of cells remaining. Future studies undertaken to address some of the questions raised by the present results could be designed differently. The hypothesis that persisting peripheral CTLp are not harmful because they are naïve or low affinity cells could be tested by modified CTLp assays which identify the activation status or affinity for donor antigen of CTLp. Alternatively, the use of monoclonal antibodies to activation markers could be useful in defining activation status (377, 429). The transient decrease in donor-reactive CTLp frequencies in the first few months after transplantation could be investigated further, perhaps by more frequent sampling during this period to determine the kinetics of the changes. More detailed examination of the kinetics of individual limiting dilution assays would be informative, particularly in those patients who exhibited decreased reactivity towards their donors. It would also be of interest to test the same group of patients at later time intervals after transplantation to determine if the proportion of patients with decreased reactivity to donor cells in the CTLp assay increases with time as has been suggested by other studies (374, 409). The occurrence of chronic rejection could be followed in these patients. Identifying an association between chronic rejection and the continued presence of donor-reactive CTLp could be useful in patient management. However, confirmation of the occurrence of chronic rejection in patients who are hyporesponsive to donor antigens in the CTLp assay would suggest that assays of direct allorecognition have limited usefulness in post-transplant patient monitoring.

CONCLUSIONS

The Matrix 96 was comparable to the gamma counter for the measurement of ^{51}Cr-release in cell culture supernatants. When used to count CTLp assays of 45 samples from cardiac transplant recipients the Matrix 96 was found to be a rapid and convenient method for processing large numbers of limiting dilution assays.

Investigation of NK-like activity in CTLp assay cultures suggested that this type of cytotoxicity did not contribute to the CTLp frequency estimates, in agreement with most other reports. Knowledge of the receptors and ligands involved in the regulation of NK- and CTL-mediated lysis is increasing rapidly, however, and will provide a fuller understanding of the interactions between the different killer lymphocytes and their targets.

Donor-reactive CTLp frequencies decreased by 3 to 7 months after transplantation in most of the cardiac transplant recipients studied, but increased to pre-transplant levels by 9 to 15 months after transplantation. Decreased donor-specific CTLp frequencies were found in three patients by 9 to 15 months after transplantation. This is consistent with several other studies in which decreases in donor-reactive CTLp frequencies were found in a minority of cardiac and renal transplant recipients. The clinical relevance of decreased CTLp frequency after organ transplantation needs further elucidation.

Pre-transplant donor-reactive CTLp frequencies were not predictive of acute rejection. Donor-reactive CTLp frequencies were not increased during acute rejection, but donor-

reactive CTLp frequencies in samples taken at 9 to 15 months after transplantation were negatively correlated with acute rejection. Monitoring of donor-reactive CTLp frequencies in peripheral blood does not, therefore, appear to be useful in the study of acute rejection processes after cardiac transplantation.

BIBLIOGRAPHY

1. Liu C-C, Young LHY, Young JD-E. Lymphocyte-mediated cytolysis and disease. N Engl J Med 1996; 335: 1651-1659.

2. Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on ^{51}Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology 1968; 14: 181-196.

3. Blomberg K, Granberg C, Hemmila I, Lovgren T. Europium-labelled target cells in an assay of natural killer cell activity. I. A novel non-radioactive method based on time-resolved fluorescence. J Immunol Methods 1986; 86: 225-229.

4. Auto-Gamma 5000 series Systems Service Manual. Publication No. 169-3009. Packard Instrument Co. 1983, Meriden CT.

5. Granberg C, Blomberg K, Hemmila I, Lovgren T. Determination of cytotoxic T lymphocyte activity by time-resolved fluorometry using europium-labelled concavalin A-stimulated cells as targets. J Immunol Methods 1988; 114: 191-195.

6. Korzeniewski C, Callewaert DM. An enzyme-release assay for natural cytotoxicity. J Immunol Methods 1983; 64: 313-320.

7. Diem K, Lentner C, eds. Geigy Scientific Tables. 7th ed. Macclesfield: Geigy Pharmaceuticals, 1970: 292.

8. Helman EZ, Ting P. Counting of beta and gamma emitters used in clinical radioimmunoassays and competitive-binding assays by liquid scintillation counters. Clin Chem 1973; 19: 191-196.

9. Mariani E, Monaco MCG, Sgobbi S, de Zwart JF, Mariani AR, Facchini A. Standardization of a micro-cytotoxicity assay for human natural killer cell lytic activity. J Immunol Methods 1994; 172: 173-178.

10. Potter CG, Gotch F, Warner GT, Ostrup J. Lymphocyte proliferation and cytotoxic assays using flat-bed scintillation counting. J Immunol Methods 1987; 105: 171-177.

11. Matrix 96™ Direct Beta Counter Operation Manual. Publication No. 169-4067 Rev. B. Packard Instrument Co., Inc. 1990, Meriden CT.

12. Hillman GG, Roessler N, Fulbright RS, Pontes JE, Haas GP. ^{51}Cr-release assay adapted to a 96-well format sample reading. BioTechniques 1993; 15: 744-748.

13. Hillman GG, Roessler N, Fulbright RS, Pontes JE, Haas GP. Application of the direct beta counter Matrix 96 for cytotoxic assays: simultaneous processing and reading of 96 wells using a ^{51}Cr-retention assay. Cancer Immunol Immunother 1993; 36: 351-356.

14. Bland JM, Altman DG. Statistical methods for assessing the agreement between two methods of clinical measurement. Lancet 1986; i: 307-310.

15. Sharrock CEM, Kaminski E, Man S. Limiting dilution analysis of human T cells: a useful clinical tool. Immunol Today 1990; 11: 281-286.

16. Kabelitz D, Herzog W-R, Zanker B, Wagner H. Human cytotoxic T lymphocytes. I. Limiting-dilution analysis of alloreactive cytotoxic T-lymphocyte precursor frequencies. Scand J Immunol 1985; 22: 329-335.

17. Kaminski E, Hows J, Goldman J, Batchelor R. Optimising a limiting dilution culture system for quantitating frequencies of alloreactive cytotoxic T lymphocyte precursors. Cell Immunol 1991; 137: 88-95.

18. Mestre M, Massip E, Bas J, *et al.* Longitudinal study of the frequency of cytotoxic T cell precursors in kidney allograft recipients. Clin Exp Immunol 1996; 104: 108-114.

19. Hu H, Robertus M, de Jonge N, *et al.* Reduction of donor-specific cytotoxic T lymphocyte precursors in peripheral blood of allografted heart recipients. Transplantation 1994; 58: 1263-1268.

20. Snell GD, Russell E, Fekete E, Smith P. Resistance of various inbred strains of mice to tumour homoiotransplants, and its relation to the *H-2* allele which each carries. J Natl Cancer Inst 1953; 14: 485-491.

21. Billingham RE, Brent L, Medawar PB, Sparrow EM. Quantitative studies on tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. Proc Roy Soc Br 1954; 143: 43-58.

22. Gorer PA, Lyman S, Snell GD. Studies on the genetic and antigenic basis of tumour transplantation. Linkage between a histocompatibility gene and 'fused' in mice. Proc Roy Soc Br 1948; 135: 499-505.

23. Cuncliffe V, Trowsdale J. The molecular genetics of human chromosome 6. J Med Genet 1987; 24: 649-658.

24. Abbas AK, Lichtman AH, Pober JS. Cellular and molecular immunology. 2nd ed. Philadelphia: WB Saunders Co., 1994: 47.

25. Klein J. The major histocompatibility complex of the mouse. Science 1979; 203: 516-521.

26. Bjorkman PJ, Parham P. Structure, function and diversity of class I major histocompatibility complex molecules. Annu Rev Biochem 1990; 59: 253-288.

27. Giles RC, Capra JD. Structure, function, and genetics of human class II molecules. Adv Immunol 1985; 37: 1-71.

28. Bodmer JG, Marsh SGE, Albert ED, *et al.* Nomenclature for factors of the HLA system, 1998. Eur J Immunogen 1999; 26: 81-116.

29. Ceppellini R, Curtoni ES, Mattiuz PL, Miggiano V, Scudeller G, Serra A. Genetics of leukocyte antigens: a family study of linkage and segregation. In: Curtoni ES, Mattiuz PL, Tosi RM, eds. Histocompatibility Testing 1967. Copenhagen: Munksgaard, 1967: 149-187.

30. Springer TA, Strominger JL, Mann D. Partial purification of detergent-soluble HL-A antigen and its cleavage by papain. Proc Natl Acad Sci USA 1974; 71: 1539-1543.

31. Goodfellow PM, Jones EA, van Heyningen V, *et al.* The β_2-microglobulin gene is on chromosome 15 and not in the HL-A region. Nature 1975; 254: 267-269.

32. Springer TA, Kaufman JF, Terhorst C, Strominger JL. Purification and structural characterisation of human HLA-linked B-cell antigens. Nature 1977; 268: 213-218.

33. Charron DJ, McDevitt HO. Characterization of HLA-D-region antigens by two-dimensional gel electrophoresis. J Exp Med 1980; 152: 18S-36S.

34. Tragardh L, Rask L, Wiman K, Fohlman J, Peterson PA. Complete amino acid sequence of pooled papain-solubilized HLA-A, -B, and -C antigens: relatedness to immunoglobulins and internal homologies. Proc Natl Acad Sci USA 1980; 77: 1129-1133.

35. Larhammar D, Schenning L, Gustafsson K, Wiman K, Claesson L, Rask L, Peterson PA. Complete amino acid sequence of an HLA-DR antigen-like beta chain as predicted from the nucleotide sequence: similarities with immunoglobulins and HLA-A, -B, and -C antigens. Proc Natl Acad Sci USA 1982; 79: 3687-3691.

36. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. Nature 1987; 329: 506-512.

37. Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RJ, Strominger JL, Wiley DC. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 1993; 364: 33-39.

38. Hughes AL, Nei M. Patterns of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 1988; 335: 167-170.

39. Hughes AL, Nei M. Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc Natl Acad Sci USA 1989; 86: 958-962.

40. Rothbard JB, Gefter ML. Interactions between immunogenic peptides and MHC proteins. Annu Rev Immunol 1991; 9: 527-566.

41. Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. Annu Rev Immunol 1989; 7: 601-624.

42. Nagy ZA, Baxevanis CN, Ishii N, Klein J. Ia antigens as restriction molecules in Ir-gene controlled T-cell proliferation. Immunol Rev 1981; 60: 59-83.

43. Rose ML. HLA antigens in tissues. In: Dyer P, Middleton D, eds. Histocompatibility testing: a practical approach. Oxford: Oxford University Press, 1993; 191-210.

44. David-Watine B, Israel A, Kourilsky P. The regulation and expression of MHC class I genes. Immunol Today 1990; 11: 286-292.

45. Glimcher LH, Kara CJ. Sequences and factors: a guide to MHC class II transcription. Annu Rev Immunol 1992; 10: 13-49.

46. Cecka JM. The role of HLA in renal transplantation. Hum Immunol 1997; 56: 6-16.

47. Opelz G, Wujciak T, Mytilineos J, Sherer S. Revisiting HLA matching for kidney transplantation. Transplant Proc 1993; 25: 173-175.

48. Zerbe TR, Arena VC, Kormos RL, Griffith BP, Hardesty RL, Duquesnoy RJ. Histocompatibility and other risk factors for histological rejection of human cardiac allografts during the first three months following transplantation Transplantation 1991; 52: 485-490.

49. Opelz G, Wujciak T. The influence of HLA compatibility on graft survival after heart transplantation. N Engl J Med 1994; 330: 816-819.

50. Smith JD, Rose ML, Pomerance A, Burke M, Yacoub MH. Reduction of cellular rejection and increase in longer-term survival after heart transplantation after HLA-DR matching. Lancet 1995; 346: 1318-1322.

51. Loveland B, Simpson E. The non-MHC transplantation antigens: neither weak nor minor. Immunol Today 1986; 7: 223-229.

52. Goulmy E. Minor histocompatibility antigens. From T cell recognition to peptide identification. Hum Immunol 1997; 54: 8-14.

53. Abbas AK, Lichtman AH, Pober JS. Cellular and molecular immunology. 2nd ed. Philadelphia: WB Saunders Co., 1994: 15.

54. Marrack P, Kappler JW. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. Adv Immunol 1986; 38: 1-30.

55. Jorgensen JL, Reay PA, Ehrich EW, Davis M. Molecular components of T-cell recognition. Annu Rev Immunol 1992; 10: 835-873.

56. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu Rev Immunol 1988; 6: 629-662.

57. Haas W, Pereira P, Tonegawa S. Gamma/delta cells. Annu Rev Immunol 1993; 11: 637-685.

58. Davis MM. T cell receptor gene diversity and selection. Annu Rev Biochem 1990; 59: 475-496.

59. Kruisbeek AM. Development of αβ T cells. Curr Opin Immunol 1993; 5: 227-234.

60. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Human leucocyte differentiation antigens detected by monoclonal antibodies. Specification - Classification - Nomenclature. Berlin: Springer-Verlag, 1984.

61. Littman DR. The structure of the CD4 and CD8 genes. Annu Rev Immunol 1987; 5: 561-584.

62. Swain SL. T cell subsets and the recognition of MHC class. Immunol Rev 1983; 74: 129-142.

63. Reinherz EL, Schlossman SF. The differentiation and function of human T lymphocytes. Cell 1980; 19: 821-827.

64. Krensky AM, Reiss CS, Mier JW, Strominger JL, Burakoff SJ. Long-term human cytolytic T-cell lines allospecific for HLA-DR6 antigen are OKT4+. Proc Natl Acad Sci USA 1982; 79: 2365-2369.

65. Swain SL, Dennert G, Wormsley S, Dutton RW. The Lyt phenotype of a long-term allospecific T cell line: both helper and killer activities to IA are mediated by Ly-1 cells. Eur J Immunol 1981; 11: 175-180.

66. Salgame P, Abrams JS, Clayberger C, *et al.* Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. Science 1991; 254: 279-282.

67. Bendelac A, Schwartz RH. $CD4^+$ and $CD8^+$ T cells acquire specific lymphokine secretion potentials during thymic maturation. Nature 1991; 353: 68-71.

68. Bloom BR, Salgame P, Diamond B. Revisiting and revising suppressor T cells. Immunol Today 1992; 13: 131-136.

69. Parker DC. T cell-dependent B cell activation. Annu Rev Immunol 1993; 11: 331-360.

70. Reth M. Antigen receptors on B lymphocytes. Annu Rev Immunol 1992; 10: 97-121.

71. Banchereau J, Rousset F. Human B lymphocytes: phenotype, proliferation, and differentiation. Adv Immunol 1992; 52: 125-262.

72. Burton DR, Woof JM. Human antibody effector function. Adv Immunol 1992; 51: 1-84.

73. Fitzgerald-Bocarsly P, Herberman R, Hercend T, *et al.* A definition of natural killer cells. In: Ades EW, Lopez C, eds. Natural Killer Cells and Host Defense. 5th Int. Natural Killer Cell Workshop, Hilton Head, SC, 1988. Basel: Karger, 1989: XIII.

74. Trinchieri G. Biology of natural killer cells. Adv Immunol 1989; 47: 187-376.

75. Ortaldo JR, Oldham RK, Cannon GC, Herberman RB. Specificity of natural cytotoxic reactivity of normal human lymphocytes against a myeloid leukemia cell line. J Natl Cancer Inst 1977; 59: 77-82.

76. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 1986; 319: 675-678.

77. Croft M. Activation of naïve, memory and effector T cells. Curr Opin Immunol 1994; 6: 431-437.

78. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. Annu Rev Immunol 1989; 7: 445-480.

79. Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annu Rev Immunol 1996; 14: 233-258.

80. Inaba K, Steinman RM. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. J Exp Med 1984; 160: 1717-1735.

81. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392: 245-252.

82. Weiss A, Imboden JB. Cell surface molecules and early events involved in human T lymphocyte activation. Adv Immunol 1987; 5: 223-252.

83. Chan AC, Shaw AS. Regulation of antigen receptor signal transduction by protein tyrosine kinases. Curr Opin Immunol 1996; 8: 394-401.

84. Liu J. FK506 and cyclosporin, molecular probes for studying intracellular signal transduction. Immunol Today 1993; 14: 290-295.

85. Jain J, Valge-Archer VE, Sinskey AJ, Rao A. The AP-1 site at 150 bp, but not the NF-κB site, is likely to represent the major target of protein kinase C in the interleukin 2 promoter. J Exp Med 1992; 175: 853-862.

86. Weiss A, Littman DR. Signal transduction by lymphocyte antigen receptors. Cell 1994; 76: 263-274.

87. Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. Science 1989; 243: 355-361.

88. Minami Y, Kono T, Miyazaki T, Taniguchi T. The IL-2 receptor complex: its structure, function, and target genes. Annu Rev Immunol 1993; 11: 245-267.

89. Smith KA. Interleukin-2: inception, impact, and implications. Science 1988; 240: 1169-1176.

90. Voss SD, Sondel PM, Robb RJ. Characterization of the interleukin 2 receptors (IL-2R) expressed on human natural killer cells activated *in vivo* by IL-2: association of the p64 IL-2R γ chain with the IL-2R β chain in functional intermediate-affinity IL-2R. J Exp Med 1992; 176: 531-541.

91. Taniguchi T. Cytokine signaling through nonreceptor protein kinases. Science 1995; 268: 251-255.

92. Cantrell DA, Smith KA. The interleukin-2 T-cell system: a new cell growth model. Science 1984; 224: 1312-1316.

93. Kurnick JT, Warrens AR, Moscicki RA, Leary CP. T-cell subsets in human lymphocytes maintained in IL-2 medium after PHA or mixed lymphocyte reaction activation. Clin Immunol Immunopathol 1983; 27: 444-451.

94. Hemler ME. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu Rev Immunol 1990; 8: 365-400.

95. Berke G. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. Annu Rev Immunol 1994; 12: 735-773.

96. Cantor H, Boyse EA. Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly+ cells in the generation of killer activity. J Exp Med 1975; 141: 1390-1399.

97. Keene JA, Forman J. Helper activity is required for the in vivo generation of cytotoxic lymphocytes. J Exp Med 1982; 155: 768-782.

98. Wagner H, Rolinghoff M. T-T cell interactions during *in vitro* cytotoxic allograft responses. I. Soluble products from activated Ly1+ T cells trigger autonomously antigen-primed Ly2,3+ T cells to cell proliferation and cytolytic activity. J Exp Med 1978; 148: 1523-1538.

99. Gromo G, Geller RL, Inverardi L, Bach FH. Signal requirements in the step-wise functional maturation of cytotoxic T lymphocytes. Nature 1987; 327: 424-426.

100. Widmer MB, Grabstein KH. Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor. Nature 1987; 326: 795-798.

101. Okada M, Kitahara M, Kishimoto S, Matsuda T, Hirano T, Kishimoto T. IL-6/BSF-2 functions as a killer helper factor in the in vitro induction of cytotoxic T cells. J Immunol 1988; 141: 1543-1549.

102. Alderson MR, Sassenfeld HM, Widmer MB. Interleukin 7 enhances cytolytic T lymphocyte generation and induces lymphokine-activated killer cells from human peripheral blood. J Exp Med 1990; 172: 577-587.

103. Stern AS, Podalski FJ, Hulmes JD, *et al.* Purification to homogeneity and partial characterisation of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. Proc Natl Acad Sci USA 1990; 87: 6808-6812.

104. Guerder S, Matzinger P. A fail-safe mechanism for maintaining self-tolerance. J Exp Med 1992; 176: 553-564.

105. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 1998; 393: 474-478.

106. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. Help for cytotoxic-T-cell responses is mediated by CD40 signalling. Nature 1998; 393: 478-480.

107. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature 1998; 393: 480-483.

108. Inaba K, Young JW, Steinman RM. Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. J Exp Med 1987; 166: 182-194.

109. Young JW, Steinman RM. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. J Exp Med 1990; 171: 1315-1332.

110. van Gool SW, Zhang Y, Kasran A, de Boer M, Ceuppens JL. T helper-independent activation of human CD8+ cells: the role of CD28 costimulation. Scand J Immunol 1996; 44: 21-29.

111. Bass HZ, Yamashita N, Clement LT. Heterogeneous mechanisms of human cytotoxic T lymphocyte generation. I. Differential helper cell requirement for the generation of cytotoxic effector cells from CD8+ precursor subpopulations. J Immunol 1992; 149: 2489-2495.

112. Eijsvoogel VP, van Rood JJ, du Toit ED, Schellekens PT. Position of a locus determining mixed lymphocyte reaction distinct from the known HL-A loci. Eur J Immunol 1972; 2: 413-418.

113. Eijsvoogel VP, Schellekens PT, du Bois MJ, Zeijlemaker WP. Human cytotoxic lymphocytes after alloimmunization in vitro. Transplant Rev 1976; 29: 125-145.

114. Dutton RW, Bradley LM, Swain SL. T cell memory. Annu Rev Immunol 1998; 16: 201-223.

115. Trowbridge IS, Thomas ML. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. Annu Rev Immunol 1994; 12: 85--116.

116. Bradley LM, Croft M, Swain SL. T-cell memory: new perspectives. Immunol Today 1993; 14: 197-199.

117. Sprent J, Tough DF. Lymphocyte life-span and memory. Science 1994; 265: 1395-1400.

118. Croft M, Bradley LM, Swain SL. Naïve versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J Immunol 1994; 152: 2675-2685.

119. Flynn K, Mullbacher A. Memory alloreactive cytotoxic T cells do not require costimulation for activation in vitro. Immunol Cell Biol 1996; 74: 413-420.

120. Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990; 248: 1349-1356.

121. Lombardi G, Sidhu S, Batchelor R, Lechler R. Anergic T cells as suppressor cells in vitro. Science 1994; 264: 1587-1589.

122. Essery G, Feldmann M, Lamb JR. Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. Immunology 1988; 64: 413-417.

123. Mescher MF. Molecular interactions in the activation of effector and precursor cytotoxic T lymphocytes. Immunol Rev 1995; 146: 177-210.

124. Griffiths GM. The cell biology of CTL killing. Curr Opin Immunol 1995; 7: 343-348.

125. Duke RC, Persechini PM, Chang S, Liu CC, Cohen JJ, Young JD. Purified perforin induces target cell lysis but not DNA fragmentation. J Exp Med 1989; 170: 1451-1456.

126. Thornberry NA, Lazebnik Y. Caspases: enemies within. Science 1998; 281: 1312-1316.

127. Cohen JJ, Duke RC, Fadok VA, Sellins KS. Apoptosis and programmed cell death in immunity. Annu Rev Immunol 1992; 10: 267-293.

128. Nagata S, Golstein P. The Fas death factor. Science 1995; 267: 1449-1456.

129. Ashkenazi A, Dixit VM. Death receptors: Signaling and modulation. Science 1998; 281: 1305-1308.

130.Zinkernagel RM, Moskosphidis D, Kundig T, Oehen S, Pircher H, Hengartner H. Effector T-cell induction and T-cell memory versus peripheral deletion of T cells. Immunol Rev 1993; 133: 199-223.

131. Lanier LL, Phillips JH. Inhibitory MHC class I receptors on NK cells and T cells. Immunol Today 1996; 17: 86-91.

132. Lanier LL. NK cell receptors. Annu Rev Immunol 1998; 16: 359-393.

133. Braud VM, Allan DS, O'Callaghan CA, *et al.* HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 1998; 391: 795-799.

134. Burshtyn DN, Yang W, Yi T, Long EO. A novel phosphotyrosine motif with a critical amino acid at position -2 for the SH2 domain-mediated activation of the tyrosine phosphatase SHP-1. J Biol Chem 1997; 272: 13066-13072.

135. Vely F, Olivero S, Olcese L, *et al.* Differential association of phosphatases with hematopoietic co-receptors bearing immunoreceptor tyrosine-based inhibition motifs. Eur J Immunol 1997; 27: 1994–2000.

136. Siliciano RF, Pratt JC, Schmidt RE, Ritz J, Reinherz EL. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature 1985; 317: 428–430.

137. Moretta A, Poggi A, Pende D, *et al.* CD69-mediated pathway of lymphocyte activation: anti-CD69 monoclonal antibodies trigger the cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing T cell receptor αβ. J Exp Med 1991; 174: 1393–1398.

138. Galandrini R, De Maria R, Piccoli M, Frati L, Santoni A. CD44 triggering enhances human NK cell cytotoxic functions. J Immunol 1994; 153: 4399–4407.

139. Lanier LL, Chang C, Phillips JH. Human NKR-P1A: a disulfide linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. J Immunol 1994; 153: 2417–2428.

140. Cantoni C, Biassoni R, Pende D, *et al.* The activating form of CD94 receptor complex: CD94 covalently associates with the Kp39 protein that represents the product of the NKG2-C gene. Eur J Immunol 1998; 28: 327-338.

141. Brumbaugh KM, Perez-Villar JJ, Dick CJ, Schoon RA, Lopez-Botet M, Leibson PJ. Clonotypic differences in signaling from CD94 (Kp43) on NK cells lead to divergent cellular responses. J Immunol 1996; 157: 2804–2812.

142. Biassoni R, Cantoni C, Falco M, *et al.* The human leukocyte antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med 1996; 183: 645–50.

143. Moretta A, Sivori S, Vitale M, *et al.* Existence of both inhibitory (p58) and activatory (p50) receptors for HLA-C molecules in human natural killer cells. J Exp Med 1995; 182: 875-884.

144. Bottino C, Sivori S, Vitale M, *et al.* A novel surface molecule homologous to the p58/p50 family of receptors is selectively expressed on a subset of human natural killer cells and induces both triggering of cell functions and proliferation. Eur J Immunol 1996; 26: 1816–1824.

145. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. Nature 1998; 391: 703-707.

146. Renard V, Cambiaggi A, Vely F, *et al.* Transduction of cytotoxic signals in natural killer cells: a general model of fine tuning between activatory and inhibitory pathways in lymphocytes. Immunol Rev 1997; 155: 205-221.

147. Oshimi Y, Oda S, Honda Y, Nagata S, Miyazaki S. Involvement of Fas ligand and Fas-mediated pathway in the cytotoxicity of human natural killer cells. J Immunol 1996; 157: 2909-2915.

148. Ozer H, Strelkauskas AJ, Callery RT, Schlossman SF. The functional dissection of human peripheral null cells with respect to antibody-dependent cellular cytotoxicity and natural killing. Eur J Immunol 1979; 9: 112-118.

149. Perussia B, Trinchieri G, Jackson A, *et al.* The Fc receptor for IgG on human natural killer cells: phenotypic, functional, and comparative studies with monoclonal antibodies. J Immunol 1984; 133: 180–189.

150. Lotze MT, Grimm EA, Mazumder A, Strausser JL, Rosenberg SA. Lysis of fresh and cultured autologous tumor cells by human lymphocytes cultured in T-cell growth factor. Cancer Res 1981; 41: 4420-4425.

151. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA. Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumour cells by interleukin 2-activated autologous human peripheral blood lymphocytes. J Exp Med 1982; 155: 1823-1838.

152. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon. Relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J Exp Med 1986; 164: 814-825.

153. Trinchieri G, Matsumoto-Kobayashi M, Clark SC, Seehra J, London L, Perussia B. Response of resting human peripheral blood natural killer cells to interleukin 2. J Exp Med 1984; 160: 1147-1169.

154. London L, Perussia B, Trinchieri G. Induction of proliferation *in vitro* of resting human natural killer cells: IL-2 induces into cell cycle most peripheral blood NK cells, but only a minor subset of low density T cells. J Immunol 1986; 137: 3845-3854.

155. Cuturi MC, Anegon I, Sherman F, Loudon R, Clark SC, Perussia B, Trinchieri G. Production of hematopoietic colony-stimulating factors by human natural killer cells. J Exp Med 1989; 169: 569-583.

156. Tsudo M, Goldman CK, Bongiovanni KF, Chan WC, Winton EF, Yagita M, Grimm EA, Waldmann TA. The p75 peptide is the receptor for interleukin 2 expressed on large granular lymphocytes and is responsible for the interleukin 2 activation of these cells. Proc Natl Acad Sci USA 1987; 84: 5394-5398.

157. Siegel JP, Sharon M, Smith PL, Leonard WJ. The IL-2 receptor β chain (p70): role in mediating signals for LAK, NK, and proliferative activities. Science 1987; 238: 75-78.

158. Heaton KM, Grimm EA. Differential inhibition of lymphokine-activated killing, proliferation, and cytokine secretion by humanized antibodies against the low- and intermediate-affinity interleukin-2 receptors. Hum Immunol 1995; 42: 274-280.

159. Luger TA, Krutman J, Kirnbauer R, *et al.* IFN-β_2/IL-6 augments the activity of human natural killer cells. J Immunol 1989; 143: 1206-1209.

160. Stotter H, Custer MC, Bolton ES, Guedez L, Lotze MT. IL-7 induces human lymphokine-activated killer cell activity and is regulated by IL-4. J Immunol 1991; 146: 150-155.

161. Wolf SF, Temple PA, Kobayashi M, *et al.* Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J Immunol 1991; 146: 3074-3081.

162. Chan SH, Perussia B, Gupta JW, *et al.* Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. J Exp Med 1991; 173: 869-879.

163. Robertson MJ, Soiffer RJ, Wolf SF, *et al.* Response of human natural killer (NK) cells to NK stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. J Exp Med 1992; 175: 779-788.

164. Brunda MJ, Tarnowski D, Davatelis V. Interaction of recombinant interferons with recombinant interleukin-2: differential effects on natural killer cell activity and interleukin-2-activated killer cells. Int J Cancer 1986; 37: 787-793.

165. Toledano M, Mathiot C, Michon J, *et al.* Interferon-γ (IFN-γ) and interleukin-2 in the generation of lymphokine-activated killer cell cytotoxicity - IFN-γ-induced suppressive activity. Cancer Immunol Immunother 1989; 30: 57-64.

166. Chouaib S, Bertoglio J, Blay J-Y, Marchiol-Fournigault C, Fradelizi D. Generation of lymphokine-activated killer cells: Synergy between tumor necrosis factor and interleukin-2. Proc Natl Acad Sci USA 1988; 85: 6875-6879.

167. Naume B, Shalaby R, Lesslauer W, Espevik T. Involvement of the 55- and 75-kDa tumor necrosis factor receptors in the generation of lymphokine-activated killer cell activity and proliferation of natural killer cells. J Immunol 1991; 146: 3045-3048.

168. Brooks B, Chapman K, Lawry J, Meager A, Rees RC. Suppression of lymphokine-activated killer (LAK) cell induction mediated by interleukin-4 and transforming growth factor-β1: effect of addition of exogenous tumour necrosis factor-alpha and interferon-gamma, and measurement of their endogenous production. Clin Exp Immunol 1990; 82: 583-589.

169. Naume B, Gately MK, Desai BB, Sundan A, Espevik T. Synergistic effects of interleukin 4 and interleukin 12 on NK cell proliferation. Cytokine 1993; 5: 38-46.

170. Acha-Orbea H, Groscurth P, Lang R, Stitz L, Hengartner H. Characterization of cloned cytotoxic lymphocytes with NK-like activity. J Immunol 1983; 130: 2952-2959.

171. Austyn JM, Wood KJ. Principles of cellular and molecular immunology. Oxford: Oxford University Press, 1993: 589.

172. Matis LA, Sorger SB, McElligott DL, Fink PJ, Hedrick SM. The molecular basis of alloreactivity in antigen-specific major histocompatibility complex-restricted T cell clones. Cell 1987; 51: 59-69.

173. Braciale T, Andrew M, Braciale V. Simultaneous expression of H2 restricted and alloreactive recognition by a cloned line of influenza virus-specific cytotoxic T lymphocytes. J Exp Med 1981; 153: 1371-1376.

174. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. Annu Rev Immunol 1993; 11: 385-402.

175. Hilton R, Lombardi G, Lechler R. MHC molecules in transplantation and tolerance. In: Browning MJ, McMichael AJ, eds. HLA and MHC: genes, molecules and function. Oxford: BIOS Scientific Publishers Ltd., 1996: 407-431.

176. Falk K, Rotzschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides from MHC molecules. Nature 1991; 351: 290-296.

177. van Bleek GM, Nathenson SG. The structure of the antigen-binding groove of major histocompatibility complex class I molecules determines specific selection of self-peptides. Proc Natl Acad Sci USA 1991; 88: 11032-11036.

178. Engelhard VH. Structure of peptides associated with class I and class II MHC molecules. Annu Rev Immunol 1994; 12: 181-207.

179. Lechler RI, Lombardi G, Batchelor JR, Reinsmoen N, Bach FH. The molecular basis of alloreactivity. Immunol Today 1990; 11: 83-88.

180. Bevan MJ. High determinant density may explain the phenomenon of alloreactivity. Immunol Today 1984; 5: 128-130.

181. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. Immunol Today 1994; 15: 32-38.

182. Sayegh MH, Watschinger B, Carpenter CB. Mechanisms of T cell recognition of alloantigen. The role of peptides. Transplantation 1994; 57: 1295-1302.

183. Susskind B, Iannotti MR, Shornick MD, Steward NS, Gorka J, Mohanakumar T. Indirect allorecognition of HLA class I peptides by CD4+ve cytolytic T lymphocytes. Hum Immunol 1996; 46: 1-9.

184. Benichou G, Malloy KM, Tam RC, Heeger PS, Fedoseyeva EV. The presentation of self and allogeneic MHC peptides to T lymphocytes. Hum Immunol 1998; 59: 540-548.

185. Mason DW, Morris PJ. Effector mechanisms in allograft rejection. Annu Rev Immunol 1986; 4: 119-145.

186. Kissmeyer-Nielsen F, Olsen S, Petersen VP, Fjeldborg O. Hyperacute rejection of kidney allografts associated with pre-existing humoral antibodies against donor cells. Lancet 1966; 2: 662-665.

187. Trento A, Hardesty RL, Griffith BP, Zerbe T, Kormos RL, Bahnson HT. Role of the antibody to vascular endothelial cells in hyperacute rejection in patients undergoing cardiac transplantation. J Thorac Cardiovasc Surg 1988; 95: 37-41.

188. Binah O. Immune effector mechanisms in heart transplant rejection. Cardiovasc Res 1994; 28: 1748-1757.

189. Rose AG, Cooper DK, Human PA, Reichenspurner H, Reichart B. Histopathology of hyperacute rejection of the heart: experimental and clinical observations in allografts and xenografts. J Heart Lung Transplant 1991; 10: 223-234.

190. Weil R 3rd, Clarke DR, Iwaki Y, *et al.* Hyperacute rejection of a transplanted human heart. Transplantation 1981; 32: 71-72.

191. Kemnitz J, Cremer J, Restrepo-Specht I, *et al.* Hyperacute rejection in heart allografts. Case studies. Pathol Res Pract 1991; 187: 23-29.

192. Dunn MJ, Rose ML. Antibody mediated rejection following cardiac transplantation. In: Rose ML, Yacoub MH, eds. Immunology of Heart and Lung Transplantation. London: Edward Arnold, 1993: 200-215.

193. Lavee J, Kormos RL, Duquesnoy RJ, *et al.* Influence of panel-reactive antibody and lymphocytotoxic crossmatch on survival after heart transplantation. J Heart Lung Transplant 1991; 10: 921-930.

194. Cooper DK. Clinical survey of heart transplantation between ABO blood group-incompatible recipients and donors. J Heart Transplant 1990; 9: 376-381.

195. Singh G, Thompson M, Griffith B, *et al.* Histocompatibility in cardiac transplantation with particular reference to immunopathology of positive serologic crossmatch. Clin Immunol Immunopathol 1983; 28: 56-66.

196. Smith JD, Danskine AJ, Laylor RM, Rose ML, Yacoub MH. The effect of panel reactive antibodies and the donor specific crossmatch on graft survival after heart and heart-lung transplantation. Transplant Immunol 1993; 1: 60-65.

197. Brasile L, Zerbe T, Rabin B, Clarke J, Abrams A, Cerilli J. Identification of the antibody to vascular endothelial cells in patients undergoing cardiac transplantation. Transplantation 1985; 40: 672-675.

198. Dunn MJ, Rose ML, Latif N, *et al.* Demonstration by Western blotting of anti-heart antibodies before and after cardiac transplantation. Transplantation 1991; 51: 806-812.

199. Kobashigawa JA, Kirklin JK, Naftel DC, *et al.* Pretransplantation risk factors for acute rejection after heart transplantation: a multiinstitutional study. J Heart Lung Transplant 1993; 12: 355-366.

200. Kubo SH, Naftel DC, Mills RM Jr, *et al.* Risk factors for late recurrent rejection after heart transplantation: a multi-institutional, multivariable analysis. J Heart Lung Transplant 1995; 14: 409-418.

201. Billingham ME, Cary NRB, Hammond ME, *et al.* A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: heart rejection study group. J Heart Transplant 1990; 9: 587-593.

202. Hoshinaga K, Mohanakumar T, Goldman MH, *et al.* Clinical significance of in situ detection of T lymphocyte subsets and monocyte/macrophage lineages in heart allografts. Transplantation 1984; 38: 634-637.

203. Strom TB, Tilney NL, Paradysz JM, Bancewicz J, Carpenter CB. Cellular components of allograft rejection: identity, specificity, and cytotoxic function of cells infiltrating acutely rejecting allografts. J Immunol 1977; 118: 2020-2026.

204. Marboe CC, Knowles DM 2nd, Chess L, Reemtsma K, Fenoglio JJ Jr. The immunologic and ultrastructural characterization of the cellular infiltrate in acute cardiac allograft rejection: prevalence of cells with the natural killer (NK) phenotype. Clin Immunol Immunopathol 1983; 27: 141-151.

205. Rose ML, Yacoub MH. Immunocytochemical analysis of transplanted heart and lung. In: Rose ML, Yacoub MH, eds. Immunology of heart and lung transplantation. London: Edward Arnold, 1993: 166-180.

206. Rose ML, Gracie JA, Fraser A, Chisholm PM, Yacoub MH. Use of monoclonal antibodies to quantitate T lymphocyte subpopulations in human cardiac allografts. Transplantation 1984; 38: 230-234.

207. Weintraub D, Masek M, Billingham ME. The lymphocyte subpopulations in cyclosporine-treated human heart rejection. J Heart Transplant 1985; 4: 213-216.

208. Ibrahim S, Dawson DV, van Trigt P, Sanfilippo F. Differential infiltration by CD45RO and CD45RA subsets of T cells associated with human heart allograft rejection. Am J Pathol 1993; 142: 1794-1803.

209. Griffiths GM, Namikawa R, Mueller C, *et al.* Granzyme A and perforin as markers for rejection in cardiac transplantation. Eur J Immunol 1991; 21: 687-693.

210. Legros-Maida S, Soulie A, Benvenuti C, *et al.* Granzyme B and perforin can be used as predictive markers of acute rejection in heart transplantation. Eur J Immunol 1994; 24: 229-233.

211. Duquesnoy RJ, Demetris JD. Immunopathology of cardiac transplant rejection. Curr Opin Immunol 1995; 10: 193-206.

212. Smith JD, Danskine AJ, Rose ML, Yacoub MH. Specificity of lymphocytotoxic antibodies formed after cardiac transplantation and correlation with rejection episodes. Transplantation 1992; 53: 1358-1362.

213. Schuurman HJ, Meyling FH, Wijngaard PL, van der Meulen A, Slootweg PJ, Jambroes G. Endomyocardial biopsies after heart transplantation. The presence of immunoglobulin/immune complex deposits. Transplantation 1989; 48: 435-438.

214. Hammond EH, Yowell RL, Nunoda S, *et al.* Vascular (humoral) rejection in heart transplantation: pathologic observations and clinical implications. J Heart Transplant 1989; 8: 430-443.

215. Weis M, von Scheidt W. Cardiac allograft vasculopathy: a review. Circulation 1997; 96: 2069-2077.

216. Gao S-Z, Schroeder JS, Alderman EL, *et al.* Prevalence of accelerated coronary artery disease in heart transplant survivors: comparison of cyclosporin and azathioprine regimens. Circulation 1989; 80 (suppl III): III-100-III-105.

217. Uretsky BF, Murali S, Reddy PS, *et al.* Development of coronary artery disease in cardiac transplant patients receiving immunosuppressive therapy with cyclosporin and prednisone. Circulation 1987; 76: 827-834.

218. Yeung AC, Davis SF, Hauptmann PJ, *et al.* Incidence and progression of transplant coronary artery disease over 1 year: results of a multicenter trial with use of intravascular ultrasound. J Heart Lung Transplant 1995; 14: S215-S220.

219. Crisp SJ, Dunn MJ, Rose ML, Taylor PM, Yacoub MH. Antiendothelial antibodies after heart transplantation: the accelerating factor in transplant associated coronary artery disease? J Heart Lung Transplant 1994; 13: 81-91.

220. Reed EF, Hong B, Ho E, Harris PE, Weinberger J, Suciu-Foca N. Monitoring of soluble HLA alloantigens and anti-HLA antibodies identifies heart allograft recipients at risk of transplant-associated coronary artery disease. Transplantation 1996; 61: 566-572.

221. Salomon RN, Hughes CC, Schoen FJ, Payne DD, Pober JS, Libby P. Human coronary transplantation-associated arteriosclerosis. Evidence for a chronic immune reaction to activated graft endothelial cells. Am J Pathol 1991; 138: 791-798.

222. Hosenpud JD, Morris TE, Shipley GD, Mauck KA, Wagner CR. Cardiac allograft vasculopathy. Preferential regulation of endothelial cell-derived mesenchymal growth factors in response to a donor-specific cell-mediated allogeneic response. Transplantation 1996; 61: 939-948.

223. Austyn JM. The initiation of immune responses and allograft rejection. In: Rose ML, Yacoub MH, eds. Immunology of heart and lung transplantation. London: Edward Arnold, 1993: 22-41.

224. Lafferty KJ, Prowse SJ, Simeonovic CJ. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. Annu Rev Immunol 1983; 1: 143-173.

225. Hart DN, McKenzie JL. Interstitial dendritic cells. Int Rev Immunol 1990; 6: 127-138.

226. Hart DNJ, Fabre JW. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. J Exp Med 1981; 154: 347-361.

227. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leucocytes from cardiac allografts into host spleens: a novel pathway for the initiation of rejection. J Exp Med 1990; 171: 307-314.

228. Rose ML, Page C, Hengstenberg C, Yacoub MH. Identification of antigen presenting cells in normal and transplanted human heart: importance of endothelial cells. Hum Immunol 1990; 28: 179-185.

229. Forbes RD, Parfrey NA, Gomersall M, Darden AG, Guttman RD. Dendritic cell-lymphoid cell aggregation and major histocompatibility antigen expression during rat cardiac allograft rejection. J Exp Med 1986; 164: 1239-1258.

230. Pober JS, Orosz CG, Rose ML, Savage COS. Can graft endothelial cells initiate a host anti-graft immune response? Transplantation 1996; 61: 343-349.

231. Kirby JA, Cunningham AC. Intragraft antigen presentation: the contribution of bone-marrow derived, epithelial and endothelial presenting cells. Transplant Rev 1997; 11: 127-140.

232. Bishop DK, Shelby J, Eichwald EJ. Mobilization of T lymphocytes following cardiac transplantation. Evidence that CD4-positive cells are required for cytotoxic T lymphocyte activation, inflammatory endothelial development, graft infiltration, and acute allograft rejection. Transplantation 1992; 53: 849-857.

233. Bolton EM, Gracie JA, Briggs JD, Kampinga J, Bradley JA. Cellular requirements for renal allograft rejection in the athymic nude rat. J Exp Med 1989; 169: 1931-1946.

234. Hall BM, de Saxe I, Dorsch SE. The cellular basis of allograft rejection in vivo. III. Restoration of first-set rejection of heart grafts by T helper cells in irradiated rats. Transplantation 1983; 36: 700-705.

235. van Emmerik NEM, Loonen EHM, Vaessen LMB, *et al*. The avidity, not the mere presence, of primed cytotoxic T-lymphocytes for donor human leukocyte class II antigens determines their clinical relevance after heart transplantation. J Heart Lung Transplant 1997; 16: 240-249.

236. Hall BM, Dorsch S, Roser B. The cellular basis of allograft rejection in vivo. II. The nature of memory cells mediating second set heart graft rejection. J Exp Med 1978; 148: 890-902.

237. Herbert J, Roser B. Lymphocyte subpopulations and memory of MHC antigens. I. Quantitative aspects of neonatal heart graft rejection in normal and immune rats. Transplantation 1987; 43: 556-560.

238. Steinmuller D. Which T cells mediate allograft rejection? Transplantation 1985; 40: 229-233.

239. Hall BM, Dorsch SE. Cells mediating allograft rejection. Immunol Rev 1984; 77: 31-59.

240. Madsen JC, Peugh WN, Wood KJ, Morris PJ. The effect of anti-L3T4 monoclonal antibody treatment on first-set rejection of murine cardiac allografts. Transplantation 1987; 44: 849-852.

241. Herbert J, Roser B. Strategies of monoclonal antibody therapy that induce permanent tolerance of organ transplants. Transplantation 1988; 46 (2, Suppl): 128S-134S.

242. Gracie JA, Bolton EM, Porteous C, Bradley JA. T cell requirements for the rejection of renal allografts bearing an isolated class I MHC disparity. J Exp Med 1990; 172: 1547-1557.

243. Bradley JA, Gracie JA, Porteous C, Bolton EM. The role of $CD4^+$ and $CD8^+$ T cells in rejection of class I MHC disparate renal allografts. Transplant Proc 1991; 23: 266-267.

244. Heidecke CD, Kupiec-Weglinski JW, Lear PA, *et al.* Interactions between T lymphocyte subsets supported by interleukin 2-rich lymphokines produce acute rejection of vascularised cardiac allografts in T cell deprived rats. J Immunol 1984; 133: 582-588.

245. Hall BM, Gurley KE, Dorsch SE. The possible role of cytotoxic T cells in the mediation of first-set allograft rejection. Transplantation 1985; 40: 336-339.

246. Bolton EM, Armstrong HE, Briggs JD, Bradley JA. Cellular requirements for first-set renal allograft rejection. Transplant Proc 1987; 19: 321-323.

247. Mintz B, Silvers WK. Histocompatibility antigens on melanoblasts and hair follicle cells. Cell-localized homograft rejection in allophenic skin grafts. Transplantation 1970; 9: 497-505.

248. Tyler JD, Galli SJ, Snider ME, Dvorak AM, Steinmuller D. Cloned LYT-2^+ cytolytic T lymphocytes destroy allogeneic tissue in vivo. J Exp Med 1984; 159: 234-243.

249. Rosenberg AS, Singer A. Evidence that the effector mechanism of skin allograft rejection is antigen-specific. Proc Natl Acad Sci USA 1988; 85: 7739-7742.

250. Hall BM. Cells mediating allograft rejection. Transplantation 1991; 51: 1141-1151.

251. Bradley JA, Bolton EM. The T-cell requirements for allograft rejection. Transplant Rev 1992; 6: 115-129.

252.Bradley JA, Mowat AM, Bolton EM. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. Immunol Today 1992; 13: 434-438.

253. Duquesnoy RJ, Traeger JDK, Zeevi A. Propagation and characterization of lymphocytes from transplant biopsies. Crit Rev Immunol 1991; 10: 455-480.

254. Dallman MJ, Wood KJ, Morris PJ. Specific cytotoxic T cells are found in the nonrejected kidneys of blood-transfused rats. J Exp Med 1987; 165: 566-571.

255. Weber T, Zerbe T, Kaufman C, *et al.* Propagation of alloreactive lymphocytes from histologically negative endomyocardial biopsies from heart transplant patients. Association with subsequent histological evidence of allograft rejection. Transplantation 1989; 48: 430-435.

256. Ouwehand AJ, Baan CC, Roelen DL, *et al.* The detection of cytotoxic T cells with high-affinity receptors for donor antigens in the transplanted heart as a prognostic factor for graft rejection. Transplantation 1993; 56: 1223-1229.

257. Vaessen LM, Baan CC, Ouwehand AJ, *et al.* Differential avidity and cyclosporine sensitivity of committed donor-specific graft-infiltrating cytotoxic T cells and their precursors. Relevance for clinical cardiac graft rejection. Transplantation 1994; 57: 1051-1059.

258. van Emmerik NEM, Vaessen LMB, Balk AHMM, Bos E, Claas FHJ, Weimar W. Progressive accumulation of CTL with high avidity for donor antigens during the development of acute cardiac rejection. Transplantation 1996; 62: 529-536.

259. Jutte NHPM, Knoop CJ, Heijse P, *et al.* Cytotoxicity of graft-derived lymphocytes: specific for donor heart endothelial cells? J Heart Lung Transplant 1997; 16: 209-215.

260. Barry WH. Mechanisms of immune-mediated myocyte injury. Circulation 1994; 89: 2421-2432.

261. Ansari AA, Sundstrom JB, Kanter K, *et al.* Cellular and molecular mechanisms of human cardiac myocyte injury after transplantation. J Heart Lung Transplant 1995; 14: 102-112.

262. Hunt SA. Current status of cardiac transplantation. JAMA 1998; 280: 1692-1698.

263. Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunol Today 1992; 13: 136-142.

264. Li B, Sehajpal PK, Khanna A, *et al.* Differential regulation of transforming growth factor beta and interleukin 2 genes in human T cells: demonstration by usage of novel competitor DNA constructs in the quantitative polymerase chain reaction. J Exp Med 1991; 174: 1259-1262.

265. Khanna AK, Hosenpud JD. Cyclosporin induces the expression of the cyclin inhibitor p21. Transplantation 1999; 67: 1262-1268.

266. Salomon DR, Limacher MC. Chronic immunosuppression and the treatment of acute rejection. In: Hosenpud JD, Cobanoglu A, Norman DJ, Starr A, eds. Cardiac transplantation. A manual for health care professionals. New York: Springer-Verlag, 1991: 139-168.

267. Santiago Delphin EA. Principles of clinical immunosuppression. Surg Clin North Am 1979; 59: 283-298.

268. Tsai SY, Carlstedt-Duke J, Weigel NL, *et al.* Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell 1988; 55: 361-369.

269. Yamamoto KR. Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 1985; 19: 209-252.

270. Paliogianni F, Boumpas DT. Glucocorticoids regulate calcineurin-dependent trans-activating pathways for interleukin-2 gene transcription in human T lymphocytes. Transplantation 1995; 59: 1333-1339.

271. Paliogianni F, Raptis A, Ahuja SS, Najjar SM, Boumpas DT. Negative transcriptional regulation of human interleukin 2 (IL-2) gene by glucocorticoids through interference with nuclear transcription factors AP-1 and NF-AT. J Clin Invest 1993; 91: 1481-1489.

272. Davies JD, Waldmann H. Monoclonal antibodies in transplantation. In: Rose ML, Yacoub MH, eds. Immunology of heart and lung transplantation. London: Edward Arnold, 1993: 93-116.

273. Rebellato LM, Gross U, Verbanac KM, Thomas JM. A comprehensive definition of the major antibody specificities in polyclonal rabbit antithymocyte globulin. Transplantation 1994; 57: 685-694.

274. Taub RN. Effects of heterologous antilymphocyte serum on lymphoid cells labeled with 5-iodo-2-deoxyuridine-125-I. Fed Proc 1970; 29: 142-144.

275. Lance EM. The mechanism of action of anti-lymphocyte serum. Studies of antibody eluate. J Exp Med 1969; 130: 49-76.

276. Martin WJ. Assay for the immunosuppressive capacity of antilymphocyte serum. I. Evidence for opsonization. J Immunol 1969; 103: 979-989.

277. Merion RM, Howell T, Bromberg JS. Partial T-cell activation and anergy induction by polyclonal antithymocyte globulin. Transplantation 1998; 65: 1481-1489.

278. Bonnefoy-Berard N, Vincent C, Revillard JP. Antibodies against functional leukocyte surface molecules in polyclonal antilymphocyte and antithymocyte globulins. Transplantation 1991; 51: 669-673.

279. Monaco AP, Wood ML, Gray JG, Russell PS. Studies on heterologous anti-lymphocyte serum in mice. II. Effect on the immune response. J Immunol 1966; 96: 229-238.

280. Monaco AP, Wood ML, Russell PS. Some effects of purified heterologous antihuman lymphocyte serum in man. Transplantation 1967; 5: 1106-1114.

281. Muller TF, Grebe SO, Neumann MC, *et al.* Persistent long-term changes in lymphocyte subsets induced by polyclonal antibodies. Transplantation 1997; 64: 1432-1437.

282. Carey JA, Frist WH. Use of polyclonal antilymphocytic preparations for prophylaxis in heart transplantation. J Heart Transplant 1990; 9: 297-300.

283. Gilbert EM, Dewitt CW, Eiswirth CC, *et al.* Treatment of refractory cardiac allograft rejection with OKT3 monoclonal antibody. Am J Med 1987; 82: 202-206.

284. Charlton B, Auchinloss Jr H, Fathman CG. Mechanisms of transplantation tolerance. Annu Rev Immunol 1994; 12: 707-734.

285. Billingham RE, Brent L, Medawar PB. Actively acquired tolerance to foreign cells, twin diagnosis and the freemartin condition in cattle. Nature 1953; 172: 603-606.

286. Kirk AD, Burkly LC, Batty DS, *et al.* Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. Nature Med 1999; 5: 686-693.

287. Starzl TE, Demetris AJ, Trucco M, *et al.* Cell migration and chimerism after whole-organ transplantation: the basis of graft acceptance. Hepatology 1993; 17: 1127-1152.

288. Burlingham WJ, Grailer AP, Fechner JH, *et al.* Microchimerism linked to cytotoxic T lymphocyte functional unresponsiveness (clonal anergy) in a tolerant renal transplant recipient. Transplantation 1995; 59: 1147-1155.

289. Lechler R, Bluestone JA. Transplantation tolerance - putting the pieces together. Curr Opin Immunol 1997; 9: 631-633.

290. Starzl TE, Demetris AJ, Murase N, Trucco M, Thomson AW, Rao AS. The lost chord: microchimerism and allograft survival. Immunol Today 1996 17: 577-588.

291. Hutchinson IV. Immune mechanisms of long-term graft acceptance. Adv Nephrol Necker Hosp 1996; 25: 17-38.

292. Kahan BD, Kerman RH, Van Buren CT, Flechner SM, Golden DL, Lewis RM. Clinical outcome in 36 patients after at least one and up to 5 years of steroid withdrawal based upon specific mixed lymphocyte reaction hyporesponsiveness toward the living related donor. Transplant Proc 1989; 21: 1579-1580.

293. Reinsmoen NL. Posttransplant donor antigen-specific hyporeactivity in human transplantation. Transplant Rev 1995; 9: 17-28.

294. Wijngaard PLJ, Schuurman H-J, Gmelig Meyling FHJ, Jambroes G, Borleffs JCC. Transplantation tolerance in heart transplant recipients as demonstrated by unresponsiveness in cell-mediated lympholysis. Hum Immunol 1992; 34: 167-172.

295. Reinsmoen NL, Kaufman D, Matas A, Sutherland DER, Najarian JS, Bach FH. A new in vitro approach to determine acquired tolerance in long-term kidney allograft recipients. Transplantation 1990; 50: 783-790.

296. Bach F, Hirschhorn K. Lymphocyte interaction: a potential histocompatibility test in vitro. Science 1964; 143: 813-14.

297. Bain B, Vas MR, Lowestein L. The development of large immature mononuclear cells in mixed leukocyte cultures. Blood 1964; 23: 108-116.

298. Thorsby E, Piazza A. Joint report from the Sixth International Histocompatibility Workshop Conference. II. Typing of HLA-D (LD-1 or MLC) determinants. In: Kissmeyer-Nielsen F, ed. Histocompatibility Testing 1975. Munksgaard: Copenhagen, 1975: 414-458.

299. Jorgensen F, Lamm LU, Kissmeyer-Nielsen F. MLC results among unrelated can be predicted. Tissue Antigens 1975; 5: 262-265.

300. Termijtelen A, Erlich HA, Braun LA, *et al.* Oligonucleotide typing is a perfect tool to identify antigens stimulatory in the mixed lymphocyte culture. Hum Immunol 1991; 31: 241-245.

301. Olerup O, Moller E, Persson U. HLA-DP incompatibilities induce significant proliferation in primary mixed lymphocyte cultures in HLA-A, -B, -DR and -DQ compatible individuals: Implications for allogeneic bone marrow transplantation. Tissue Antigens 1990; 36: 194-202.

302. Rubin AL, Stenzel KH, Hirschhorn K, Bach F. Histocompatibility and immunologic competence in renal homotransplantation. Science 1964; 143: 815-816.

303. Sybesma JPHB, Overbeeke M, Borst-Eilers E, Heynen CJJ, de Planque BA, Zegers BJM. HL-A typing in families of potential kidney recipients. Mixed lymphocyte cultures in a case of crossing-over between the two HL-A loci. Vox Sang 1973; 25: 141-150.

304. Bach ML, Engstrom MA, Bach FH, Etheredge EE, Najarian JS. Specific tolerance in human kidney allograft recipients. Cell Immunol 1972; 3: 161-166.

305. Rashid A, Sengar DP, Harris JE. Relationship of donor-specific mixed lymphocyte culture reactivity to graft function in recipients of cadaveric renal allografts. Clin Exp Immunol 1979; 36: 521-527.

306. Charpentier BM, Lang P, Martin B, Fries D. Specific recipient-donor unresponsiveness mediated by a suppressor cell system in human kidney allograft tolerance. Transplantation 1982; 33: 470-477.

307. Reinsmoen NL, Matas AJ. Evidence that improved late renal transplant outcome correlates with the development of in vitro donor antigen-specific hyporeactivity. Transplantation 1993; 55: 1017-1023.

308. Reinsmoen NL, Hertz MI, Kubo S, Bolman RM, Matas AJ. Immune factors correlating with improved long-term graft outcome in kidney, lung and heart transplants. Transplant Proc 1993; 25: 3277-3288.

309. Solliday S, Bach FH. Cytotoxicity: specificity after in vitro sensitization. Science 1970; 170: 1406-1409.

310. Breuning MH, Breur B, Engelsma M, Ivanyi P. Activation of cytotoxic T lymphocytes in HLA-A, -B- and C-identical responder-stimulator combinations. I. Variations in generation of anti-class II CTL in primary mixed lymphocyte cultures. Tissue Antigens 1984; 24: 81-89.

311. Report from the European CML study group on the Third European CML workshop. Human histocompatibility testing by T cell mediated lympholysis: a European standard CML technique. Tissue Antigens 1980; 16: 335-367.

312. Stiller CR, Sinclair NR, Abrahams S, *et al.* Anti-donor immune responses in prediction of transplant rejection. N Engl J Med 1976; 294: 978-982.

313. Goulmy E, Persijn G, Blokland E, D'Amaro J, van Rood JJ. Cell-mediated lympholysis studies in renal allograft recipients. Transplantation 1981; 31: 210-217.

314. Pfeffer PF, Thorsby E, Hirschberg H. Donor-specific decreased cell-mediated cytotoxicity in recipients of well functioning, one HLA haplotype-mismatched kidney allografts. Transplantation 1983; 35: 156-160.

315. Thomas J, Thomas F, Mendez-Picon G, Lee H. Immunological monitoring of long-surviving renal transplant recipients. Surgery 1977; 81: 125-131.

316. Burlingham WJ, Grailer A, Sollinger HW. Changes in donor-specific cell-mediated lympholysis (CML) response associated with success of early steroid withdrawal in DST-azathioprine-treated renal transplant recipients. Transplant Proc 1989; 21: 1818-19.

317. Liburd EM, Pazderka V, Kovithavongs T, Dossetor JB. Evidence for suppressor cells and reduced CML induction by the donor in transplant patients. Transplant Proc 1978; 10: 557-561.

318. Thomas J, Thomas F, Johns C, Lee HM. Considerations in immunological monitoring of long-term transplant recipients. Transplant Proc 1978; 10: 569-572.

319. Dossetor JB, Liburd EM, Kovithavongs T, Pazderka V. Suppressor cells and loss of in vitro killer cell inductiveness to donor cells in the peripheral blood lymphocytes of allograft recipients. Transplant Proc 1979; 11: 1260-1262.

320. Goulmy E, Stijnen T, Groenewoud AF, *et al.* Renal transplant patients monitored by the cell-mediated lympholysis assay. Evaluation of its clinical value. Transplantation 1989; 48: 559-563.

321. van Besouw NM, Vaessen LMB, Daane CR, *et al.* Peripheral monitoring of direct and indirect alloantigen presentation pathways in clinical heart transplant recipients. Transplantation 1996; 61: 165-167.

322. Muluk SC, Clerici M, Via CS, Weir MR, Kimmel PL, Shearer GM. Correlation of in vitro CD4+ T helper cell function with clinical graft status in immunosuppressed kidney transplant recipients. Transplantation 1991; 52: 284-291.

323. Thomas JM, Thomas FT, Hoffman S, Johns C, Mendez-Picon G, Lee HM. Donor-specific CML hyporesponsiveness after successful renal transplantation: studies on the mechanism. Transplant Proc 1979; 11: 1258-1259.

324. Spencer A, Szydlo RM, Brookes PA, *et al.* Bone marrow transplantation for chronic myeloid leukaemia with volunteer unrelated donors using ex-vivo or in-vivo T-cell depletion: major prognostic impact of HLA class I identity between donor and recipient. Blood 1995; 86: 3590-3597.

325. Sharrock CEM, Man S, Wanachiwanawin W, Batchelor JR. Analysis of the alloreactive T cell repertoire in man. Transplantation 1987; 43: 699-703.

326. Reisaeter AV, Brinchmann JE, Lundin KEA, Thorsby E. Monozygotic twins display differences in their allospecific cytotoxic T cell repertoire. Scand J Immunol 1996; 44: 638-642.

327. Schwarer AP, Jiang YZ, Brookes PA, *et al.* Frequency of anti-recipient alloreactive helper T-cell precursors in donor blood and graft-versus-host disease after HLA-identical sibling bone-marrow transplantation. Lancet 1993; 341: 203-205.

328. Imami N, Brookes PA, Lombardi G, *et al.* Association between interleukin-4-producing T lymphocyte frequencies and reduced risk of graft-versus-host disease. Transplantation 1998; 65: 979-988.

329. Lefkovits I, Waldmann H. Limiting dilution analysis of the cells of the immune system. I. The clonal basis of the immune response. Immunol Today 1984; 5: 265-268.

330. de St. Groth SF. The evaluation of limiting dilution assays. J Immunol Methods 1982; 49: R11-R23.

331. Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J Immunol 1981; 126: 1614-1619.

332. Strijbosch LWG, Buurman WA, Does RJMM, Zinken PH, Groenewegen G. Limiting dilution assays. Experimental design and statistical analysis. J Immunol Methods 1987; 97: 133-140.

333. Moretta A, Pantaleo G, Moretta L, Mingari MC, Cerottini J-C, Mingari MC. Direct demonstration of the clonogenic potential of every human peripheral blood T cell. Clonal analysis of HLA-DR expression and cytolytic activity. J Exp Med 1983; 157: 743-754.

334. Moretta A, Pantaleo G, Moretta L, Mingari MC, Cerottini J-C. Quantitative assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T cell populations. J Exp Med 1983; 158: 571-585.

335. Man S, Lechler RI, Batchelor R, Sharrock CEM. Individual variation in the frequency of HLA class II-specific cytotoxic T lymphocyte precursors. Eur J Immunol 1990; 20: 847-854.

336. Zhang L, van Bree S, van Rood JJ, Claas FHJ. The effect of individual HLA-A and -B mismatches on the generation of cytotoxic T lymphocyte precursors. Transplantation 1990; 50: 1008-1010.

337. Breur-Vriesendorp BS, Vingerhoed J, van Twuyver E, de Waal LP, Ivanyi P. Frequency analysis of HLA-specific cytotoxic T lymphocyte precursors in humans. Transplantation 1991; 51: 1096-1103.

338. Barnardo MC, Davey NJ, Bunce M, *et al.* A correlation between HLA-C matching and donor anti-recipient cytotoxic T-lymphocyte precursor frequency in bone marrow transplantation. Transplantation 1996; 61: 1420-1423.

339. Grundschober C, Rufer N, Sanchez-Mazas A, *et al.* Molecular characterization of HLA-C incompatibilities in HLA-ABDR-matched unrelated bone marrow donor-recipient pairs. Sequence of two new Cw alleles (Cw*02023 and Cw*0707) and recognition by cytotoxic T lymphocytes. Tissue Antigens 1997; 49: 612-623.

340. Rufer N, Breur-Vriesendorp B, Tiercy J-M, *et al.* HLA-B35-subtype mismatches in ABDR serologically matched unrelated donor-recipient pairs. Hum Immunol 1994; 41: 96-101.

341. Zhang L, Rinke de Wit TF, Li SG, van Rood JJ, Claas FH. Subtypes of HLA-A1 defined on the basis of CTL precursor frequencies. Hum Immunol 1990; 27: 80-89.

342. Lopez-Larrea C, Arnaiz-Villena A. The "public" determinants HLA-Bw4/Bw6 do not generate cytotoxic T lymphocytes (CTLs). Cell Immunol 1989; 123: 441-444.

343. Tekolf WA, Shaw S. Primary *in vitro* generation of cytotoxic cells specific for human minor histocompatibility antigens between HLA-identical siblings. J Immunol 1984; 132: 1756-1760.

344. Kaminski E, Hows J, Man S, *et al.* Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantation. Transplantation 1989; 48: 608-613.

345. de Beuger M, Bakker A, Bontkes H, van Rood JJ, Goulmy E. High frequencies of cytotoxic T cell precursors against minor histocompatibility antigens after HLA-identical BMT: absence of correlation with GVHD. Bone Marrow Transplant 1993; 11: 363-368.

346. Kaminski ER, Hows JM, Goldman JM, Batchelor JR. Pretransfused patients with severe aplastic anaemia exhibit high numbers of cytotoxic T lymphocyte precursors probably directed at non-HLA antigens. Br J Haematol 1990; 76: 401-405.

347. Kaminski E, Sharrock C, Hows J, *et al.* Frequency analysis of cytotoxic T lymphocyte precursors - possible relevance to HLA-matched unrelated donor bone marrow transplantation. Bone Marrow Transplant 1988; 3: 149-165.

348. Lantz O, Alard P, Chavanel G, Fries D, Senik A, Charpentier B. EBV cell-lines (LCL) and E- cells as stimulator cells for limiting dilution analysis (LDA) of alloreactive IL-2-secreting cells (IL-2-SC) and cytotoxic precursors (CTLp). Comparison of their stimulator capacity and ability to generate specific alloreactivity. J Immunol Methods 1991; 137: 121-132.

349. Orosz CG, Horstemeyer B, Zinn NE, Bishop DK. Development and evaluation of a limiting dilution analysis technique that can discriminate in vivo alloactivated cytotoxic T lymphocytes from their naïve CTL precursors. Transplantation 1989; 47: 189-194.

350. Roelen DL, van Beelen E, van Bree SPMJ, van Rood JJ, Volker-Dieben HJ, Claas FHJ. The presence of activated donor HLA class I-reactive T lymphocytes is associated with rejection of corneal grafts. Transplantation 1995; 59: 1039-1042.

351. Kabelitz D, Zanker B, Zanker C, Heeg K, Wagner H. Human cytotoxic T lymphocytes. II. Frequency analysis of cyclosporin A-sensitive alloreactive cytotoxic T-lymphocyte precursors. Immunology 1987; 61: 57-62.

352. Bouma GJ, Hollander DAMJ, van der Meer-Prins EMW, *et al.* In vitro sensitivity to prednisolone may predict kidney rejection after steroid withdrawal. Transplantation 1996; 62: 1422-1429.

353. van Emmerik NEM, Daane CR, Knoop CJ, *et al.* The avidity of allospecific cytotoxic T lymphocytes (CTL) determines their cytokine production profile. Clin Exp Immunol 1997; 110: 447-453.

354. Seeley JK, Golub SH. Studies on cytotoxicity generated in human mixed lymphocyte cultures. I. Time course and target spectrum of several distinct concomitant cytotoxic activities. J Immunol 1978; 120: 1415-1422.

355. Bolhuis RL, Schellekens H. Induction of natural killer cell activity and allocytotoxicity in human peripheral blood lymphocytes after mixed lymphocyte culture. Scand J Immunol 1981; 13: 401-412.

356. Grimm EA, Owen-Schaub L. The IL-2 mediated amplification of cellular cytotoxicity. J Cell Biochem 1991; 45: 335-339.

357. Seeley JK, Masucci G, Poros A, Klein E, Golub SH. Studies on cytotoxicity generated in human mixed lymphocyte cultures. II. Anti-K562 effectors are distinct from allospecific CTL and can be generated from NK-depleted T cells. J Immunol 1979; 123: 1303-1311.

358. Kabelitz D. Human cytotoxic lymphocytes. IV. Frequency and clonal specificity of CD8+CD16- (Leu2+Leu11-) and CD16+CD3- (Leu11+Leu4-) cytotoxic lymphocyte precursors activated by alloantigen or K562 stimulator cells. Cell Immunol 1989; 121: 298-305.

359. Truman C, Haque K, Donaldson C, *et al.* Natural killer cells contribute to frequency estimates of cytotoxic T lymphocyte precursors. Eur J Immunogen 1997; 24: 41 (abstract).

360. Ciccone E, Pende D, Viale O, *et al.* Evidence of a natural killer (NK) cell repertoire for (allo) antigen recognition: definition of five distinct NK-determined allospecificites in humans. J Exp Med 1992; 175: 709-718.

361. Litwin V, Gumperz J, Parham P, Phillips JH, Lanier LL. Specificity of HLA class I antigen recognition by human NK clones: evidence for clonal heterogeneity, protection by self and non-self alleles, and influence of the target cell type. J Exp Med 1993; 178: 1321-1336.

362. Young NT, Rust NA, Dallman MJ, Cerundolo V, Morris PJ, Welsh KI. Independent contributions of HLA epitopes and killer inhibitory receptor expression to the functional alloreactive specificity of natural killer cells. Hum Immunol 1998; 59: 700-712.

363. Valiante NM, Uhrberg M, Shilling HG, *et al.* Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. Immunity 1997; 7: 739-751.

364. Ciccone E, Viale D, Pende M, *et al.* Specific lysis of allogeneic cells after activation of CD3- lymphocytes in mixed lymphocyte culture. J Exp Med 1988; 168: 2403-2408.

365. Colonna M, Brooks EG, Falco M, Ferrara GB, Strominger JL. Generation of allospecific natural killer cells by stimulation across a polymorphism of HLA-C. Science 1993; 260: 1121-1124.

366. Kahan BD. Immunological monitoring: utility and limitations. Transplant Proc 1985; 17: 1537-1545.

367. van Besouw NM, Daane CR, Vaessen LMB, *et al.* Nonspecific immune reactivity of peripheral blood mononuclear cells is related to graft vascular disease. Transplant Proc 1997; 29: 2544-2545.

368. Reader JA, Burke MM, Counihan P, *et al.* Noninvasive monitoring of human cardiac allograft rejection. Transplantation 1990; 50: 29-33.

369. Loonen L, Vaessen L, Balk A, *et al.* Long-term survival of heart grafts in the presence of donor-specific cytotoxic T-cell precursors (CTLp) in the peripheral blood. Transpl Int 1994; 7 (Suppl 1): S596-S598.

370. Vaessen LMB, Baan CC, Daane CR, *et al.* Immunological monitoring in peripheral blood after heart transplantation: frequencies of T-helper cells and precursors of cytotoxic T cells with high avidity for donor antigens correlate with rejection. Transplant Proc 1995; 27: 485-487.

371. DeBruyne LA, Ensley RD, Olsen SL, *et al.* Increased frequency of alloantigen-reactive helper T lymphocytes is associated with human cardiac allograft rejection. Transplantation 1993; 56: 722-727.

372. DeBruyne LA, Renlund DG, Bishop DK. Evidence that human cardiac allograft acceptance is associated with a decrease in donor-reactive helper T lymphocytes. Transplantation 1995; 59: 778-783.

373. Cattell EL, Cunningham AC, Bal W, Taylor RM, Dark JH, Kirby JA. Limiting dilution analysis: quantification of IL-2 producing allospecific lymphocytes after renal and cardiac transplantation. Transpl Immunol 1994; 2: 300-307.

374. Hornick PI, Mason PD, Yacoub MH, Rose ML, Batchelor R, Lechler RI. Assessment of the contribution that direct allorecognition makes to the progression of chronic cardiac transplant rejection in humans. Circulation 1998; 97: 1257-1263.

375. Suitters AJ, Rose ML, Dominguez MJ, Yacoub MH. Selection for donor-specific cytotoxic T lymphocytes within the allografted human heart. Transplantation 1990; 49: 1105-1109.

376. Vaessen LMB, Baan CC, Ouwehand AJ, *et al.* Acute rejection in heart transplant patients is associated with the presence of committed donor-specific cytotoxic lymphocytes in the graft but not in the blood. Clin Exp Immunol 1992; 88: 213-219.

377. Tugulea S, Ciubotariu R, Colovai AI, *et al.* New strategies for early diagnosis of heart allograft rejection. Transplantation 1997; 64: 842-847.

378. Ciubotariu R, Liu Z, Colovai AI, *et al.* Persistent allopeptide reactivity and epitope spreading in chronic rejection of organ allografts. J Clin Invest 1998; 101: 398-405.

379. Zanker B, Jooss-Rudiger J, Franz H-E, Wagner H, Kabelitz D. Evidence that functional deletion of donor-reactive T lymphocytes in kidney allograft recipients can occur at the level of cytotoxic T cells, IL-2-producing T cells, or both. Transplantation 1993; 56: 628-632.

380. van Twuyver E, de Hoop J, Ten Berge RJM, *et al.* Comparison of T cell responses in patients with a long-term surviving renal allograft versus a long-term surviving liver allograft. It's a different world. Transplantation 1996; 61: 1392-1397.

381. Lantz O, Alard P, Ben Aribia MH, *et al.* Persistence of donor-specific IL-2-secreting cells and cytotoxic T lymphocyte precursors in human kidney transplant recipients evidenced by limiting dilution analysis. J Immunol 1990; 144: 3748-3755.

382. Miller LW, Schlant RC, Kobashigawa J, Kubo S, Renlund DG. Cardiac transplantation: complications. J Am Coll Cardiol 1993; 22: 41-51.

383. Leclercq G, De Smedt M, Plum J. Direct evidence for partial target specificity in lymphokine-activated killer thymocytes. Scand J Immunol 1990; 32: 373-379.

384. Amos DB. Cytotoxicity testing. In: Ray JG Jr, Hare DB, Pedersen PD, Mullaly DI, eds. NIAID manual of tissue typing techniques. Washington, D.C., 1976: 25-28. (DHEW publication No. (NIH) 77-545).

385. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantations. Tissue Antigens 1992; 39: 225-235.

386. Bunce M, Taylor CJ, Welsh KI. Rapid HLA-DQB typing by eight polymerase chain reaction amplifications with sequence-specific primers (PCR-SSP). Hum Immunol 1993; 37: 201-206.

387. Altman DG. Practical Statistics for Medical Research. London: Chapman & Hall, 1991.

388. Naume B, Espevik T. Immunoregulatory effects of cytokines on natural killer cells. Scand J Immunol 1994; 40: 128-134.

389. Danzer SG, Kirchner H, Rink L. Cytokine interactions in human mixed lymphocyte culture. Transplantation 1994; 57: 1638-1642.

390. Bolhuis RLH, Ronteltap CPM. Generation of natural killer (NK) cell activity after mixed lymphocyte culture (MLC): activation of effector cells in NK cell-depleted cell populations. Immunol Lett 1980; 1: 191-196.

391. Mazumder A, Grimm EA, Rosenberg SA. Characterization of the lysis of fresh human solid tumors by autologous lymphocytes activated in vitro with phytohaemagglutinin. J Immunol 1983; 130: 958-964.

392. Shimizu Y, Kuroki T, Shiomi S, Kobayashi K, Monna T. Proliferation and cytotoxicity of lectin-induced lymphokine-activated killer (LILAK) cells. J Gastroenterol and Hepatol 1991; 6: 485-490.

393. Fan J, Nishanian P, Breen EC, McDonald M, Fahey JL. Cytokine gene expression in normal human lymphocytes in response to stimulation. Clin Diagn Lab Immunol 1998; 5: 335-340.

394. Grabstein K, Dower S, Gillis S, Urdal D, Larsen A. Expression of interleukin 2, interferon-γ, and the IL 2 receptor by human peripheral blood lymphocytes. J Immunol 1986; 136: 4503-4508.

395. Arai K, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T. Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem 1990; 59: 783-836.

396. Paliard X, de Waal Malefijt R, Yssel H, Blanchard D, Chretien I, Abrams J, de Vries J, Spits H. Simultaneous production of IL-2, IL-4 and IFN-gamma by activated human CD4+ and CD8+ T cell clones. J Immunol 1988; 141: 849-855.

397. Gillis S, Ferm MM, Ou W, Smith KA. T cell growth factor: parameters of production and a quantitative microassay for activity. J Immunol 1978; 120: 2027-2032.

398. Smith KA, Gillis S, Baker PE, McKenzie D, Ruscetti FW. T-cell growth factor-mediated T-cell proliferation. Ann N Y Acad Sci 1979; 332: 423-432.

399. Kataoka T, Takaku K, Magae J, *et al.* Acidification is essential for maintaining the structure and function of lytic granules of CTL. Effect of concanamycin A, an inhibitor of vacuolar type H(+)-ATPase, on CTL-mediated cytotoxicity. J Immunol 1994; 153: 3938-3947.

400. Hanabuchi S, Koyanagi M, Kawasaki A, *et al.* Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. Proc Natl Acad Sci USA 1994; 91: 4930-4934.

401. Langhorne J, Fischer Lindahl K. Limiting dilution analysis of CTL precursors. In: Lefkovits I, Pernis B, eds. Immunological Methods. Vol. II. New York: Academic Press, 1981: 221-240.

402. Pei J, Farrell C, Hansen JA, *et al.* Evaluation of the limiting dilution cytotoxic T lymphocyte precursor frequency (fCTLp) assay in a multicentre study. In: Charron D, ed. Genetic diversity of HLA. Functional and medical implication. Proceedings of the Twelfth International Histocompatibility Workshop and Conference. Paris: EDK, 1997: Vol II, 577-579.

403. Kenyon NS, Flye MW, Mohanakumar T. Enumeration of cytotoxic T-lymphocyte precursor frequencies in normals and renal allograft recipients using limiting dilution analysis: evidence for HLA-DR restricted regulatory cells of cytotoxic T-lymphocytes. Transplant Proc 1989; 21: 336-337.

404. Wang XN, Proctor SJ, Dickinson AM. Frequency analysis of recipient-reactive helper and cytotoxic T lymphocyte precursors using a combined single limiting dilution assay. Transpl Immunol 1996; 4: 247-251.

405. O'Shea J, Madrigal A, Davey N, *et al*. Measurement of cytotoxic T lymphocyte precursor frequencies reveals cryptic HLA class I mismatches in the context of unrelated donor bone marrow transplantation. Transplantation 1997; 64: 1353-1356.

406. Roelen D, Datema G, van Bree S, Zhang L, van Rood J, Claas F. Evidence that antibody formation against a certain HLA alloantigen is associated not with a quantitative but with a qualitative change in the cytotoxic T cells recognizing the same antigen. Transplantation 1992; 53: 899-903.

407. Bouma GJ, van der Meer-Prins EMW, van der Woude FJ, van Rood JJ, Claas FHJ. Relevance of pretransplant donor-specific T cell allorepertoire for human kidney graft survival. Transplantation 1995; 59: 969-976.

408. Steinmann J, Kaden J, May G, Schroder K, Herwartz C, Muller-Ruchholtz W. Failure of in vitro T-cell assays to predict clinical outcome after human kidney transplantation. J Clin Lab Anal 1994; 8: 157-162.

409. Beik AI, Higgins RM, Lam FT, Morris AG. Clinical significance of selective decline of donor-reactive IL-2-producing T lymphocytes after renal transplantation. Transpl Immunol 1997; 5: 89-96.

410. Roelen DL, van Bree FP, Schanz U, van Rood JJ, Claas FH. Differential inhibition of primed alloreactive CTLs in vitro by clinically used concentrations of cyclosporin and FK506. Transplantation 1993; 56: 190-195.

411. Roelen DL, van Bree J, Witvliet MD, *et al.* IgG antibodies against an HLA antigen are associated with activated cytotoxic T cells against this antigen, IgM are not. Transplantation 1994; 57: 1388-1392.

412. Deacock SJ, Lechler RI. Positive correlation of T cell sensitization with frequencies of alloreactive T helper cells in chronic renal failure patients. Transplantation 1992; 54: 338-343.

413. Kahan BD. Cyclosporin. N Engl J Med 1989; 321: 1725-1738.

414. Herzog W-R, Zanker B, Irschick E, *et al.* Selective reduction of donor-specific cytotoxic T lymphocyte precursors in patients with a well-functioning kidney allograft. Transplantation 1987; 43: 384-389.

415. van der Mast BJ, van Besouw NM, Hepkema BG, *et al.* Mutual tolerance after liver and not after heart transplantation? Evaluation of patient-anti-donor and donor-anti-patient responses by mixed lymphocyte culture. Transpl Immunol 1998; 6: 33-38.

416. Bishop GA, Sharland AF, McCaughan GW. High-dose/activation-associated tolerance model for allografts: lessons from spontaneous tolerance of transplanted livers. Curr Op Organ Transplant 1999; 4: 58-64.

417. Mason PD, Robinson CM, Lechler RI. Detection of donor-specific hyporesponsiveness following late failure of human renal allografts. Kidney Int 1996; 50: 1019-1025.

418. Liu Z, Colovai AI, Tugulea S, *et al.* Indirect recognition of donor HLA-DR peptides in organ allograft rejection. J Clin Invest 1996; 98: 1150-1157.

419. Jutte NHPM, Heyse P, Daane CR, *et al.* Prophylactic therapy with OKT3 does not affect donor specific reactivity of peripheral blood lymphocytes from heart transplant recipients. Transplant Immunol 1994; 2: 22-26.

420. Hattler BG, Miller J, Johnson MC. Cellular and humoral factors governing canine mixed lymphocyte cultures after renal transplantation. II. Cellular. Transplantation 1972; 14: 47-56.

421. Miller J, Hattler BG. Reactivity of lymphocytes in mixed culture in response to human renal transplantation. Surgery 1972; 72: 220-228.

422. Hu H, de Jonge N, Doornewaard H, *et al.* Cytotoxic T lymphocytes infiltrating the human cardiac allograft show a restriction in T-cell receptor Vβ gene usage: a study on serial biopsy and blood specimens. J Heart Lung Transplant 1994a; 13: 1058-1071.

423. Bishop KD, Ferguson RM, Orosz CG. Differential distribution of antigen-specific helper T cells and cytotoxic T cells after antigenic stimulation in vivo. A functional study using limiting dilution analysis. J Immunol 1990; 144: 1153-1160.

424. Poindexter NJ, Steward NS, Shenoy S, *et al.* Renal allograft infiltrating lymphocytes: frequency of tissue specific lymphocytes. Hum Immunol 1997; 55: 140-147.

425. Hadley GA, Mohanakumar T. Kidney cell-restricted recognition of MHC class I alloantigens by human cytolytic T cell clones. Transplantation 1993; 55: 400-404.

426. Kahan BD. Current controversies and new horizons. Specific immunological unresponsiveness of long-term organ transplant recipients. In: Brent L, Sells RA, eds. Organ transplantation: current clinical and immunological concepts. London: Bailliere Tindall, 1989: 137-143.

427. Ouwehand AJ, Baan CC, Groeneveld K, *et al.* Altered specificity of alloreactive cardiac graft-infiltrating cells by prophylactic treatment with OKT3 or horse antilymphocyte globulin. Transplantation 1993; 55: 154-158.

428. Baan CC, Vaessen LMB, Loonen EHM, *et al.* The effect of antithymocyte globulin therapy on frequency and avidity of allospecific committed CTL in clinical heart transplants. Transplant Proc 1995; 27: 482-484.

429. Van Emmerik NEM, Knoop CJ, Vaessen LMB, *et al.* C1.7 monoclonal antibody designates high-avidity CD4+ cytotoxic T lymphocytes involved in clinical heart rejection. Transplantation 1998; 66: 135-138.