Development Of A Sample Bank And Clinical Database For The Retrospective Analysis Of Unrelated Bone Marrow Transplants: A Pilot Study Of 138 Transplants Using RSCA For High Resolution HLA Matching

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Abstract

The Anthony Nolan Register provides donors for allogeneic stem cell transplantation for haematological disorders. A clinical database and sample bank were established for the ongoing analysis of transplants from Register donors. Clinical data and blood samples were collected from donors and patients transplanted in the UK from 1996 onwards.

DNA was extracted from all samples received, and used for the detection of HLA mismatches at six loci (HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1) by RSCA, and HA-1 by SSP. 138 donor/patient pairs were selected for a pilot study. HLA matching between patient and donor, along with the diagnosis of the patient, donor and patient age, CMV status and gender, and the T cell depletion status of the transplant were analysed for their effect on transplant outcome. The outcome variables studied included overall survival, disease free survival, transplant related mortality, relapse incidence, and the occurrence of acute and chronic GvHD.

Patients with AML had decreased overall and disease free survival, and patients with CML had increased risk of relapse. Transplant from a same sex donor increased the risk of death or relapse, and male patients receiving stem cells from female donors showed reduced overall survival. Developing acute GvHD increased the risk of transplant related mortality. A mismatch at HLA class I and at class II was associated with unfavourable outcome for all survival variables. The inclusion of HLA-DPB1 matching did not alter the effects seen when other mismatches were present. Matching at the allele level for all loci gave an increased risk of relapse compared with patients mismatched with their

donor for HLA-DPB1 only, indicating that a mismatch for HLA-DPB1 alone may protect from relapse.

The data from this study was used to calculate the numbers required for a definitive study, for the optimisation of donor selection from the Register.

Contents

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Chapter 1

Introduction

Bone marrow transplantation (BMT) between unrelated individuals is now a routine treatment for a variety of haematological and immunological diseases. One of the main complications following this therapeutic procedure, however, is rejection of either the host tissue by the bone marrow graft (graft-versus-host disease), or of graft tissue by the host (graft rejection). Graft-versus-host disease (GvHD) comes about when the T cells of the allogeneic bone marrow recognise the cells of the recipient as foreign, causing a severe inflammatory response (Deeg 1993). These conditions occur due to interactions between donor and recipient immune systems. Graft rejection can be avoided through suppression of the patient's immune system, and it has also been shown that removing T cells from the bone marrow graft can reduce the extent of graft-versus-host disease seen (Hale and Waldmann 1994). The Major Histocompatibility Complex (MHC) antigens are the main targets of the graft versus host and rejection responses. Therefore, patients and donors are typed and matched for their MHC encoded HLA molecules prior to transplantation in order to try and limit an allo-reactive response.

The history of bone marrow transplantation

Mouse models

The effects of radiation on humans following the use of atomic bombs during World War II led to research experiments on mice, to identify the effects of radiation and discover potential curative treatments. Through experiments where mice were exposed to whole body irradiation, it was found that protection of the spleen with lead foil, or subsequent infusion of autologous

bone marrow protected from radiation damage (Jacobson *et al.* 1951; Lorenz *et al.* 1951). These experiments were developed further, showing that if marrow were successfully transplanted between mouse strains, a skin graft could be given from the same donor and not rejected (Main and Prehn 1955). The skin would only be accepted if given from the donor strain, and the marrow in the transplanted mice had the same immunogenetics as that of the donor (Ford *et al.* 1956; Trentin 1956).

When transplants were carried out between mice of different strains, it was found that in some cases, the bone marrow elicited an immune response against the recipient, giving a secondary disease, now known as graft-versus-host disease (GvHD) (Barnes *et al.* 1956). The severity of this disease was found to be due to genetic differences between the donor and recipient (Uphoff 1957), which agreed with discoveries previously seen in solid organ transplant rejection (Gorer *et al.* 1948; Snell 1948). These genetic factors are discussed later.

To try to reduce the severity and occurrence of the GvHD, immune suppressive drugs such as methotrexate (MTX) were administered with the bone marrow, resulting in a reduced GvHD response (Lochte *et al.* 1962; Uphoff 1958). In an effort to move away from using lethal dose irradiation for myeloablation, other methods of T-cell depletion were developed, using immune suppressive drugs, such as cyclophosphamide (Santos and Owens 1969), and also antibodies specific for lymphocytes and thymocytes (Brent *et al.* 1967). Once the complications following allogeneic bone marrow transplantation could be controlled in the mouse, this treatment was used in an attempt to cure firstly immune deficiency disorders (Steinmuller and Motulsky 1967), and later autoimmune diseases in murine models of human diseases (van Bekkum 1993).

Canine transplantation

Although bone marrow transplantation could be successfully carried out in mouse models of human disease, the transition from this to human transplantation was not simple. The mice used were inbred and raised under laboratory conditions, not representative of the human situation. Therefore,

experiments had to be carried out in outbred animal populations to give a greater experience of the genetic variability faced. Larger animals were also required to test the level of radiation and the dose of immune suppressive drugs that could be given safely. These experiments were carried out in the dog, as these animals were readily available and inexpensive to keep in a laboratory environment. Large families could be studied, with the availability of matched littermate pairs. In early experiments, similar GvHD responses were seen to those already observed in mouse BMT (Thomas *et al.* 1962), leading to the discovery of the genes of the canine major histocompatibility complex, the Dog Leukocyte Antigens (DLA) (Epstein *et al.* 1968). One of the discoveries that came from these experiments was that dogs receiving previous donor blood transfusions developed acute rejection of bone marrow when subsequently transplanted from littermates (Storb and Deeg 1986). This transfusion-induced sensitisation was overcome by the use of alkylating agents (such as Cyclophosphamide) in conjunction with antithymocyte globulin. Canine transplantation was also key in the use of other prophylactic immune suppressive drugs used in combination, such as cyclosporin along with methotrexate, a combination which was shown in dogs to give a good protection against the development of GvHD (Deeg *et al.* 1984; Deeg *et al.* 1982).

Bone marrow transplantation in humans

While experiments were being carried out in dogs, to identify methods of transplantation that could be safely carried out in individuals with different genetic backgrounds, some human transplantation had already been attempted. After the first discoveries in mice that marrow could be successfully engrafted after total body irradiation, it was attempted for the treatment of human haematological malignancies (Thomas *et al.* 1957). Although engraftment was not achieved in most cases, it was seen that the actual process could be tolerated in humans, leading the way for further investigation. The first successful bone marrow engraftment was seen in two acute lymphoblastic leukaemia (ALL) patients transplanted, after total body irradiation (TBI), with marrow from their

identical twin (Thomas *et al.* 1959). Engraftment was maintained but the leukaemia recurred within a few months, indicating that TBI alone was not enough to eradicate the leukaemia. This also supported the views seen in murine transplants where it was thought that to cure leukaemia, some genetic differences were required (Barnes *et al.* 1956). Autologous transplantation was also attempted in acute lymphocytic leukaemia (ALL) patients, with marrow stored from a previous remission. Remission was achieved, but the patient later relapsed, perhaps because there were still some leukaemic cells present in the graft, or a lack of antileukaemic response (McGovern *et al.* 1959). Due to the staggering lack of success in the maintenance of engraftment, studies were carried out to test the tolerance level for radiation exposure, and chemotherapy alone and in combination in the hope of extending the life of the bone marrow graft (Buckner *et al.* 1974).

Transplant work in animal models, and blood transfusion anomalies led the way to the discovery of the Major Histocompatibility Complex, which explained many of the transplant results seen in these early experiments. Using the knowledge gained from early transplantation experiments, and new found information about histocompatibility antigens, more success was had with transplantation in patients with diseases that did not require extensive conditioning before transplantation to avoid graft rejection (Bach *et al.* 1968; De Koning *et al.* 1969; Gatti *et al.* 1968). Following these encouraging results, matched sibling transplants were also performed in leukaemic patients given lethal total body irradiation (Thomas *et al.* 1971), and later the first unrelated transplant for a patient with aplastic anaemia (Speck *et al.* 1973). Unfortunately, this and following unrelated transplants were unsuccessful (Horowitz *et al.* 1975; Lohrmann *et al.* 1975; O'Reilly *et al.* 1977), and the spirits of the transplant physicians were severly dampened. The first successful unrelated bone marrow transplant was finally carried out in a child with ALL in second remission, although the patient eventually relapsed and died 2 years post transplant (Hansen *et al.* 1980). These transplants began the path towards the way stem cell transplants are carried out today.

Other sources of stem cells

Sources of stem cells for allogeneic transplantation today have extended to umbilical cord blood and mobilised peripheral blood from adult donors. An HLA matched cord blood unit, once identified, can be acquired rapidly as it is cryopreserved, and already typically intermediate or high resolution typed for HLA-A, -B and -DRB1 loci at collection (Barker *et al.* 2002). Total nucleated cell dose of a cord blood graft has shown to be critical with regard to engraftment and survival post transplant (Gluckman *et al.* 1997; Rubinstein *et al.* 1998), therefore, the fixed cell count of the cord blood unit presents a limiting factor especially for adult patients. However, cord blood has a higher frequency of progenitor cells compared with adult peripheral blood or bone marrow (Broxmeyer *et al.* 1992). Despite early findings that a 0-3 HLA antigen mismatch did not increase the risk of developing acute or chronic GvHD (Gluckman *et al.* 1997; Laughlin *et al.* 2001), larger studies have reported significantly higher rates of acute GvHD with mismatched grafts (Rubinstein *et al.* 1998). The naive status of cord blood lymphocytes has raised concerns for a reduced graft versus leukaemia effect after cord blood transplant (Linch and Brent 1989), but studies so far have not shown a relationship with increased relapse compared with bone marrow transplants (Barker *et al.* 2001a; Rocha *et al.* 2001). Comparisons between cord blood and marrow stem cell transplants in children have determined no significant difference with respect to survival (Barker *et al.* 2001a; Rocha *et al.* 2001). The main challenges with respect to adult patients are associated with the cell dose available form a cord blood unit. Studies are ongoing to try and overcome this problem with ex vivo expansion of cord blood haematopoietic cells (Jaroscak *et al.* 2003), and the transplantation of more than one cord blood unit (Barker *et al.* 2001b).

Recombinant granulocyte colony-stimulating factor (G-CSF) - mobilised peripheral blood stem cells (PBSC) are now widely used for allogeneic transplantation from HLA-identical related donors. Results have shown improved immune reconstitution compared to related bone marrow transplants (Bensinger *et al.* 2001; Champlin *et al.* 2000), and reduced risk of relapse in

patients with CML in first chronic phase (Elmaagacli *et al.* 1999). In studies of unrelated PBSC transplant, neutrophil and platelet recovery were more rapid in recipients of PBSC compared with bone marrow (Elmaagacli *et al.* 2002; Fauser *et al.* 2000; Ringden *et al.* 1999). With accelerated immune reconstitution demonstrated by a reduced risk for interstitial pneumonia cause by CMV following PBSC transplant (Trenschel *et al.* 2000). Although it was originally though there would be an increased risk of acute GvHD with PBSC due to the larger number of lymphocytes present compared to bone marrow (Korbling *et al.* 1995), a lower incidence of severe acute GvHD has been reported in unrelated transplants compared with bone marrow in CML patients (Elmaagacli *et al.* 2002), but not in related transplants with other disease groups (Bensinger *et al.* 2001; Champlin *et al.* 2000). Reduced incidence of transplant related mortality in recipients of PBSC has also been reported, thought to be due to superior immune reconstitution and lower rate of severe acute GvHD (Elmaagacli *et al.* 2002). PBSC transplantation also offers practical advantages for the donor over conventional allogeneic bone marrow transplants, namely the ability to harvest stem cells in an outpatient setting, thereby avoiding hospitalisation and the risks of general anaesthesia.

Graft versus Host disease

The first description of graft-versus-host disease was published in 1955, in a mouse model, where it was originally named secondary disease, to separate it from the primary disease of radiation sickness seen post allogeneic transplant (Barnes and Loutit 1955). Later described as a disease resulting from the presence of immunocompetant donor cells in an immunocompromised host, it was termed graft versus host disease (Billingham and Brent 1957), the effect of which was also later seen in human transplantation (Graw *et al.* 1970; Mathe *et al.* 1963). HLA disparity between the haematopoietic cell donor and transplant recipient is the most powerful factor governing the severity and kinetics of GvHD. Donor T lymphocytes proliferate and differentiate in response to disparate histocompatibility antigens in host tissues, and directly, or through secondary mechanisms, attack recipient cells, producing the signs and

symptoms of GvHD. It has been shown that the incidence of acute GvHD increases with HLA disparity between donor and recipient (Beatty *et al.* 1985; Kernan *et al.* 1993).

Graft versus host disease can be split into two parts, acute GvHD, which encompasses the dermatitis, hepatitis and enteritis seen to develop within 100 days post transplant, and chronic GvHD, which describes a more diverse and widespread syndrome developing after day 100.

In 1966, Billingham defined the criteria for the development of GvHD (Billingham 1966):

1. "The graft must contain immunologically competent cells.
2. The host must possess important transplantation alloantigens that are lacking in the donor graft, and is, therefore, capable of stimulating it antigenically.
3. The host must be incapable of mounting an effective immunological reaction against the graft, at least for sufficient time for the latter to manifest its immunological capabilities; that is, it must have the security of tenure."

The afferent phase of GvHD consists of antigen presentation, activation of T cells, clonal proliferation and differentiation (reviewed in Ferrara and Deeg 1991), resulting in host cell death. In the efferent phase, activated lymphocytes release cytokines, leading to cell death directly or through recruitment and activation of secondary effectors such as NK cells (Piguet *et al.* 1987). NK cells can express inhibitory receptors that signal killing when they do not bind their specific ligand, for example, an HLA class I molecule (Bottino *et al.* 1995). If donor and recipient are mismatched for this ligand, killing by NK cells may be induced (Colonna *et al.* 1993), contributing to the GvH effect. NK cells have been found in cutaneous GvHD (Acevedo *et al.* 1991).

Administration of high-dose chemotherapy is followed by an increase in circulating cytokines (known as "cytokine storm"), which are thought to

increase the ability of graft immune cells to recognize host antigens (reviewed in (Ferrara 1993), and can also lead to localized tissue damage, thus exposing cryptic antigens in certain organs (eg, the MIC antigens in the gut) which may activate NK and T cells (Bauer *et al.* 1999). Administration of non-myeloablative but immune-suppressive chemotherapy followed by allogeneic transplants (ie, mini-dose transplant, transplant-light) decreases the original cytokine storm and tissue damage, leading to lower incidence of GVHD, hopefully maintaining a graft versus tumor effect (Slavin *et al.* 1998).

Graft versus leukaemia effect

Although the effects of GvHD can be detrimental to the patient, it has been observed that in some cases, alongside GvHD, a second effect can occur which is favourable to transplant outcome (Weiden *et al.* 1979). Termed the Graft versus leukaemia effect (GvL) (Bortin *et al.* 1973), it has been postulated that some histocompatibility antigen mismatches between donor and recipient may lead to recognition of disparate antigens on the malignant cells of the patient by the T cells of the donor (Wang *et al.* 2002), leading to eradication of the disease. One of the limitations of successful transplantation in advanced malignant disease is the relapse rate. Although relapse may be due to an insufficient conditioning regime, the absence of a GvL effect may also contribute. When outcomes have been compared between allogeneic and syngeneic transplants, the risk of relapse was higher in the syngeneic transplant group, suggesting that an antileukaemic effect is associated with allogeneic marrow (Horowitz *et al.* 1990; Ringden and Horowitz 1989). In an attempt to reduce the incidence and severity of GvHD, T cell depletion has been used to great success (Hale, Waldmann 1994; Hale and Waldmann 1996). However, it has been found that the incidence of relapse is higher in recipients of T cell depleted allogeneic transplants when compared with unmodified marrow (Apperley *et al.* 1988; Goldman *et al.* 1988; Martin *et al.* 1988). CML in chronic phase is highly susceptible to GvL effects mediated by allogeneic T cells, as evidenced by a

5.14-fold increased risk of relapse associated with T cell depletion of donor marrow prior to HLA-identical allogeneic bone marrow transplantation (Horowitz *et al.* 1990).

The most compelling evidence for a GvL effect in humans is that, in some cases, patients with relapsed disease can be brought back into remission through an infusion of donor lymphocytes post transplant (Collins Jr *et al.* 1997; Kolb *et al.* 1990; Kolb *et al.* 1995). The effector cells that mediate a GvL effect may include CD8+ CTL that recognise tumour antigens in association with MHC class I antigens, CD4+ T cells that recognise tumour antigens in association with MHC class II antigens, or NK/LAK cells that mediate an antitumour effect through cytokine secretion and without MHC restriction (Sosman *et al.* 1989).

The challenge is to induce a GvL effect while reducing the GvHD response as much as possible. This has been attempted by reducing the number of CD3+ cells infused in the donor lymphocyte infusion (DLI) (Giralt *et al.* 1995; Mackinnon *et al.* 1995), altering post-transplant imuune suppression (Sullivan *et al.* 1989), and the use of IL-2 therapy to stimulate the likely effectors of the GvL effect (Soiffer *et al.* 1994).

Tolerance

During T cell education in the thymus, thymocytes are deleted if they have a strong affinity for self-MHC (reviewed in Nossal 1994). Preventing the emergence of autoimmune T cells from the thymus represents the central mechanism of self-tolerance. However, this mechanism of central tolerance induction cannot explain the lack of response seen against antigens encountered in the periphery that are not expressed in the thymus. Peripheral tolerance is maintained by mechanisms that act on mature lymphocytes that encounter self-antigens in peripheral tissues. Some self-antigens may induce neither central nor peripheral tolerance but are simply ignored by the immune system. Such "clonal ignorance" (Ohashi *et al.* 1991), may be because the self-antigen is physically separated from immunocompetant lymphocytes or because the antigen is presented to lymphocytes in the absence of the second signals that are needed to trigger effective immune responses. For an immune response to

occur, lymphocytes must usually be exposed to two types of stimulus. The first signal, an antigen, ensures the specificity of the response, second signals include costimulators and cytokines, usually produced as a result of the initial innate immune response to a pathogen (Fearon and Locksley 1996). Because self-antigens do not normally elicit innate immune reactions, they may be ignored by the immune system.

It is widely believed that self-antigen recognition without costimulation induces functional anergy, although it is not known which factors determine whether a self-antigen is functionally ignored or induces anergy. The frequently noted association between infections and autoimmunity has been attributed to the activation of anergic self-reactive lymphocytes by adjacent cells reacting to microbial antigens but may also result from the activation of autoreactive lymphocytes that have remained ignorant of the antigen until second signals are up-regulated as a consequence of the infection. The best characterised co-stimulatory signalling components are the B7 molecules (CD80/CD86) expressed on antigen presenting cells, and their receptor, CD28, expressed on T cells. Ligation of CD28 by B7 molecules or anti-CD28 antibodies co-stimulates the growth of niave T cells, whereas anti-B7 antibodies that inhibit the binding of B7 to CD28 inhibit T cell responses (de Boer *et al.* 1992). Self-antigens may also induce tolerance in the mature T lymphocyte if the self-antigens trigger mechanisms that actively block lymphocyte activation or induce apoptosis, for example, with CTLA-4-mediated inhibition. A T cell co-receptor for B7 molecules, called CTLA-4, was shown to function primarily to shut off T cell activation (Krummel and Allison 1995). CTLA-4 is induced on T cells after activation, and upon binding B7 on the APC, it transduces signals that inhibit the transcription of IL-2 and the progression of T cells through the cell cycle (Krummel and Allison 1996). CTLA-4 blocks signals transduced by CD28, suggesting that these two B7-recognising molecules function as mutual antagonists.

In a transplant setting, the aim is to induce immunological tolerance to the engrafted tissue. This has been shown in mice where a mixed chimera has been

achieved post allogeneic bone marrow transplantation, where haematopoietic cells from both donor and recipient locate to the thymus and hence delete both host-reactive and donor reactive T cells, resulting in a peripheral T cell repertoire that is both host and donor tolerant (Manilay *et al.* 1998; Tomita *et al.* 1994). Due to the presence of both host and donor-derived APCs in the thymus of a mixed chimera, the deletion of both host and donor-reactive T cells occurs to a greater extent than in a full chimera (Yoshikai *et al.* 1990). In human transplantation, the risk of GvHD and relapse are great barriers to the induction of a mixed chimera and other avenues have required exploration. Animal models involving second signal blockade (Adams *et al.* 2001; Wekerle *et al.* 2000), have shown encouraging results that may be applicable to allogeneic bone marrow transplantation in humans.

Factors affecting transplant outcome

In unrelated bone marrow transplantation, the most important factor with respect to out come is HLA matching (Madrigal *et al.* 1997). However, if more than one HLA matched donor is available, other factors must be taken into account to ensure the choice of donor will give the best outcome possible. It has been seen in HLA identical sibling bone marrow transplantation that certain characteristics of the recipient and donor can be associated with poor transplant outcome. Risk factors include age of the donor and recipient, with transplants performed with older donors and recipients having an increased risk of acute and chronic GvHD (Ochs *et al.* 1994; Weisdorf *et al.* 1991). Also, transplantation of a male recipient with marrow from a female donor is thought to increase the risk of developing GvHD, due to recognition of male-specific minor antigens (Vogt *et al.* 2000). This is especially relevant in multiparous women, or those who have had previous blood transfusions, who may have already had exposure to a male antigen (Wiesdorf *et al.* 1991). Treatment of the bone marrow graft or the recipients with T-cell-depleting antibodies is thought to reduce the risk of graft-versus-host disease, but can increase the risk of disease relapse in some individuals (Apperley *et al.* 1986; Hale, Waldmann 1994). Through studies carried out on the rapidly increasing number of unrelated

transplants performed it has been found that these and other factors could influence the outcome of allogeneic bone marrow transplantation, but still the most important selection criteria rested with the MHC (Madrigal *et al.* 1997).

Disease

Factors to consider include, type and subtype of disease, stage of disease at diagnosis and transplant and the time from diagnosis to transplant. Disease stage at time of transplant is the most important factor influencing outcome in all disease categories, more advanced disease is associated with increase in transplant related mortality and higher incidence of relapse (Gratwohl and Hermans 2000).

Chronic Leukaemia

Chronic Myeloid Leukaemia can be separated into three stages, chronic phase (CP), accelerated phase (AP) and blast crisis (BC). Characteristic of CML is the Philadelphia chromosome (Nowell and Hungerford 1960), a cytogenetic abnormality present in the malignant cell population, t(9;22), forming fusion genes $p210^{BCR\text{-}ABL}$ or $p190^{BCR\text{-}ABL}$ (Rowley 1973). Disease in CP, spleen size, percentage of circulating blasts, platelet count and age are the features that have shown prognostic significance (Sokal *et al.* 1984). Chromosomal abnormalities additional to Philadelphia are thought to give a poor prognosis (Sokal *et al.* 1988), but unpredictable onset of malignant transformation means these factors are of little help in deciding on treatment.

Until now, the main cure for chronic phase CML has been bone marrow transplantation. Therefore, this disease has been the most widely studied with respect to unrelated bone marrow transplantation, and has a very good success rate with overall survival at approximately 50%, and relapse at 15% at 3 years post transplant (Beatty *et al.* 1989; Devergie *et al.* 1997; Dini *et al.* 1998; Marks *et al.* 1993; Spencer *et al.* 1995). Due to the slow progression of CML, it was originally thought there was no urgency in finding a matched donor, giving more time to find an exact match. However, potential problems associated with GvHD for CML patients have since shown to be offset by early transplantation,

within first year of diagnosis, to ensure best chance of long-term disease free survival. For CML patients, factors found to have a significant adverse effect on survival included patient age over 50 and long interval from diagnosis to transplantation (Hansen *et al.* 1998). It is thought for successful transplantation in patients with a matched donor, increased cell dose should be considered to minimize the risk of graft failure (Petersdorf *et al.* 1997). In a risk assessment of CML patients from the EBMT, it was found that disease stage had a significant effect on overall survival and transplant related mortality (Gratwohl *et al.* 1998). In the same study, relapse incidence was only associated with a stage of disease that was accelerated phase or greater.

Until recently, interferon-α (IFN-α) was used to treat patients with CML-CP who were not eligible for allogeneic stem cell transplants. However, a new drug, Imatinib mesylate (STI571), has been developed, which inhibits the kinase activity of all proteins that contain ABL, inhibiting cellular growth and inducing apoptosis in CML (Deininger *et al.* 1997; Druker *et al.* 1996; le Coutre *et al.* 1999). It was first used in 1998 to treat CML patients, and it induced complete haematologic responses in over 95% of patients with CML resistant to treatment with IFN-α but still in CP, and it induced major cytogenetic responses in over 40% (Druker *et al.* 2001). Imatinib mesylate has also been used in relapsed CML post transplant with failed response to DLI, giving positive results (Olavarria *et al.* 2002). However, further research is required before Imatinib mesylate can be considered as a replacement for allogeneic stem cell transplantation as a proportion of patients disease has become resistant to the drug (Roche-Lestienne *et al.* 2002).

Acute Leukaemia

Most patients with acute leukaemia have been treated successfully with various chemotherapeutic regimes, but for a subset of patients who are refractory to treatment or where remission is not achieved, bone marrow transplantation is a treatment option (Biggs *et al.* 1992). Reports of disease free survival at 5 years post transplant, for AML and ALL in second complete remission, have shown

rates of 27% ± 11% and 37% ± 11% respectively (Sierra *et al.* 1997). In patients with acute leukaemia, the stage of the disease at the time of transplant is a primary determinant of overall outcome. For patients with high-risk acute leukaemia, disease free survival was better for adults in CR. Results have shown to be improved if the patient is transplanted in remission or early first relapse (Kernan *et al.* 1993).

Acute Myeloid Leukaemia

AML can be diagnosed through the identification of >30% leukaemic myeloblasts in the peripheral blood and bone marrow (reviewed in Lowenberg *et al.* 1999). Based on morphology and chromosomal abnormalities, the disease is separated into subtypes M0-M7 based on the French-American-British (FAB) classification scheme (Bennett *et al.* 1985a; Bennett *et al.* 1985b). Allogeneic transplantation is generally an option for patients who have an intermediate risk of relapse after chemotherapy, whereas for those in first remission it unnecessary as relapse is 30-40%, and high risk and older patients, do less well after transplantation often without benefit (Grimwade *et al.* 1998). It has been shown in multicentre randomised trials that allogeneic stem cell transplantation is the most effective strategy for preventing relapse in first CR of AML (Woods *et al.* 2001; Zittoun *et al.* 1995). In adult patients, however, this does not necessarily lead to improved disease free survival due to the significant level of transplant related mortality (Cassileth *et al.* 1998; Harousseau *et al.* 1997). In a study of 97 patients who received allogeneic transplants for AML from HLA identical siblings, no significant difference was seen between patients grafted in CR1 as compared with those engrafted in CR2 for event free survival, transplant related mortality, or GvHD (Robin *et al.* 2003). This is contrary to the reports of other studies, where long term leukaemia free survival has been seen in patients transplanted in first complete remission (Zittoun *et al.* 1995), with a much reduced leukaemia free survival rate in patients transplanted in 2CR (Clift *et al.* 1998). However, the overall survival rate of patients with refractory AML who undergo transplantation have been reported to be as low as 10-15% (Edenfield and Gore 1999; Greinix *et al.* 2002).

Acute Lymphoblastic Leukaemia

In contrast to AML cells, ALL cells lack specific morphological or cytochemical features, so the diagnosis depends on immunophenotyping. ALL can be of B lymphocyte or T lymphocyte origin and specific genetic abnormalities are found in the blast cells of 60-75% of patients with ALL (reviewed in Pui and Evans 1998). Transplantation is usually considered for those who have no response to the initial induction treatment and those who have a second remission after haematological relapse (Appelbaum 1997), but some patients with unfavourable prognosis can be transplanted in first remission (Sebban *et al.* 1994). The duration of CR2 in ALL has been shown to be short, e.g. Median <1 year in children relapsing on therapy or within 6 months of discontinuing therapy, so BMT must be performed rapidly to be effective (Henze *et al.* 1991). Two thirds of the patients diagnosed with ALL are children, who have a far better prognosis than adults where transplantation is generally the only option (Parker *et al.* 1997). Patients who are generally candidates for allogeneic BMT are those with risk factors predicting treatment failure after chemotherapy, such as high white cell counts, age >30 years, cytogenetic abnormalities, and a long interval to achieve first remission (Chao *et al.* 1991; Sebban *et al.* 1994; Wingard *et al.* 1990). The risk of relapse is a major problem in ALL, possibly due to the selection of patients with high risk leukaemia, with a trend for lower risk of relapse seen in patients grafted in CR1-CR2, but not so in patients with advanced disease (Zikos *et al.* 1998). In ALL patients, acute GvHD has shown a favourable effect on relapse and in turn, transplant related mortality (Doney *et al.* 1991).

Other malignant disease

Myelodysplastic syndrome (MDS) refers to a heterogeneous group of closely related clonal haematopoietic disorders. All are characterised by a cellular marrow, with impaired morphology and maturation (dysmyelopoiesis), and peripheral blood cytopenias, resulting from ineffective blood cell production. French-American-British (FAB) Cooperative Group, classified the Myelodysplastic disorders into 5 subgroups, differentiating them from acute

myeloid leukemia. Two subgroups of refractory anaemia characterized by 5% or less myeloblasts in bone marrow exist: (1) refractory anaemia (RA) and (2) RA with ringed sideroblasts (RARS). Two subgroups of refractory anaemia's with greater than 5% myeloblasts exist: (1) RA with excess blasts (RAEB), defined as 6-20% myeloblasts, and (2) RAEB in transformation (RAEB-T), with 21-30% myeloblasts. The higher the percentage of myeloblasts, the shorter the clinical course and the closer the disease is to acute myelogenous leukaemia. The fifth type of MDS, the most difficult to classify, is called chronic myelomonocytic leukaemia (CMML) (Bennett *et al.* 1982).

Allogeneic bone marrow transplantation is the definitive therapy for MDS. In patients with less advanced MDS (i.e., patients with RA or RARS), the 3-year disease-free survival (DFS) rate ranged from 40% to 60%, with a relapse rate of 10% to 20% and a mortality rate of about 30% (Deeg *et al.* 2000). Compared with patients with MDS who are at low risk, patients with advanced MDS who underwent allogeneic BMT (i.e., those with RAEB or RAEB-T) had a much lower DFS rate (0% to 20%) and high relapse and mortality rate (30% to 60%) (de Witte *et al.* 2000b). In MDS or MDS-related AML, transplant outcome is related to disease morphology, patient age, disease duration, CMV status, blast count and neutrophil count (Anderson *et al.* 1996).

Since the median age of MDS is between 60 and 70 years, patients who can benefit from BMT are limited in number, which may explain the higher DFS and mortality. In addition, elderly patients usually have a lower tolerance to myeloablative treatment and total body irradiation, although a recent study suggested that allogeneic and syngeneic marrow transplantation can be successfully carried out in patients with MDS who are 55 to 65 years of age (Deeg *et al.* 2000).

Multiple Myeloma is an incurable but highly treatable form of cancer of the blood and immune system. It is characterized by the accumulation of malignant plasma cells in the bone marrow and excess monoclonal immunoglobulin (Ig) in the serum and/or urine. The cause of Myeloma is unknown. When B cells respond to an infection, they mature and change into plasma cells. Plasma cells

produce and release immunoglobulins to attack and help kill pathogens such as bacteria. When plasma cells grow out of control, they can produce a tumour. These tumours can grow in several sites, particularly in the bone marrow. When these tumours grow in multiple sites, they are referred to as multiple myeloma.

Traditional approaches for treatment of multiple myeloma have included systemic chemotherapy and radiation to the affected sites, however, less than 15% of patients have survived more than 10 years under these conditions (reviewed in Alexanian and Dimopoulos 1994). High dose chemotherapy followed by allogeneic bone marrow transplantation has been shown to induce a graft versus tumour effect (Lokhorst *et al.* 1997; Tricot *et al.* 1996), but 5 year survival rates are only 25%, due to a high treatment related mortality of 40-50% (Bensinger *et al.* 1996; Bjorkstrand *et al.* 1996; Gahrton *et al.* 1995). Stage of disease, time from diagnosis to transplant, and the number of cycles of chemotherapy the patient had received before transplant were found to be risk factors for poor transplant outcome (Bensinger *et al.* 1996).

Non-Hodgkin's lymphoma (NHL) is a malignant growth of B or T cells in the lymph system. The Non-Hodgkin's lymphomas encompass over 29 types of lymphoma. The distinctions are based on the type of cancer cells. Lymphomas can be grouped by cell type and rate of growth. The United States National Cancer Institute now splits lymphomas into aggressive (fast growing) and indolent (slow growing) although T-Cell, B-Cell, Large Cell, or Follicular Cell can also group them. The main groups are low-grade NHL, intermediate-grade NHL, high-grade NHL (immunoblastic subtypes), lymphoblastic lymphoma, Burkitt's lymphoma and Hodgkin's disease (Institute 1982). In comparison with autografts, allogeneic bone marrow transplants have resulted in a lower relapse rate in the treatment of lymphoma (Copelan *et al.* 1990; Mandigers *et al.* 1998). However, it is thought that the high treatment related mortality observed in allogeneic bone marrow transplantation for NHL might outweigh the benefits of a lower relapse rate (Peniket *et al.* 2003).

Non-malignant diseases

The aim of transplantation for many non-malignant diseases is to replace the missing or malfunctioning cells of the immune system to avoid the problems of infection or the need for transfusion. Non-malignant diseases including several immunodeficiencies, have been treated with some success with unrelated bone marrow transplantation (Buckley *et al.* 1999; Mullen *et al.* 1993). Factors important for improved transplant outcome include early diagnosis, resulting in healthier patients at the time of transplantation (Myers *et al.* 2002). Also, prevention of disease and transplantation related complications such as infection and GvHD. The main advantage of allogeneic transplantation for immunodeficiencies, is that little or no myeloablation or immunosuppression is required, so engraftment may not a problem. However, for some non-malignant conditions such as aplastic anaemia engraftment can be a significant problem.

SCID, Severe Combined Immunodeficiency, is a primary immune deficiency. The defining characteristic is a severe defect in both the T- & B-lymphocyte systems. This can result in the onset of one or more infections within the first few months of life. These infections are usually serious, and can even be life threatening, they may include pneumonia, meningitis or bloodstream infections.

Immunodeficiency with Hyper-IgM (HIM) is a rare primary immunodeficiency characterized by the production of normal to increased amounts of IgM antibody of questionable quality and an inability to produce sufficient quantities of IgG and IgA. Individuals with HIM are susceptible to recurrent bacterial infections and are at an increased risk of autoimmune disorders and cancer at an early age. X-linked immunodeficiency with hyperimmunoglobulin M (XHIM) is caused by the absence of CD40 ligand (CD154), which is normally expressed on activated $CD4^+$ T lymphocytes.

Wiskott-Aldrich syndrome (WAS) is a condition with variable expression, but commonly includes immunoglobulin M (IgM) deficiency. WAS always causes persistent thrombocytopenia and, in its complete form, also causes small

platelets, atrophy, cellular and humoral immunodeficiency, and an increased risk of autoimmune disease and haematological malignancy. In one study of 154 patients with WAS, only 30% had a classic presentation with thrombocytopenia, small platelets, eczema, and immunodeficiency; although 84% had clinical signs and symptoms of thrombocytopenia, 20% had only haematological abnormalities, 5% had only infectious manifestations, and none had eczema exclusively. WAS is an X-linked recessive genetic condition; therefore, this disorder is found almost exclusively in boys.

The Hurler syndrome is an autosomal recessive disease that belongs to a group of diseases called mucopolysaccharidoses. Storage of abnormal quantities of this material (mucopolysaccharide) in different body tissues is responsible for the symptoms and appearance of the disease. Bone marrow transplantation is the only long-term therapy known to prolong the life of patients born with the Hurler syndrome (Fleming *et al.* 1998; Hobbs *et al.* 1981), who would usually within the first decade of life. Age at the time of transplant is an important factor, with those transplanted before the age of 2 years have an improved transplant outcome with marrow from an unrelated donor than those transplanted over the age of 2 (Peters *et al.* 1996).

CMV status

A major complication of bone marrow transplantation is infection post transplant. The patient has been treated for their primary disease with therapeutic agents, such as chemotherapy, which results in immune suppression. During preparative regimes for the bone marrow transplant, immune suppressive drugs and usually irradiation are given to further suppress the immune system to avoid rejection of the allogeneic graft. Once the patient is immune suppressed they can be open to effects from latent viruses and infection from opportunistic pathogens. One of the more severe infections, which can lead to morbidity and mortality in bone marrow transplant recipients, is cytomegalovirus (CMV) (Broers *et al.* 2000). CMV is a virus that can become latent after primary infection. It infects cells involved in immune responses, particularly bone marrow progenitors, endothelial cells,

polymorphonuclear leukocytes and monocytes (Taylor Wiedeman *et al.* 1991). The human CMV virus has many ways of evading immune recognition, allowing it to become a permanent resident of the cells of its' host. Upon infection, host cell protein synthesis becomes dramatically reduced, and some CMV genes have been shown to prevent the cell-surface expression of HLA class I (Barnes and Grundy 1992). For example, the US6 gene binds directly to the TAP complex and therefore inhibits peptide translocation from the cytosol to the ER (Lehner *et al.* 1997). Although this mechanism can evade recognition by T -cells it should be noted that these cells could now be putative targets for NK cell attack. CMV infection has also shown to have immunosuppressive effects on lymphocyte proliferative responses (Meyers *et al.* 1986).

Infected healthy individuals show no symptoms of CMV infection, but viral DNA can be detected by PCR of known viral sequences, or the expression of the CMV lower matrix phosphoprotein pp65 can be detected in the peripheral leukocytes (Bacigalupo *et al.* 1995). These individuals are regarded as CMV seropositive. When an individual is immune suppressed, for example, in a bone marrow transplant situation, or through HIV infection, the virus is no longer controlled and can become symptomatic. For recipients of allogeneic bone marrow transplantation, the most life threatening manifestation of CMV infection is interstitial pneumonitis, which inevitably results in death (Meyers *et al.* 1986).

There is a poor correlation between viral titre and disease severity in these patients (Slavin *et al.* 1994), which has lead to the hypothesis that the pathogenesis of CMV pneumonitis involves tissue damage caused by the immune system. CMV pneumonitis has also been shown to be associated with the occurrence and increased severity of GvHD (Meyers *et al.* 1986).

If a seropositive patient is transplanted, or a seropositive donor is donating bone marrow to a seronegative recipient, the recipient can be prophylactically treated with antiviral therapy, in order to try to control any viral reactivation (Goodrich *et al.* 1991).

The use of fluconazole for the prevention of fungal infection (Slavin *et al.* 1995),

and gancyclovir given at time of engraftment or onset of CMV antigenemia for the prevention of CMV disease (Goodrich *et al.* 1991), improved the safety and efficacy of allogeneic grafts.

CMV seropositivity in patients has been seen as an independent risk factor for increase risk of GvHD and incidence of transplant related mortality (Broers *et al.* 2000; Craddock *et al.* 2001). CMV-specific T cell proliferation is significantly less likely to occur in recipients of unrelated BMT, who are therefore at greater risk of developing CMV disease (Krause *et al.* 1997). However, a CMV-seropositive donor has not been found as a risk factor of CMV infection in the recipient post transplant (Miller *et al.* 1986). The use of Gancyclovir in seropositive patients has been associated with increased survival (Hansen *et al.* 1998).

Age

The success of allogeneic bone marrow transplantation relies partly on the ability of naïve progenitor cells of the donor marrow to migrate to, and be educated by, the thymus of the recipient. It can be hypothesised that, due to involution of the thymus beginning in teenage years and culminating by the age of 30 years, younger patients will have greater capacity for this education to occur (Botnick *et al.* 1982). Both recipient and donor age can influence the transplant outcome. Younger recipients transplanted with stem cells from younger donors (less than 30 years) have been reported to have the greater overall survival rate, which could be due to their higher tolerance level for transplant related complications (Gratwohl *et al.* 1995). In a study from Genoa, patients over 35 had increased transplant related mortality when a standard conditioning regimen was used (Bacigalupo 2000).

Many haematological malignancies increase in incidence with advancing age, such as the acute leukaemias, myelodysplastic syndrome and non-Hodgkins lymphoma (Molina and Storb 2000). Also, unfavourable chromosomal abnormalities are more common in older patients (Faderl *et al.* 1998; Grimwade *et al.* 1998), leading to a poorer overall prognosis. Advanced age may translate into poorer outcome after allogeneic transplantation, and the age of the

recipient has been shown to be an independent risk factor for transplant related mortality (Gratwohl *et al.* 1998). In a retrospective study carried out by the EBMT, advanced age was identified as an independent poor prognostic factor in AML and ALL, but with development and changes in transplant protocols over time, an improvement in transplant related mortality has been observed (Frassoni *et al.* 1996). The effect of age on the development of acute GvHD is controversial. In some studies, the risk of developing acute GvHD and the severity of acute GvHD was associated with increased age of the donor (Doney *et al.* 1991), and the patient (Nash *et al.* 1992; Weisdorf *et al.* 1991). In other studies, however, age has not been associated with increased risk of acute GvHD (Hagglund *et al.* 1995; McGlave *et al.* 2000; Ringden *et al.* 1998).

Increased patient age has been observed as a risk factor in the development of chronic GvHD in many studies (Carlens *et al.* 1998; Ochs *et al.* 1994), including and excluding the previous manifestation of acute GvHD (Atkinson *et al.* 1990). This effect, however, can be reduced with increased GvHD prophylaxis, or from T cell depleted transplants, which can lower the incidence of GvHD and also increase survival rates in older patients (Aschan and Ringden 1994).

The NMDP have shown that increased donor age was a significant risk factor for overall and disease-free survival, independent of the age of the transplant recipient (Kollman *et al.* 2001). No significant effect of donor or recipient age on the risk disease relapse was found in the same study.

With the development of unrelated cord blood transplantation (i.e. the 'youngest' donor possible), it is now recognised that perhaps this entirely naive population of cells do not cause GvHD, even across HLA mismatched barriers (Gluckman *et al.* 1997). Due to the low number of cells available in cord blood donations, it was thought to only be possible to transplant a very small (and therefore young) individual, who would be likely to have the thymic capacity for education of the T-cells (Mackall *et al.* 1995). The average number of $CD34^+$ cells in a 100ml cord blood unit is $1.2x10^6$, and the ideal number of CD34+ cells though to give a successful transplant outcome is $>8x10^6$/kg of patient (D'Arena *et al.* 1996). Further work has now shown that it is possible to

transplant an adult donor with cord blood stem cells with an acceptable level of GvHD post transplant, but not completely without a GvHD response (Laughlin *et al.* 2001).

Gender and parity of female donors

It has been observed that female recipients of male bone marrow have a higher risk of graft rejection than when transplanted with bone marrow from a female donor (Voogt *et al.* 1990). A similar effect has also been seen in the greater risk of developing GvHD in male recipients of female grafts. The association of a gender mismatch with graft rejection and (GvHD) in bone marrow transplantation could be due to so-called, male-specific, minor histocompatibility antigens (mHag) (Goulmy *et al.* 1983; Goulmy *et al.* 1996). These are generally HLA class I restricted peptides, derived from male-specific proteins, which are encoded by genes found on the Y chromosome (Meadows *et al.* 1997; Wang *et al.* 1995). One such set of mHag, the H-Y antigens, are expressed on the surface of haematopoietic cells and can be recognised by H-Y specific cytotoxic T-cells, *in vitro* (Voogt *et al.* 1988). In some studies, a gender mismatch has been shown to increase the incidence of GvHD (Gratwohl *et al.* 1995), especially in male recipients receiving marrow from a female, where the T-cells present in the female marrow may recognise a peptide encoded by the male H-Y chromosome, presented by matched HLA class I molecules on the recipient cells (Vogt *et al.* 2000; Wang *et al.* 1995). The presence of these HLA class I-presented peptides in a sex-mismatched transplant has been shown to induce a graft-versus-host disease response (Goulmy *et al.* 1996; Rufer *et al.* 1998). The parity of female donors and sex of donor and recipient have been shown as significant risk factors for *de novo* chronic GvHD, with the combination of the parous female donor and male recipient having the highest risk for chronic GvHD development (Wagner *et al.* 2000). In a study of donor characteristics affecting transplant outcome, the NMDP found no significant association between donor sex and parity of female donors with relapse. The sex of the donor had no effect on overall or disease free survival, regardless of the effect of the recipient (Kollman *et al.* 2001). In an EBMT study of sibling

transplants from female donors, male recipients showed an increase in transplant related mortality (Gratwohl *et al.* 2001).

For the aforementioned reasons, a young male donor is highly sought after. However, currently, only 30% of potential donors on the Anthony Nolan Register are male. This could be due to recruitment strategies; the fact that some women may have more time for donation; or that women feel more comfortable about needles or being in a clinical environment.

Conditioning and GvHD prophylaxis

Conventional conditioning regimens result in an almost complete elimination of patient lympho-haematopoietic tissue. The aim of conventional conditioning is tumour ablation and rejection prophylaxis, and it was originally believed ablation of the marrow was required to create space for the donor cells, although it is now realised donor cells can clear recipient cells for the space needed (Storb *et al.* 1997). However, the side effects of this treatment are severe, including: nausea and alopecia, organ damage, severe immunodeficiency, infertility, growth retardation and secondary malignancy (reviewed in Vindelov 2001). The primary means of conditioning is total body irradiation (TBI) along with chemotherapy agent, Cyclophosphamide (CY), which is immunosuppressive but not myeloablative. It has been shown in canine experiments that the TBI dose required for stable engraftment could be reduced from the myeloablative dose of 9.2 Gy to the non-myeloablative dose of 2.0 Gy (McSweeney and Storb 1999), and in human studies, a fractionated dose of TBI compared with a single dose has improved event free survival in AML patients (Deeg *et al.* 1986; Thomas *et al.* 1982). Alkylating agents such as Busulphan or Melphalan can be used in combination with one another and other chemotherapy agents as an alternative to TBI for myeloablation, although there are still many side effects. There is evidence in animals that stable donor chimerism can be achieved with sublethal doses of TBI and drugs (Storb *et al.* 1997). A reduced intensity conditioning regimen needs to be sufficient for engraftment, but retain sufficient of the patient's lympho-haematopoietic tissue to support a mixed chimerism and be protective in the short term (McSweeney,

Storb 1999). A mixed chimerism results in a state of bi-directional (donor/patient) tolerance, while at the same time reactivity against third party antigens remains intact. A combination of reduced intensity conditioned transplant followed by donor lymphocyte infusions has shown this to be an alternative to the conventional conditioning regimen (Massenkeil *et al.* 2003).

One of the most widely used methods for the depletion of T-cells from bone marrow for the prevention of GvHD and graft rejection is through the use of CAMPATH-1 antibodies. CAMPATH-1M and -1G antibodies are specific for CD52, a cell surface marker expressed on all human lymphocytes which is a good target for cell lysis by antibody with human complement (Hale *et al.* 1983; Hale *et al.* 1998). CAMPATH-1 antibodies have been used either *ex-vivo* ("in the bag") or *in-vivo* (treating the recipient), in the attempt to control GvHD and prevent rejection following allogeneic bone marrow transplantation, by removing alloreactive T-cells (Hale, Waldmann 1994). It is thought that these effector T-cells in the graft have been educated on the histocompatibility background of the donor and will therefore recognise any mismatches of minor or major histocompatibility antigens between the donor and recipient, leading to graft versus host disease. As well as being associated with a reduction in acute and chronic GvHD (Kernan *et al.* 1993; McGlave 1993), T cell depletion appears to be linked with reduced post transplantation complications such as hepatic veno-occlusive disease and pulmonary dysfunction (Ho *et al.* 2001; Moscardo *et al.* 2001; Soiffer *et al.* 2001), and has been associated with delayed neutrophil engraftment (Hale, Waldmann 1994). T cell depleted transplants also leave the patient susceptible to opportunistic infection post transplant (Pirsch and Maki 1986).

The main disadvantage of T cell depletion is its interference with the graft versus leukaemia (GVL) mechanism, leading to an increased relapse rate in leukaemia patients (Marmont *et al.* 1991). In a single centre study, comparing T cell depleted transplants with T cell replete transplants, the overall survival rates were not significantly different between the two patient groups, despite the higher risk or relapse (Schots *et al.* 2001). Transplant related mortality was

lower in the T cell depleted transplant group in the same study. T cell depletion of the graft, therefore, can reduce the risk of developing GvHD, but can increase the risk of relapse. This can be overcome to some extent by the introduction of donor lymphocyte infusions post transplant, in an effort to introduce enough lymphocytes to control or eradicate disease, without inducing a GvHD effect (Bacigalupo *et al.* 1997; Collins Jr *et al.* 1997; Dazzi *et al.* 2000; Giralt *et al.* 1995; Guglielmi *et al.* 2002; Kolb *et al.* 1995).

The History of the Major Histocompatibility Complex

Both B and T lymphocytes recognise antigens through their cell surface receptors. B cells, however, can capture free antigen via their immunoglobulin receptors, whereas T cells are only able to recognise antigens on a cell surface, in association with Major Histocompatibility Complex (MHC) molecules. The phenomenon of the MHC molecule restriction of T cell recognition was published by Nobel Prize winners, Doherty and Zinkernagel, in 1974 (Zinkernagel and Doherty 1974b), however the road to the discovery of this system began many years before with the work of Gorer, Snell and Medawar in rodents and rabbits.

Discovery of Murine MHC (H-2)

Peter Gorer, working at the Lister Institute in London, discovered the mouse MHC H-2 system, when attempting to identify blood groups in inbred mice (Gorer, 1936). By immunising rabbits with mouse blood cells, he produced antibodies specific for antigens he called I, II, III and IV. Through studies with mouse tumours transplanted between the same and different strains of mice, it was discovered that a difference of antigen II was responsible for the rapid rejection of allogeneic skin and tumour grafts (Gorer 1937a; Gorer 1937b; Gorer 1938). George Snell, who at the same time was also working with mice in Maine, USA, tried to isolate these 'histocompatibility antigens', by producing congenic pairs of inbred mouse strains (which have a small genetic region from another strain, but which are otherwise identical to the original inbred strain.

Congenic strains are derived by backcrossing to a parental inbred strain for at least ten generations while selecting for heterozygosity at a particular locus). Each pair should differ by an individual allele, and when a tumour was transplanted from one to the other, the rejection episode was directed at this allele expressed by the tumour (Snell 1948). The rejection episodes could be distinguished as strong or weak, and this led to the definition of 'major' or 'minor' histocompatibility antigens. The major antigen described by Snell was identical to the "antigen II" described by Gorer around the same time, and they finally combined their work (Gorer *et al.* 1948), and named what they thought was a single antigen in the mouse, H-2 (Snell 1948). Gorer continued to study the gene and found that it was actually a cluster that could be divided into several serological specificities, with antibodies from the sera of one individual able to agglutinate the leukocytes of another (Gorer and Mikulska 1954; Gorer and Mikulska 1959).

Discovery of Human MHC (Human Leukocyte Antigen)

The first human MHC antigens were reported by Jean Dausset (Dausset 1954; Dausset and Nenna 1952), and named MAC (Dausset 1958). He found antibodies that agglutinated donor white cells in the sera of leukopenic patients who had received many blood transfusions (Dausset, Nenna 1952). Similar work was also being carried out by Rose Payne, at Stanford University, USA, and Jon van Rood in Leiden, The Netherlands, where both found that pregnancy had immunised mothers against leukocyte antigens inherited by the child from the father (Payne and Rolfs 1958, van Rood, 1958 #292; van Rood *et al.* 1958). Van Rood named these antigens 4a and 4b (van Rood and van Leeuwen 1963), and Payne christened the system of three specificities she had found, LA (Payne *et al.* 1964).

Nomenclature of Human MHC and the history of Tissue typing

As many investigators were reporting different tissue specific antigens, and methods of testing them, a forum was needed for them to compare their data.

To this end, the first International Histocompatibility Workshop (IHWS) was established. It was held in Washington, in 1964, and organised by Bernard Amos. Alarmingly, none of the data collected correlated between the groups! Workshops were subsequently held in 1965 in the Netherlands, where reagents and a cell panel were formed, which could be used by all participants and in 1967 in Italy. Finally, by the third workshop, it was established that there was a single major complex of genes encoding all these human leukocyte specific antigens. It took a year for the workshop nomenclature committee to agree on a name for the complex, which was "Human Leukocyte locus A" (HL-A) and was later changed to "Human Leukocyte Antigen" (HLA) (Curtoni, 1967) (Klein 1986). The serological methods used to distinguish these HLA antigens demonstrated antibody-mediated immunity, however, a test was needed which would define the cellular response to histocompatibility.

It was with the development of the Mixed Lymphocyte Culture (MLC) technique (Bach and Hirschhorn 1964; Bach and Voynow 1966) that the MHC class II antigens were discovered. The reactivity seen when peripheral blood mononuclear cells from individuals were mixed, was seen to be induced by a locus closely related to the class I loci (Amos and Bach 1968). Using homozygous cells in the MLC test led to the discovery of several new antigens termed MLC, LD and eventually HLA-D at the 1975 Histocompatibility Workshop (Bradley *et al.* 1973; Dupont *et al.* 1973; Mattiuz *et al.* 1975; Mempel *et al.* 1973; van den Tweel *et al.* 1973). Some further identification of class II molecules was carried out using cells that were β-2-microglobulin deficient, and therefore could not express HLA class I molecules. These were originally named DC1 and DC2 and were found to be in strong linkage with HLA-D antigens already found (Tosi *et al.* 1978). Sera from multiparous women was again utilised to determine specificity of HLA-DR, and using these, the MLC, and other in house tests, the class II locus HLA-DQ was also discovered (Ceppellini *et al.* 1971; Duquesnoy *et al.* 1979; Park *et al.* 1978). Finally, HLA-DP was discovered using a secondary MLC, the Primed Lymphocyte Test (PLT) (Shaw *et al.* 1980; Termijtelen *et al.* 1980).

Serology

Until the fourth Histocompatibility Workshop, histocompatibility laboratories had been identifying different HLA antigens using their own antisera, and subsequently, using the complement dependant cytotoxicity reaction (van Rood *et al.* 1975). At the fourth histocompatibility workshop, held in Los Angeles, January 1970, Paul Terasaki introduced the first standard tissue typing technique, the lympho-micro-cytotoxicity assay (Mittal *et al.* 1968; Terasaki and McClelland 1964; Terasaki *et al.* 1966). In this cell cytotoxicity assay, selected allo-antisera and late monoclonal antibodies were mixed with viable lymphocytes. Specific antibodies bound to the polymorphic protein moiety of the HLA molecule expressed on the cell surface. Exogenous complement (usually rabbit) was added to the well which resulted in lysis of cells to which antibody had bound. The damaged cells could then take up a vital or fluorescent dye, and be identified through inverted phase contrast or fluorescent microscopy.

Antisera for the typing trays had to be selected with the greatest of care, and sera from pregnant women were the predominant source. The trays had to contain antibodies to all antigens common in the population to be tested, with preferably one antigen being recognized by at least two antisera. If no monospecific antisera were available, a combination of sera, with different cross-reactivity patterns were used, meaning different patterns could be picked out to identify certain HLA specificity. Controls were used to confirm the presence of known epitopes, for example in HLA-B typing, all B locus antigens carry either the Bw4 or Bw6 determinant, encoded by $\alpha\alpha$77-83 on the α1 domain of the class I heavy chain (Bjorkman *et al.* 1987a).

Antibodies detect amino acid differences on the surface of the HLA molecule, distal from the cell membrane. Serological specificities, therefore, are not homologous to all allele types, meaning many molecular subtypes cannot be detected by serology (Middleton *et al.* 1988). For example, 3 HLA-A2 antigens can be detected using serological methods, but at least 78 HLA-A2 alleles have been detected using molecular typing methods (Robinson *et al.* 2000; Robinson

et al. 2003).

The microcytotoxicity assay was found to be most useful in the determination of HLA-A and -B antigens, but did not give very reproducible results with HLA-C. Anti-C sera were scarce and many C locus alleles may not have serological equivalents (Bunce and Welsh 1994). HLA class II specificities could only be identified on B-lymphocytes. To obtain a B lymphocyte enriched population from PBLs it was necessary to deplete the T cells. This was carried out through sheep red blood cell agglutination, using nylon wool separation, or using magnetic beads loaded with B or T cell specific antibodies for example. HLA class II typing was further complicated by the expression of varying numbers of gene products that can be expressed at the cell surface. An HLA-DR haplotype can express one or two -DR molecules depending on the specificity (Svensson *et al.* 1996). Also, due to the high linkage disequilibrium between HLA-DR and -DQ, some antisera were directed against class II haplotypes instead of specific antigens (Carlsson *et al.* 1987). The development of monoclonal antibodies against the class II antigens has made class II typing by serological methods easier, but cross reactivity, due to shared epitopes, still hampers the method.

Cellular Assays

The majority of cellular assays to detect the minor and major histocompatibility difference between individuals have been derived from the observation that lymphocytes from two unrelated individuals could stimulate each other when cultured in vitro for a period of time (Bain *et al.* 1964). The stimulation seen was primarily due to differences in HLA-DR antigens, resulting in cellular activation and blast transformation (Al-Daccak *et al.* 1990b; Baxter-Lowe *et al.* 1992). The technique developed to identify the determinants involved became known as the mixed lymphocyte culture (MLC) or reaction (MLR) (Bach, Hirschhorn 1964; Bain *et al.* 1964). When patient and donor cells were mixed in the MLC, $CD4^+$ T cells proliferated and cytotoxic $CD8^+$ T cells were generated, meaning that responses within the assay could either be measured through cell expansion or killing. It was recognized that these different populations of

lymphocytes were stimulated by various MHC differences (Bach *et al.* 1973). Cell proliferation can be measured through the incorporation of ^{3}H-Thymidine into the DNA of dividing cells, or by identifying and measuring the cytokines secreted by the activated cells. Cytotoxicity can be measured by the amount of Chromium released by the lysed cells.

For a standard "one-way" MLR assay, three cell samples were used, stimulator, responder and third party (of known HLA type). To emulate a graft versus host response, the patient cells would be the stimulators and the donor cells would be the responders. Each set of cells was divided into two aliquots, one of each of which was irradiated with 3000 rads, to prevent them from responding (Bach, Voynow 1966). Experiments were set up using combinations of irradiated and non-irradiated stimulators and responders, including the third party cells and responder cells as a positive and negative control respectively. Cells were harvested and the amount of thymidine uptake measured by a β counter. If the test response of the responder against the irradiated stimulators was significantly greater than the reactivity of responder cells alone, the test was considered positive.

HLA-DR mismatches predominate in the MLR (Al-Daccak *et al.* 1990a; Baxter-Lowe *et al.* 1992), along with HLA-DQ mismatches (Clay *et al.* 1989). However, positive MLRs can occur in HLA-DR and -DQ matched pairs, suggesting roles for other loci such as HLA-DP (Clay *et al.* 1989), and HLA-A and -B (Termijtelen and van Rood 1981a). The effect of HLA-DP mismatch on the MLR was at first controversial (Pawelec *et al.* 1982; Termijtelen and van Rood 1981b), but it was later determined that some HLA-DP mismatches could induce proliferation (Al-Daccak *et al.* 1990b; Cesbron *et al.* 1990; Olerup *et al.* 1990).

Although the MLR has been used in the selection of donors for bone marrow transplantation, it is now accepted that it does not predict GVHD and clinical outcome after BMT from unrelated donors (Al-Daccak *et al.* 1990b; Hows *et al.* 1986; Mickelson *et al.* 1993; Segall *et al.* 1996). This may be because the MLR mainly detects $CD4^{+}$ lymphocytes and $CD8^{+}$ lymphocytes to a lesser extent, which are an important effector cell population in the GVHD response. It has

been suggested that the MLR be used in the selection of donors alongside other tissue typing techniques in order to exclude known mismatched donors before the MLR stage (Clay *et al.* 1989).

The CTLp (Cytotoxic T lymphocyte precursor) assay was used as a quantitative HLA matching test that confirms HLA identity between donor and recipient. An alloreactive CTLp is a naive T cell that has the potential to differentiate into a mature CTL after encountering the specific alloantigen recognized by its T cell receptor. The CTLp assay is dominated by alloreactive $CD8^+$ CTLs recognizing HLA class I differences on the foreign stimulators cells, with a minor contribution from $CD4^+$ CTLs (Kabelitz *et al.* 1985; Moretta *et al.* 1983). The CTLp is an example of a limiting dilution assay (LDA), meaning the limiting factor must be the number of mononuclear cells added which contain the precursor. PBMCs from patient (stimulator), donor (responder) and third party of known HLA type (control) were split and irradiated. Responder cells were diluted into varying concentrations and mixed with irradiated stimulator cells. At the same time, fresh stimulator and control cells were plated separately, and stimulated with phytohaemagglutinin (PHA) as targets for the responders. On the day of assay, these PHA blasts were incubated with Sodium chromate (^{51}Cr). On addition of the PHA blasts to the cytotoxicity assay plate, they were killed by any specific cytotoxic T lymphocytes. This killing was measured by the release of chromium from the target cells.

The CTLp could define HLA differences between donor and patient that were indistinguishable by serological methods (Rufer *et al.* 1993). For example, HLA-B44.1 and B44.2 were indistinguishable by serology, but CTLs specific for the mismatched antigen could be detected in a CTLp assay. This mismatch involves a single amino acid difference at position 156, and it has been shown that transplants involving this mismatch have resulted in GvHD and rejection (Fleischhauer *et al.* 1990; Keever *et al.* 1994). High CTLp frequency was shown to predict severe acute GvHD post HLA-matched unrelated donor BMT in some cases (Kaminski *et al.* 1989; Roosnek *et al.* 1993), but not others (Fussell *et al.* 1994; Montagna *et al.* 1996). Due to these mixed findings, the CTLp was still

used in donor selection, but thought to be most useful in cases where more than one matched unrelated donor was available.

Another LDA, the HTLp (Helper T lymphocyte precursor) assay, has been used to identify the frequency of IL-2 secreting alloreactive T helper cells (Vie and Miller 1986). The HTLp response has been attributed to alloreactive CD4+ HTLs recognising HLA class II differences, and also a significant contribution of CD8+ HTLs recognizing HLA class I (Jooss *et al.* 1989; Schwarer *et al.* 1994; Theobald and Bunjes 1993). The HTLp has been shown to correlate with acute GvHD after unrelated donor BMT (Schwarer *et al.* 1994). Although these cellular assays were found to be useful in demonstrating an *in vivo* response *in vitro*, in some cases, they were not ideal as actual tissue typing techniques. They were labour-intensive and time-consuming to perform and the numbers of donor and recipient cells available were limited. They are now thought to be more useful tools in the final stages of donor selection, when more than one unrelated donor is available for transplant, in the hope that an unfavourable response can be detected.

Biochemical Techniques

Along with cellular assays, the application of biochemical techniques, such as one- and two-dimensional isoelectric focusing (IEF) in polyacrylamide gels helped to identify some of the serologically undetectable subtypes (Yang *et al.* 1985; Yang *et al.* 1984). IEF works on the principle that, for any protein there is a characteristic pH, called the isoelectric point, at which the protein has no net charge and therefore will not move in an electric field. Proteins are electrophoresed in polyacrylamide gel in which a pH gradient is established by a mixture of special buffers. Each protein moves to a point in the gradient that corresponds to its isoelectric point and stays there (Dale and Latner 1968). Complex mixtures of proteins cannot be resolved well on one-dimensional gels, but two-dimensional gel electrophoresis, combining two different separation methods, can be used to resolve proteins in a two-dimensional protein map (Kenrick and Margolis 1970). In the first step, native proteins are separated in a gel on the basis of their intrinsic charge using isoelectric focusing. In the second

step, this gel is placed on top of a gel slab and the proteins are subjected to SDS-PAGE in a direction perpendicular to that used in the first step. Each protein migrates to form a discrete spot. As with the cellular assays, however, these techniques were cumbersome for routine operation, and difficult to standardise. Also, some HLA antigens were found to be difficult to separate due to their similar IEF patterns (Levine and Yang 1995; Vega *et al.* 1985).

The serological, cellular and biochemical techniques mentioned could not definitively identify HLA alleles, meaning it was not possible to evaluate the effect of HLA matching at the allelic level, or determine which incompatibilities may not affect outcome. DNA based techniques for the detection of allelic differences have provided the ability to investigate the relationship between HLA sequence disparity and transplant outcome.

DNA based HLA typing methods

Restriction Fragment Length Polymorphism (RFLP)

RFLP relies on the behaviour of restriction endonucleases, enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Following digestion of the DNA template, the fragments produced were separated by agarose gel electrophoresis. Following transfer to a membrane, fragments could be identified through hybridization with a homologous probe, usually labeled with ^{32}P. RFLP was first used for the analysis of HLA sequences in 1982 (Wake *et al.* 1982), and was a successful method of typing HLA class II alleles that could not be determined using serological techniques (Paulsen *et al.* 1985). Isolation of sufficient DNA for RFLP analysis was time-consuming and labor intensive. However, the development of the polymerase chain reaction (PCR) (Mullis *et al.* 1986), meant small amounts of DNA template could be amplified to the levels required for RFLP analysis (Higuchi *et al.* 1988). When the PCR step was included, the product was used as a template for endonuclease digestion, the fragments were separated by agarose gel electrophoresis and visualized through ethidium

bromide staining. This process was called PCR-RFLP and was applied to both HLA class I and class II typing (Al-Daccak *et al.* 1990b; Tatari *et al.* 1995). The specificity of the technique could be developed by the use of a greater number of endonucleases, or through a number of secondary amplifications with group specific primers (Nomura *et al.* 1991; Ota *et al.* 1991).

For RFLP analysis to be performed successfully, some idea of the sequence of the allele is required. Also, some combinations of alleles in heterozygous individuals cannot be distinguished (Olerup 1990).

Sequence specific primer extension (SSP)

Using PCR, it became possible to make many copies of a specific area of the gene of interest, rendering the number of copies produced sufficient to be visualized on a gel. Sequence specific primers (SSP), for HLA typing (Wu *et al* 1989), were developed, which were complimentary to an allele, or group of alleles specific for a serologically defined antigen. The terminal 3′ nucleotide of the primers matched the target DNA sequence, meaning it could be amplified with *Taq* polymerase. If the primers were not complimentary to the DNA sequence, amplification would not be successful. Following amplification, the PCR products could be visualized as specifically sized bands on an agarose gel with Ethidium Bromide incorporation. As the result of the reaction is either "a PCR product" or "no PCR product", an internal positive control was added to each reaction. This constituted a primer pair specific for a "housekeeping" gene (Olerup and Zetterquist, 1992), which was a gene that should be present in every sample, for example, β-actin. Therefore, regardless of whether the SSP reaction was positive or negative, the control should always amplify. If no positive control band was seen, the SSP reaction had failed. In PCR-SSP, many of these PCR reactions were performed on a single sample simultaneously, and the pattern of alleles amplified corresponded to the genotype for a single locus, or many loci depending on the number of reactions employed. One of the biggest challenges in highly polymorphic genes was to locate a sufficient number of allele-specific sites to provide the necessary specificity for the PCR-

SSP reactions, meaning combinations of sequence-specific primer mixes must be used (Bunce *et al.* 1995). To obtain a high resolution typing, many PCR-SSP reactions must be employed, which can be arduous. Some laboratories use a two-step approach, using a low-resolution method for typing beforehand (Yu *et al.* 1997). The method is limited, in that it identifies only known polymorphisms that are utilized to predict the entire sequence. If an unknown allele is present, it may be missed.

Sequence specific oligonucleotide probes (SSOP)

The specificity of SSOP (sequence specific oligonucleotide probes) was initiated through the detection system and not the initial amplification as with PCR-SSP, described in the previous section. In this method, a generic PCR amplification was performed, specific only for a given HLA locus. A battery of probes was then used, determining an allele-specific pattern of sequence motifs (Saiki *et al.* 1986). Each probe had a DNA sequence complimentary to a small area of the gene that showed differences between alleles. The probes were labeled with digoxigenin (DIG) at the 5′ end, for detection with an enzyme-linked anti-digoxigenin antibody, and subsequently an enzyme-specific fluorescent substrate. The PCR reaction was performed and the product for each patient sample was dot blotted onto a nitrocellulose membrane, each membrane being specific for a different probe. The membranes were then exposed to UV light to denature the double-stranded template, and incubated with the DIG-labelled probes. After any excess probe was washed off, the membranes were incubated with the anti-DIG antibody, which bound to the DIG on any probes bound to the DNA in the membrane. Following another washing step, the substrate was added and the films exposed to X-ray film. Once developed, the patterns of the dots seen were compared to the known patterns for each allele.

PCR-SSOP was found to be cheap to run and gave a high throughput, meaning many samples could be processed at the same time. However, the technique is quite complex, in that the number of alleles to be detected, and the presence of heterozygous combinations can make interpretation difficult. To resolve these

issues, many probes are required, making the technique more cumbersome and analysis of the results problematical. Because the probes used are based on known polymorphisms, reagents need to be constantly updated, and unknown polymorphism could remain unidentified.

Conformational analysis of DNA

The principle of conformation-based methods relies on the study of the behaviour of DNA fragments in polyacrylamide gel, under electrophoresis (PAGE). The mobility of the DNA fragment in PAGE depends upon its size and sequence.

SSCP

The single strand conformation polymorphism (SSCP) method takes advantage of the defined secondary structure of single stranded DNA. The structure is sequence-specific in solution, and under certain conditions can be separated and defined (Orita *et al.* 1989). Through differences in the mobility of the single stranded DNA in non-denaturing PAGE, it is possible to identify different sequences.

The method involves group specific amplification by PCR, melting of the PCR products and analysis of the single strands on non-denaturing PAGE. Separation depends on the nucleotide sequence and length of the DNA fragments and also the optimization of electrophoretic conditions to maximize the differential migration between different single-stranded fragments. The resolution of the technique can be improved by the addition of glycerol to the gel, reduction of temperature, lengthening of the gel and the use of different polyacrylamide matrices (Dean and Gerrard 1991).

SSCP has been used as a donor selection tool for matching with patient (Clay *et al.* 1995; Pursall *et al.* 1996), and also as a typing tool in combination with SSP (Lo *et al.* 1992). SSCP analysis has also been used in the detection of novel HLA alleles (Blasczyk *et al.* 1995a; Blasczyk *et al.* 1995b; Blasczyk *et al.* 1995c).

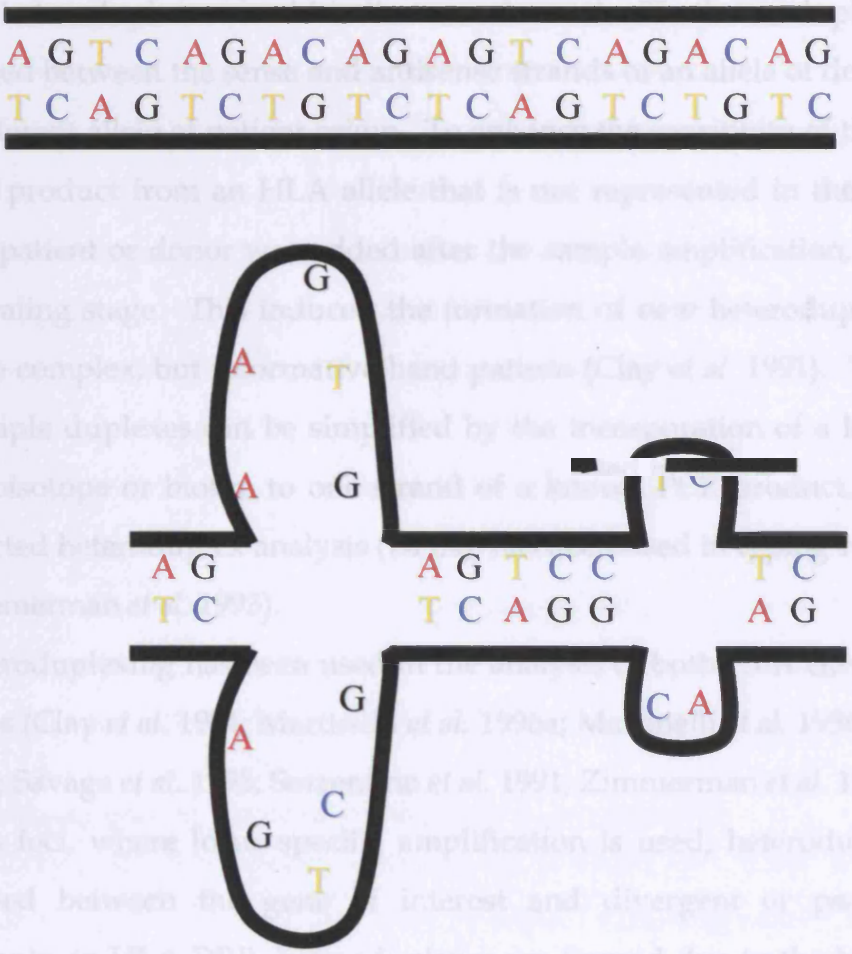
SSCP is simple and inexpensive, complex instrumentation is not required and

high sample throughput can be achieved (Hoshino *et al.* 1992). However, the use of SSCP for HLA analysis has its drawbacks, for example, the optimal size fragment for analysis is only 150-200 bp, although larger fragments can be used when detection of all potential single nucleotide substitutions is not essential for the analysis (Sheffield *et al.* 1993). Also, there is the potential for one DNA fragment to adopt several different stable conformations under the same conditions, resulting in complex banding patterns. Banding patterns can also be confusing due to inter- and intra-gel variability that could be due to changes in electrophoretic conditions, gel temperature, running time or gel composition.

Heteroduplexing

DNA heteroduplexes are formed mainly during the final stages of the PCR reaction, between co-amplified alleles of a particular locus, or between loci due to primer cross-reactivity at sites with similar sequences (Wood *et al.* 1991). The resulting double-stranded molecules contain regions where some of the nucleotide sequences on each strand are complimentary to one another, and in some areas the strands are mismatched, forming loops or bulges along the DNA, see figure 1-1.

Figure 1-1. Cartoon of a DNA homoduplex and heteroduplex. A homoduplex is formed between complimentary strands of DNA. When strands are non-complimentary, under certain conditions, mismatches occur forming loops and bulges.



These mismatches along the length of the DNA fragment cause it to kink, producing a "drag" effect when the molecule is subjected to non-denaturing PAGE. The homoduplexes formed are more streamlined and can travel quickly through the gel, whereas the heteroduplexes travel more slowly. This not only allows separation between the homoduplex and heteroduplex, but also between heteroduplexes with different base pair mismatches and hence different conformations.

The banding pattern produced was originally used in an HLA setting to match donors and recipient in a crossmatch test. DNA samples from patient and

donor were mixed and amplified together in the same PCR reaction. The resulting band pattern was compared to those obtained when each patient or donor sample was amplified alone under the same conditions. Matching bands indicated HLA identity, whereas mismatched bands or the production of any new heteroduplexes would indicate a mismatch. The heteroduplexes would be formed between the sense and antisense strands of an allele of donor origin and a different allele of patient origin. To enhance the sensitivity of the method, the PCR product from an HLA allele that is not represented in the tissue type of that patient or donor was added after the sample amplification, but before the annealing stage. This induced the formation of new heteroduplexes, giving a more complex, but informative band pattern (Clay *et al.* 1991). The analysis of multiple duplexes can be simplified by the incorporation of a label, such as a radioisotope or biotin, to one strand of a known PCR product. This process, directed heteroduplex analysis (DHA), has been used in typing HLA-DQ alleles (Zimmerman *et al.* 1993).

Heteroduplexing has been used in the analysis of both HLA class I and class II genes (Clay *et al.* 1994; Martinelli *et al.* 1996a; Martinelli *et al.* 1996b; Savage *et al.* 1996; Savage *et al.* 1995; Sorrentino *et al.* 1991; Zimmerman *et al.* 1993). For some HLA loci, where locus specific amplification is used, heteroduplexes may be formed between the gene of interest and divergent or pseudogenes, for example, in HLA-DRB, heteroduplexes are formed due to the hybridization of divergent DRB genes on all DR haplotypes except DR8 (Wood *et al.* 1991).

The use of the heteroduplexing method was greatly improved with the development of the universal heteroduplex generator (UHG) (Clay *et al.* 1994), This synthetic sequence mimics the genomic DNA sequence but contains controlled nucleotide substitutions, deletions, or insertions at nucleotide positions opposite to, and contiguous with, known mutation sites within the genomic DNA. This controlled variation in the UHG greatly enhances electrophoretic sorting of single-base substitutions in the samples and increases the resolution between homoduplex and heteroduplex DNA. The UHG has been successfully utilized in heteroduplex analysis screening for the selection of

unrelated donors (Bidwell and Hui 1990; Clay *et al.* 1991), and also in conjunction with SSCP for HLA-A, -B and -C allotype matching (Pursall *et al.* 1996).

Heteroduplex analysis is a rapid, safe and cost-effective method that can be used in any routine laboratory, with the only post-PCR manipulation being polyacrylamide electrophoresis. The main advantages heteroduplex analysis has over SSCP are that each double-stranded duplex adopts a unique conformation under constant conditions, meaning only one band is detected for each, and longer fragments can be analysed. However, if a heteroduplex differs by only one or two bases from the homoduplex, the heteroduplex signal is difficult to distinguish due to the greater intensity of the homoduplex signal when analysed by PAGE.

Reference strand mediated conformation analysis (RSCA), is a heteroduplexing technique also including a second DNA sample for the introduction of extra duplexes. This DNA molecule is fluorescently labelled (the FLR) and allows the detection of only those duplexes that contain the labelled strand of the FLR. The principles of the RSCA method will be discussed in more detail later.

Denaturing and temperature gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) has been shown to detect differences in the melting behavior of small DNA fragments (200-700 bp) that differ by as little as a single base substitution (Fischer and Lerman 1979; Fischer and Lerman 1980). When a DNA fragment is subjected to an increasingly denaturing physical environment, it partially melts. As the denaturing conditions become more extreme, the partially melted fragment completely dissociates into single strands. Rather than partially melting in a continuous zipper-like manner, most fragments melt in a step-wise process. Discrete portions or domains of the fragment suddenly become single-stranded within a very narrow range of denaturing conditions. The rate of mobility of DNA fragments in acrylamide gels changes as a consequence of the physical shape of the fragment. Partially melted fragments migrate much more slowly during electrophoresis through the polyacrylamide matrix than completely double-

stranded fragments (Fischer, Lerman 1980). When a double-stranded fragment is electrophoresed into a gradient of increasingly denaturing conditions, it partially melts and undergoes a sharp reduction in mobility because it changes shape. In practice, the denaturants used are heat (a constant temperature of 60°C) and a fixed ratio of formamide (ranging from 0-40%) and urea (ranging from 0-7 M).

When the fragment completely denatures, its mobility again becomes a function of size. Complete strand separation can be prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is accomplished during PCR amplification using a PCR primer with a 5' tail consisting of a sequence of 40 GC (Myers *et al.* 1985). The position in the gradient where a domain of a DNA fragment melts and thus nearly stops migrating is dependent on the nucleotide sequence in the melted region. Sequence differences in otherwise identical fragments often cause them to partially melt at different positions in the gradient and therefore 'stop' at different positions in the gel. By comparing the melting behavior of the polymorphic DNA fragments side-by side on denaturing gradient gels, it is possible to detect fragments that have mutations in the first melting domain.

DGGE has been rarely used in HLA typing, usually after group-specific amplification, or in conjunction with another method (Knapp *et al.* 1997; Tamouza *et al.* 1997).

DGGE requires specific conditions to be maintained for the particular system under investigation and it may be difficult to reproduce these conditions between gel runs. Also, two alleles that have a different sequence, but similar melting points, may not be resolved as they may produce identical gel retardation and resulting band pattern.

Sequence Based Typing

Many methods have been developed for sequence based typing (SBT), but the one mainly in use in histocompatibility laboratories is the dideoxy-mediated chain termination method, utilizing chain-terminating dideoxynucleotide triphosphates (ddNTPs) (Sanger *et al*, 1977). A generic PCR was performed,

specific for the locus of interest, and the PCR product denatured, through heating, to produce single stranded DNA. A sequencing primer was then annealed to the single stranded DNA in a PCR reaction with ddNTPs. When the chain of nucleotides extended in the 5′ to 3′ direction, a ddNTP became incorporated instead of a regular dNTP, meaning the sequence was terminated. Each ddNTP was labelled with a different fluorescent dye; meaning four separate sets of chain-terminated fragments were produced. As each chain-terminated fragment was still bound to it's single stranded DNA template, heating was required to separate them. The chain terminated fragments were then separated by polyacrylamide gel electrophoresis (PAGE) using an automated sequencing machine, which would assign each base depending on the fluorescent labeled dye, and produce a matching sequence. With the development of sequence analysis software, it was then possible to match the sequences produced to a database, containing all known sequences to date of each HLA allele (Lefranc 2002; Robinson *et al.* 2000).

As this method determines the actual sequence of nucleotides in the gene of interest, theoretically it should give the highest resolution typing. However, the main problem facing this technique in HLA typing is the existence of a large number of identical sequence motifs that are present in different alleles. The computer software and analysis methods are limited in that they cannot determine the cis or trans orientations of the nucleotide sequences. Therefore, heterozygous ambiguities are an increasing problem for direct sequencing of HLA genes, where many combinations of some groups of alleles cannot be resolved, even when group-specific amplification is used (Rozemuller and Tilanus 1996). To resolve these ambiguities, other techniques, or additional steps are required, making this procedure difficult to perform on a routine basis (Voorter *et al.* 1998).

Genetic Organisation of HLA

The Major Histocompatibility Complex (MHC) situated on the short arm of chromosome six (6p21.3) (Lamm *et al.* 1974) encodes the Human Leukocyte Antigen (HLA) genes in humans. The MHC consists of three major

Histocompatibility gene clusters, HLA class I, II and III (Trowsdale *et al.* 1991). HLA class I and class II antigens are classical transplantation antigens, whereas the class III region encodes for genes such as complement components and clotting factor B (Carroll *et al.* 1984), cytochrome P-450 and the 21 hydroxylase genes (Carroll *et al.* 1985). A simplified map of the human MHC can be seen in Figure 1-2.

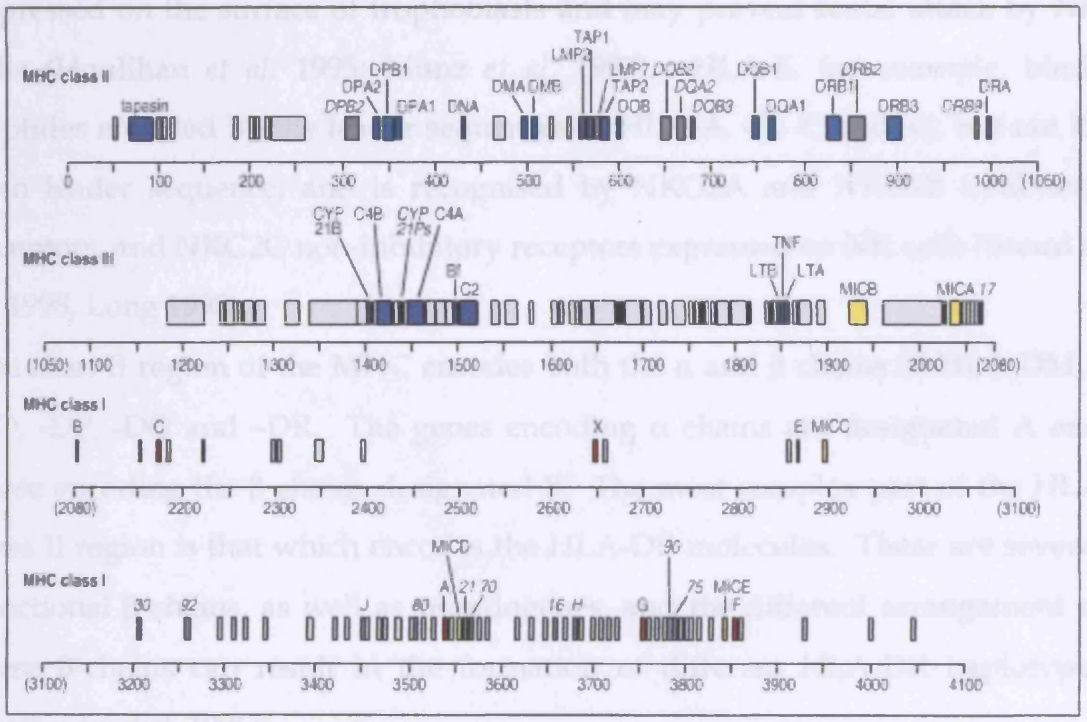


Figure 1-2. Simplified map of the MHC. HLA class I genes are shown in red, with HLA class II genes in blue. MHC class I associated genes are highlighted in yellow. Adapted from Immunobiology, the Immune System in Health and Disease (Janeway *et al.* 1999).

The class I region of the human MHC contains three 'classical' loci, HLA-A, -B and -C and the class I-like genes, which encode class I molecules such as HLA-E, -F and -G. These non-classical class I genes exhibit far fewer alleles than the classical genes, and the function is less well defined compared with classical class I molecules. It is thought the non-classical class I antigens may be targets for Natural Killer (NK) cell inhibitory receptors, for example, HLA-G which is expressed on the surface of trophoblasts and may prevent foetal attack by NK cells (Houlihan *et al.* 1995; Munz *et al.* 1997). HLA-E, for example, binds peptides encoded by the leader sequences of HLA-A, -B, -C and -G, but not its own leader sequence, and is recognised by NKG2A and NKG2B inhibitory receptors, and NKG2C non-inhibitory receptors expressed on NK cells (Braud *et al.* 1998; Long 1998).

The class II region of the MHC encodes both the α and β chains of HLA-DM, -DO, -DP, -DQ and -DR. The genes encoding α chains are designated A and those encoding the β chains designated B. The most complex part of the HLA class II region is that which encodes the HLA-DR molecules. There are several functional β-chains, as well as pseudogenes, and the different arrangement of these β-chains can result in the formation of different HLA-DR haplotypes (Svensson *et al.* 1996).

Structure of HLA class I

HLA class I molecules are made up of an MHC encoded heavy chain, non-covalently associated with a non-polymorphic light chain, Beta-2-microglobulin (β_2m), which is not encoded within the MHC, and a peptide of between 9 and 12 amino acids in length. The first HLA molecular structure to be determined was that of HLA-A2, using X-ray crystallography (Bjorkman *et al.* 1987b). The α1 and α2 domains of the heavy chain form a groove for binding the peptide, the domains are each divided into an α helix and 4 β-strands, which together form the peptide-binding groove. The α3 domain is associated with β_2m, positioned beneath the peptide-binding groove (see Figure1-3).

The class I gene is made up of 7 or 8 exons; the first constitutes a 5' untranslated extracellular domains, α1, α2 and α3, which protrude from the cell surface. The

α3 domain of class I, encoded by exon 4, has a conserved sequence, required for its interactions with β_2m and the TCR co-receptor, CD8 (Salter *et al.* 1989). The fifth exon encodes the transmembrane region, which anchors the molecule into the plasma membrane of the cell. This then extends into exons 6-7 (or in some circumstances 8) which encode the cytoplasmic tail (Strachan 1987). A cartoon of the class I HLA molecule can be seen in figure 1-3.

Figure 1-3a. Ribbon diagram of an HLA class I molecule. HLA class I molecules are composed of a heavy chain consisting of the α1, α2 and α3 regions, transmembrane region and cytoplasmic tail, and β_2-microglobulin as a light chain. N and C refer to the amino and carboxy termini of the polypeptide chains respectively (adapted from Janeway *et al.* 1999)

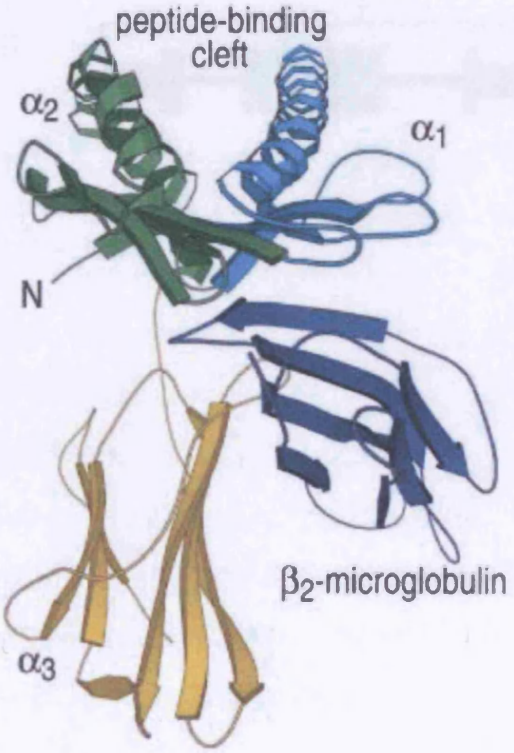
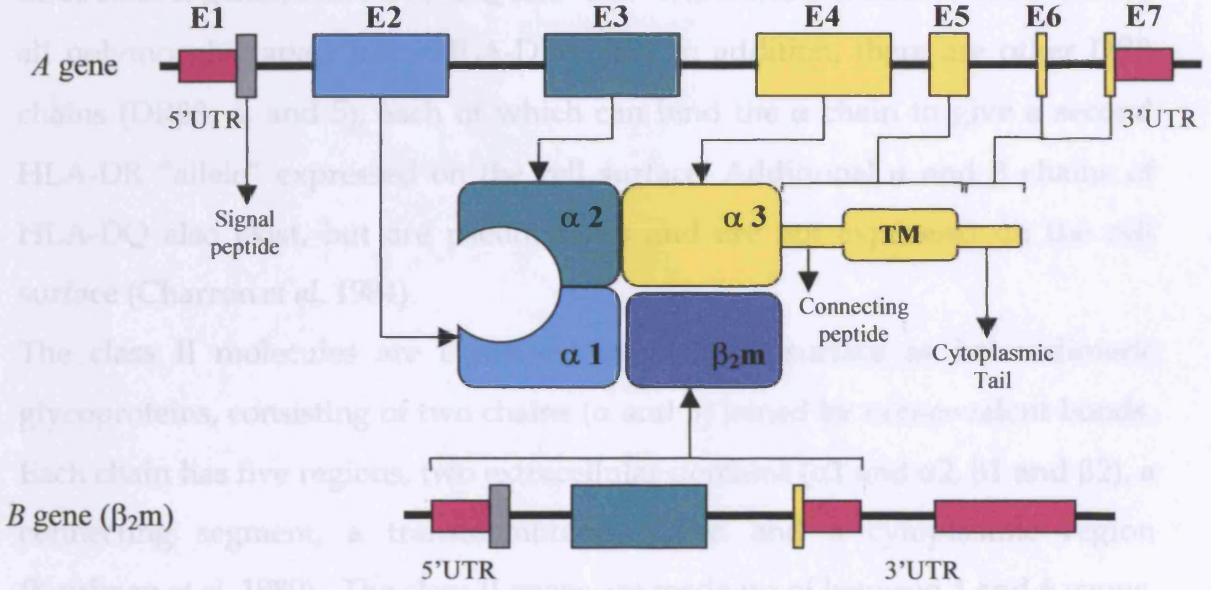


Figure 1-3b. Schematic diagram of the HLA class I molecule. The correspondence between exons of genes and domains of proteins. E = exon, TM = transmembrane region, UTR = untranslated region (adapted from Klein and Horejsi 1997)



Structure of HLA class II

HLA class II genes are slightly more complex than class I as they are made up of two potentially polymorphic heavy chains as opposed to one. There are three class II genes, HLA-DR, -DQ and -DP. The α and β chains of these loci are all polymorphic apart from HLA-DRα, and in addition, there are other DRβ chains (DRβ3, 4, and 5), each of which can bind the α chain to give a second HLA-DR "allele" expressed on the cell surface. Additional α and β chains of HLA-DQ also exist, but are pseudogenes and are not expressed on the cell surface (Charron *et al.* 1984).

The class II molecules are expressed on the cell surface as heterodimeric glycoproteins, consisting of two chains (α and β) joined by non-covalent bonds. Each chain has five regions, two extracellular domains (α1 and α2, β1 and β2), a connecting segment, a transmembrane region and a cytoplasmic region (Kaufman *et al.* 1980). The class II genes are made up of between 4 and 6 exons, exon 1 codes for the leader sequence, exons 2 and 3 encode the extracellular domain, and exons 4-6 encode the connecting segment, and the transmembrane and cytoplasmic regions. The α and β chains of the HLA class II molecules show similar structure to HLA class I, although they are associated with one another rather than with β_2m (Fremont *et al.* 1996). The peptide binding groove is shaped slightly differently to that of HLA class I, with open-ends rather than those which are closed off, giving less restriction to the size of peptide which can be accommodated (Rammensee 1995). A schematic representation of the HLA class II molecule can be seen in figure 1-4.

Figure 1-4a. Ribbon diagram of an HLA class II molecule. HLA class II molecules consist of an α and β chain. N and C refer to the amino and carboxy termini of the polypeptide chains respectively (adapted from Janeway *et al.* 1999).

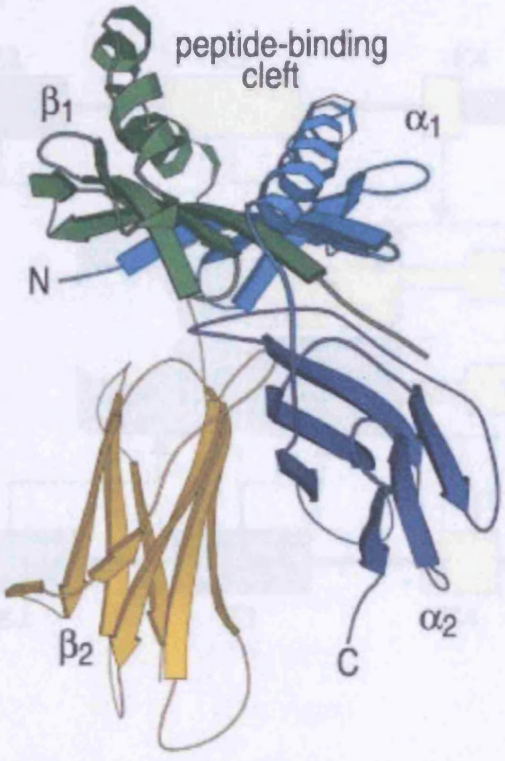
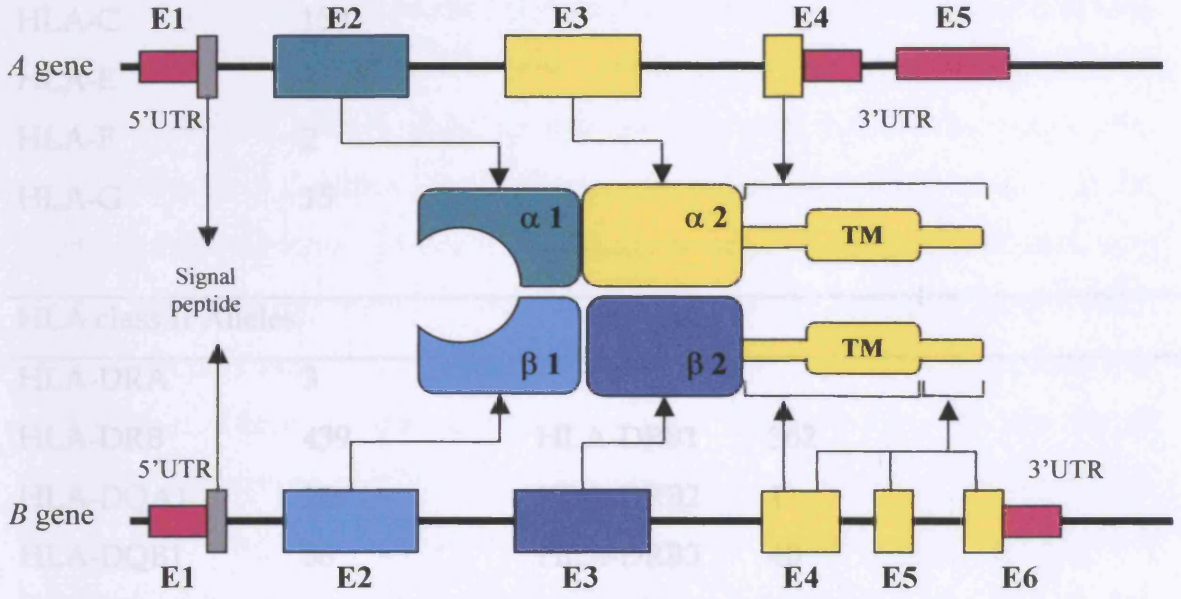


Figure 1-4b. Schematic diagram of the HLA class II molecule. The correspondence between exons of genes and domains of proteins. E = exon, TM = transmembrane region, UTR = untranslated region (adapted from Klein, Horejsi 1997)



Polymorphism of HLA

The HLA genes are extremely polymorphic, with over 1700 alleles currently sequenced (Robinson *et al.* 2003). The number of sequenced alleles for each locus is described in table 1-1.

Table 1-1. The number of HLA alleles currently sequenced (Robinson *et al.* 2003)

HLA class I Alleles			
HLA-A	303		
HLA-B	559		
HLA-C	150		
HLA-E	6		
HLA-F	2		
HLA-G	15		
HLA class II Alleles			
HLA-DRA	3		
HLA-DRB	439	HLA-DRB1	362
HLA-DQA1	25	HLA-DRB2	1
HLA-DQB1	56	HLA-DRB3	40
HLA-DPA1	20	HLA-DRB4	12
HLA-DPB1	107	HLA-DRB5	17
HLA-DMA	4	HLA-DRB6	3
HLA-DMB	6	HLA-DRB7	2
HLA-DOA	8	HLA-DRB8	1

The polymorphism of the HLA genes comes about through genetic exchange, recombination, and point mutation. Genetic exchange can be seen at the chromosome level, between alleles of different, but homologous, loci, a process called gene conversion. It can also be seen at the allelic level, between alleles of the same locus, allele conversion (Madrigal *et al.* 1992; Parham *et al.* 1988). Recombination can occur through the cross over of two alleles, for example, the HLA-A*6901 allele structure suggests that it was formed through the recombination of exons 1 and 2 of HLA-A*6801 and the remainder of HLA-A*0201(Holmes and Parham 1985).

One hypothesis for the polymorphic nature of the HLA molecules is their specific function. The role of the HLA molecule is to be able to specifically bind

as many pathogen peptides as possible in order to present them to a cytotoxic T cell, which will in turn eliminate the infected cell (Zinkernagel, Doherty 1974b). It is also important for the HLA molecule to bind non-pathogenic peptides for thymic education and the development of the T-cell repertoire (Ignatowicz *et al.* 1996). The peptides bind to the HLA molecule using specific anchor residues that fit into "pockets" within the peptide binding site, therefore the polymorphism of the binding groove pockets determine which peptides residues can bind to which HLA molecule (Bjorkman *et al.* 1987b). Most of the polymorphism observed in the HLA system is targeted in the peptide-binding groove, affecting the peptide binding specificity of the molecule (Saper *et al.* 1991). This polymorphism allows the HLA molecules to bind a large repertoire of peptides. These are then presented to, and recognised by, specific T cell receptors. Due to the polymorphic nature of the HLA genes, most individuals will be heterozygous for a given locus. One set of genes, or a haplotype, is inherited from each parent, therefore giving the possibility of any one of four possible haplotype combinations in one offspring. This, as we will see later, makes the possibility of finding a sibling donor for HLA identical bone marrow transplantation rather complicated.

Linkage Disequilibrium

As previously mentioned, HLA alleles are inherited as haplotypes, and there is variation between the frequencies of alleles present in different populations. This occurs through natural selection over time, where populations are subjected to various diseases and only a section of the population has HLA molecules, which are capable of binding and presenting the appropriate peptide from the pathogen. These individuals would eradicate the pathogen and survive, whereas those without the appropriate HLA molecule would not (Hill *et al.* 1991; Hughes and Nei 1988).

When the frequency of particular haplotypes is studied, it can be seen that a given haplotype occurs at a higher frequency than expected when compared with the gene frequency of the separate alleles (Grange *et al.* 1981). This is due to genetic linkage disequilibrium, which depends on the amount of possible

recombination between loci, i.e. cross over can occur between chromosomes. There is generally more opportunity for cross over to occur between two loci which are separated by a large distance on the chromosome compared with those which are situated close to one another (Hiller *et al.* 1978). There are also regions known as recombinatorial hotspots present on the chromosome, which allow cross over even thought the two loci may be close to one another (Baisch and Capra 1993). Linkage disequilibrium within the MHC is most prevalent for genes found close together, for example the HLA-B and -C, or HLA-DR and -DQ genes. Also, this haplotype may have been "advantageous" at some time, which may account for its higher frequency (Proust *et al.* 1982).

A common haplotype in the Caucasoid population is HLA-A1 (allele frequency 15%), -B8 (allele frequency 13.7%) and DRB1*03 (allele frequency 12.4%) with the expected haplotype frequency being 0.25% (Kimura and Sasazuki 1992). The actual frequency of this haplotype in the Caucasoid population is 6.4%, due to the lack of recombination between these loci.

This phenomenon is not true for all HLA loci, or all haplotypes, however; HLA-DPB1 is situated on the chromosome close to DQB1, but there is a recombinatorial hotspot situated between them, leading to a large amount of crossover (Baisch, Capra 1993; Howell *et al.* 1993). This means it is very rare to find a common haplotype, which includes an HLA-DPB1 allele. Thus, HLA-DP is usually excluded when looking for an HLA matched unrelated donor as it is almost impossible to find one with the same HLA-DBP1 alongside matching haplotypes for the other HLA alleles.

For many years, the high level of linkage between HLA-B and -C suggested there was no need to type the C locus, as if the B locus were matched, this would be too. However, as HLA typing techniques became of increased specificity it was found that more than one HLA-C allele can be in linkage with the same HLA-B allele, so now typing for both loci has become more commonplace (Prasad *et al.* 1999).

Function of HLA molecules

HLA molecules are found on the surface of all nucleated cells and their natural

function is to act as a restriction element for the recognition of pathogenic antigens by T cells for the distinction of normal 'healthy' tissue from infected tissue (Zinkernagel and Doherty 1974a). As mentioned previously, the HLA molecules expressed on the cell surface can also act as targets for allorecognition (Schendel *et al.* 1992; Termijtelen 1990).

The function of the HLA molecules is to bind peptide fragments derived from pathogens and transport them to the cell surface for presentation to T-cells. HLA class I molecules present pathogenic peptides on the surface of cells infected with virus or bacteria, whose replication cycle occurs within the host cell (Früh and Yang 1999; Knittler *et al.* 1998; Pamer and Cresswell 1998; Roosnek *et al.* 1988; van Endert 1999). These HLA:peptide complexes are recognised by cytotoxic T-cells expressing CD8 (McMichael *et al.* 1986). Infected cells are killed, preventing the formation of new viral particles, or eliminating further infection from cytosolic bacteria. HLA class I molecules can also be bound by receptors on NK cells.

HLA class II molecules bind and present peptides from pathogens and their products, which have been internalised, or are present in vesicular cell compartments (Barlow *et al.* 1998; Machamer and Cresswell 1982; Malcherek *et al.* 1998; Panina-Bordignon *et al.* 1991). These pathogens are internalised by phagocytosis or endocytosis depending on the type of pathogen. Macrophages, for example, have intracellular vesicles where some classes of pathogen, such as mycobacteria and *Leishmania* spp. can replicate. On activation of the macrophage, the proteins of these pathogens are degraded by proteases to form peptides, which are presented on the cell surface, bound to HLA class II molecules. This will initiate a response from Th1 type CD4-expressing T-cells that will release cytokines to induce the macrophage to destroy the pathogen. B cells can internalise pathogens through receptor-mediated endocytosis, and again peptides from these pathogens are transported and presented by HLA class II molecules on the cells surface. Th2 type CD4-expressing T cells bind to these HLA-peptide complexes and activate the B cell to produce antigen-specific antibodies to neutralise or destroy the pathogens. $CD4^+$ cells are also

important in providing help to $CD8^+$ cells, there are 2 different mechanisms for activating a CD8 T cell. In one model, there is a CD4 cell that sees Ag on the surface of an APC. The CD4 cell is then triggered to express CD40L, which activates CD40 on the APC. The APC then upregulates expression of CD80 (B7.1) or CD86 (B7.2) that stimulate the CD4 T cell through CD28. The T cell then produces IL-2. This IL-2 can activate a CD8 T cell by providing a second, costimulatory signal, assuming the CD8 cell is already interacting with Ag/MHCI complex on an APC. This process occurs in the lymph nodes. Following activation, the CD8 cell leaves the lymph node and migrates to the tissue where it can carry out its effector functions.

The Discovery of HLA Function

Doherty and Zinkernagel identified restriction of T cell recognition, by the MHC, in 1974 (Zinkernagel, Doherty 1974b). Two different mouse strains were innoculated with lymphocytic choriomeningitis virus (LCMV) and the immune lymphocytes of the infected mice were then cultured with ^{51}Cr-labelled LCMV-infected target cells, and cell lysis measured by the amount of ^{51}Cr released. The target cells used were from the 'L' cell line derived from an $H\text{-}2^k$ mouse. The L cells were killed by the cytotoxic T cells (CTL) of the mouse strain CBA ($H\text{-}2^k$) and the F1 offspring of this mouse e.g. (CBA x C57BL/6)F1, but not the strains BALB/c ($H\text{-}2^d$) or C57BL/6 ($H\text{-}2^b$). All strains infected with the virus died, therefore there was no problem with the infectivity of the virus. Zinkernagel and Doherty gave two hypotheses for this finding, either some strains did not produce CTL, or all strains produced CTL, but the cells only killed H-2 compatible targets. Further tests revealed the latter to be true. Thus, the conclusion was drawn that T cells were capable of recognising viral antigens (non-self) and MHC molecules on stimulating/target cells (self).

HLA class I antigen processing and presentation

HLA class I molecules usually bind peptides from intracellularly derived self or viral proteins, whereas HLA class II molecules bind peptides from extracellularly derived proteins. Proteins that come from infecting viruses are

processed, and presented on the cell surface in the same way as self-peptides, as the virus takes over the cell's normal biosynthetic pathway to make its own proteins (Lwoff 1966). The proteins formed in the cytosol are broken down by proteases present in a complex called the proteosome. This structure is made up of 28 subunits, three of which are known as LMP2, LMP7 and MECL-1. LMP2 and LMP7 are encoded within the MHC, and their expression, along with that of HLA, is regulated by the cytokine, interferon-gamma (IFNγ), as is that of MECL-1 which is encoded on chromosome 16 (Larsen *et al.* 1993). When the expression of LMP2, LMP7 and MECL-1 is up regulated, they substitute for their homologous subunits of the proteosome, γ, MBI and Z respectively (Eluteri *et al.* 1997). These substitutions seem to alter the specificity of the proteosome, which results in the cleavage of peptides after hydrophobic and basic residues, producing peptides with carboxy-terminal residues optimal for binding HLA class I molecules (Bouvier and Wiley 1994). The peptides are transported into the endoplasmic reticulum (ER) in order to bind the HLA molecules. This occurs via the transporter associated with antigen processing (TAP). This heterodimer is made up of two molecules (TAP-1 and TAP-2, which are encoded within the MHC), each of which has a hydrophobic transmembrane domain, which protrudes into the lumen of the endoplasmic reticulum, and an ATP-binding cassette domain, which lies within the cytoplasm. This TAP complex forms a channel through which the peptides enter the lumen of the endoplasmic reticulum where they will be bound to HLA class I molecules. The newly synthesised HLA class I molecules in the ER are bound to a chaperone protein, calnexin, which maintains their correct structure (Suh *et al.* 1996). Beta-2-microglobulin (β_2m) is also present in the ER and binds to the folded HLA molecule. The calnexin molecule is displaced and the complex binds calreticulin, another chaperone protein (Solheim *et al.* 1997). Tapasin, a protein associated with TAP-1, connects the HLA:β_2m complex with TAP, making it easily accessible for the peptides entering the ER (Elliott 1997). Once an appropriate peptide has bound, the HLA:β_2m:peptide complex is released from its chaperone proteins and leaves the ER, to be transported to the

cell surface. HLA molecules not bound to a peptide cannot leave the ER, as the peptide is needed to stabilise the complex, which would dissociate quickly on reaching the cell surface, were the peptide not present.

HLA class II antigen processing and presentation

Peptides presented by HLA class II molecules are formed in a less specific manner, as the size and characteristics of the peptide do not seem to be as strictly defined as those required for binding HLA class I molecules (Siklodi *et al.* 1998). These class II binding peptides originate from proteins present extracellularly. The antigen presenting cell (APC) internalises the pathogen or extracellular protein into an intracellular vesicle known as an endosome (Chapman 1998). As this vesicle migrates further inside the cell, it becomes more acidic, inducing the activation of proteases such as cathepsins B, D, S and L, which eventually break the protein down into peptides (Mizuochi *et al.* 1994; Riese *et al.* 1996). HLA class II molecules are present in the ER alongside the HLA class I molecules. This poses a potential problem, as the smaller peptides destined for class I binding, or newly formed larger proteins could possibly bind to the empty class II molecules non-specifically. This is prevented by the binding of the invariant chain (Ii), part of which blocks the HLA class II peptide binding groove, preventing binding of any peptides present in the ER (Hitzel and Koch 1996; Machamer, Cresswell 1982). As well as preventing the binding of spurious peptides, Ii also helps target delivery of the HLA class II molecules to the endosomes, where they can be loaded with peptide (Malcherek *et al.* 1998; Xu *et al.* 1995). The vesicle containing the HLA class II molecules and the endosome fuse, and proteases in the low pH endosome cleave fractions of Ii away from the HLA class II molecule, leaving one small fragment in the peptide binding groove, known as CLIP (class II associated invariant chain peptide) (Machamer, Cresswell 1982; Wu and Gorski 1996). These events occur in the MIIC (MHC class II compartment), where there is a class II-like molecule present called HLA-DM (Ferrari *et al.* 1997). HLA-DM differs from the other class II molecules in that it is not expressed at the cell surface and does not require a peptide for stabilisation. HLA-DM acts as a catalyst for exchange

between CLIP and peptides present in the endosome, into the peptide binding groove of HLA class II (Sherman *et al.* 1995). It also helps in the selection of peptides for each class II molecule, binding to separate HLA:peptide complexes, and removing peptides which have a low binding efficiency, and exchanging them for those which bind in a more stable manner. Once the peptide with the best binding affinity for the class II HLA molecule is bound, the molecule can travel to, and be stabilised on, the cell surface.

T cell development and education

HLA class I complexes are recognised by T cells which express the CD8 co-receptor, and HLA class II complexes are recognised by T cells which express the CD4 co-receptor. The haematopoietic stem cell, formed in the bone marrow, may form committed thymic progenitors, which then migrate to the thymus to mature. Progenitor cells enter the cortex of the thymus with very few cell surface markers and no T cell receptor rearrangement. There were originally two models predicting how thymocytes develop into α:β or γ:δ receptor expressing T cells. The first states that TCR-γ and TCR-δ genes rearrange preferentially or at the same time as D-Jβ gene segments, and only those which produce compatible γ and δ chains terminate gene rearrangement and become γ:δ T cells (Livak *et al.* 1995). The second model denotes successful rearrangement of TCR-β chain as the deciding factor, which gives signals through the pTα:β complex, initiating a rearrangement and deleting the δ-chain.

The end-point of the maturation pathway for α:β T cells is to express CD4 or CD8 and be specific for immunogenic peptides presented by self HLA, but unreactive toward self peptides presented by self HLA. The immature progenitors, which express no T cell surface markers are therefore, at this stage, known as 'double negative' (DN) thymocytes as they do not express CD4 or CD8 (Petrie *et al.* 1990). The DN thymocytes interact with the thymic stroma and undergo differentiation and proliferation. The DN thymocytes then begin to express some cell surface markers as they proceed through the thymic cortex,

firstly CD44, an adhesion molecule, and later CD25, which is the a chain of the interleukin-2 receptor (IL-2R) (Shortman and Wu 1996). As the DN thymocytes develop from $CD44^{low}$ to $CD25^{+}$, rearrangement of the T cell receptor (TCR) β-chain occurs. These β-chains then go on to pair with the surrogate α-chain, pTα, which allows the formation of a pre-TCR. The thymocyte loses expression of CD44 and CD25 at this stage and the pre-TCR joins with CD3 that is present in the ER and this complex is transported to the cell surface via the *cis*-Golgi. Once the pre-TCR and CD3 reach the cell surface, this stops further β-chain gene rearrangement and induces the cell surface expression of both CD4 and CD8. The thymocyte is now double positive (DP), and needs to interact with APCs present in the cortex to allow only those cells restricted by self HLA but unreactive with self peptide, to enter the periphery. The first stage is to exclude all those cells which cannot recognise self HLA, 'positive selection' (von Boehmer 1994). Only those cells which can recognise self HLA get through to the next stage and the remaining die. These successfully positively selected cells then rearrange their α-chain genes which eventually replaces the pre-TCR forming the α:β TCR (Petrie *et al.* 1993). They also undergo 'negative selection', eliminating the cells which respond too strongly to self peptide:self HLA complexes (reviewed in Nossal 1994). Finally, depending on whether the TCR is specific for HLA class I or class II, the thymocyte loses the expression of CD4 or CD8, emerging into the thymic medulla and out into the periphery as a single positive T cell, with a CD8 co-receptor if it is specific for HLA class I and a CD4 co-receptor if specific for class II. There are two main theories for how this occurs, the instructive model and the stochastic or selection model. The instructive model, states that a signal through a class II specific T cell receptor and CD4 shuts of CD8 expression and *vice versa* (Bommhardt *et al.* 1997). The stochastic model states that the CD4 or CD8 expression is generated randomly, and the thymocytes only pass through negative selection and become single positive when the co-receptor is matched for the class I or class II specificity of the T cell receptor (Fowlkes and Schweighoffer, 1995).

In the periphery, $CD8^{+}$ T-cells recognise intracellularly-derived peptides

presented by HLA class I molecules, and $CD4^+$ T-cells recognise extracellularly-derived peptides presented by HLA class II molecules as previously discussed. In the allogeneic bone marrow transplant situation, HLA:peptide complexes are recognised in the same manner, which can lead to cell death if the HLA molecule, peptide, or both are recognised as foreign.

NK cell recognition of HLA

When CTL's bind HLA:peptide complexes, an activatory signal is received by the T cell, which causes it to kill the APC, however, when an inhibitory receptor expressed on the surface of the NK cell binds HLA molecules, killing can be prevented, depending on which activatory and inhibitory receptors participate in the signalling (Moretta *et al.* 1995). This inhibitory phenomenon was first discovered in experiments by Kärre *et al* (Ljunggren and Kärre 1985), where murine NK cells showed greater killing of tumour cells which had lost their cell surface HLA expression, than those which bore HLA molecules - this is known as the "missing-self" theory. The loss of HLA expression can occur in virally infected cells as well as tumour cells, as a means of escape from T cell recognition.

NK cells express two types of receptors on their surface, inhibitory and activatory (Moretta *et al.* 1995). The receptors can be divided into two groups. The first are C-type (Ca^{2+}-dependant)-lectin-like receptors, which in humans are heterodimers consisting of CD94 and NKG2, both type 2 integral membrane proteins (Lanier *et al.* 1994). The second group of inhibitory receptors are immunoglobulin-type monomers consisting of type 1 integral membrane proteins known as KIRs (killer immunoglobulin-like receptors). The KIRs recognise certain epitopes expressed by HLA-B and -C molecules, and some HLA-A also (Colonna and Samaridis 1995), whereas the CD94:NKG2-A, and -B inhibitory receptors, and NKG2-C activatory receptor recognise HLA-E molecules which bind peptides from the signal sequences of HLA-A, -B and -C (Braud *et al.* 1998). Inhibitory KIRs which have two immunoglobulin (Ig) domains can recognise HLA-C molecules, dependent on whether they have a Serine and a Asparagine, or an Asparagine and a Lysine at amino acid positions

77 and 80 in their α1 helix (Colonna *et al.* 1993). Those containing three Ig domains recognise HLA-B antigens which have the Bw4 sequence motif at positions 77-83 of the α1 helix (Gumperz *et al.* 1995).

For a receptor to be able to transmit an inhibitory signal, it needs to have two immune receptor tyrosine-based inhibitory motifs (ITIM). Crosslinking of the receptor induces tyrosine phosphorylation of the ITIM, which then recruits tyrosine phosphatase SHP-1. In contrast, activatory receptors have ITAMs (immune receptor tyrosine-based activatory motifs), that can bind lck and adaptor molecule pp36, which becomes intensely phosphorylated when activatory receptors are engaged. However, when the inhibitory KIR engages HLA class I, and SHP-1 becomes activated, this is thought to then prevent the phosphorylation of pp36. Failure to phosphorylate pp36 prevents complex formation with phosphorylated PLC-γ and Grb2, an essential event for IP_3 generation and the calcium cascade, which leads to the release of cytotoxic granules onto the target cell (Valiante *et al.* 1996).

Each individual should express at least one NK cell inhibitory receptor, which is specific for their own HLA molecules, to prevent lysis of their own cells. In an allogeneic transplant situation, it is possible that one of the class I molecules which are recognised by the receptors is mismatched, and the HLA molecule expressed will not be recognised by the NK receptor, resulting in lysis of the allogeneic cell. This could be important in both rejection and graft versus host responses. A study by Velardi's group has shown, in mice, an NK-mediated graft versus leukaemia response without GvHD if NK cells were transfused before T cell depleted marrow (Ruggeri *et al.* 2002).

HLA class I-like genes

Along with HLA, there are also some "class I-like" genes that map within the HLA, such as the MIC (MHC class I chain related) genes (Groh *et al.* 1996). The MIC genes (MICA and MICB) were found during a search of the HLA-B region of the HLA, for other genes encoding expressed proteins (Bahram *et al.* 1994), and are situated between the major histocompatibility loci HLA-B and HLA-DR. The MIC genes have a similar structure to that of HLA class I, with an α1,

α2 and α3 external domains, a transmembrane region and a carboxyl-terminal cytoplasmic tail, including two pairs of cysteines in the α2 and α3 domains which form intradomain disulphide loops. In comparison with class I, however, the intron separating the leader sequence and the exon encoding α1 is much larger in the MIC genes, and the cytoplasmic tail and untranslated region are encoded within a single exon. MICA has been shown to have 15-21% sequence homology with the α1 domain, 32-36% homology with the α2 domain and 32-36% homology with the α3 domain of class I (Bahram *et al.* 1994). The MIC genes also show some polymorphism, although the function for this polymorphism is not clear, as the recognition by T cell and NK cell receptors so far investigated has been shown to be broad, and unable to distinguish between different allele products (Bauer *et al.* 1999). When the MICA structure is superimposed onto that of an HLA class I molecule, the variable residues in MICA are positioned on the edge of where the peptide binding groove would be (Bahram *et al.* 1996).

MICA has been crystallised, indicating a somewhat distorted structure in comparison with that of HLA class I antigens (Bjorkman *et al.* 1987b; Li *et al.* 1999). There is no peptide-binding groove, instead a shallow platform is present, with a loop comprised of the residues that would generally form the α2 helix in HLA class I. There is no evidence that these proteins can bind peptide, they also do not associate with β_2m (Groh *et al.* 1996). The reason for the lack of β_2m binding could be due to a number of factors: Binding of oligosaccharides instead of β_2m, steric clashes with the side chains of residues present in MICA that are different to those in HLA class I and residues of β_2m, or steric hindrance of β_2m due to salt bridges formed between some MIC residues. Although MICA does not need to bind β_2m to refold correctly, it is still not clear how it maintains a stable structure.

MIC antigens are recognised by T cells expressing the γ:δ receptor, and not the α:β receptor, which recognise HLA molecules (Groh *et al.* 1998). Due to the discovery of γ:δ T-cells in the intestinal endothelium, where MICA is constitutively expressed, it was originally thought that it was this receptor that

bound MICA (Groh *et al.* 1998), however, it was later discovered that these cells, along with NK cells, express another activatory receptor, NKG2:DAP10 (Bauer *et al.* 1999; Wu *et al.* 1999). If both these receptors were bound to MICA, it may lead to a stronger response (Hagmann 1999), however, it has since been found that only the NKG2 receptor is specific for MICA (Steinle *et al.* 2001).

The MIC antigens are upregulated when the cell is put under stress, in a similar way to some other proteins encoded by genes found within the MHC, such as heat shock protein 70 (hsp70) (Milner and Campbell 1990), due to their common promoter regions. Upregulation may occur when the cell is damaged, infected or proliferating. MICA and MICB have been found expressed on the surface of epithelial cells, and transcription has been seen in endothelial cells, but not B cells, T cells or monocytes. The nature of this expression could implicate MIC molecule upregulation on leukaemic or virally infected cells, and could be utilised as a useful target in immunotherapy. The polymorphic nature of the MIC antigens could mean that they are relevant as GvHD or rejection targets in allogeneic transplantation.

MHC and Allorecognition

Direct allorecognition occurs when the APC and the T cell are of different origins. In the unnatural setting of allogeneic bone marrow transplantation, this system can introduce problems in the form of graft rejection or graft versus host disease (GvHD). Graft rejection can occur in any transplant situation when the cells of the patient react against the cells in the transplanted tissue. Graft versus host disease only occurs when the transplanted tissue itself is capable of an immune response (Barnes *et al.* 1956). Studies on precursor frequencies for T cells capable of recognizing foreign MHC molecules have shown they can be 10-100 times higher than T cells which can recognize a peptide in the context of a self-MHC molecule (Ford and Burger 1983; Ford and Atkins 1973). There are two ways in which foreign MHC molecules can be recognized by T cells in a transplant situation; through direct and indirect allorecognition. Direct allorecognition describes the process where T cells bind intact foreign MHC molecules and bound peptide on the cell surface, recognising different amino

acids present on both the endogenous peptide and the MHC molecule (Lombardi *et al.* 1989). Alternatively, antigens may be shed from the foreign cell surface and processed and presented by self antigen presenting cells, with the T cells recognizing the amino acids of the presented peptide only (Sherwood *et al.* 1986). Indirect allorecognition can elicit both acute and chronic rejection episodes (Fluck *et al.* 1999), however, it is thought that direct allorecognition is the dominant process in acute organ rejection (Larsen *et al.* 1990a; Larsen *et al.* 1990b).

This phenomenon may be explained as a result of the T cell thymic education process. During thymic selection, all T cells are educated to self and non-self as thymocytes. Thymocytes that have too strong an affinity for self-MHC are deleted (negative selection), whilst those immature T cells with the ideal affinity for self MHC are allowed to continue through the education process (positive selection). Since the developing T cell repertoire has no *a priori* knowledge of self-MHC, T cells must be capable of relatively high affinity interactions with all potential MHC molecules. Under natural circumstances, where T cells would interact only with self-MHC molecules, T cells with high affinity are deleted through negative selection. However, in allogeneic transplantation, mature T cells encounter non-self MHC and the T cells with high affinity for such molecules have not been deleted in the thymus, accounting for the high frequency and affinity of allogeneic T cells.

HLA matching in allogeneic bone marrow transplantation

Transplant from an HLA-matched related donor is the primary technique for allogeneic transplantation, however, only 40% of otherwise eligible patients have a suitably histocompatible or closely HLA-matched related donor (Beatty *et al.* 1985). The probability of finding an HLA-A/B/Cw/DRB1/DRB3/DRB5/DQB1 compatible unrelated donor has also been shown as 38.4% (Tiercy *et al.* 2000). It is therefore important not only to identify HLA mismatches between donor and recipient, but also to determine whether one type of mismatch has a greater detrimental effect when compared with another. As knowledge of the MHC has increased, and tissue typing

methods have improved, HLA mismatching in allogeneic bone marrow transplantation has been investigated in a number of ways. Investigations into the effect of HLA mismatches on the outcome of unrelated bone marrow transplantation have been carried out retrospectively, as higher resolution typing technology, and the discovery of new HLA antigens and alleles have come about (Scott *et al.* 1998). Various different ways of examining HLA mismatching in unrelated bone marrow transplantation include the comparison of HLA class I and class II effects, the effect of individual locus mismatches, antigenic or allelic mismatches and the effect of cumulative HLA mismatches.

HLA class I vs class II mismatches

It has been shown in related transplants that when mismatching for one HLA antigen, the risk of GvHD is greater with and HLA-DR mismatch than for HLA-A or -B (Servida *et al.* 1996). Also, that the risk of acute GvHD is increased with the presence of multiple mismatches (Beatty *et al.* 1985). Initial reports in unrelated transplantation focused on HLA-DR matching, showing an increased risk of acute GvHD and decreased survival in patients who received an HLA-DR mismatched transplant (Petersdorf *et al.* 1995). It was later found that matching for both HLA-DRB1 and -DQB1 reduced the risk of acute GvHD and improved survival after unrelated BMT (Petersdorf *et al.* 1996). However, when compared with HLA identical sibling donors, the risk of acute GvHD, graft failure and mortality was still greater than with unrelated donors when matched by serology and MLC (Beatty *et al.* 1991; Davies *et al.* 1995; Hows *et al.* 1986; Kernan *et al.* 1993), therefore, it was thought that HLA class I mismatches may also play a role.

Following the demonstration that a patient mismatched for HLA-B44 exhibited acute GvHD (Keever *et al.* 1994), further investigations were carried out into the detection and effect of an HLA class I mismatch on transplant outcome (Petersdorf *et al.* 2001b; Rufer *et al.* 1993; Sasazuki *et al.* 1998; Scott *et al.* 1998). Graft failure was shown to be primarily associated with HLA class I disparity, especially where 2 or more alleles were mismatched (Petersdorf *et al.* 1998; Petersdorf *et al.* 1997). Also, HLA-A and -C mismatches have been shown to be

risk factors for acute GvHD (Sasazuki *et al.* 1998).

Petersdorf determined that HLA class I determinants governed graft acceptance, whereas class II determinants played a role in GvHD (Petersdorf *et al.* 1998). The presence of multiple class I disparities was associated with increased risk of graft failure, and the presence of class II disparities was associated with an increased risk of GvHD. These finding were contrary to what was seen in a similar study carried out on transplants for the Japanese Marrow Donor Program, where severe acute GvHD was seen with a class I mismatch, and class II matching did not significantly effect the occurrence of GvHD (Sasazuki *et al.* 1998). These findings indicate that the roles of individual HLA loci may differ according to ethnic background, and the types of mismatches found were different due to HLA differences in the two populations.

HLA allele versus HLA antigen mismatches

In a cohort of 548 patients with CML who underwent unrelated bone marrow transplantation, Petersdorf investigated the effect of antigenic versus allelic mismatches for HLA class I loci on graft failure (Petersdorf *et al.* 2001b). A greater number of nonsynonymous amino acid substitutions were found in the antigenic mismatches when compared with allelic mismatches. The allele and antigen mismatches also differed in the location of the mismatched amino acids in the α_1 and α_2 domains. Mismatching involving a single HLA-A, -B or -C allele was not associated with graft failure, but mismatching involving a single antigen mismatch or multiple HLA class I allele or antigen mismatches was associated with an increased risk of graft failure. Homozygosity of the recipient for mismatched alleles or antigens was identified as a risk factor for graft failure, and Petersdorf postulates that if donor mismatches cannot be avoided, then the preferred HLA-A, -B or -C antigen or allele mismatch should be at a locus for which the recipient is heterozygous. This would ensure a concomitant recipient mismatch to counterbalance the donor mismatch (Petersdorf *et al.* 2001b).

This approach has been criticized because 86% of the patients in the study also

had a class II mismatch (Cooper *et al.* 2002), which has been associated with GvHD (Petersdorf *et al.* 1998).

Individual locus mismatches

Many studies have been carried out to determine the effect of a single HLA locus mismatch on the outcome of unrelated transplantation (Bishara *et al.* 1995; Moreau and Cesbron 1994; Petersdorf *et al.* 1997; Petersdorf *et al.* 1995; Petersdorf *et al.* 1996; Petersdorf *et al.* 1993b). However, due to the high level of linkage disequilibrium within the MHC (Baisch, Capra 1993; Hiller *et al.* 1978), it is unusual to find a single allele mismatch between donor and recipient. A number of the studies carried out have relied on serological matching for loci other than the locus of interest for each study (Beatty *et al.* 1993; Petersdorf *et al.* 1995), meaning there could be allelic mismatches between other loci (Scott *et al.* 1998), that were not considered in the study, that could have an effect on transplant outcome.

Now that it is possible to carry out high resolution typing to identify allelic mismatches for all HLA loci, a true mismatch for a single locus can be defined on a completely matched background. However, as stated previously, it is difficult to find a single mismatch due to linkage disequilibrium between the HLA loci (Sasazuki *et al.* 1998). This linkage does not extend to HLA-DP (Baisch, Capra 1993), making it an ideal candidate to study the effect of a single locus mismatch (Varney *et al.* 1999). In unrelated transplants, HLA-DP mismatches have been reported in 75-89% of cases (Hurley *et al.* 2000; Petersdorf *et al.* 2001a; Varney *et al.* 1999). It has been determined that a mismatch for HLA-DPB1 in unrelated bone marrow transplantation can result in acute GvHD (Petersdorf *et al.* 2001a; Varney *et al.* 1999), but these studies had conflicting results with regard to the effect on patient survival. To look at the effect of mismatches of other individual loci on the background of otherwise HLA allele matched pairs, very large studies need to be undertaken. Two groups have investigated the effect of individual locus mismatches on an otherwise HLA matched background in 440 and 300 unrelated transplants

(Petersdorf *et al.* 1998; Sasazuki *et al.* 1998). Sasazuki found HLA-A and HLA-C mismatches to be strong risk factors for the development of severe acute GvHD (Sasazuki *et al.* 1998), whereas Petersdorf found the numbers of single HLA-A, -B and -C mismatches to be too small for meaningful comparison (Petersdorf *et al.* 1998). Sasazuki also found that matching for HLA-C increased the risk of leukaemic relapse and HLA-A mismatching significantly affected the survival rate (Sasazuki *et al.* 1998), but the effect of HLA class II mismatches did not appear to have a significant effect on outcome. It was suggested that the reduced risk of relapse with an HLA-C mismatch indicated a role for NK cells in a graft versus leukaemia response, implying that donor NK cells killed patient leukaemia cells which did not express the necessary HLA-C ligand to bind the NK receptor and inhibit killing (Sasazuki *et al.* 1998).

Cumulative HLA mismatches

The effect of linkage disequilibrium means there may be multiple HLA class I and II mismatches between serologically matched patients and donors when typed retrospectively at high resolution (Grundschober *et al.* 1997; Petersdorf *et al.* 2001b; Petersdorf *et al.* 1995; Rufer *et al.* 1995; Santamaria *et al.* 1994; Tiercy *et al.* 1991). The risk of acute GvHD is greater in the presence of multiple mismatches (Beatty *et al.* 1985), and severe acute GvHD is a greater risk for patients with class I plus class II mismatches when compared with single mismatches at any of the class I loci (Petersdorf *et al.* 1998). Also, patients with multiple class I mismatches and those with both class I and class II mismatches show significantly lower survival than matched patients (Petersdorf *et al.* 1998). A single class I or class II mismatch had little demonstrable effect on survival (Petersdorf *et al.* 1998). Disparity for 2 or more HLA class I alleles is associated with graft failure (Petersdorf *et al.* 1998), as is a mismatch for both class I and class II alleles (Petersdorf *et al.* 1997).

Qualitative mismatches

Now that it is possible to identify an allelic mismatch at any given locus, it should be possible to identify the impact of different mismatches, or find which

mismatches are "permissive". Due to the low number of genotypically matched unrelated donors available to a single patient, the goal should be to establish minimal matching criteria that enables transplantation to be conducted safely without excluding donors for patients who have no other therapeutic option. This means comparing the effect of mismatches at different loci in a qualitative rather than quantitative manner. This hierarchical effect of mismatches at different loci has been successfully demonstrated in the risk of graft failure of kidney transplantation (Gilks *et al.* 1987). This model allowed the relative risk of each mismatch combination to be estimated independently by assessing the effect of 0, 1 or 2 mismatches at each locus, which in this case showed that mismatching for HLA-DR had a greater effect than mismatching for HLA-B, which in turn was greater than HLA-A.

Minor histocompatibility antigens

T cells recognise MHC molecules complexed with specific peptides. There are thought to be three main ways this recognition occurs, the T cell either recognises epitopes on the peptide alone, the MHC molecule alone, or both the peptide and the MHC molecule together. The correct peptide is still required to be bound to a specific MHC molecule, even if it is not directly recognised by the T cell, because the binding of the peptide can alter the shape of the part of the MHC molecule exposed to the T cell, and different amino acids can be seen depending on its orientation (Bjorkman *et al.* 1987b). When the donor and recipient are completely matched for all their HLA molecules, the proteins present in the cells of each individual will still be different and can be recognised as foreign peptides restricted by self HLA molecules, when the cell is actually of host origin, resulting in cell killing. Any HLA mismatches can also be recognised directly, or as peptides presented by self-HLA molecules on the donors own cells.

Minor histocompatibility antigens (mHag) are peptide components of proteins which differ between individuals. In the case of mHags, the peptide alone is recognised, because the HLA molecule presenting the peptide is usually matched between the patient and donor (Goulmy *et al.* 1996). With high

resolution HLA typing and matching techniques now available for any polymorphic gene (Argüello *et al.* 1998b), it may be possible to completely match individuals for all polymorphic HLA genes. In a completely HLA matched situation, mHag mismatching may become more of a problem, as has been shown in HLA identical sibling transplantation (Goulmy *et al.* 1996).

The first evidence of responses against minor histocompatibility antigens was described in mouse experiments, where individuals transplanted with tumours matched for their MHC had a rejection episode, though less acute than those with known MHC mismatches (Counce *et al.* 1956; Snell 1948). In humans, the first defined mHags were male-specific, since found to be encoded by genes on the Y-chromosome (Goulmy *et al.* 1978; Goulmy *et al.* 1983; Goulmy *et al.* 1976; Goulmy *et al.* 1977; Wang *et al.* 1995). Peptides from these, such as H-Y, can be recognised when presented by a few different HLA antigens, namely HLA-A1, -A2, -B7 and -B60 and is expressed on a broad range of cell lineages of both haematopoietic and non-haematopoietic origin (Goulmy 1997).

The presence of a further set of autosomal mHAgs has been described by Goulmy *et al*, and called HA-1, HA-2, HA-3, HA-4 and HA-5 (Van Els *et al.* 1992). HA-1, HA-2 and HA-3 were found to occur at a frequency of 69-95% in a healthy population, while HA-4 and HA-5 were only present in 7-16% of individuals tested (Van Els *et al.* 1992). A mismatch for the diallelic, HLA-A*0201-restricted, HA-1 has been shown to induce a graft-versus-host response in HLA-A*0201 matched transplants.

Hypotheses for the project

That an information database could be established, for the Anthony Nolan Bone Marrow Trust, to store demographic and clinical information pertaining to each unrelated transplant performed for later statistical analysis with a view to analyse factors affecting the outcome of transplants from donors provided by the Trust.

That a novel HLA matching and typing tool, Reference Strand mediated Conformation Analysis, could be used to determine the level of matching at 6 HLA loci, between donor and recipient and the effect of this matching analysed with respect to outcome in the form of overall survival, disease free survival, transplant related mortality, relapse incidence and occurrence of acute and chronic GvHD.

The ultimate aim is to use the processes and the database developed to collect DNA, PBMCs and plasma samples and clinical information on all patients who undergo a transplant with a donor provided by the Anthony Nolan Registry from 1996 onwards. The data collected can then be used for more definitive studies. The information gained from this primary analysis will be used to calculate the number of samples required for the next study.

Chapter 2

Materials and Methods

Sample collection

Two 25ml aliquots of whole blood were collected from both recipients and donors originally in Heparin as an anticoagulant. Later a second medium was used; RPMI 1640 (BioWhittaker), containing 0.5mM β-mercaptoethanol, and 0.6% Tri-sodium citrate. These samples were used for the isolation of Peripheral Blood Mononuclear Cells (PBMC). A further 10ml sample was collected in EDTA (1.6 mg/ml of blood) for DNA extraction. Samples were transported by post, or courier, depending on the location of the transplant or harvest centre, with the aim of having the samples arrive in the laboratory within 2 days.

Processing and storage of samples

PBMCs were isolated from Heparinised or Sodium Citrated blood using the Lymphoprep™ gradient cell separation system. The heparinised samples were diluted 1:1 with RPMI 1640 and 10-15ml layered onto each 10ml of Lymphoprep™, centrifuged at 974g (without brake) for 22 minutes and the layer of PBMCs transferred into 20ml fresh RPMI. The cells were then washed in a total volume of 50ml RPMI 1640 and pelleted by centrifugation at 652g for 10 minutes and were then given two further washes in 50ml fresh RPMI 1640. Samples collected in Sodium Citrate which were less than 24 hours old were treated as above. Samples collected in Sodium Citrate, which had been in

transit for more than 24 hours, were defibrinated. 100µl 1M Calcium Chloride was added per 5ml of blood and the samples were transferred to sterile glass conical flasks containing glass beads. The flasks were shaken manually until a clot formed between the platelets, red blood cells and beads. The remaining liquid phase containing the PBMCs was layered onto Lymphoprep and treated as previously described. The PBMCs were stored in liquid nitrogen (N_2) at a density of $10x10^6$ cells/ml in foetal calf serum (FCS, Serum Supreme™, BioWhittaker) with 10% DMSO (BDH) for later use. The whole blood collected in EDTA was separated in two. One fraction of whole blood was stored at -70°C. The remainder was separated into buffy coat and plasma by centrifugation at 515g for 5 minutes, and stored at -70°C for DNA extraction and functional analyses respectively.

DNA Extraction

DNA was extracted from the blood sample collected in EDTA using a salting-out technique (Miller *et al.* 1988). Between 1 and 2ml of whole blood was mixed with 13-14ml of Red Cell Lysis Buffer (RCLB, 10mM Tris pH 8.0, 5mM $MgCL_2$, 1mM NaCl) in a 15ml tube (Falcon) and incubated at room temperature for 10 minutes. The cell lysate was then centrifuged for 10 minutes at 2500g. The supernatant was carefully discarded, leaving approximately 0.5ml, and the pellet released into suspension by vortex. This process was repeated until all red blood cells were lysed, indicated by a clear supernatant and white cell pellet after centrifugation. Distilled water (240µl) and 80µl Enzyme Buffer (10mM Tris, 10mM EDTA, 50mM NaCl) was added and the cell pellet dissociated through manipulation using a 1000µl Gilson pipette. Thirty microlitres of 10mg/ml Proteinase K (Flowgen) was added along with 20µl 10% SDS. The tube was incubated in a water bath at 55°C for 55 minutes. The lysate was transferred to a sterile 1.5ml microcentrifuge tube. Fifty microlitres of 5M NaCl was added and the tube vortexed at high speed for 15 seconds. The solution was centrifuged at 1500g for 10 minutes. The supernatant was then transferred to a new sterile 1.5ml microcentrifuge tube containing 750µl 99% Ethanol, on

ice. The solution was gently mixed until strands of genomic DNA were seen to precipitate. This DNA was extracted from the tube by spooling onto a sealed glass pasteur pipette and washed with 1ml of 70% Ethanol before being allowed to air dry. Once DNA strands appeared clear, indicative of thorough drying, the DNA was dissolved in 50-100µl of 1M Tris pH 8.0, 0.5M EDTA and the optical density measured at 260nm and 280nm to determine purity and concentration. Ideal purity was given by OD_{260nm}:OD_{280nm} = 1.6 - 1.8 and an ideal concentration of more than 500ng/µl. Samples of acceptable purity and concentration were stored at -20°C until use, when an aliquot was diluted in sterile distilled water to 200ng/µl and stored at 4°C. Samples which did not yield sufficient purity or concentration were concentrated and purified using GFX™ columns (Pharmacia Biotech™).

Purification using GFX™ columns

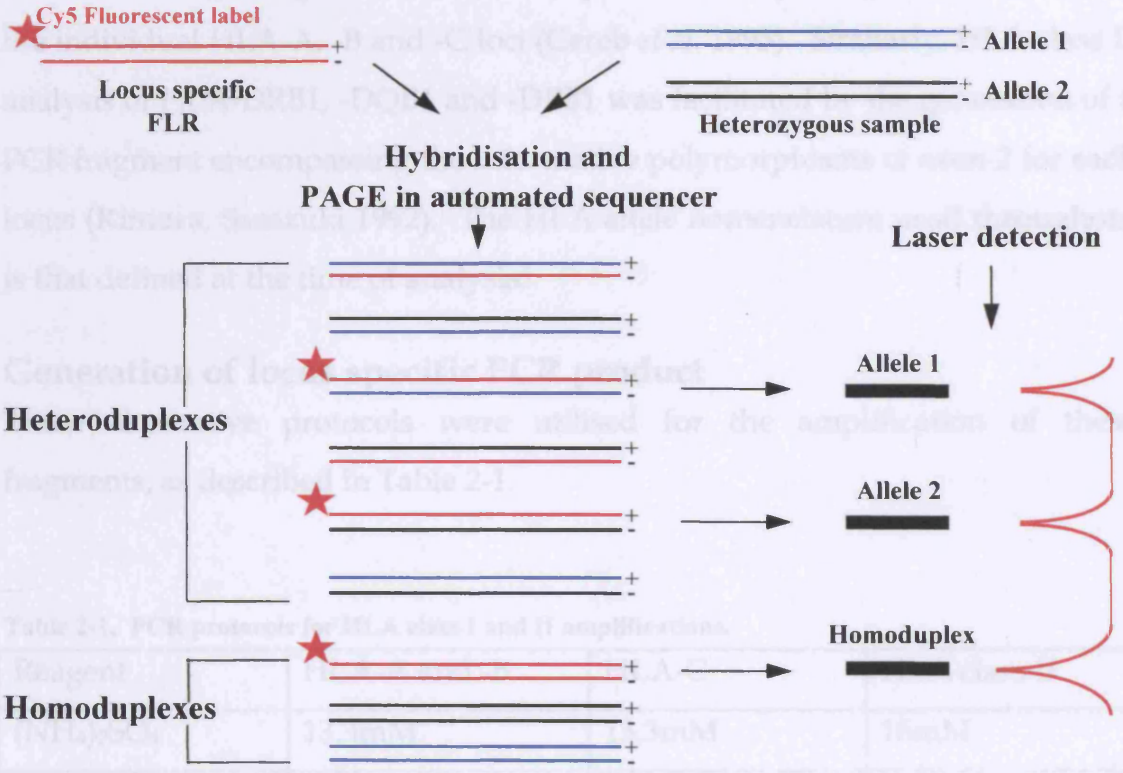
GFX™ purification (Pharmacia Biotech) uses an ion exchange technique for the purification of genomic DNA. At high salt concentration the nucleic acid binds to the glass fibre matrix in the GFX™ column. The DNA was washed with an ethanol-based solution to dissolve impurities, but not the bound DNA. The DNA is then eluted in the buffer of choice, which due to its reduced salt concentration will allow release of the DNA from the matrix. DNA or PCR products were purified following manufacturer's protocols. Briefly, one GFX™ column was used per sample to be purified, this was placed into a labelled 2ml waste collection tube. 500µl of chaotropic Capture Buffer was added to the column and 100µl of the sample to be purified was added and mixed. The column with tube were centrifuged at 12000g for 30 seconds, and the liquid in the collection tube discarded. 500µl of ethanol-based Wash Buffer was added to the column and it was centrifuged as before. The column was then transferred to a labelled 1.5ml microcentrifuge tube and 50µl (or less, depending on final concentration required) elution buffer (1M Tris pH 8.0, 0.5M EDTA for DNA, 1X PCR buffer for PCR product) was added and the column incubated at room temperature for one minute. The column and microcentrifuge tube were

centrifuged for a further minute, the eluted DNA/PCR product collected in the microcentrifuge tube and the column discarded. DNA was stored in concentrated form at -20°C until diluted for use.

Reference Strand mediated Conformation Analysis

RSCA is a DNA based technique that was developed at the Anthony Nolan Research Institute, allowing the detection of polymorphisms in gene sequences, and has been mainly applied for high resolution HLA class I and class II typing (Argüello *et al.* 1998b). RSCA is a heteroduplexing technique, which relies on the behaviour of double stranded DNA, in polyacrylamide gel, for the separation of different base pair sequences. A heteroduplex is formed between a fluorescently-labelled "known" single stranded DNA fragment, and an unlabelled "unknown" single stranded DNA fragment. Any mismatches between the two fragments in the heteroduplex will cause loops and bulges where the bases are uncomplimentary, whereas completely matched fragments will form a smooth, streamlined homoduplex. The mismatched fragments will run slowly through the gel, due to their uneven shape, whereas the matched homoduplex will travel uninhibited through the gel. This means the homoduplex would run faster through the gel than the heteroduplexes. Due to the specific flurochrome labelling, only the duplexes containing the labelled strand of the FLR were detectable with laser excitation of the Cy5 on ALFexpress DNA sequencing apparatus, see figure 2-1.

Fig 2-1. Schematic of Reference Strand mediated Conformation Analysis. This figure was adapted from Argüello *et al* (Argüello *et al*. 1998a). Homozygous FLR was hybridised to unlabelled sample PCR product and the resulting duplexes separated by PAGE on the ALFexpress automated sequencer (Pharmacia Biotech)



The first step in the RSCA process is to amplify a specific area of the gene of interest using PCR. This area is identified through the analysis of sequence data and sequence specific primers are designed to sit either side. For HLA class I analysis, locus specific primers positioned within intron 1 and intron 3 allowed the PCR amplification of an amplicon encompassing exons 2 and 3 of the individual HLA-A, -B and -C loci (Cereb *et al.* 1995). Similarly, HLA class II analysis of HLA-DRB1, -DQB1 and -DPB1 was facilitated by the generation of a PCR fragment encompassing the informative polymorphisms of exon 2 for each locus (Kimura, Sasazuki 1992). The HLA allele nomenclature used throughout is that defined at the time of analysis.

Generation of locus specific PCR product

Three alternative protocols were utilised for the amplification of these fragments, as described in Table 2-1.

Table 2-1. PCR protocols for HLA class I and II amplifications.

Reagent	HLA-A and -B	HLA-C	HLA class II
$(NH_4)_2SO_4$	13.3mM	13.3mM	16mM
$MgCl_2$	0.84mM	0.42mM	1.5mM
dNTP	74µM	74µM	200µM
each primer	25 pmol	25 pmol	25 pmol
Taq polymerase	1.25 units	1.25 units	0.75 units
DNA	200-500ng	200-500ng	200-500ng

Primers used for HLA-DPB1 and -DQB1 amplification were described in the 11th Histocompatibility Workshop (Kimura, Sasazuki 1992), along with other -DQB1 specific primers designed in house. Generic primers, designed by Pel-Freez™, were used for HLA-DRB1 amplification. Flanking HLA-DRB1 group specific primers were designed in house and were used in conjunction with the generic primers in a nested PCR to avoid amplification of other HLA-DRB

genes and to give a PCR product of the correct size for use in RSCA. Sequences of all primers are described in the Appendix I.

PCR cycling was carried out in a PTC-200 Peltier Thermocycler (MJ Research, Watertown, Massachusetts, USA). To confirm the presence of amplification product, 3µl from each 25µl PCR reaction was run on a 1.5% agarose gel in 1X TBE, at 120 volts for 45 minutes (see fig 2-2). PCR products were visualised by including 0.5ng/ml Ethidium Bromide in the agarose gel and viewing under ultra-violet light on a transilluminator. Size of PCR product was determined by the inclusion of a molecular weight marker (XIV, Boehringer Mannheim) with products ranging from 100 bp to over 2KB, each band separated by 100 bp (see figure 2-2).

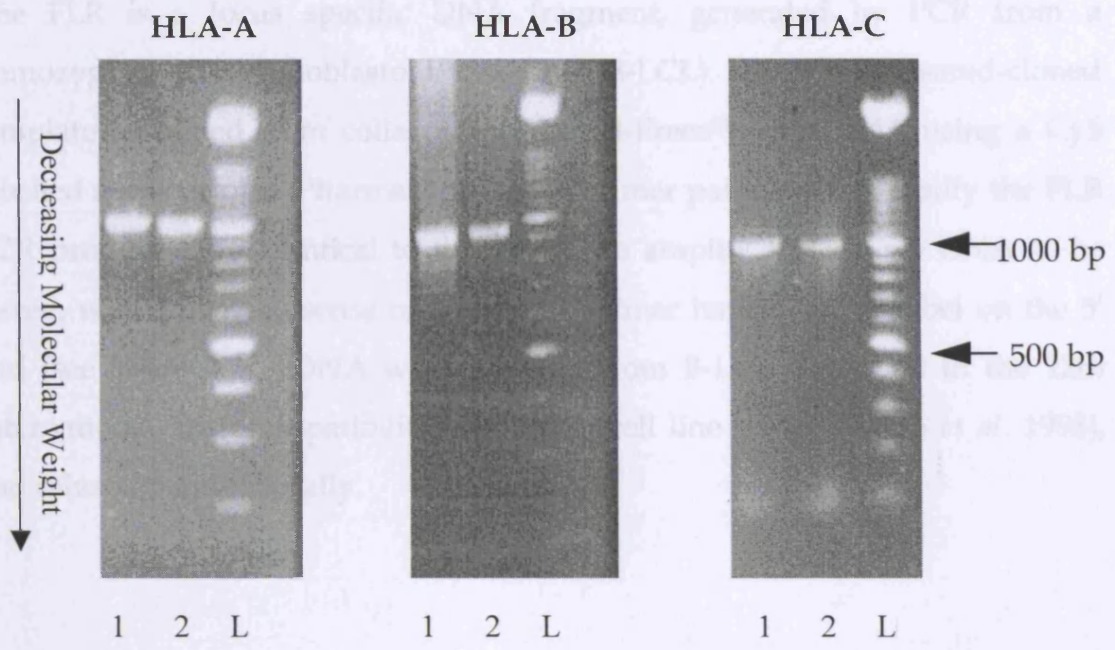


Figure 2-2. Successful PCR amplification. HLA-A, -B and -C PCR products had an expected length of 979 bp, 940 bp and 909 bp respectively (lanes marked 1 and 2 in each gel shown). The DNA molecular weight marker used in all agarose gels was 100 bp marker XIV (Boehringer Mannheim, L), with fragments of 100 bp – 1500 bp, at 100 bp intervals, with a larger band at the top of the marker of 2642 bp.

Formation of FLR (fluorescent labelled reference)

The FLR is a locus specific DNA fragment, generated by PCR from a homozygous B lymphoblastoid cell line (B-LCL) DNA, or plasmid-cloned template (obtained from collaborators at Pel-Freez™ Inc. USA), using a Cy5 labelled sense primer (Pharmacia™). The primer pair used to amplify the FLR PCR product were identical to those used to amplify the sample DNA to be tested, with either the sense or anti-sense primer having a Cy5 label on the 5′ end (see Table 2-2). DNA was extracted from B-LCL described in the 12th International Histocompatibility Workshop cell line panel (Marsh *et al.* 1998), and others obtained locally.

Table 2-2. B-LCL's used for the formation of FLRs

Locus	Template	FLR	Cy5 label (5')
HLA-A	B-LCL STEINLIN	A*0101	sense strand
	Plasmid Template	A*0102	sense strand
	B-LCL AMALA	A*0217	sense strand
	Plasmid Template	A*0205	sense strand
HLA-B	SP0010	B*4402	sense strand
	RSH	B*4201	sense strand
	JBUSH	B*3801	sense strand
HLA-C	BOB	B*51011	sense strand
	WJR076	B*5701	sense strand
	BM92	B*5701	antisense strand
	HOM-2a	B*27052	antisense strand
	AMALA	Cw*0303	sense strand
	STEINLIN	Cw*0701	sense strand
HLA-DRB1	KAS116	DRB1*0101	sense strand
	SPL	DRB1*08021	sense strand
	REN	DRB1*1001	sense strand
	PLH	DRB1*0701	sense strand
HLA-DQB1	AMALA	DQB1*03011	antisense strand
	BTB	DQB1*0402	sense strand
	BTB	DQB1*0402	antisense strand
HLA-DPB1	QBL	DPB1*0202	sense strand
	PLH	DPB1*1501	sense strand

Formation of duplexes

For PCR positive samples, 3-5µl of amplification product was hybridised to 1µl FLR. The mixture of PCR product and FLR was heated to 95°C for 5 minutes which denatures the double stranded DNA, then cooled to 55°C for 5 minutes which allows non-specific re-annealing of the sense and antisense strands of the sample PCR and FLR fragments, forming multiple duplexes. The samples were then cooled to 15°C for 5 minutes. Homoduplexes formed between the sense and antisense strands of the original DNA amplification products, and heteroduplexes formed between the sense strands of the FLR and the antisense strands of the samples, and vice versa. However, due to the specific flurochrome labelling, only the duplexes containing the labelled strand of the FLR were detectable with laser excitation of the Cy5 on ALFexpress DNA sequencing apparatus.

Electrophoresis

Between 1µl and 2µl of appropriate loading buffer was added to each hybridisation product and 2µl of this mixture was loaded onto non-denaturing polyacrylamide gel. For HLA class I analysis a 6% gel was optimal: 9.6ml Long Ranger gel solution (FMC BioProducts), 8ml 10X TBE buffer (BioWhittaker), 48µl TEMED (Amersham Pharmacia Biotech) and 480µl 10% Ammonium persulphate (Amersham Pharmacia Biotech) in a total volume of 80ml. For HLA class II analysis MDSequagel™ (National Diagnostics) was used at 0.5X: 20ml, 8ml 10X TBE buffer (BioWhittaker), 48µl TEMED (Amersham Pharmacia Biotech) and 480µl 10% Ammonium persulphate (Amersham Pharmacia Biotech) in a total volume of 80ml. Gel length was 21cm to laser detection and gel thickness was 0.5mm. RSCA was performed using the ALFexpress Automated Sequencer (Amersham Pharmacia Biotech). For each transplant pair, the recipient sample was loaded in the first of three lanes, the donor sample in the second lane and a pool of the two in the third lane. Using this loading method it was possible to distinguish a real mismatch, as an extra peak appears in the mixture lane. This allows elimination of artefacts due to lane-to-

lane variability. Electrophoretic conditions were 30 W constant power at a temperature of 40°C maintained with an external cooling system, for 400-580 minutes, depending on the mobility times of the duplexes. Gels could be re-used up to five times on addition of fresh running buffer (1X TBE) to buffer reservoirs. As the fluorescently labelled duplexes crossed the fixed laser in the Sequencer, the Cy5 fluorescent label was excited. A signal was detected by the sequencer, and recorded in a software-generated results file where it was represented as a peak in the electropherogram (see Figure 2-1).

Formation of top and bottom markers

The problems usually associated with heteroduplex techniques effecting difficulties in direct sample-to-sample comparison include intra- and inter-gel variability, and resolution of DNA fragments of similar mobility. These problems have been overcome using RSCA. Inter and intra gel variability is avoided by using markers in every lane, and locus specific ladders in each gel. Where necessary, internal markers are formed from duplexes which, due to conformational differences, run faster than the homoduplex and slower than the slowest running heteroduplex observed. They are added to the loading buffer (15% Ficoll + 1.2% dextran blue) and therefore can be included in every lane.

HLA-A markers

For HLA-A, the top and bottom marker are the heteroduplex and homoduplex (respectively) of the hybridisation of a hybrid HLA-A*2501 exon 2 and Patr-A exon 3 chimp MHC molecule, created by overlap extension (Pel-Freez™, patent pending) unlabelled PCR product and a short A*0102 Cy5 sense strand labelled PCR product. Initially PCR products were obtained using a nested PCR protocol as the primers sit inside those used for sample and standard FLR amplification (5AIn1-46 and 3A36L). Pel-Freez™ have now generated plasmid cloned template for both these amplicons. The template for the top marker allele was provided already dilute from Pel-Freez™ and this was amplified using the same set of primers, unlabelled. 40µl of Cy5 labelled short A*0102

PCR was hybridised to 120µl top marker allele PCR product. 10µl of Cy5 sense labelled short A*0102 product was added post hybridisation to increase the intensity of the bottom marker as most will have been used in the hybridisation, and this was diluted 1:1 with Ficoll loading buffer (15% Ficoll, 1.2% dextran blue). For use, 2µl was added to 4µl of test product/FLR hybrid and 2µl loaded on the gel.

HLA-B markers

For HLA-B, the top and bottom marker are the heteroduplex and homoduplex of a hybridisation of short HLA-B*4501 unlabelled PCR product and short HLA-B*4201 Cy5 sense strand labelled PCR product. These PCR products were generated using a nested PCR protocol as the primers sit inside those used for sample and standard FLR amplification (5Bin1:41-62 and 3BIn3-12L). The B-LCL RSH (HLA-B*4201) was amplified using standard B locus primers and the product checked on agarose gel. A 1:100 dilution was made and used as a template for a nested PCR using Cy5 sense labelled 5B short and unlabelled 3B short primers, giving a Cy5 sense labelled short B*4201 PCR product. The template for the HLA-B*4501 top marker allele was provided already dilute from Pel-Freez™ and this was amplified using the same set of primers unlabelled. 40µl of Cy5 labelled short B*4201 PCR product was hybridised to 120µl top marker allele PCR product. 10µl of Cy5 sense labelled short B*4201 product was added to the hybridisation product to increase the intensity of the bottom marker as most will have been used in the hybridisation, and this was diluted 1:1 with Ficoll loading buffer (15% Ficoll, 1.2% dextran blue). For use, 2µl were added to 4µl of test product/FLR hybrid and 2µl loaded on the gel.

HLA-C markers

For HLA-C the top and bottom marker are the heteroduplex and homoduplex of a hybridisation of Cw*1601 unlabelled PCR product and long B*5701 Cy5 sense strand labelled PCR product. DNA from B-LCL PF97387 (HLA-Cw*1601) was amplified using HLA-C specific primers 5Cin1-61 (Cereb *et al.* 1995) and

3Cin3-12L. This PCR product was performed as a 200µl reaction and purified and concentrated on a GFX™ column to a final volume of 100µl. After PCR and GFX™ purification, 1-3µl of product was checked using an agarose gel to show successful PCR and purification. DNA from B-LCL WJR076 (HLA-Cw*5701) was amplified using HLA-B specific primers Cy5 sense labelled 5Bin1-62 and 3Bin3:37-84. Before hybridisation to the Cw*1601 PCR product, the FLR was run in a Long Ranger polyacrylamide gel in the ALFexpress to ensure no contamination was present. 6µl of the B*5701 FLR was hybridised to 18µl of the concentrated Cw*1601 PCR product and the resulting duplexes tested by PAGE on the ALFexpress. To form the HLA-C specific loading buffer, hybridisation product and Ficoll loading buffer were combined 1:1, with 1.5µl extra B*5701 FLR added to increase the intensity of the bottom marker as most will have been used in the hybridisation. 2µl was added to each sample to be tested.

External Standard Ladders

For HLA-A and -B, external ladders were prepared for each of the 5 FLRs used (2 for HLA-A and 3 for -B). After amplification of well-defined control cell line DNA, exhibiting alleles of known gel mobility, hybrids were generated with the relevant locus-specific references. These hybrids were pooled and stored at -20°C until required. After addition of the relevant internal marker loading buffer, the ladders could then be loaded into at least two gel lanes prior to electrophoresis. The heteroduplex peaks of defined mobility present in the external standard ladder, in combination with specialised computer software, allowed the calculation of a standard curve to evaluate test product duplexes hybridised to the same FLR. For HLA-C, a ladder utilising only one FLR hybridised to HLA-C allele PCR product was used; to be run alongside all the FLRs.

HLA-A ladder

For HLA-A analysis, one ladder was used for each different FLR - HLA-A*0102 from the B-LCL DAUDI and HLA-A*0205 from the B-LCL AM. PCR products

were amplified using standard HLA-A primers (unlabelled 5AIn1-46 and 3A36L) from B-LCL WT100BIS (A*1101), DEM (A*0201) and TERESAKI 995 (A*2402), for the A*0102 ladder, and AMAI (A*6802), LWAGS (A*3301), LBUF (A*3001) and TERASAKI 995 (A*2402) for the A*0205 ladder. Each PCR product was purified and concentrated using a GFX™ column as before. The PCR products were hybridised individually (3:1) with the A*0102 or A*0205 FLR and mixed after checking their purity and mobility values on Long Ranger gel using RSCA on the ALFexpress.

HLA-B ladder

For HLA-B analysis, one ladder was used for each different FLR - HLA-B*4201 from the B-LCL RSH, HLA-B*4402 from the B-LCL SP0010 and HLA-B*3801 from the B-LCL JAP-NF. Ladder components were amplified using standard HLA-B primers (unlabelled 5Bin1:41-62 and 3Bin3-12L) from B-LCL PF04015 (B*0801), EA (B*0702), CALOGERO (B*4002), PLH (B*4701), PITOUT (B*44031), AKIBA (B*52011) and DOP-ND (B*44031 and B*1516), for the B*4201 ladder, PITOUT (B*44031), PLH (B*4701), LK707 (B*52011 and B*7301), BM21 (B*4101), EJ32B (B*1801), EA (B*0702) AND OMW (B*4501) for the B*4402 ladder and AMAI (B*5301), LK707 (B*52011 and 7301), CALOGERO (B*4002), IBW9 (B*1402), EA (B*0702), OMW (B*4501) and BOLETH (B*1501) for the B*3801 ladder. Each PCR product was purified and concentrated using a gfx™ column as before. The PCR products were hybridised individually (3:1) with the B*4201, B*4402 or B*3801 FLR and mixed after checking their purity and mobility values on Long Ranger gel using RSCA on the ALFexpress.

HLA-C ladder

For HLA-C analysis, a single ladder was used for all FLRs. The FLR used in the formation of the ladder was B*5101 from the B-LCL BOB amplified with Cy5 sense labelled 5Bin1:41-62 and 3Cin3-12L. This was then hybridised (1:3) to PCR products from B-LCLs HOM 2a (Cw*0102), RSH (Cw*1701), DEM (Cw*0602), CJO A (Cw*0401), PF97387 (Cw*1601) and BOB (Cw*1502) in turn,

which were amplified using unlabelled 5Cin1-61 and 3Cin3-12L primers and purified and concentrated using GFX™ columns. The hybridisation products were tested by PAGE in the ALFexpress and then pooled. For analysis, 4µl of ladder was added to 2µl of ficoll loading buffer with 2µl loaded on the gel.

HLA-DPB1 ladder

Three ladders for each FLR were generated by Daniel Ramon, at the Anthony Nolan Research Institute (Ramon *et al.* 1998). This number of ladders was required as no top and bottom markers were available for HLA-DP analysis. The DPB1*0202 and DPB1*1501 FLRs were generated as previously described. The DPB1*0202 FLR was hybridised (1:3) to PCR products from DPB1*0401, DPB1*02012, DPB1*0402, DPB1*1901, DPB1*0601 and DPB1*1401, to make ladder 1, DPB1*1601, DPB1*2101, DPB1*0301, DPB1*0901 and DPB1*1501 to make ladder 2, and DPB1*01011, DPB1*0501, DPB1*2001, DPB1*1701, DPB1*1001 and DPB1*1401 to make ladder 3. The DPB1*1501 FLR was hybridised (1:3) to PCR products from DPB1* 2001, DPB1* 02012, DPB1* 0301, DPB1* 0501, DPB1* 0401 and DPB1* 1901 to make ladder 4, DPB1* 0402, DPB1* 01011, DPB1* 0601, DPB1* 1701 and DPB1* 0202 to make ladder 5 and DPB1* 2301, DPB1* 1301, DPB1* 0901, DPB1* 1001, DPB1* 2101 and DPB1* 1401 to make ladder 5. For analysis, 4µl of ladder was added to 2µl of ficoll loading buffer with 2µl loaded on the gel.

Assignment of a mobility scale

Gels were analysed using Allelelinks™ fragment analysis software on the ALFexpress. The migration of the different duplexes were measured as the time taken for each to travel from the well until traversing the laser. As with all gel systems there could be variability in the gel, meaning two identical duplexes in different lanes could have slightly different time values. This problem was overcome by utilising a part of the software, which allows the assignation of an arbitrary value to certain peaks, allowing complete alignment of the gel.

For analysis, the bottom marker was given a value of 1000 and the top marker was given a value of 2000. As these markers appeared in every lane, the gel could then be aligned and analysed using these arbitrary values instead of time, giving a scale of mobility. Further alignment of the HLA class I gels was achieved using the ladders, which were ideally loaded either side of the area of the gel containing the matching FLR. Each component of the ladders had a known mobility, and these could be assigned in the same way as the values for the markers were, allowing even more accurate alignment of the gel, for more comprehensive analysis. The alignment of the gel with markers and ladders is represented in figure 2-3.

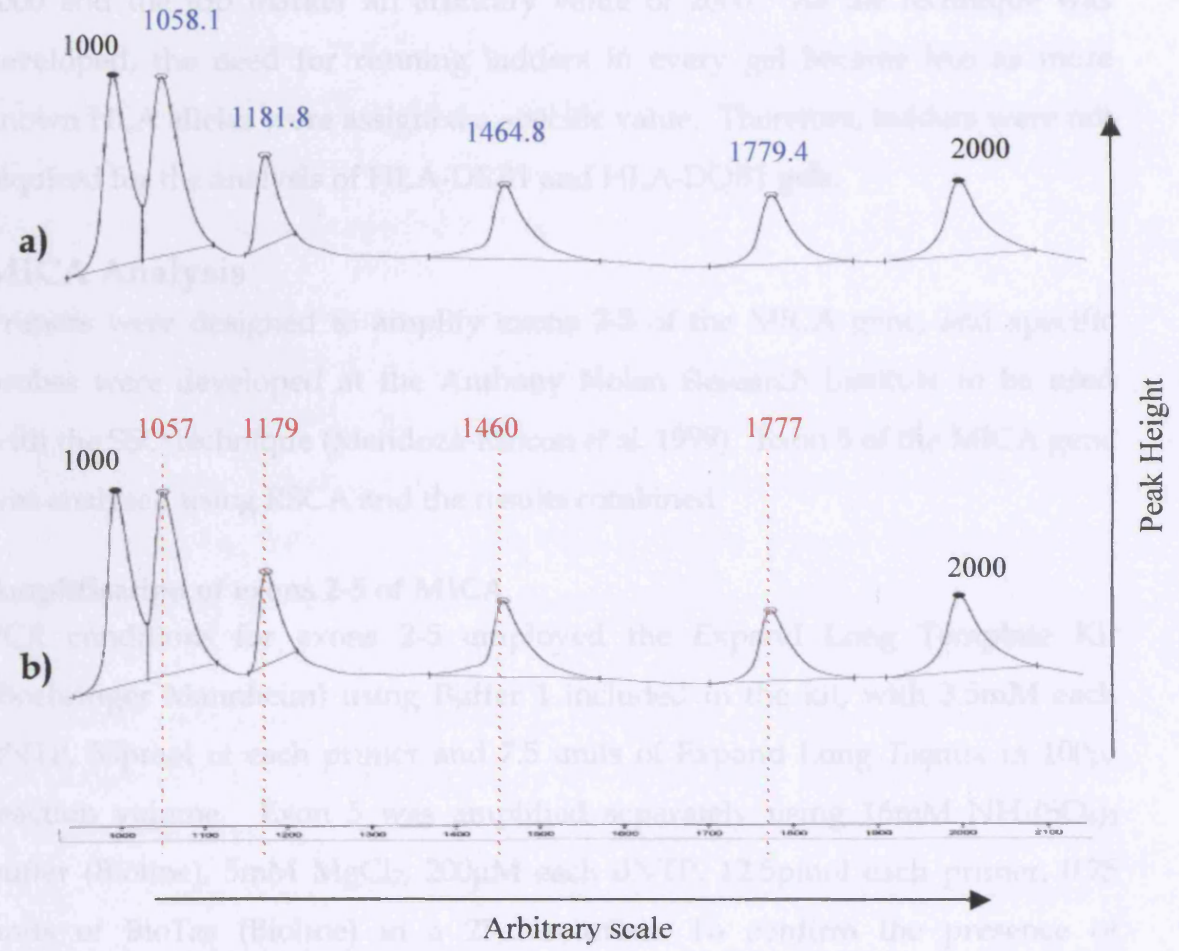


Figure 2-3. Alignment of the gel using markers and ladders.

(a) Lanes are aligned by the assignation of an arbitrary value of 1000 to the bottom marker and 2000 to the top marker.

(b) Lanes can be further aligned by adjusting the position of the ladders to a standard value, which is automatically applied to the rest of the gel, therefore avoiding gel to gel variation.

For HLA class II, the peak created by the labelled primer in the FLR PCR was given an arbitrary value of 1 and the homoduplex peak an arbitrary value of 1000 and the top marker an arbitrary value of 2000. As the technique was developed, the need for running ladders in every gel became less as more known HLA alleles were assigned a specific value. Therefore, ladders were not required for the analysis of HLA-DRB1 and HLA-DQB1 gels.

MICA Analysis

Primers were designed to amplify exons 2-5 of the MICA gene, and specific probes were developed at the Anthony Nolan Research Institute to be used with the SSO technique (Mendoza-Rincon *et al.* 1999). Exon 5 of the MICA gene was analysed using RSCA and the results combined.

Amplification of exons 2-5 of MICA

PCR conditions for exons 2-5 employed the Expand Long Template Kit (Boehringer Mannheim) using Buffer 1 included in the kit, with 3.5mM each dNTP, 35pmol of each primer and 7.5 units of Expand Long *Taq*mix in 100µl reaction volume. Exon 5 was amplified separately using 16mM $NH_4(SO_4)_2$ buffer (Bioline), 3mM $MgCl_2$, 200µM each dNTP, 12.5pmol each primer, 0.75 units of Bio*Taq* (Bioline) in a 25µl reaction. To confirm the presence of amplification product, 3µl from each PCR reaction was run on a 1.5% agarose gel in 1X TBE, at 120 volts for 45 minutes.

Labelling of probes

A Di-digoxygenin (DIG) labelling kit was used (Boehringer Mannheim), to label each oligonucleotide probe. 100pmol of oligo was added to 5X Tailing buffer, 25mM $CoCl_2$, 1mM DIG/ddUTP, and 50 units of *T. Transferase* all supplied in the kit. This mix was incubated at 37°C for 20 minutes and then the reaction stopped with 2µl of a mixture of 1µl Glycogen solution (supplied in the kit) and 200µl 0.2M EDTA (pH7.8 - 8.0). The oligo was precipitated with 2.5µl 4M LiCl and 75µl 100% Ethanol, and incubated at -70°C for 30 minutes. The oligo was removed by centrifugation at 12,000g for 15 minutes and air-dried. The oligo was resuspended in 100µl dH_2O ready for use.

Positive controls for oligotyping

Through previous sequencing of various B-LCL DNA for MICA, positive controls were available for most probes. For those where positive controls were not available, pseudo controls were formed. This involved amplification of DNA from the B-LCL JY using the probe as a sense primer and the closest intronic antisense primer in a PCR reaction, the conditions for which were as described for the amplification of MICA exon 5.

Sequence specific oligotyping of MICA exons 2-4

PCR products were denatured at 95°C and rapidly cooled on ice. Using the HYDRA 96 (Robbins Scientific), 2µl of each PCR product was dotted onto 25 labelled nylon positively charged membranes (Boehringer Manheim), along with controls for each probe. The PCR products were hybridised to the membrane using ultraviolet light. Each membrane was blocked with a solution of 4X SSPE, 0.1% Lauroylsarcosite and 1% Blocking solution (Boehringer Manheim), then exposed to one of 25 DIG-labelled oligonucleotide probes specific for MICA nucleotide motifs at a concentration of 10pmol in 5ml 3M Tetramethylammonium, 20mM Tris pH 8.0 and 2mM EDTA (TMAC). The membranes were washed thoroughly with 2X SSPE and 1% SDS and TMAC solution at specific temperatures of 58°C or 60°C to remove excess probe. After washing, filters were neutralised with 1X Neutralisation Solution (Boehringer Manheim). Membranes were incubated at 37°C for 40 minutes in 0.1M Tris pH 7.5, 0.15M NaCl, 1% Blocking reagent (Boehringer Manheim) and an anti-DIG Fab antibody fragment, labelled with Alkaline Phosphatase (Boehringer Manheim). After washing with 1X Neutralisation Solution (Boehringer Manheim) and, 0.1M Tris pH 7.5, 0.15M NaCl and 50mM $MgCl_2$ The membranes were exposed to a chemiluminescent substrate, Disodium3-(4-methoxyspiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclo(3.3.1.1^{3,7})decan]-4-yl)phenyl phosphate (CSPD) (Boehringer Mannheim). Membranes were then exposed to Kodak X-ray film for 5-20 minutes. The film was developed and results analysed manually and using the Typetool™ computer package (LabScan Systems Inc. USA).

RSCA analysis of MICA exon 5

Two FLRs were used in the analysis of MICA exon 5, (called 5A and 5D). These were formed from B-LCLs EJ32B and PF97387 DNA respectively by exon 5 amplification with sense Cy5 labelled primers Amplification and hybridisation were carried out as previously described. Ladders were formed containing heteroduplexes, which represented the six expected positions in RSCA for MICA exon 5. Each position was given a reference (5A, 5D etc) which corresponded to a "probe" on the Typetool™ analysis carried out on the SSO results of MICA exons 2-4. With this additional information it was possible to assign a MICA type to each sample using the Typetool™ software.

Sequence specific PCR detection of HA-1

For HA-1 analysis a kit developed by Olerup SSP™ (Robbins Scientific) was used. It contained primers developed by Professor Els Goulmy's group in The Netherlands, which had been previously published (Wilke *et al.* 1998). Thirty nanograms of DNA was added to the PCR Master Mix, provided with the kit, which contained nucleotides, buffer, glycerol and cresol red. Ten microlitres of this mix was then added to two PCR tubes, each containing a primer pair specific for the HA-1^H or HA-1^R allele (to give a PCR product of 210 bp) and a control primer pair matching non-allelic sequences (to give a PCR product of 515 bp and 430 bp respectively). After amplification, the entire PCR product was loaded on a 2% agarose gel and run for 45 minutes at 150 volts (see figure 3-5, Chapter 3).

Detection of HA-1 specific T cells

Phycoerythrin-labelled HLA-A*0201/HA-1^H tetramers were made in order to detect whether HA-1 specific T cells were present in patients with Graft versus Host Disease (GvHD). Tetramers can be used for the detection of specific T cells in a heterogeneous sample. A tetramer is formed from 4 monomers (HLA heavy chain - specific peptide - β_2m) bonded via Biotin to a Streptavidin molecule fluorescently labelled with Phycoerythrin. Specific T cells bind to the HLA/peptide molecules and can be detected in conjunction with specific

surface markers by flow cytometry on a Fluorescence Activated Cell Sorter (FACS).

HLA-A*0201/HA-1^{H} Tetramer formation

Cloning of HLA-A*0201 and β_2m

This work was carried out by Geraldine Aubert and Christina Zamoyska at the Anthony Nolan Research Institute. Exons 1-3 of HLA-A*0201 cDNA were amplified with primers containing restriction sequences for the *Nco I* and *Bam HI* restriction enzymes and cloned into a PCR2.1 plasmid (Invitrogen). The HLA-A*0201 was then cut from the plasmid with *Nco I* and *Bam HI* restriction enzymes (Gibco) and subcloned into an inducible expression vector, peT3d (Novagen), containing a C-terminal sequence coding for a biotinylation target. This vector was then transfected into pLysS *Escherichia Coli* (*E.Coli*) for expression of the protein.

The β_2m gene, cloned in another expression vector was a generous gift from Professor Don Wiley, Harvard University, USA, which was also transfected into *E. Coli* XL1 Blue (Promega).

The HA-1^{H} peptide was synthesised by Applied Biosystems using standard Fmoc chemistry.

Expression of proteins

Stocks of *E. Coli* pLysS containing HLA-A*0201:Bsp2 and β_2m expression vectors were streaked onto 1.2% agar (Gibco) 2XYT (16g Tryptone, 10g Yeast Extract, 5g NaCl in 1L H_2O) culture plates, with chloramphenicol (34μg/ml in ethanol)/ ampicillin (50μg/ml) and ampicillin (50μg/ml) antibiotics, respectively. The A*0201:Bsp2 vector and the β_2m vector contained resistance genes for ampicillin, and the *E. Coli* pLysS had resistance for chloramphenicol. The plates were incubated at 37°C overnight.

One isolated colony from each plate was added to 5mls 2XYT medium in a sterile 15ml centrifuge tube containing the appropriate antibiotics and incubated for six hours at 37°C in a shaking incubator. These *E. coli* pLysS

suspensions were then expanded into one litre in a sterile 2L conical flask under the same conditions, until an optical density of OD_{500} = 0.4 was reached (approximately 4 hours). When this stage of growth (mid-exponential growth phase) was reached, 100mg Isopropyl β-D-Thiogalactopyranoside (IPTG) was added to induce protein expression and the culture incubated for a further 4 hours.

Induction of protein expression was checked using a Sodium dodecyl sulphate (SDS) polyacrylamide gel.

SDS -PAGE

For protein separation, stacking SDS-PAGE was used. This consists of a double layer gel with the top layer having a lower acrylamide concentration than the bottom. The lower gel was 15% acrylamide (Protogel™, National Diagnostics) with 0.5% SDS and 1.5M Tris buffer pH 8.0, and the upper gel was 4.5% acrylamide with 0.4% SDS and 0.5M Tris buffer pH 8.0. Once the gel was polymerised, proteins to be tested were mixed with SDS loading buffer (0.0625M Tris pH 8.0, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) and denatured at 100°C for 5 minutes before loading. The gel was run for 60 minutes at 30 kV in 0.025M Tris buffer with 0.192M Glycine and 0.1% SDS, and then removed from the glass plates and stained with 0.25% Coomassie Brilliant Blue, 45% Methanol, 10% Acetic acid, followed by de-staining in 10% Methanol with 7% Acetic acid. The results of this analysis are shown in figure 2-4.

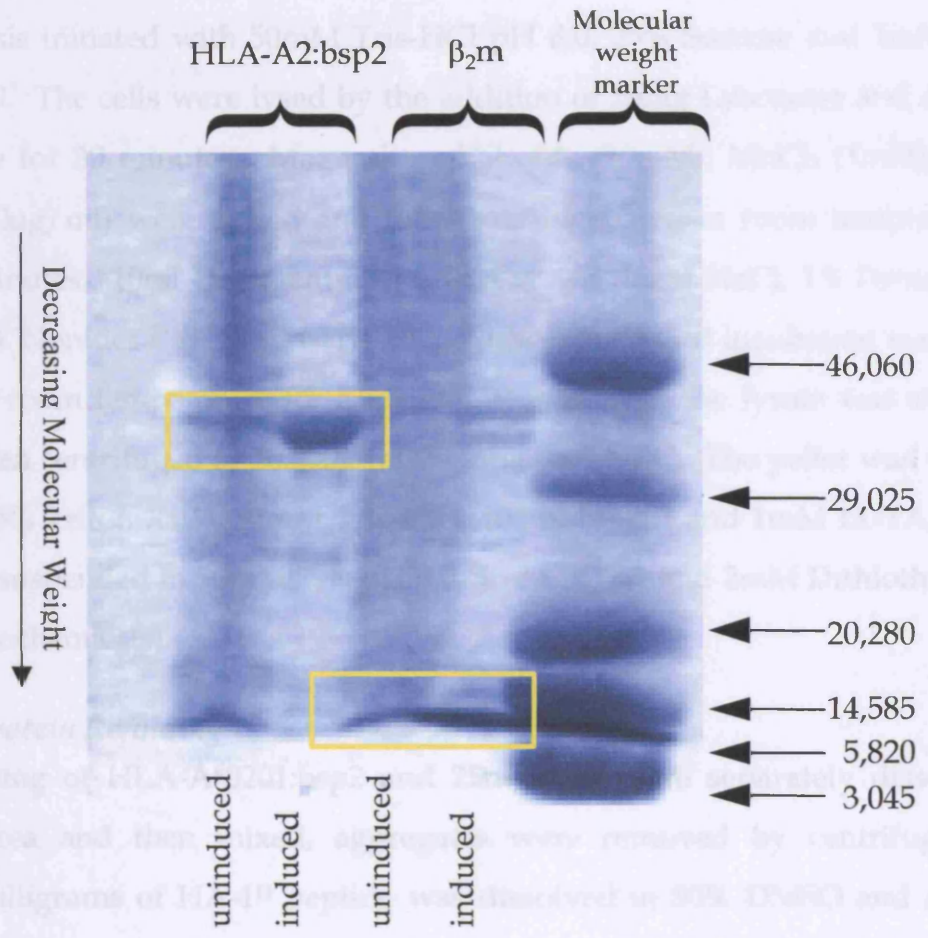


Fig 2-4. Detection of the induction of protein expression by SDS-PAGE. The first two lanes show the lysed *E. Coli* before and after expression of the HLA-A*0201:bsp protein and the second two lanes show the lysed *E. Coli* before and after expression of the β_2m, with IPTG. The areas of interest can be seen in the yellow boxes, where a strong band is present in the lanes where the protein has been expressed. The final lane contains a molecular weight marker to ensure that the proteins are the expected size.

Harvesting proteins

The *E. coli* were pelleted by centrifugation at 4,000g for 10 minutes and then lysis initiated with 50mM Tris-HCl pH 8.0, 25% Sucrose and 1mM EDTA pH 8.0. The cells were lysed by the addition of 25mg Lysosyme and incubated on ice for 30 minutes. Magnesium Chloride (10mM), $MnCl_2$ (1mM) and DNase (10μg/ml) were added and the lysate incubated at room temperature for 30 minutes. 10ml Detergent buffer was added (0.2M NaCl, 1% Deoxycholic acid, 1% Nonidet P40, 20mM Tris-HCl, 2mM EDTA) and incubation was carried out at room temperature for a further 10 minutes. The lysate was sonicated and then centrifuged at 10,000g for 20 minutes at 4°C. The pellet was washed with 0.5% Triton X100, 50mM Tris-HCl, 100mM NaCl and 1mM EDTA, then finally resuspended in 50mM Tris pH 8.0, 5mM EDTA and 2mM Dithiothreitol (Alexis Biochemicals).

Protein Refolding

30mg of HLA-A*0201:bsp2 and 25mg β_2m were separately dissolved in 8M Urea and then mixed, aggregates were removed by centrifugation. Ten milligrams of HA-1^H peptide was dissolved in 50% DMSO and added to the other proteins. This protein solution was then added to 200ml of 4°C refolding buffer (100mM Tris-HCl pH 8.0, 400mM L-arginine-HCl, 2mM EDTA, 5mM reduced Glutathione, 0.5mM oxidised Glutathione and 0.5mM Phenylmethlysulfonyl fluoride (PMSF) and incubated, with continuous stirring at 4°C for 24 hours.

A further 15mg HLA-A*0201:bsp2 was added and incubation continued, stirring for 24 - 48 hours. Correct refolding was tested using specific antibodies in a dot blot technique after FPLC purification.

The monomer solution was filtered first through a 0.2 micron filter to remove any large aggregates and then vacuum filtered using an Amicon™ stir cell through a YM10 microfiltration membrane with a molecular weight cut off of 10,000 (Millipore). A Vivaspin 20ml concentration column (Vivascience) was then used to concentrate the monomers to 1ml, by centrifugation. This

concentrate was again filtered with a 0.2 micron Vivaspin 0.5ml filtration column (Vivascience) to remove any aggregates which may have formed, and purified on a fast protein liquid chromatography (FPLC) gel filtration column in 10mM Tris pH 8.0, 5mM NaCl, and the monomer fractions collected (the FPLC trace can be seen in figure 2-5). The fractions in each peak were then pooled and concentrated using Vivaspin 0.5ml concentrator columns, to a final volume of 200µl. Each fraction was analysed with a dot blot technique to determine correct refolding.

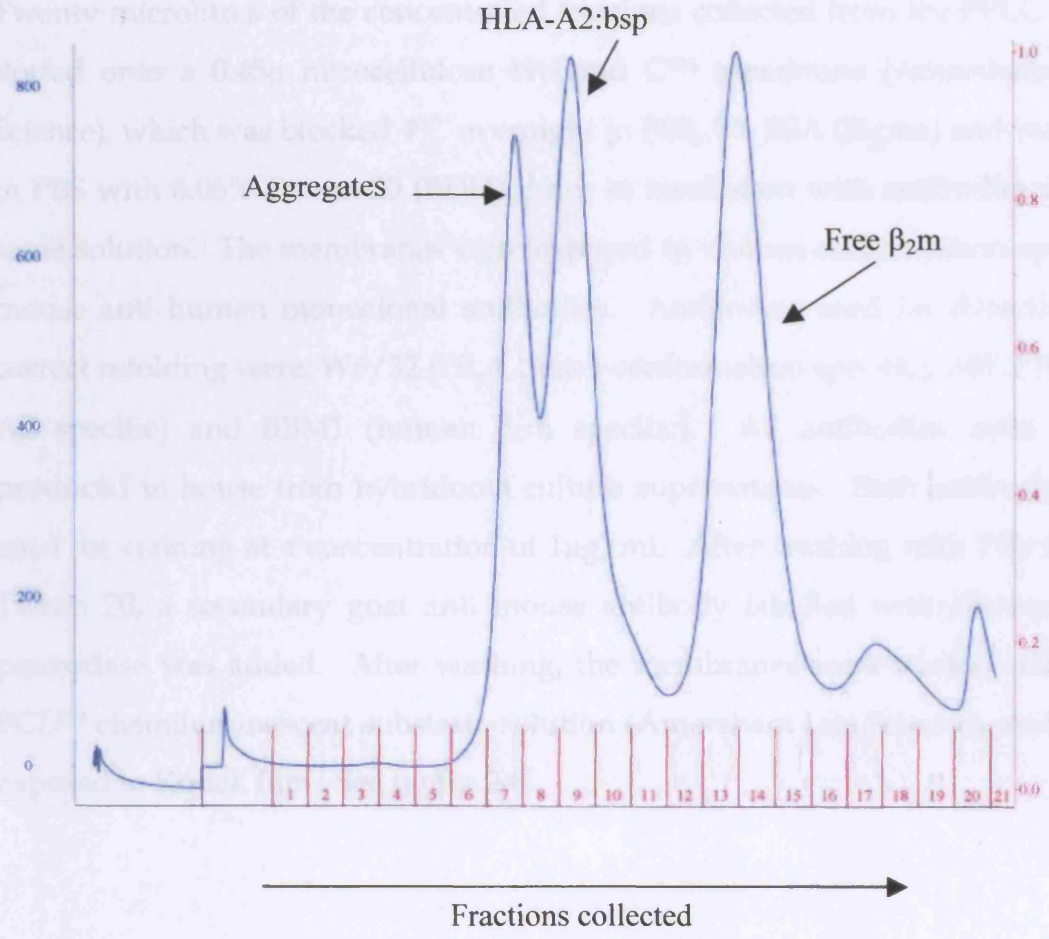
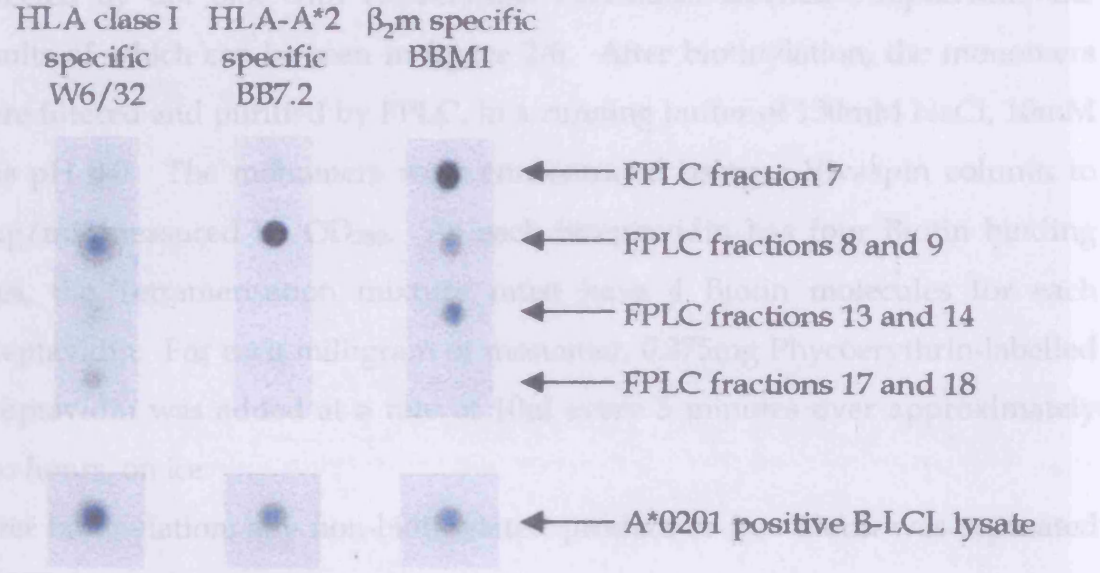


Figure 2-5. FPLC Purification of monomers. Each fraction was collected and those contained in the same peak were pooled. A 200µl sample was taken for dot blotting and the remaining fractions were concentrated ready for biotinylation.

Dot blotting for the detection of correctly folded proteins

Twenty microlitres of the concentrated fractions collected from the FPLC were dotted onto a 0.45μ nitrocellulose Hybond C™ membrane (Amersham Life Science), which was blocked 4°C overnight in PBS, 5% BSA (Sigma) and washed in PBS with 0.05% Tween 20 (BDH), prior to incubation with antibodies in the same solution. The membranes were exposed to various conformation-specific mouse anti human monoclonal antibodies. Antibodies used for detection of correct refolding were; W6/32 (HLA class I conformation specific), BB7.2 (HLA-A2 specific) and BBM1 (human β_2m specific). All antibodies used were produced in house from hybridoma culture supernatants. Each antibody was used for staining at a concentration of 1μg/ml. After washing with PBS 0.05% Tween 20, a secondary goat anti mouse antibody labelled with Horseradish peroxidase was added. After washing, the membranes were treated with the ECL™ chemiluminescent substrate solution (Amersham Life Science), and then exposed to Kodak film. See figure 2-6.

Figure 2-6. Confirmation of correct refolding by dot blot. Twenty microlitres of the pooled fractions that represented a peak in the FPLC purification (see figure 2-5) were dotted onto a nitrocellulose membrane. Specific monoclonal antibodies were added, with a secondary, Peroxidase-labelloed antibody, and after addition of luminescent substrate, the membranes were exposed to X-ray film.



Biotinylation and Tetramerisation

A kit was used for Biotinylation of the monomers (Avidity LLC). Bir A enzyme (Biotin protein ligase) and kit components Biomix A and Biomix B, were mixed with protease inhibitors (Pepstatin A 5µg/µl, Benzamidine 5mM, E64 10µM and Elastatinal 10µg/ml) and incubated at 30°C overnight. Biotinylation was detected by dot blot with Horseradish Peroxidase-labelled Streptavidin, the results of which can be seen in figure 2-6. After biotinylation, the monomers were filtered and purified by FPLC, in a running buffer of 150mM NaCl, 10mM Tris pH 8.0. The monomers were concentrated using a Vivaspin column to 2mg/ml, measured by OD_{280}. As each Streptavidin has four Biotin binding sites, the Tetramerisation mixture must have 4 Biotin molecules for each Streptavidin. For each milligram of monomer, 0.375mg Phycoerythrin-labelled Streptavidin was added at a rate of 10µl every 5 minutes over approximately two hours, on ice.

After biotinylation, any non-biotinylated product or free Biotin was separated by FPLC as before.

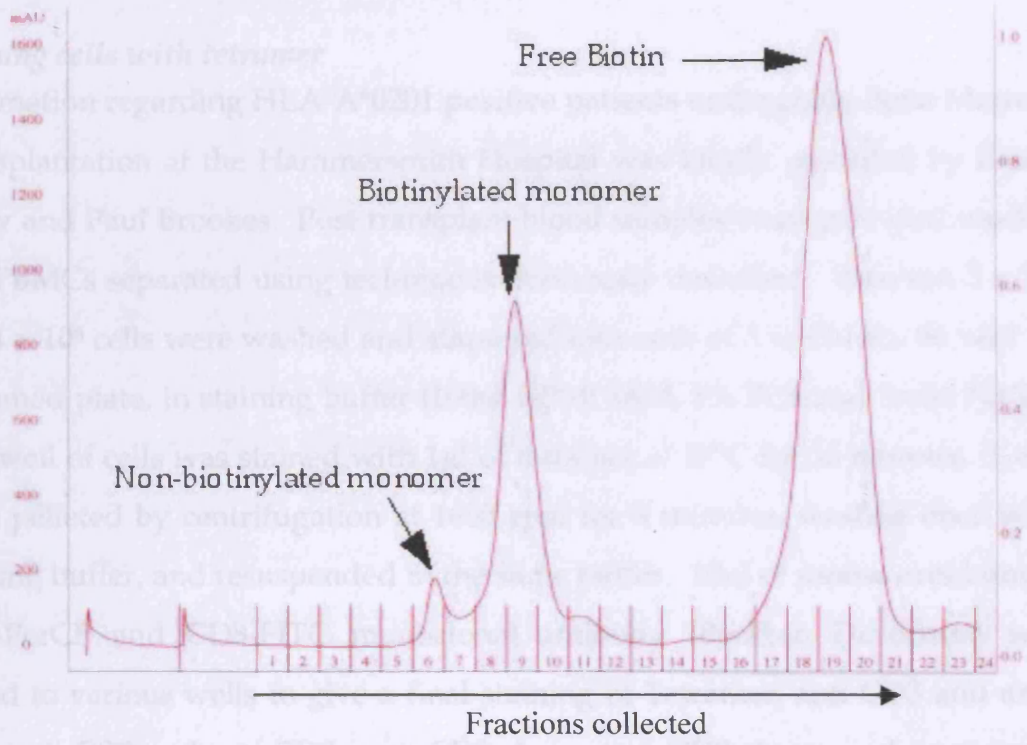


Figure 2-7. Separation of free biotin. Biotin, which had not been attached to any of the monomers with the BirA enzyme, was removed by FPLC. The peaks containing biotinylated product were collected and pooled ready for tetramerisation.

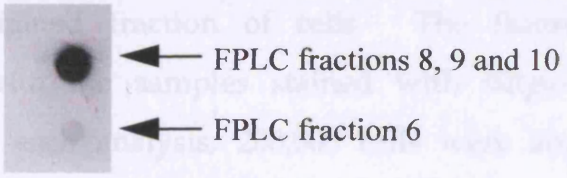


Figure 2-8. Detection of Biotinylation with Streptavidin - Peroxidase. Fractions of the monomer before and after biotinylation were dotted on the Hybond C membrane as before (with blocking carried out in Marvel™ instead of BSA) and probed with Horseradish Peroxidase labelled Streptavidin to detect biotinylated monomers. The membranes were then exposed to X-ray film.

Detection of HLA-A*0201:HA-1^H specific CTLs

Staining cells with tetramer

Information regarding HLA-A*0201 positive patients undergoing Bone Marrow Transplantation at the Hammersmith Hospital was kindly provided by Linda Casey and Paul Brookes. Post transplant blood samples were provided weekly and PBMCs separated using techniques previously described. Between 5 x 10^5 and 1 x 10^6 cells were washed and aliquoted into each of 5 wells of a 96 well V-bottomed plate, in staining buffer (100µl RPMI 1640, 5% FCS and 5mM NaN_3). One well of cells was stained with 1µl of tetramer at 37°C for 30 minutes. Cells were pelleted by centrifugation at 1600 rpm for 3 minutes, washed once with staining buffer, and resuspended in the same buffer. 10µl of mouse anti human CD3-PerCP and CD8-FITC monoclonal antibody (Beckton Dickinson) was added to various wells to give a final staining of Tetramer, anti-CD3 and anti-CD8; anti-CD3 and anti-CD8; anti-CD3 alone; anti-CD8 alone; and no staining (as a negative control). The cells were incubated with the antibodies at 4°C for 30 minutes and washed twice in staining buffer. The cells were fixed with 100µl RPMI 1640, 1% formaldehyde to prevent internalisation of antibodies and tetramer.

Analysis of Tetramer-stained cells by flow cytometry

The detectors on the Becton Dickinson FACScan™ flow cytometer were set using the unstained fraction of cells. The fluorescence detection was compensated with the samples stained with single fluorescently labelled antibodies. For each analysis, 200,000 cells were acquired and analysis of tetramer positive cells carried out on $CD8^+$ positive cells, which were previously gated on CD3.

Formation of HA-1 specific CTL

In order to test the efficacy of the HLA-A*0201/HA-1^H specific tetramers it was essential to have a positive control, in the order of an HLA-A*0201/HA-1^H specific cell line. Antigen presenting cells were required, in the order of potential dendritic cells from an HLA-A*0201/HA-1^H negative individual (HA-

1R homozygous) which could be pulsed with HA-1^H peptide and present the peptide to T cells of the same individual in an autologous manner.

Formation of potential dendritic cells

PBMCs were obtained from the peripheral blood of A*0201/HA-1H positive individuals as previously described. Potential dendritic cells were generated following a protocol described in a current study being undertaken by the European Union Concerted Action on Peptide Sensitisation (EUCAPS). On day -7, Twenty million cells per well in 3ml of Ex-Vivo medium (BioWhittaker) were plated in a six well plate, and incubated for 2 hours at 37°C at 5% CO_2. All non-adherant cells were removed by washing and retained for later use as responder cells. Cells were maintained in 2.5ml Ex-Vivo medium with 800 U/ml GM-CSF (R&D Systems) and 500 U/ml IL-4 (R&D Systems). On day -5, 2.5 ml Ex-Vivo medium with 1600 U/ml GM-CSF and 1000 U/ml IL-4 was added. On day -3, 2.5ml medium was removed and replaced with medium containing the same cytokine cocktail. On day 0, the potential dendritic cells were harvested and resuspended by washing with Ex-Vivo medium. It would have been ideal to have tested the phenotype of these cells, however, the number of cells available was insufficient to carry out this analysis.

T cell stimulation

The potential dendritic cells were resuspended in 1ml Aim-V medium (BioWhittaker) and pulsed with 50µg/ml HA-1^H peptide overnight at 37°C in 5% CO_2. One millilitre of RPMI with 10% autologous serum, 1U/ml IL-2 and 1U/ml IL-12, and 10 x 10^6 autologous PBMCs was added. On day +5, 10U/ml IL-2 was added. On day +7, autologous monocytes were procured from 10 x 10^6 PBMC's layered for 2 hours, harvested and pulsed for 2 hours with peptide, then added to the culture. This series of events was repeated until enough T lymphocyte-like cells were available for staining with tetramer.

Statistical analysis

Calculation of Kaplan-Meier Survival Curves

All survival data was calculated by the Kaplan-Meier method, using the SPSS™, version 10.0 (SPSS Inc., Chicago, IL, USA) statistical analysis program. The equation used for this analysis was:

$$P[T > t_i] = i\Pi i=1 [1 - di / ni]$$

Where $P[T \geq t_i]$ is the probability of survival to a specific time, T.

Where ${}^{i}\Pi_{I=1} [1 - d_i / n_i]$ is the cumulative product of the conditional probabilities of survival at each time point.

Where d is the number of events which occur at a particular time point and n is the risk of these events occurring at that time point, t.

Basically, the equation is worked out in stages. Initially, the time points are calculated at which an event occurred and put in chronological order, with the number of events, and censored points occurring at this each time point.

The number of patients at risk of the event occurring at that time point is calculated by the total number of patients (at the first time point at which an event occurs) minus the number of censored patients at that time plus the number of patients for which an event occurred. The probable survival at each time point is then calculated by one minus the number of events occurring at that time point over the number of patients calculated to be at risk. The probable survival is then added in a cumulative manner and plotted against each time point to give a survival curve. Confidence intervals were calculated at one year for most outcomes.

Significance Testing using the Log Rank Test

To test whether there was a significant difference between the variables tested and transplant outcome, the non-parametric Log Rank Test was utilised.

Ninety-five percent confidence intervals were used, meaning that if $p < 0.05$, there was a significant difference on outcome between the variables. Those calculations involving HLA matching were tested in a pairwise fashion with HLA matched individuals.

Regression Model

The Cox proportional hazards model was used to determine the independent effects of factors on the outcome of unrelated bone marrow transplantation. This was calculated using the SPSS™, version 10.0 (SPSS Inc., Chicago, IL, USA) statistical analysis program. The equation used was:

$$\lambda_i(t) = \lambda_0(t)\exp\{\beta_1 x_1 + \beta_2 x_2 + ... + \beta_n x_n\}$$

Where $\lambda_i(t)$ is the hazard for individual i at time t, and $\lambda_0(t)$ is an arbitrary baseline hazard.

Where x_1.....x_n are the variables in the model and β_1.....β_n are the corresponding coefficients.

The relative hazard is assumed to be constant over time in this model and by measuring the covariant against log(time) it is possible to determine whether it has a significant effect on outcome.

Estimating sample size

Estimating sample size is important in the design of a study, and the quality of the estimate ultimately depends on the quality of the information used to derive it. The information used in calculating the sample size should reflect as closely as possible the type of data that will be gathered from the study in question. Ideally, studies should be large enough to detect reliably the smallest possible differences in the primary outcome. Sample size must be planned carefully to ensure that the research time, effort and support costs invested in any study are not wasted.

Power increases with sample size, therefore, a larger sample has a greater

ability than a small sample to detect a clinically important effect if it exists. A balance is required between sample size and the factors that affect it. In order to calculate the sample size required it is necessary to have an idea of the results expected in the study. The larger the sample size, the smaller the sampling error.

The statistical null hypothesis (H_0) is the opposite to what you believe to be true.

Using statistical theory, it is necessary to show from the data collected that H_0 is false and should be rejected. This is know as Reject Support testing, because rejecting the null hypothesis supports the findings.

Result agrees with H_0 = correct acceptance

Result disagrees with H_0 = correct rejection

The type 1 error rate (α) must be <0.05 and Type 2 error rate must be <0.2 (β). Statistical power should be at least 0.8 (1 - Type 2 error rate) to detect a reasonable departure from the null hypothesis. As will be seen in this study, the sample size was too low and the experiments carried out lack the precision to provide reliable answers to some the questions it is investigating. The probability of accepting H0 is 1-α. The probablility of incorrectly rejecting H0 is β. The probablility of correctly rejecting H_0 is 1-β.

		State of the World	
		HO	H1
Decision	H_0	Correct Acceptance	Type II Error β
	H_1	Type I error α	Correct Rejection

The minimum information needed to calculate sample size for a study in which a specific event is being counted includes the power, the level of significance, the underlying event rate in the population under investigation and the size of the effect sought.

The number of participants required in each intervention group, *m*, is given by:

$$m = 2 \times \frac{[z_{(1-\alpha/2)} + z_{(1-\beta)}]^2}{\triangle^2}$$

where $z_{(1-\alpha/2)}$ and $z_{(1-\beta)}$ represent percentage points of the normal distribution for statistical significance level and power, respectively (1.96 and 0.8416), and Δ represents the standardised difference (i.e. the treatment difference divided by its standard deviation) (Rosner, 1990; Altman, 1990; Juszczak, 2003)

The power of a study is its ability to detect a true difference in outcome between the standard or control group (eg. matched) and the group including the factor to be studied (eg. mismatched). This is usually chosen to be 80%. By definition, a study power set at 80% accepts a likelihood of one in five (that is, 20%) of missing such a real difference.

The chosen level of significance sets the likelihood of detecting a variable effect when no effect exists (leading to a so-called "false-positive" result) and defines the threshold "p value". Results with a p value above the threshold lead to the conclusion that an observed difference may be due to chance alone, while those with a p value below the threshold lead to rejecting chance and concluding that the factor has a real effect. The level of significance is most commonly set at 5% (that is, p = 0.05). This means the investigator is prepared to accept a 5% chance of erroneously reporting a significant effect.

The effect of the factor in the study can be expressed as an absolute difference. That is, the difference between the rate of the event (eg. death) in the control group (eg. HLA matched) and the rate in the variable group (eg. HLA class I mismatched), or as a relative reduction, that is, the proportional change in the event rate with the variable. If the rate in the control group is 63% and the rate in the variable group is 42%, the absolute difference is 21%; the relative reduction with the variable is 21%/63%, or 33%.

Checklist for determining sample size for a definitive study

1. Estimate the event rate in the control group by extrapolating from a population similar to the population expected in the study.
2. Determine, for the primary outcome, the smallest difference that will be of clinical importance.
3. Determine the clinically justifiable power for the particular study (80%).
4. Determine the significance level or probability of a "false positive" result that is scientifically acceptable ($p = 0.05$).

The data produced in this pilot study may be used in a sample size power calculation in order to estimate the number of samples required for definative studies.

Chapter 3

Method Development

Introduction

In order to detect the factors that affected the outcome of unrelated bone marrow transplantation, a system was required for the collection, storage and processing of the samples. Also, when using techniques for analysis of these samples, some modifications were required in order to obtain ultimate results. This chapter describes some of the problems found in the initiation and progression of the study, and how they were overcome.

Sample Collection and Storage

Project initiation

Blood samples were collected pre- unrelated donor bone marrow transplant from transplant recipients and their unrelated donors. It was established which transplant centres had carried out transplants using unrelated donors provided by the Anthony Nolan Bone Marrow Trust Register over the previous two years (1994 -1996), by studying transplant records on the Anthony Nolan Bone Marrow Trust (ANBMT) database. The Consultant Haematologist of each Transplant Centre and Harvest Centre was contacted (see Appendix II, Letter 1), given details of the study and asked whether they would be willing to participate. The Consultant was asked to nominate an internal contact that had direct interaction with the recipient or donor and was able to take a blood sample, this was usually the Bone Marrow Transplant Co-ordinator.

A project proposal (see Appendix III), and copies of all letters, and forms, were approved by the Royal Postgraduate Medical School and Royal Free Hospital

Ethics Committees. The responsibility lay with the transplant centre to explain the details of the study to the recipient. An Ethically Approved Consent Form, which gave a brief explanation to the patient was enclosed with each sample collection box to be signed by the patient or donor and the phlebotomist, to verify informed consent (see Appendix IV).

Sample Collection

Collaboration was established between the ANBMT Operations Department and the Research Institute. Details of transplants between donors and recipients resident in the UK were then routinely forwarded from the Operations Department to the Research Institute. Blood samples were requested from the harvest centre by the Operations Department at the time of donor medical examination, usually 14 days before harvest. Patient samples were requested by letter from myself and obtained from the transplant centre when the patient was admitted to begin their pre-transplant conditioning regimen. Transport boxes with pre-paid return envelopes, the relevant forms described, and blood sample collection tubes containing anticoagulant were sent with the sample request.

All blood samples were originally collected with Sodium-Heparin (10 units per ml of blood); however, it was discovered that the cells sent by post in this anticoagulant had a poor viability. On investigation of other research organisations that had attempted sample collection by post, it was found that the Imperial Cancer Research Institute had seen similar problems, and had developed a more suitable transport medium (Marsh 1996). Subsequently, blood samples were collected in this transport medium (Tri-sodium citrate (0.6%) in RPMI 1640 (BioWhittaker), with 0.5mM β-mercaptoethanol), which was prepared at the Anthony Nolan Research Institute under sterile conditions. Use of this medium resulted in greater cell viability, although sample-to-sample variation was still inevitable. To further avoid the deterioration of the sample during transit a courier system was introduced for those hospitals providing the most samples.

Samples collected with Heparin as an anticoagulant also gave poor quality PCR

product, due to the inhibition of *Taq* polymerase, even with the use of Heparinase (Sigma) in the PCR, used to denature the Heparin. Blood to be used for DNA extraction was subsequently collected in EDTA (1.6 mg per ml of blood) as an anticoagulant which, at low concentrations, did not inhibit *Taq polymerase*, and gave improved PCR results.

Establishment of the database of clinical information

Data Collection and Storage

The fields created in the Filemaker Pro™ (FileMaker Inc, Santa Clara, CA database) reflected those of the Bone Marrow donor recruitment form (figure 3-1), the transplant form (figure 3-2), the prescription for marrow collection (figures 3-3, 3-4 and 3-5) provided to each transplant centre to follow up the patients who had received a transplant from a donor provided by the Anthony Nolan Registry. An example of the main database page for each transplant can be seen in figure 3-6.

Fields duplicated from the "Formal recruitment of a bone marrow donor" form (figure 3-1), included details about the patient's name, date of birth, transplant centre ID number, CMV status, blood group, weight, disease status (including diagnosis) and number of days conditioning. Fields were also made for the original HLA typing data of the patient and the donor (see figure 3-6).

Fields were made for details of the transplant centre and transplant coordinator contact details in a linked database, with the link based on the Transplant Centre field (figure 3-6). A field was made for the result of any CTLp analysis carried out, but was rarely used as not many centres requested this type of analysis.

Fields duplicated from the transplant form (figure 3-2) included donor name, donor number (from the Anthony Nolan register), whether the donor had previous pregnancies, live births or blood transfusions and the harvest date (figure 3-6).

Figure 3-1. Bone marrow donor recruitment form.

THE ANTHONY NOLAN BONE MARROW TRUST
THE ROYAL FREE HOSPITAL,

FORMAL RECRUITMENT OF A BONE MARROW DONOR

PATIENT DATA:

Patient name:	Date of birth:	Sex:
Patient ID #:	Blood group:	CMV:
Registry:	Height:	Weight (kg):
Diagnosis:	Current disease status:	

Serology	A	A	B	B	C	C	DR	DR	DQ	DQ
DNA Typing:										

A	B	C	DRB1	DRB 3/4/5	DQB1	DPB1
A	B	C	DRB1	DRB 3/4/5	DQB1	DPB1

TRANSPLANT CENTRE:

Hospital:	Contact name:
Address:	Fax no:
	Phone no:
Preferred harvest date:	
Alternative dates: (1)	(2)
Number of days of conditioning prior to transplant: (Conditioning of patient should not be undertaken until the registry has confirmed the donor to be medically fit and the results of all screening tests are known and have been reported to the transplant centre).	

DONOR DATA:

Donor ID#:						Typing date:				
Serology	A	A	B	B	C	C	DR	DR	DQ	DQ
DNA Typing:										

A	B	C	DRB1	DRB 3/4/5	DQB1	DPB1
A	B	C	DRB1	DRB 3/4/5	DQB1	DPB1

CELLULAR ASSAY RESULTS:

MLC - Not performed:	MLC - performed: GvH HvG RR % Date:	
CTLp	HTLP	Other

Signature:	Date:

Figure 3-2. Transplant form.

TRANSPLANT FORM

DONOR			**PATIENT/DONEE:**
Name:			Reference A
Date of Birth:			Reference B
Address:			Diagnosis:
Tel. Day:			Status (= coded age)
Tel. Home:			Conditioning:
Donor No.			
Occupation:			Marrow Request: **As attached**
Pregnancies:	Live Births:		
Previous Blood Transfusions:			
Smoker:			
GP:			
Address:			
Tel:			
HARVEST CENTRE:			**TRANSPLANT CENTRE:**
Doctor:			
Hospital:			Hospital:
Tel:			
Date of Harvest:			Invoice No.
Date of Admittance:			Invoice Date:
Pick Up/Delivery Arrangements: To be advised			Signed: Date:

The pages of the database that contained follow-up information, were duplicates of all the fields in the follow-up forms (figures 3-3 to 3-5). Each form had a box linked to the main database page (figure 3-6), which contained the survival status of the patient. This allowed knowledge of whether the patient was alive or not without needing to scroll through each update sheet.

Extra fields were added to the main database page in order to track the collection of samples and consent, and also information about the samples when they had been processed (figure 3-6). Information regarding the weight of the donor, CMV status and blood type were collected from records at the Anthony Nolan Trust, or by approaching the harvest centre directly.

FOLLOW-UP OF RECIPIENT HAVING RECEIVED BONE MARROW FROM AN UNRELATED DONOR PROVIDED THROUGH THE ANTHONY NOLAN RESEARCH CENTRE (ANRC)

SIX WEEKS POST TRANSPLANTATION

RECIPIENT LAST NAME:	RECIPIENT FIRST NAME:	ANRC REF:
RECIPIENT LOCAL REF:	TRANSPLANT CENTRE:	PHYSICIAN:
PRIMARY DIAGNOSIS:	TRANSPLANT DATE: DAY/MONTH/YEAR	ANRC DONOR REF:

Please provide the following information:

1. Is the recipient alive?	YES/NO	If DECEASED please state: (i) date of death: .... (ii) principal cause of death: .... (iii) contributory cause(s) of death: ....

2. What was the disease status at the time of transplant?	....

3. Did the marrow engraft?	YES/NO	If YES, please give date of engraftment: (3rd day of 3 consecutive readings of NTS > 0.5×10^9/L) .... DAY/MONTH/YEAR

4. Has the recipient received a further bone marrow transplant or lymphocyte infusion (autologous or otherwise)?	YES/NO	If YES, please specify source of marrow or lymphocytes: .... DATE: .... DAY/MONTH/YEAR

FORM COMPLETED BY .... DATE .... (DAY/MONTH/YEAR)

Figure 3-3. Patient update form – 6 weeks post transplant.

Figure 3-4. Patient update form – 3 months post transplant.

(page 1) FOLLOW-UP OF RECIPIENT HAVING RECEIVED BONE MARROW FROM AN UNRELATED DONOR PROVIDED THROUGH THE ANTHONY NOLAN RESEARCH CENTRE (ANRC)

THREE MONTHS OR 100 DAYS POST TRANSPLANTATION

RECIPIENT LAST NAME:	RECIPIENT FIRST NAME:	ANRC REF:
RECIPIENT LOCAL REF:	TRANSPLANT CENTRE:	PHYSICIAN:
PRIMARY DIAGNOSIS:	TRANSPLANT DATE: DAY/MONTH/YEAR	ANRC DONOR REF:

Please provide the following information:

1. Is the recipient alive?	YES/NO	If DECEASED please state: (i) date of death: . . . . . . (ii) principal cause of death: . . . . . . (iii) contributory cause(s) of death: . . . .
2. Please give brief details of conditioning:	Chemotherapy:	
	Radiotherapy:	Dose of TBI . . . . . . Rate . . . . . . No. of fractions . . . . .
	Additional:	(e.g. Campath IgG or ATG) . . . . .
3. Was the bone marrow T-cell depleted?	YES/NO	If YES, please state: (i) method . . . . . . (ii) approximate no. of T-cells removed/Kg . . . . .
4. What post transplant immunosuppression was used?		

(Page 2) **THREE MONTHS OR 100 DAYS POST TRANSPLANTATION** (continued)

5. Did the marrow engraft?	YES/NO	If YES, please give date of engraftment: (3rd day of 3 consecutive readings of NTS > 0.5 x 10^9/L) . . . . . DAY/MONTH/YEAR
6. Has recipient experienced recurrence of the original disease?	YES/NO/NOT APPLICABLE	If YES, please give date: . . . . . DAY/MONTH/YEAR
7. Has the recipient experienced acute GvHD?	YES/NO	If YES, to what extent? (please grade according to table) . . . . .
8. Has the recipient experienced serious infections? (e.g. Interstitial pneumonia, Cerebral toxoplasmosis, Invasive fungal infection)	YES/NO	If YES, please specify: (and whether ongoing or resolved) . . . . .
9. i) Is this the recipient's first bone marrow transplant?	YES/NO	If NO, please specify: . . . . .
ii) Has the recipient received a further bone marrow transplant or lymphocyte infusion?	YES/NO	If YES, please specify source of marrow or lymphocytes (autologous or otherwise): . . . . .
11. Please state Karnofsky/Lansky rating:		. . . . .
12. Is patient hospitalized?	YES/NO	If out-patient please state date of patient's Last visit . . . . . Next visit . . . . .

FORM COMPLETED BY . . . . . DATE . . . . . (DAY/MONTH/YEAR)

Figure 3-5. Patient update form – 6 months post transplant.

(page 1) FOLLOW-UP OF RECIPIENT HAVING RECEIVED BONE MARROW FROM AN UNRELATED DONOR PROVIDED THROUGH THE ANTHONY NOLAN RESEARCH CENTRE (ANRC)

SIX MONTHS POST TRANSPLANTATION

RECIPIENT LAST NAME:	RECIPIENT FIRST NAME:	ANRC REF:
RECIPIENT LOCAL REF:	TRANSPLANT CENTRE:	PHYSICIAN:
PRIMARY DIAGNOSIS:	TRANSPLANT DATE: DAY/MONTH/YEAR	ANRC DONOR REF:

Please provide the following information:

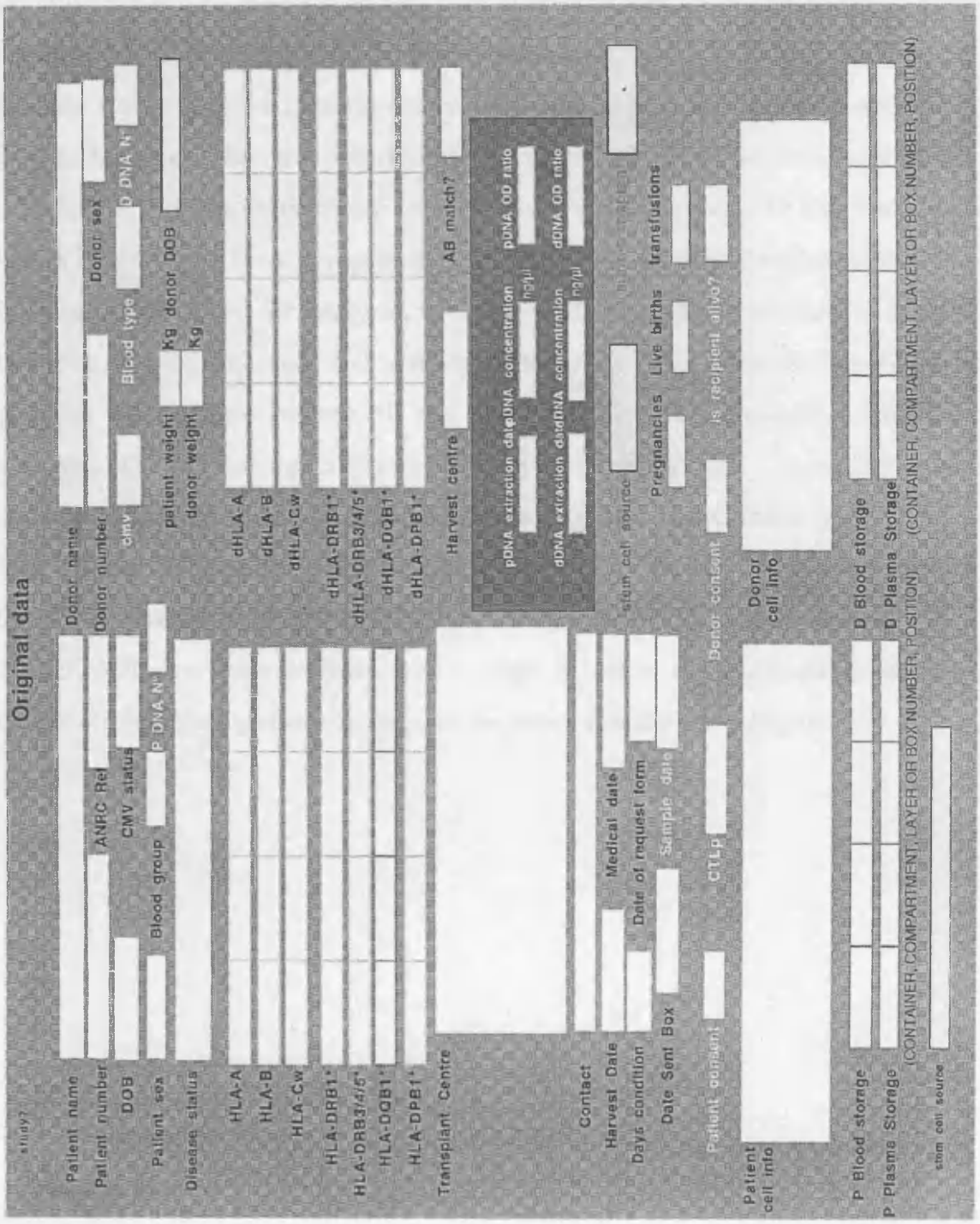
1. Is the recipient alive?	YES/NO If DECEASED please state: (i) date of death: . . . . . . (ii) principal cause of death: . . . . . . (iii) contributory cause(s) of death: . . . .
2. Has recipient experienced graft failure?	YES/NO If YES, please give date: . . . . . . DAY/MONTH/YEAR
3. Has recipient relapsed?	YES/NO/NOT APPLICABLE If YES, please give date: . . . . . . DAY/MONTH/YEAR
4. Has the recipient experienced chronic GvHD?	YES/NO If YES, to what extent? . . . .

(Page 2) **SIX MONTHS POST TRANSPLANTATION** (continued)

5. Has recipient experienced any infections since the 3-month report?	YES/NO If YES, please specify: (and whether ongoing or resolved) . . . .
6. Has the recipient received a further bone marrow transplant or lymphocyte infusion (autologous or otherwise)?	YES/NO If YES, please specify source of marrow or lymphocytes: . . . . DATE: . . . . . . DAY/MONTH/YEAR
7. Please state Karnofsky/Lansky rating:	. . . . .
8. Is patient hospitalized?	YES/NO If NO, please state date of patient's: Last visit . . . . . Next visit . . . . .

FORM COMPLETED BY . . . . . DATE . . . . . (DAY/MONTH/YEAR)

Figure 3-6. Main database page completed for each transplant.



The original follow up form was sent to the transplant centre 6 weeks post transplant (figure 3-3), and different forms sent at 3 months (figure 3-4) and 6 months (figure 3-5) post transplant, then every following 6 months with similar forms. Once the hard copy forms were returned, by fax or mail, the information provided was entered into the relevant fields in the database. It was soon found, however, that the information given on these forms was sometimes incomplete, and the consecutive forms did not always agree. To this end, once RSCA analysis had been completed, a further single form that encompassed the information required for analysis, was sent to the transplant centres for the 138 transplants to be included in this study (figure 3-7). This form was created as a page in the database where all the most up to date information could be accessed without having to look at many different pages. From the fields present in the main database page (figure 3-6) and the Clinical data request form (figure 3-7), data was extracted electronically into the computer program SPSS™, version 10.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis.

The clinical data request form was a page made in the database to try and collect all the missing data required at the time of study close (figure 3-7).

Figure 3-7. Clinical data request form.

THE ANTHONY NOLAN BONE MARROW TRUST

For the purpose of a research project, looking at molecular typing of donor-recipient pairs, being undertaken at the Anthony Nolan Bone Marrow Trust by Professor Alejandro Madrigal, the following information is needed.

Please fill in the blank spaces and return as soon as possible to:

Senior Lecturer in Haematology
Royal Free Hospital

Fax:

p DNA number

Patient name

Patient number

Hospital

BMT Date

Contact

Diagnosis

Disease stage

Radiotherapy conditioning

Additional conditioning

T cell depletion of graft

Chemotherapy conditioning

details of infections

Date of engraftment or days to engraftment (day neutrophils >0.5)

Acute GVHD

Grade

date of graft rejection

date of relapse, molecular and cytogenetic

Is recipient alive?

date and cause of death

Chronic GVHD and extent

Methods used to validate and maintain quality of the data

The clinical data collected for the final set of 138 patients was manually validated when first entered into the FileMaker Pro database, which was possible as the data set was relatively small. Data was deemed to be correct if the information was obtained in writing from the transplant centre. A sanity check was carried out to ensure dates were consecutive, for example, birth date occurred before transplant date, and date of last follow up or relapse occurred after the transplant date. Also, that the age of the donor was greater that 18 and any categorical data was coded correctly. All data manually entered into the database was checked by a second person. Once the data was transferred into SPSS for analysis, electronic validation was carried out, some examples of which are described in table 3-1.

Table 3-1. Examples of electronic validation of data in SPSS database.

Field	Definition	Data Type	Validation Rule
PATIENTID	Unique patient number	Number	Must be a whole number and UNIQUE per patient forming a primary KEY
DONORID	Unique donor number	Number	Data Type set to number to ensure numeric values are entered
HARVEST	Harvest Date	Date/Time	DateDiff("D",[HARVEST],[Date_DEA])>0, this explicitly ensures that the date within the HARVEST field is greater than that of the DATE_DEA. The algorithm calculates the number of days for HARVEST and DATE_DEA and ensures that HARVEST is greater than that of the DATE_DEA, thus allowing valid entries.
DOBP	Date of birth of patient	Date/Time	Where (DOBP > 18) AND (DOBP < HARVEST) Format([dobp], DDMMYYYY)
PATAGE	Age of patient at transplant	Number	Data Type set to number to ensure numeric values are entered. DateDiff("Y",[DOBP],[HARVEST]) calculates the difference in years between DOBP and the bone marrow transplant date.
PAT<>27	If patient was greater or less than mean age at transplant	Number	Populated with 1 OR 0. If [PATAGE] <=27 then [PATRANGE] = 0 Else [PATRANGE] = 1.
DOBD	Date of birth of donor	Date/Time	Where [DOBD] >=18 years before [HARVEST] AND [DOBD] <[HARVEST].
DONAGE	Age of donor at transplant	Number	Data Type set to number to ensure numeric values are entered. DateDiff ("Y",[DOBD],[HARVEST]) calculates the difference in years between DOBD and the bone marrow transplant date.
DON<>36	If donor was greater or less than mean age at transplant	Number	Populated with 1 OR 0. If [DONAGE] <=36 then [DONRANGE] = 0 Else [DONRANGE] = 1.
DATE_ENG	Date of engraftment	Date/Time	Where [DATE_ENG] > [HARVEST] AND [DATE_ENG] < [DATE _DEA]

Table 3-1 continued...

ENGRAFTD	Number if days from BMT to engraftment	Number	DateDiff("D",[HARVEST],[Date_ENG])>0, this explicitly ensures that the date within the HARVEST field is greater than that of the DATE_ENG. The algorithm calculates the number of days for HARVEST and DATE_ENG and ensures that HARVEST is greater than that of the DATE_ENG, thus allowing valid entries.
DATE_AGV	Date of acute GvHD diagnosis	Date/Time	Where [DATE_AGV] > [HARVEST] AND DateDiff("D",[HARVEST],[Date_AGV])<=100
DAYS2AGV	Days from BMT to acute GvHD diagnosis	Number	DateDiff("D",[HARVEST],[Date_AGV]), this explicitly ensures that the date within the HARVEST field is greater than that of the DATE_AGV. The algorithm calculates the number of days difference between fields.
DATE_CGV	Date of chronic GvHD diagnosis	Number	DateDiff("D",[HARVEST],[Date_CGV]), this explicitly ensures that the date within the HARVEST field is greater than that of the DATE_CGV. The algorithm calculates the number of days difference between fields.
DAYS2CGV	Days from BMT to chronic GvHD diagnosis	Number	Where [DATE_CGV] > [HARVEST] AND DateDiff("D",[HARVEST],[Date_CGV])>100
RELAPSE	Date of relapse	Date/Time	Where [RELAPSE] > [HARVEST] AND [RELAPSE] < [DATE_DLI]
DAYS2REL	Days from BMT to relapse	Number	DateDiff("D",[HARVEST],[RELAPSE]) determines the number of days to relapse.
DEATHSTA	Is patient alive?	Text	List box created to ensure only values of; "0";"1"; where 0 = alive and 1 = dead; regarding patient status.
DATE_DEA	Date of last follow up	Date/Time	Where [DATE_DEA] >[HARVEST]
TIMEDEAT	Days from BMT to last follow up	Number	DateDiff("D",[HARVEST],[DATE_DEA]) determines the number of days to follow up.

One of the main problems with collecting clinical data in this manner was that it required the transplant clinician to have instant access to the data, retrospectively. As most transplant centres do not have a Data Manager employed, to research this information would mean returning to the patient's notes, and some clinicians were not able to do this (even with repeated requests from Dr Mike Potter, Haematology Consultant at the Royal Free Hospital), as they were so busy. The collection of clinical data is a major problem in all studies of this nature. Due to these problems the information returned was still not as detailed as required from most transplant centres. The ideal situation would be if I could have gone to the centre and access the notes myself, but as I am not a qualified clinician, I was not allowed to access clinical data in this manner. To avoid this problem in the future, this project has now been undertaken by a Haematology Registrar employed at the Anthony Nolan Research Institute. However, problems accessing clinical data are ongoing as some transplant centres do not allow access to their patient files.

Experimental Method Development

DNA extraction

Most of the analyses carried out during this project involved the use of genomic DNA extracted from donor and recipient whole blood samples. Due to the high throughput of samples expected it was originally thought that too much time would be taken in using standard methods, and various DNA extraction kits were employed. Kits used were advertised to give high yield, good quality DNA from whole blood, these included QIAmp DNA Blood Mini Kit™ (Qiagen) and Generation Capture Column™ (Flowgen). Both kits used a column system where 0.2ml blood sample was loaded into the top of the column in a high salt solution which lysed cells and optimised binding of the nucleic acids to the matrix in the column. Washing was carried out with ethanol based solutions and then the sample eluted in sterile distilled water.

The DNA obtained from some patient samples using both kits was of low yield and quality (determined by optical density at OD_{260} and OD_{280}, Chapter 2),

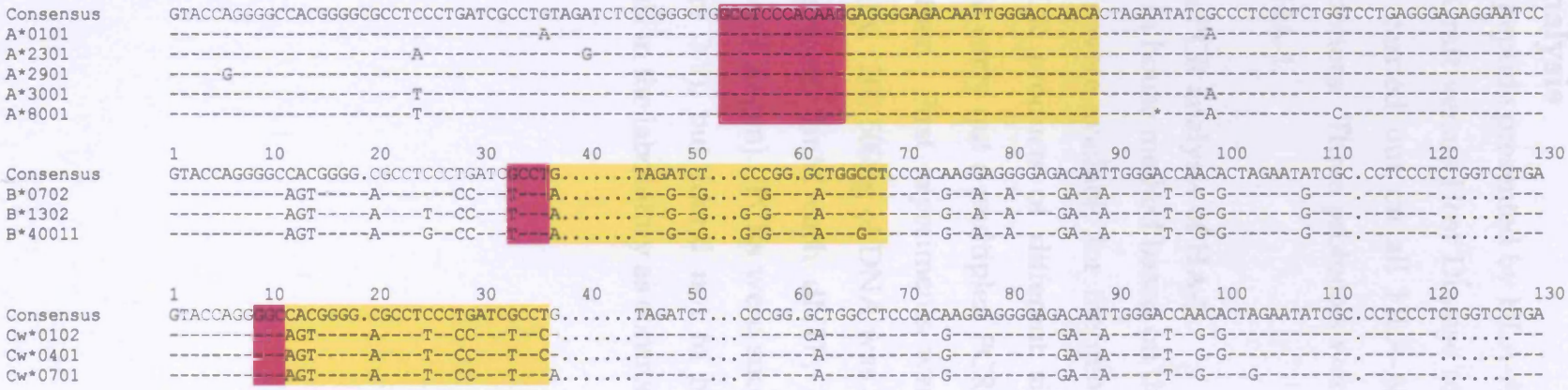
although experiments with fresh and frozen samples from healthy donors gave good quality results with a high yield. The samples that gave the lowest quality DNA were those that had been in transit for more than 24 hours, which was unavoidable. Experiments were carried out comparing extraction of the same samples with the kits and the long "salting-out" technique described in Chapter 2 (Miller *et al.* 1988). It was found that using the longer, in house technique gave better results than either of the kits for problem samples. This was due to the flexibility of the "salting-out" technique, where the quality of the product can be assessed after every stage and steps taken to improve quality. For, example, during transit some of the red blood cells in the sample adhered to the mononuclear cells. Using the kits, these residues were probably not removed as the white cell and red cell lysis occurred at the same time. Using the "salting-out" technique, the red cell residues were removed thoroughly with lysis buffer (see Chapter 2) before the white cells were lysed, giving a cleaner product. For samples that still had some contamination with protein, purification was carried out using GFX columns (Pharmacia Biotech).

PCR troubleshooting

HLA class I typing using RSCA required PCR amplification of genomic DNA extracted from donor and recipient blood samples. The expected length of the PCR products were 979, 940 and 909 respectively. During the development of the RSCA technique we had used HLA class I locus specific primers for the amplification of genomic DNA (Cereb *et al.* 1995), with great success. The DNA used in the standardisation of the technique was extracted from many B lymphocyte cell lines provided by the 12th International Histocompatibility Workshop of the World Health Organisation (Marsh *et al.* 1998). However, when the same amplification protocol was used with DNA extracted from the whole blood of recipients and donors in this study, the results were variable, even though the DNA was of reasonable quality. Various parameters of the PCR were tested, including salt concentration, amount of dNTP and brand and quantity of *Taq polymerase*. But, none of these parameters gave consistently improved results for recipient and donor samples. I studied the sequence of the

primers used in the HLA class I amplifications with the hope of lengthening one of the primers to improve annealing in the PCR reaction. In the primers described (Cereb *et al.* 1995), specificity is dictated mainly by the sense primer, therefore, the antisense primer was chosen as a vehicle for the extra nucleotides to be added. In order to maintain the promiscuity of the locus-specific primers, only a small number of nucleotides could be added, without specifically excluding some alleles (see figure 3-8). The sequences of these primers can be seen in Appendix I.

Figure 3-8. Position of extended antisense primers for HLA class I PCR. The antisense primers for the group specific PCR of HLA-A, -B and -C were extended at the 3' end to aid annealing during the PCR reaction. In this figure, the original primer position can be seen in yellow and the extension in pink. Primer sequences are listed in Appendix I.



HA-1 Analysis

HA-1 is a peptide presented by HLA-A*0201 molecules and is a minor antigenic target in Graft versus Host Disease (den Haan *et al.* 1998). Sequence Specific PCR was carried out on all HLA-A*0201 positive patients and their HLA identical donors. Three protocols were developed in order to type for the two alleles of HA-1.

Multiplex PCR analysis of HA-1

Firstly an in house method based on that described (Wilke *et al.* 1998). Two sets of primers were available for the genomic detection of the HA-1 alleles, both giving PCR products of different sizes. It would therefore ultimately be possible to carry out a multiplex PCR with each band being a positive control for the other. First experiments were carried out with each set of primers individually. 200-500ng of DNA was amplified in 1X KCl buffer (Qiagen), 1.25 µl each primer, 1mM each dNTP, 1.25mM $MgCl_2$ and 0.025 units of *Taq* polymerase (Qiagen). PCRs were successful when using B-LCL DNA controls (see figure 3-9), but found not to be reproducible when using DNA from individuals in the laboratory as controls.

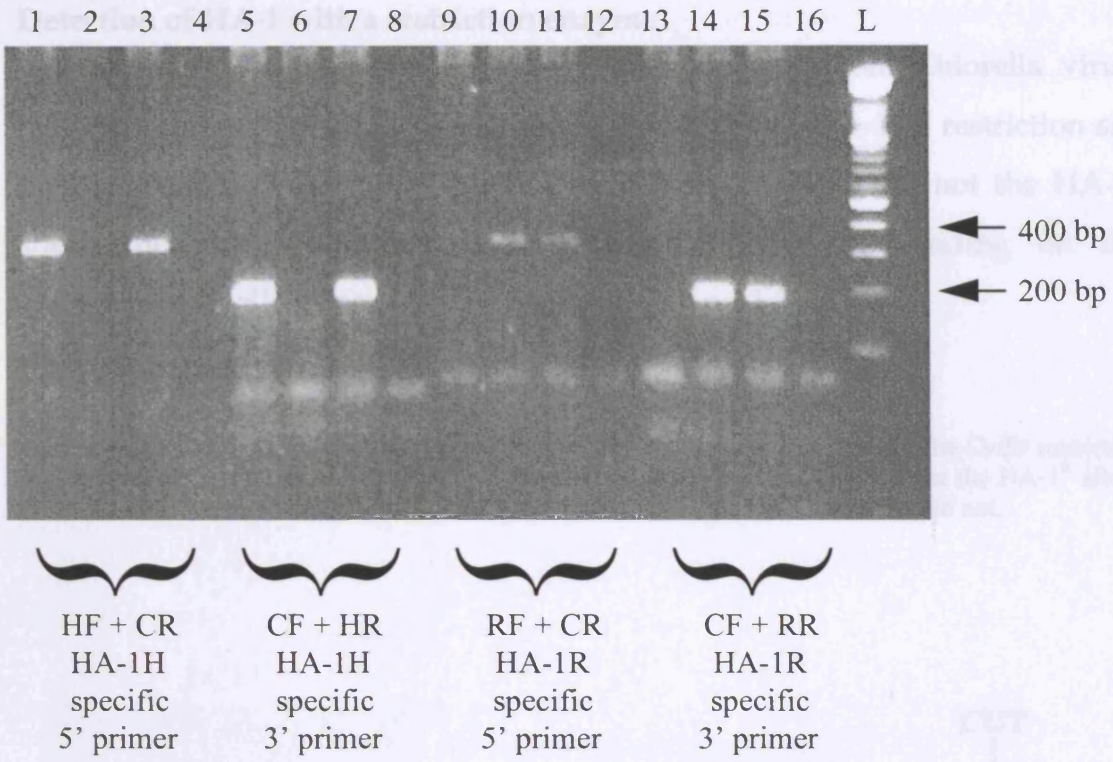


Figure 3-9. Amplification of samples with in house HA-1 specific primers. Lanes 1, 5, 9 and 13 contain amplified DNA from B-LCL KAS011 (HA-1^H, HA-1^H), lanes 2, 6, 10 and 14 contain amplified DNA from B-LCL BTB (HA-1^R, HA-1^R) and lanes 3, 7, 11 and 15 contain amplified DNA from B-LCL AMALA (HA-1^H, HA-1^R). Lanes 4, 8, 12 and 16 contain distilled water as a negative control.

Detection of HA-1 with a restriction enzyme

The second protocol employed a restriction enzyme, from Chlorella virus-infected green algae, *CviRI* (Megabase Research Products). The restriction site for the enzyme (TGCA) was present in the HA-1^H allele and not the HA-1^R allele, meaning a different pattern would be seen depending on the combination of alleles present (see Figures 3-10 and 3-11).

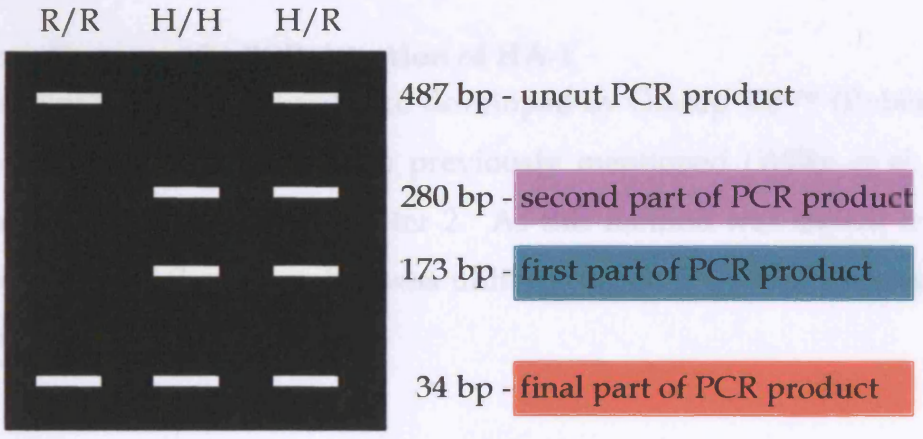
Figure 3-10. The use of *Cv1RI* restriction enzyme for the detection of HA-1. The *CviRI* restriction enzyme specific cutting motif is present in the sequence of the HA-1^H allele, but not the HA-1^R allele, therefore it was expected that the HA-1^H allele would be cut and the HA-1^R allele would not.

Figure 3-11. Expected cutting pattern of the *CviRI* restriction enzyme when generic HA-1 PCR product was used as a target. (a) cutting of the genomic DNA sequence and (b) the expected pattern seen when DNA homozygous for HA-1^{R} and HA-1^{H} were cut and when a heterozygous sample was cut.

(a)

GTGCTGCCTCCTGGACACTGGGCCGGAGCCACCGGAGCCCACTGACA
GCCGCCAGCCCGGGCGAGCTGCCCACCGAGGGTGCCGGCCCGGACGTC
GTCGAGGACATCTCCCATCTGCTGGCGGACGTGGCCCGCTTCGCTGAG
GGCCTTGAGAAACTTAAGGAGTGTGTGCTGCATGACGgtgagagccacgggg
acaccgaggcctgggtggaagacagagccagacccaagggaggatggagggagggacttggggaggctcagaa
gggagggaggctcagatggcagggagggctgtgtggaagaggccatgacagctaaggctctgagggatgtgtag
gagtttggtgggggagtccctgagcgtcactggctcaagagggtgcccactttatttttttaaaggatctgatggcaa
ttaggagggaaaggcagaggaaatgtcccatgcacaggctcagaaacacggaaacagagaatgcat

(b)

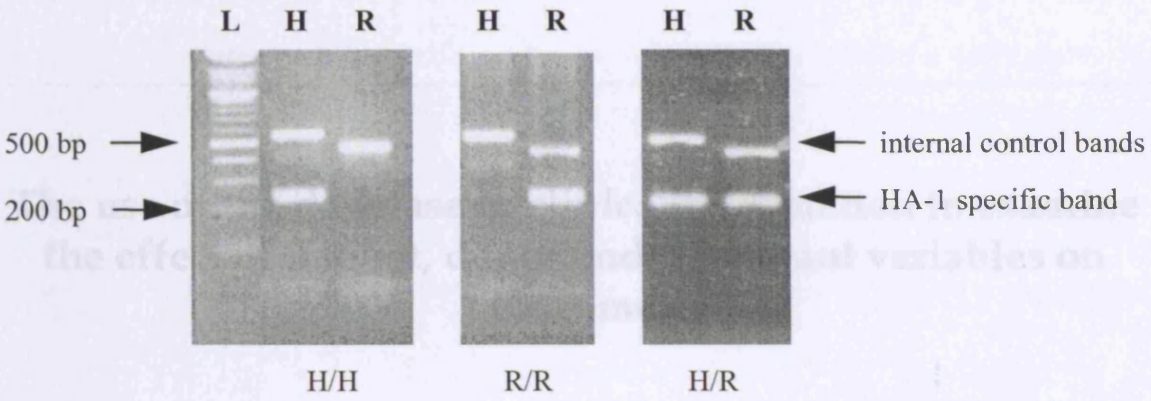


Generic amplification of the HA-1 alleles utilised generic primers capable of amplifying both HA-1 alleles. Two hundred nanograms of DNA was amplified with 1X Qiagen KCl buffer, 12.5pmol each primer, 200μM each dNTP, 4mM $MgCl_2$ and 0.75 units of *Taq* polymerase (Qiagen). To confirm the presence of amplification product, 3μl from each 25μl PCR reaction was run on a 1.5% agarose gel in 1X TBE, at 120 volts for 45 minutes. 10μl PCR product, 1.5μl enzyme buffer (10mM Tris-HCl, 150mM KCl, 10mM $MgCl_2$, 100μg/ml BSA, pH 7.8) and 10 units of *CviRI* were incubated at 37°C for 4 hours and then loaded onto a 2% agarose gel and run at 150 volts for 45 minutes. Although in theory, this protocol seemed comprehensive, in practice it was found that cutting efficiency of the enzyme was not 100% in our hands and it would therefore be difficult to detect a HA-1^H homozygous sample.

Sequence specific PCR detection of HA-1

The final protocol utilised a kit developed by Olerup SSP™ (Robbins Scientific) containing the same primers previously mentioned (Wilke *et al.* 1998). This method is described in Chapter 2. As this method was shown to be the most robust of the three tested, it was utilised for all HA-1 typing performed in this study (see figure 3-12).

Figure 3-12. Sequence specific PCR of the HA-1 alleles. Represented are a molecular size marker (L), a sample homozygous for HA-1^H, a sample homozygous for HA-1^R and a heterozygous sample.



Chapter 4

The use of the database of clinical information to examine the effect of patient, donor and transplant variables on outcome

A clinical database was established for the storage of patient and donor demographic data and clinical data corresponding to the transplant. The database was designed to contain data to be used for the analysis of the outcome of unrelated transplants performed with donors from the Anthony Nolan Register. Presented here is a primary analysis to demonstrate the type of statistical investigations that can be carried out with the data collected. There are limitations to the conclusions that can be drawn from the data due to the small number of transplants involved (n = 138) and for this reason it can only be considered an exploratory analysis.

Transplant and sample details

The number of transplants carried out with Anthony Nolan donors for patients located in the UK over the study time period (8 June 1996 - 3 September 2000) was 608. For 576 of these transplants, information was entered into the database. The remaining 32 transplants carried out were at transplant centers that had not yet agreed to participate in the study at the time that transplant was to take place, meaning a sample was not requested. 361 of the donors gave informed consent to participate in the study (see Appendix IV - Consent Form) and provided a 50ml blood sample at their medical examination before the bone marrow harvest (at the London Clinic, University College or Royal Free Hospital harvest centres - see Appendix V). 344 of the patients gave informed

consent and provided a blood sample at a hospital visit prior to the start of their conditioning regimen. All samples were processed, giving PBMCs, plasma and DNA for later analysis. For 59 patients and 129 donors, blood samples were provided with no Consent Form. These samples were processed and the DNA, plasma and PBMCs stored with a view to obtaining consent at a later date. If consent was not received, RSCA analysis was not carried out and the samples were not used in the study. At the time of the analysis, both donor and patient had given informed consent and a blood sample for 235 transplants. Of these, 138 were finally included in the study. The number of transplants provided per transplant centre can be seen in table 4-1.

Table 4-1. The number of transplants, included in the study, carried out at each transplant centre.

Transplant Centre	Transplants recorded in database	Transplants used in study
Addenbrooke's Hospital	4	0
Birmingham Children's Hospital	18	3
Birmingham Heartlands Hospital	7	2
Bristol Royal Hospital for Sick Children	67	7
Christie's Hospital	13	3
Glasgow Royal Hospital for Sick Children	8	3
Glasgow Royal Infirmary	10	5
Great Ormond Street Hospital	33	5
Hammersmith Hospital	67	50
John Radcliffe Hospital	8	1
Kings College Hospital	31	4
Leicester Royal Infirmary	8	4
Manchester Royal Infirmary	13	5
Newcastle General Hospital	16	6
Nottingham City Hospital	40	6
Our Lady's Hospital for Sick Children	2	0
Poole Hospital	1	1
Queen Elizabeth Hospital	9	1
Royal Bournemouth Hospital	1	0
Royal Free Hospital	22	3
Royal Liverpool Childrens Hospital	5	0
Royal Liverpool Hospital	13	2
Royal London Hospital	5	1
Royal Manchester Childrens Hospital	28	4
Royal Marsden Hospital	36	6
Royal Victoria Infirmary	12	2
Sheffield Childrens Hospital	8	2
St Bartholemew's Hospital	3	0
St George's Hospital Medical School	4	0
St James' University Hospital	22	3
University College Hospital	58	7
University Hospital of Wales	4	2
Total	576	138

Reasons for attrition with respect to the study

The main reasons for sample attrition from the study were poor quality of patient DNA, and lack of clinical information. DNA and cells collected at the beginning of the study were of poor quality, especially from whole blood that spent longer in transit due to the location of the transplant centre. This led to a large amount of red and white cell lysis, giving poor quality of samples by the time they were processed. Most patient samples were sent by first class mail, and could take up to 5 days, and be exposed to varying ambient temperatures depending on the time of year. These cells showed poor viability, of <80% and the DNA extracted had a purity of <1.6 (as measured by the ratio of absorbance, OD_{260nm}:OD_{280nm}), and a low concentration, making it difficult to amplify for some loci, despite attempts at purification (as described previously). These problems were overcome somewhat by the introduction of courier services to some facilities and the use of transport medium as described in the Method Development chapter. Samples were processed for PBMCs, plasma and DNA, but RSCA analysis was not carried out on patient and donor samples collected after 26 November 1999 due to time constraints.

Only those samples with PCR products available for analysis for all six loci tested (HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1) were included in the study. For the 138 transplant pairs that had complete RSCA analysis, the Clinical data request form was sent out to the transplant centres to try and obtain any missing follow-up data. All data was considered valid when it was received in writing from the transplant center on the Clinical data request form (figure 3-8). Unfortunately, one of the main problems found during the study was that the transplant teams at many of the centres changed with time, and it might take one or two rounds of follow-up with no response to find the contact had changed. Many times, the study would have to be explained to the new study nurse or transplant coordinator and they were not always as familiar with the project as the original contact, meaning some follow-up data, and original details of the transplant were unavailable. Despite persistent letters, telephone calls and faxes, patients had to be included with some clinical details missing.

One of the biggest omissions was the actual date of the diagnosis of acute or chronic GvHD. Only 22 of 71 patients with acute GvHD and 37 of 55 with chronic GvHD had a date of diagnosis, therefore, analysis was carried out using binary logistic regression where acute or chronic GvHD either did or did not occur. Thirty-one patients died before day 100, and 4 patients were not followed up after 100 days and were therefore not available for chronic GvHD analysis. Another omission that affected the analysis of the data was the CMV status of the patient and the donor. Eighteen donors and 39 patients were missing their CMV status. When interaction term analysis was carried out to assess the effect of the donor and patient CMV status combined on the outcome of transplant, this limited the number of transplants that could be analysed to 99. As data was being collected retrospectively, information such as CMV status may only have been available in the patient or donor notes, which may not have been immediately accessible. The 8 patients with non-malignant disease were excluded from any evaluations involving disease relapse, as this was not relevant to their condition. In another twelve patients, the date of disease relapse was not given, and in these cases, the date of last follow-up was used as the relapse date and treated as unvalidated data. For one donor, the actual date of birth was in question; there was a difference between the information stored on the register and that provided by the harvest centre. The age of the donor was >36 years in both cases so the data could be used although it was unvalidated.

Patient, donor and transplant characteristics

The patient and donor demographic information and transplants characteristics are listed in table 4-2.

Table 4-2. Demographics and transplant characteristics.

	Total	Match (HLA-A, -B, -C, DRB1, -DQB1 & -DPB1)	Class I mismatch only (HLA-A, -B & -C)	Class II mismatch only (HLA-DRB1 & -DQB1)	Mismatch at class I and II (HLA-A, -B or -C & HLA-DRB1, or DQB1)
Number of transplants	138	60	48	19	11
Disease					
CML	72	33	22	10	7
AML	22	11	7	3	1
ALL	24	10	10	1	3
Other malignant disease	12	6	3	3	0
Other non-malignant disease	8	0	6	2	0
Age (donor/patient)					
<=36 years/<= 27 years	30	11	12	4	3
<=36 years/>27 years	38	22	9	4	3
>36 years/<= 27 years	32	9	13	7	3
>36 years/> 27 years	38	18	14	4	2
CMV status[#] (donor/patient)					
seronegative/seronegative	51	29	15	5	2
seronegative/seropositive	15	4	7	3	1
seropositive/seronegative	19	8	7	3	1
seropositive/seropositive	14	5	6	2	1
Gender (donor/patient)					
male/male	60	26	22	10	2
male/female	31	13	11	6	1
female/male	25	9	11	2	3
female/female	22	12	4	1	5
T cell depletion					
T cell depleted	101	47	35	14	5
T cell replete	37	13	13	5	6

[#]CMV status of 18 donors and 39 patients missing

Patient's age ranged from 5 months to 52 years old (mean 27 and median 29 years) and donors ranged from 21 to 54 years old (mean 36 and median 37 years). The mean of each group was used in the statistical analyses. Seventy-two patients had CML, 65 in chronic phase, 4 in accelerated phase and 3 in blast crisis. Twenty-two patients had AML, of whom 7 were in first complete remission (CR), 14 in second CR and one transplanted with relapsed disease. There were 24 patients with ALL, 4 in first CR, 17 in second CR, 2 in fourth CR and one with relapsed disease. Twelve patients had other malignant diseases, 9 with MDS, 2 with NHL and one with Myeloma. Of the remaining 8 patients in the non-malignant disease group, 3 had SCID, 2 had Hyper IgM syndrome, 2 had Hurler syndrome and one had Wiskott-Aldrich syndrome. All HLA matching results presented were determined using RSCA.

The longest time to follow-up was 1286 days, with the shortest time being 5 days (median 217 days). Data was available on 74% of patients after 100 days, 59% of patients after 200 days and 43% of patients after 1 year. Nineteen percent of patients were followed up at 2 years and 3% at 3 years.

Sixty-nine of the 138 patients (50%) were alive at the time of last follow-up. Cause of death for each patient that died is listed in table 4-3.

Table 4-3. Cause of death of patients in the study

Cause	Number of patients (%)
Regimen-related toxicity	3 (2)
GvHD	6 (4)
Organ failure	8 (6)
Relapse	24 (17)
Infections	28 (20)

Statistical Analyses

Outcome variables investigated included overall survival, disease free survival, transplant related mortality, relapse incidence and occurrence of acute and chronic GvHD. Overall survival was determined as the survival status of the patient, where death was considered an event, and if the patient was last known to be alive, the patient event was censored at the date of last follow-up.

In the calculation of disease free survival, the patient was considered to have an event if they either had a relapse or they died. All other patients were censored at the date that they were last known to be alive and disease-free. Therefore, disease free survival was considered to be a combined endpoint, with both the time to relapse or death counting as events.

Transplant related mortality was defined as the occurrence of death as a result of the transplant procedure. Patients that died from any cause except relapse were considered to have transplant related mortality. Patients who died from relapse were censored at the date of death. Causes of death are listed in table 4-3.

A patient was considered to have relapsed if molecular or cytogenetic relapse was diagnosed. When the date of relapse diagnosis was unknown, it was considered to have occurred at the date of last follow-up. Patients who did not relapse were included until the date of final follow-up and then censored.

Incidences of acute and chronic GvHD were assessed as a binary outcome. Grade 0 or I acute GvHD were scored as negative (ie. that acute GvHD had not occurred) and grades $\geq$II were scored as positive. Any occurrence of chronic GvHD was scored as positive, independent of extent. Overall survival, leukaemia free survival, transplant related mortality and relapse incidence were calculated using standard survival methods, including Kaplan-Meier plots, log rank test and Cox proportional hazards regression. Incidences of acute and chronic GvHD were assessed using chi-squared tests and logistics regression models. These statistical analyses were carried out using the computer program SPSS, version 10.0 (SPSS Inc., Chicago, IL, USA).

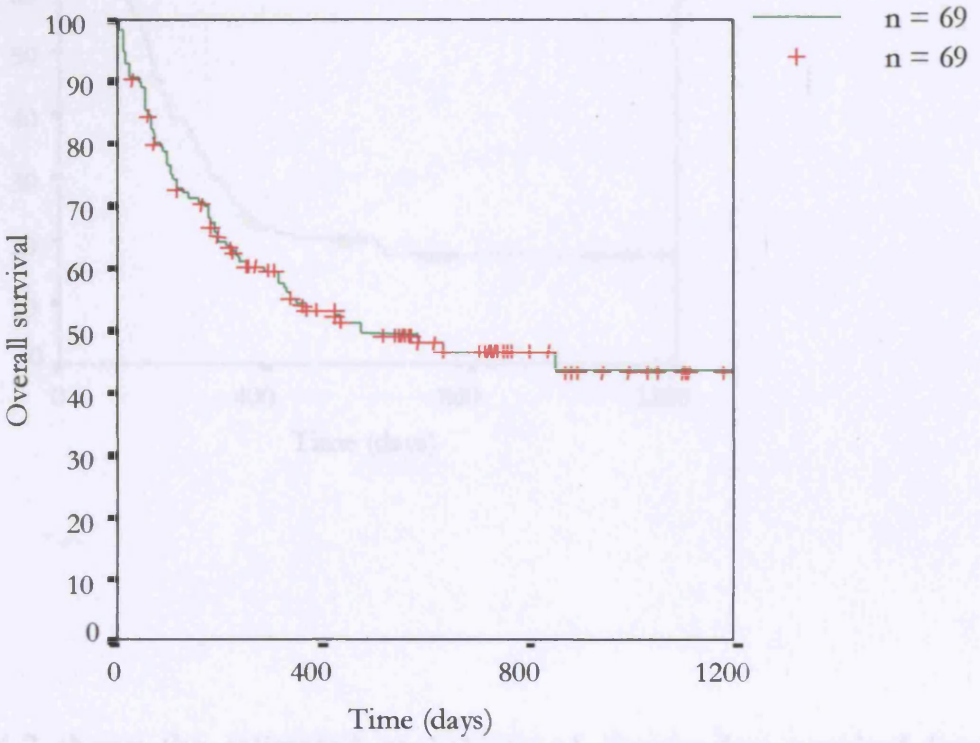
The factors included in the analyses were as follows; type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender, whether transplant was T-cell depleted or not and HLA matching status. Where appropriate, the occurrence of acute, and chronic GvHD were also included. For these primary analyses, HLA-DPB1 matching was excluded to provide data more representative of the majority of this type of study. Analysis of HLA matching including HLA-DP was investigated and will be

discussed separately in the next chapter. All HLA matching data was determined using RSCA.

Outcome analysis for all patients in the study

To give an indication of the overall outcome of all patients included in the study, Kaplan Meier analysis was performed for each outcome variable.

Figure 4-1. Probability of overall survival in all patients. A Kaplan-Meier plot showing percentage overall survival against time in days for all patients in the study (green, censored events in red).



As shown in table 4-3, 69 patients died during the course of the study. As demonstrated in figure 4-1, the estimated probability of overall survival for all patients in the study at 1 year was 54% (95% CI, 46-63%), at 2 years it was 47% (95% CI, 37-56%) and at 3 years it was 43% (95% CI, 33-54%).

Figure 4-2. Probability of disease free survival in all patients. A Kaplan-Meier plot showing percentage disease free survival against time in days for all patients in the study (green, censored events in red).

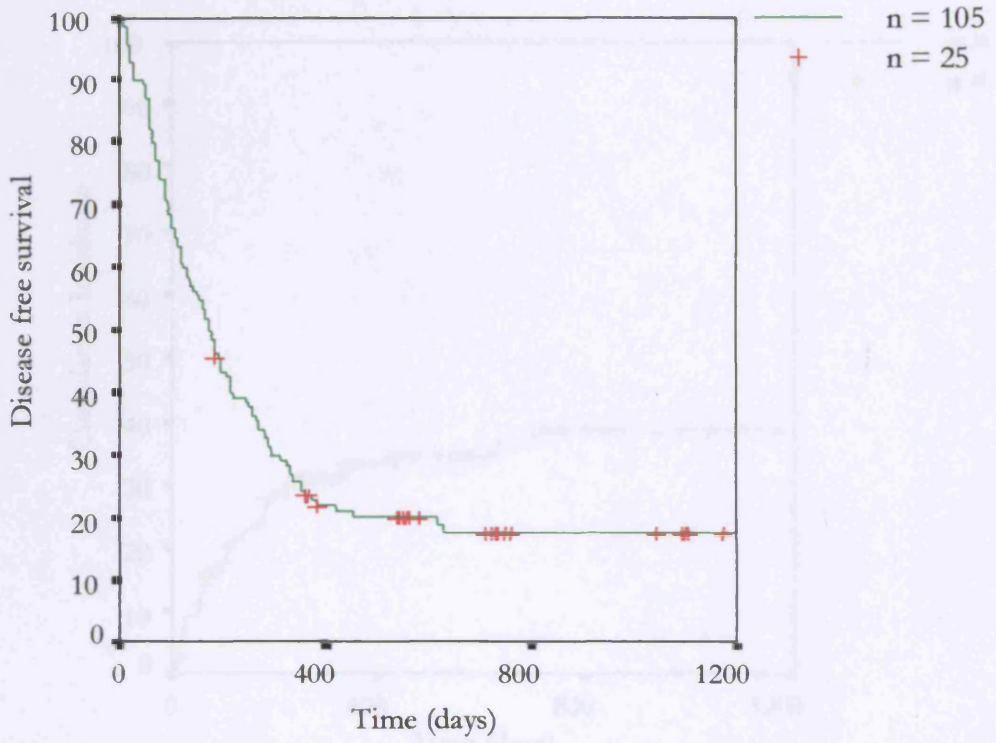
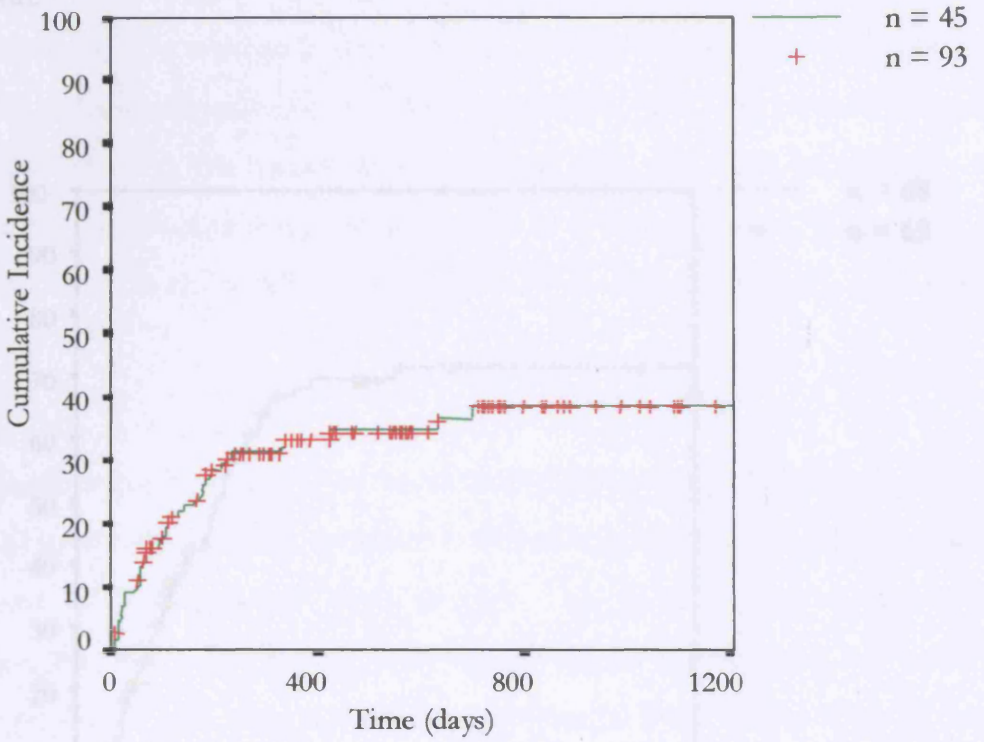


Figure 4-2 shows the estimated probability of disease free survival for all patients at risk from disease relapse (n = 130). The probability of surviving without disease relapse at 1 year was 23% (95% CI, 16-31%) and at 2 years it was 17% (95% CI, 10-24%), with the final event occurred at 632 days. Overall, 105 of 130 patients with data available for analysis either relapsed or died during the course of the study.

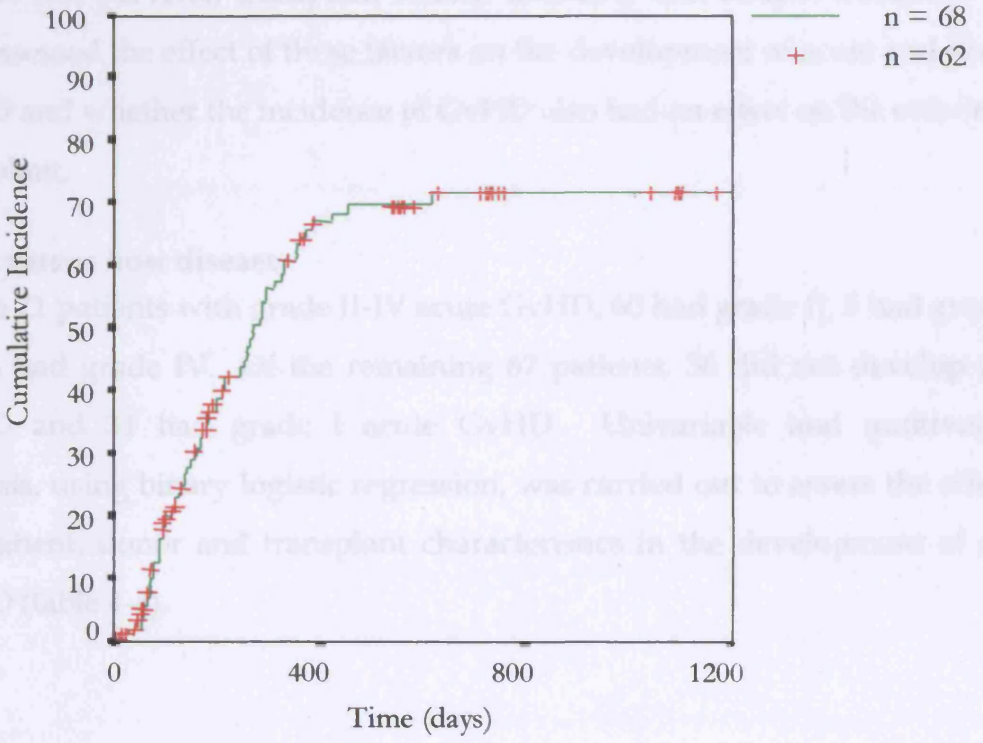
Figure 4-3. Probability of transplant related mortality of all patients. A Kaplan-Meier plot showing cumulative incidence of transplant related mortality against time in days for all patients in the study (green, censored events in red).



As shown in figure 4-3, the estimated probability of transplant related mortality at 1 year was 33% (95% CI, 25-42%) and at 2 years it was 38% (95% CI, 29-48%), with the final event occurring at 700 days. The number of transplant related deaths totalled 45 (see table 4-3).

Figure 4-4 shows the probability of relapse incidence for all patients at risk from recurring disease (n = 130).

Figure 4-4. Probability of relapse incidence of all patients. A Kaplan-Meier plot showing cumulative incidence of relapse against time in days for all patients at risk of relapse in the study (green, censored events in red).



The estimated probability of disease relapse in the study population was 64% (95% CI, 54-74%) at 1 year and 71% (95% CI, 62-81%) at 2 years (figure 4-4). The final event occurred at 619 days. Overall, 68 patients had disease relapse.

The total number of patients who developed grade II-IV acute GvHD was 71 (51%). Fifty-five (53%) of the 103 patients available for analysis after 100 days developed chronic GvHD.

Analysis of the effect of patient, donor and transplant characteristics on outcome

As previously discussed in chapter 1, many factors can influence the outcome of unrelated bone marrow transplants. In this pilot study the data collected in the clinical database was used to investigate the effect of some of the variables that prior studies have suggested a possible prognostic value, on overall survival, disease free survival, transplant related mortality and relapse incidence. We also assessed the effect of these factors on the development of acute and chronic GvHD and whether the incidence of GvHD also had an effect on the outcome of transplant.

Graft versus host disease

Of the 71 patients with grade II-IV acute GvHD, 60 had grade II, 5 had grade III and 6 had grade IV. Of the remaining 67 patients, 36 did not develop acute GvHD and 31 had grade I acute GvHD. Univariable and multivariable analysis, using binary logistic regression, was carried out to assess the effect of the patient, donor and transplant characteristics in the development of acute GvHD (table 4-4).

Table 4-4. Analysis of variables that may affect the development of acute GvHD. Binary logistic regression was used in univariable and multivariable analysis to find which factors had an independent effect on acute GvHD.

Factor		Univariable Result			Multivariable Result		
		Odds Ratio	95% Confidence Interval	p	Odds Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.04	1.00	- , -	0.53
	AML	0.39	0.15 , 1.04		0.55	0.14 , 2.07	
	ALL	0.28	0.11 , 0.75		0.28	0.06 , 1.30	
	malignant	0.57	0.17 , 1.93		0.44	0.09 , 2.06	
	non-malignant	0.19	0.04 , 1.00		0.37	0.03 , 3.95	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.10	1.00	- , -	0.10
	seropositive/seropositive	0.21	0.05 , 0.83		0.18	0.04 , 0.83	
	seropositive/seronegative	0.68	0.24 , 1.97		0.71	0.21 , 2.45	
	seronegative/seropositive	1.52	0.45 , 5.08		1.66	0.41 , 6.79	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.79	1.00	- , -	0.50
	<=36 years/<= 27 years	1.03	0.39 , 2.68		2.12	0.39 , 11.55	
	>36 years/<= 27 years	0.70	0.27 , 1.80		1.22	0.25 , 5.89	
	<=36 years/>27 years	1.11	0.45 , 2.74		0.65	0.41 , 6.79	
Gender (donor/patient)	male/male	1.00	- , -	0.43	1.00	- , -	0.19
	female/female	0.50	0.18 , 1.37		0.26	0.06 , 1.17	
	male/female	0.93	0.39 , 2.22		0.83	0.22 , 3.20	
	female/male	1.31	0.51 , 3.39		1.81	0.42 , 7.79	
T cell depletion	T-cell depletion	1.00	- , -	0.43	1.00	- , -	0.10
	no T-cell depletion	1.35	0.64 , 2.88		3.38	0.80 , 14.27	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.62	1.00	- , -	0.35
	Class I mismatch	0.64	0.30 , 1.37		0.34	0.11 , 1.12	
	Class II mismatch	1.13	0.40 , 3.19		0.80	0.15 , 4.25	
	Class I and II mismatch	1.22	0.27 , 3.57		0.58	0.06 , 5.86	

In univariable analysis, disease was the only variable significantly associated with the development of acute GvHD at the 5% level (p = 0.04, table 4-4). Compared to CML patients, those with AML had a 61% reduced odds of developing acute GvHD (Odds Ratio (OR) = 0.39; 95% CI 0.15, 1.04). Those with ALL had a 72% reduced chance (OR = 0.28; 95% CI 0.11, 0.75) and those with malignant disease had a 43% reduced chance (OR = 0.57; 95% CI 0.17, 1.93) compared with CML patients. Patients with non-malignant disease had an 81% reduced odds of developing acute GvHD (OR = 0.19; 95% CI 0.04, 1.00). This relationship association became non-significant in the multivariable analysis (p = 0.53, table 4-4).

As there was some evidence of a relationship between disease type and the occurrence of acute GvHD, this was explored further. Table 4-5 shows the percentage of patients who had acute GvHD according to disease type. It can be seen that a higher percentage of those patients with CML developed acute GvHD (64%) compared with the other disease types.

Table 4-5. The effect of disease type on the development of acute GvHD.

	Disease					
	CML	AML	ALL	Malignant	Non-malignant	Total
no aGVHD	26 (36%)	13 (60%)	16 (67%)	6 (50%)	6 (75%)	67
aGVHD	46 (64%)	9 (40%)	8 (33%)	6 (50%)	2 (25%)	71
Total	72	22	24	12	8	138

Table 4-6. Analysis of variables that may effect the development of chronic GvHD. Binary logistic regression was used in univariable and multivariable analysis to find which factors had an independent effect on chronic GvHD.

		Univariable Result			Multivariable Result		
Factor		Odds Ratio	95% Confidence Interval	p	Odds Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.08	1.00	- , -	0.32
	AML	0.76	0.24 , 2.35		0.85	0.16 , 4.49	
	ALL	0.18	0.05 , 0.63		0.24	0.03 , 1.89	
	malignant	0.39	0.10 , 1.56		0.64	0.11 , 3.78	
	non-malignant	1.18	0.20 , 7.01		5.59	0.30 , 102.64	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.05	1.00	- , -	0.04
	seropositive/seropositive	0.31	0.07 , 1.41		0.25	0.04 , 1.52	
	seropositive/seronegative	0.15	0.04 , 0.63		0.11	0.02 , 0.55	
	seronegative/seropositive	0.49	0.12 , 2.11		0.49	0.07 , 3.17	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.97	1.00	- , -	0.63
	<=36 years/<= 27 years	1.15	0.39 , 3.44		2.02	0.26 , 15.74	
	>36 years/<= 27 years	0.92	0.30 , 2.83		3.52	0.49 , 25.30	
	<=36 years/>27 years	1.15	0.39 , 3.44		2.14	0.43 , 10.62	
Gender (donor/patient)	male/male	1.00	- , -	0.42	1.00	- , -	0.24
	female/female	1.46	0.47 , 4.58		1.97	0.39 , 9.83	
	male/female	2.44	0.84 , 7.06		6.37	1.06 , 38.39	
	female/male	1.14	0.38 , 3.37		1.72	0.29 , 10.14	
T cell depletion	no T-cell depletion	1.00	- , -	0.07	1.00	- , -	0.68
	T-cell depletion	0.41	0.16 , 1.06		1.44	0.25 , 8.27	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.35	1.00	- , -	0.69
	Class I mismatch	0.71	0.30 , 1.69		0.46	0.12 , 1.80	
	Class II mismatch	2.19	0.60 , 8.01		1.02	0.10 , 10.38	
	Class I and II mismatch	1.75	0.29 , 10.54		0.47	0.02 , 12.15	

In the analysis of variables associated with the development of chronic GvHD (table 4-6), the effect of patient and donor CMV status was significant at the 5% level (p = 0.046). Compared with CMV seronegative patients transplanted with stem cells from seronegative donors, seropositive patients had a 51% reduced odds of developing chronic GvHD when transplanted with stem cells from seronegative donors (OR = 0.49; 95% CI 0.12, 2.11), and a 69% reduced chance when transplanted with stem cells from seropositive donors (OR = 0.31; 95% CI 0.07, 1.41). Seronegative donors transplanted with stem calls from seropositive donors had an 85% reduced odds of developing chronic GvHD (OR = 0.15; 95% CI 0.04, 0.63). Chi squared analysis (table 4-7) shows that 62% of seronegative patients transplanted with stem cells from a seronegative donor developed chronic GvHD, whereas all the other combinations of CMV status of patient and donor had a higher percentage of patients without chronic GvHD.

Table 4-7. The effect of CMV status of patient and donor on the development of chronic GvHD.

	CMV status of donor and patient				
	+/+	+/-	-/+	-/-	Total
no cGVHD	6 (66%)	12 (80%)	5 (56%)	16 (38%)	39
cGVHD	3 (33%)	3 (20%)	4 (44%)	26 (62%)	36
Total	9	15	9	42	75

Table 4-6 shows that multivariable analysis confirmed that the CMV status of the donor and patient was independently associated with the development of chronic GvHD (p = 0.04), showing the same relationship as univariable analysis. The analysis was repeated to include acute GvHD as a factor potentially affecting the development of chronic GvHD (table 4-8).

Table 4-8. Analysis of variables that may effect the development of chronic GvHD, including acute GvHD. Binary logistic regression was used in univariable and multivariable analysis to find which factors had an independent effect on chronic GvHD.

Factor		Univariable Result			Multivariable Result		
		Odds Ratio	95% Confidence Interval	p	Odds Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.08	1.00	- , -	0.49
	AML	0.76	0.24 , 2.35		0.84	0.14 , 5.10	
	ALL	0.18	0.05 , 0.63		0.57	0.06 , 5.47	
	malignant	0.39	0.10 , 1.56		1.02	0.14 , 7.52	
	non-malignant	1.18	0.20 , 7.01		11.27	0.44 , 289.22	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.05	1.00	- , -	0.08
	seropositive/seropositive	0.31	0.07 , 1.41		0.57	0.07 , 4.55	
	seropositive/seronegative	0.15	0.04 , 0.63		0.11	0.02 , 0.58	
	seronegative/seropositive	0.49	0.12 , 2.11		0.43	0.06 , 3.30	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.97	1.00	- , -	0.72
	<=36 years/<= 27 years	1.15	0.39 , 3.44		1.55	0.16 , 14.94	
	>36 years/<= 27 years	0.92	0.30 , 2.83		2.47	0.31 , 19.49	
	<=36 years/>27 years	1.15	0.39 , 3.44		2.41	0.44 , 13.09	
Gender (donor/patient)	male/male	1.00	- , -	0.42	1.00	- , -	0.18
	female/female	1.46	0.47 , 4.58		3.05	0.49 , 18.98	
	male/female	2.44	0.84 , 7.06		8.42	1.18 , 60.24	
	female/male	1.14	0.38 , 3.37		1.68	0.25 , 11.39	
T cell depletion	no T-cell depletion	1.00	- , -	0.07	1.00	- , -	0.74
	T-cell depletion	0.41	0.16 , 1.06		1.35	0.23 , 8.14	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.35	1.00	- , -	0.89
	Class I mismatch	0.71	0.30 , 1.69		0.60	0.13 , 2.76	
	Class II mismatch	2.19	0.60 , 8.01		1.15	0.10 , 13.86	
	Class I and II mismatch	1.75	0.29 , 10.54		0.43	0.01 , 13.48	
GVHD	no acute GvHD	1.00	- , -	<0.01	1.00	- , -	0.01
	acute GVHD	3.73	1.65 , 8.44		6.07	1.52 , 24.23	

When the analysis was repeated to include acute GvHD as a variable potentially affecting the development of chronic GvHD, the development of acute GvHD was found to have a significant effect on the development of chronic GvHD in both univariable ($p = <0.01$) and multivariable ($p = 0.01$) analyses (table 4-8). Univariable analysis showed patients with acute GvHD had an increased chance of developing chronic GvHD, 3.73 times that of a patient who had not had acute GvHD (OR = 3.73; 95% CI 1.65, 8.44). Multivariable analysis showed a similar relationship, with the acute GvHD patients having 6 times the risk of developing chronic GvHD compared with patients who did not have acute GvHD (OR = 6.07; 95% CI 1.52, 24.23). No other variables analysed in were shown to be independently associated with the development of chronic GvHD, when the incidence of acute GvHD was included as a variable.

Overall survival

Univariable and multivariable analysis was carried out using Cox Regression to investigate the effect of all factors on the overall survival of the patients in the study (table 4-9).

In univariable analysis, disease type, CMV status of donor and patient and lack of chronic GvHD were the factors found to be significantly associated with estimated reduced overall survival ($p = 0.047$, $p = 0.04$ and $p = 0.01$ respectively, table 4-9).

Table 4-9. Analysis of variables that may affect overall survival. Cox regression was used in univariable and multivariable analysis to find which factors had an independent effect on overall survival.

		Univariable Result			Multivariable Result		
Factor		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.05	1.00	- , -	<0.01
	AML	2.38	1.28 , 4.41		10.03	2.84 , 35.40	
	ALL	1.63	0.87 , 3.05		0.14	0.01 , 1.54	
	malignant	1.53	0.67 , 3.50		3.51	0.76 , 16.27	
	non-malignant	0.57	0.14 , 2.38		0.00	0.00 , 0.10	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.04	1.00	- , -	0.88
	seropositive/seropositive	2.39	1.16 , 4.92		1.37	0.37 , 5.04	
	seropositive/seronegative	1.26	0.60 , 2.67		1.76	0.40 , 7.69	
	seronegative/seropositive	2.32	1.10 , 4.91		1.09	0.22 , 5.39	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.15	1.00	- , -	0.61
	<=36 years/<= 27 years	0.62	0.30 , 1.29		1.00	0.18 , 5.44	
	>36 years/<= 27 years	1.42	0.75 , 2.66		2.45	0.59 , 9.94	
	<=36 years/>27 years	0.95	0.49 , 1.83		1.36	0.30 , 6.14	
Gender (donor/patient)	male/male	1.00	- , -	0.57	1.00	- , -	0.01
	female/female	1.04	0.52 , 2.09		7.51	1.86 , 30.43	
	male/female	1.00	0.53 , 1.90		0.35	0.09 , 1.41	
	female/male	1.51	0.81 , 2.80		0.56	0.11 , 2.83	
T cell depletion	no T-cell depletion	1.00	- , -	0.08	1.00	- , -	0.68
	T-cell depletion	1.74	0.93 , 3.25		1.47	0.23 , 9.38	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.18	1.00	- , -	<0.01
	Class I mismatch	1.53	0.88 , 2.66		11.75	3.57 , 38.66	
	Class II mismatch	1.51	0.74 , 3.08		3.03	0.71 , 13.01	
	Class I and II mismatch	2.39	1.02 , 5.57		650.61	31.27 , 13535.93	
GVHD	no acute GvHD	1.00	- , -	0.54	1.00	- , -	0.02
	acute GVHD	1.16	0.72 , 1.88		5.34	1.35 , 21.10	
	no chronic GvHD	1.00	- , -	0.01	1.00	- , -	0.01
	chronic GvHD	0.40	0.20 , 0.79		0.20	0.06 , 0.70	

A patient with AML was estimated to have more than twice the risk of dying as someone with CML (Hazard ratio (HR) = 2.38; 95% CI 1.28, 4.41), those with ALL or another malignant disease were estimated to have over a 50% greater chance of dying (HR = 1.63; 95% CI 0.87, 3.05 and HR = 1.53; 95% CI 0.67, 3.50 respectively) Those with non-malignant disease had a 43% reduced risk of death compared with CML patients (HR = 0.57; 95% CI 0.14, 2.38).

The estimated probability of overall survival at 1 year for AML patient, using Kaplan-Meier methods, was 25% (95% CI, 6-44%), CML patients was 64% (95% CI, 52-76%), ALL patients 50% (95% CI, 30-70%), patients with malignant disease 50% (95% CI, 22-78%) and those with non-malignant disease 75% (95% CI, 45-100%). The estimated probability of overall survival at 1 year for CML patients with acute phase disease was 25% (95% CI, 0-67%), blast crisis 33% (95% CI, 0-87%) and chronic phase 68% (95% CI, 56-80%).

The CMV status of the patient and the donor was also found to be a significant factor associated with overall survival in univariable analysis (p = 0.04, table 4-9). CMV seropositive patients, regardless of the CMV status of the donor, were estimated to be over 2.3 times at risk of dying than seronegative patients with a seronegative donor (HR = 2.39; 95% CI 1.16, 4.92 with a seropositive donor and HR = 2.32; 95% CI 1.10, 4.91 with as seronegative donor). CMV seronegative patients with a seropositive donor had a 26% greater risk of death than those with a seronegative donor (HR = 1.26; 95% CI 0.60, 2.67).

The estimated probability of overall survival at 1 year, calculated by Kaplan-Meier methods, for a CMV seropositive donor and patient, was 36% (95% CI, 11-61%), a seronegative patient with a seropositive donor was 47% (95% CI, 25-70%), a seropositive patient with a seronegative donor was 30% (95% CI, 5-55%) and a seronegative patient and donor was 62% (95% CI, 48-75%).

When entered into a multivariable analysis model, disease ($p = <0.01$), gender of patient and donor ($p = 0.01$), HLA matching ($p = <0.01$) and the incidence of acute ($p = 0.02$) and chronic GvHD ($p = 0.01$) were shown to be independently associated with reduced overall survival (table 4-9). A patient with AML had a risk of dying 10 times that of a CML patient (HR = 10.03; 95% CI 2.84, 35.40), and a patient with malignant disease had 3.5 times the risk (HR = 3.51; 95% CI 0.76, 16.27). Those patients non-malignant disease had a greatly reduced risk of dying compared to those with CML (HR = 0.00; 95% CI 0.00, 0.10 respectively). Interestingly, ALL patients had a greater risk of dying compared with CML patients in the univariable analysis (HR = 1.63; 95% CI 0.87, 3.05; $p = 0.05$), but the multivariable analysis showed a reduced risk (HR = 0.14; 95% CI 0.01, 1.54). This may mean that other factors had more of an effect on overall survival than disease in this case.

Female patients transplanted with stem cells from female donors had 7.5 times the risk of death compared with male patients with male donors (HR = 7.51; 95% CI 1.86, 30.43). Female patients with male donors had a 65% reduced risk of death (HR = 0.35; 95% CI 0.09, 1.41) compared to male patients. Male patients with female donors had a 44% reduced risk of death compared to those with male donors (HR = 0.56; 95% CI 0.11, 2.83).

Patients who were mismatched with their donor at one or more class I and class II loci were at a far greater risk of dying than those who were matched at all loci (HR = 650.61; 95% CI 31.27, 13535.93). Those with a class I mismatch only were almost 12 times more at risk of death than matched patients (HR = 11.75; 95% CI 3.57, 38.66). A class II mismatch only led to a 3 times greater risk of dying compared to matched patients (HR = 3.03; 95% CI 0.71, 13.01).

Patients who developed acute GvHD had over 5 times greater risk of dying

than those that did not develop acute GvHD (HR = 5.34; 95% CI 1.35, 21.10).

Disease free survival

Univariable and multivariable analysis was carried out using Cox Regression to investigate the effect of all variables on the disease free survival of the patients in the study (table 4-10).

Table 4-10. Analysis of variables that may affect disease free survival. Cox regression was used in univariable and multivariable analysis to find which factors had an independent effect on disease free survival.

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.07	1.00	- , -	0.001
	AML	0.91	0.54 , 1.53		1.07	0.48 , 2.42	
	ALL	0.55	0.32 , 0.96		0.08	0.02 , 0.41	
	malignant	0.46	0.21 , 1.01		0.18	0.05 , 0.63	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.09	1.00	- , -	0.23
	seropositive/seropositive	1.52	0.79 , 2.93		0.91	0.35 , 2.34	
	seropositive/seronegative	0.96	0.49 , 1.88		1.01	0.38 , 2.69	
	seronegative/seropositive	2.18	1.12 , 4.22		3.45	1.01 , 11.73	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.73	1.00	- , -	0.38
	<=36 years/<= 27 years	0.73	0.41 , 1.30		1.25	0.36 , 4.32	
	>36 years/<= 27 years	0.55	0.55 , 1.56		2.46	0.88 , 6.88	
	<=36 years/>27 years	0.59	0.59 , 1.61		1.31	0.52 , 3.28	
Gender (donor/patient)	male/male	1.00	- , -	0.96	1.00	- , -	0.02
	female/female	0.91	0.51 , 1.61		2.07	0.66 , 6.51	
	male/female	0.92	0.56 , 1.50		0.42	0.17 , 1.02	
	female/male	1.05	0.62 , 1.79		0.31	0.12 , 0.81	
T cell depletion	no T-cell depletion	1.00	- , -	0.71	1.00	- , -	0.30
	T-cell depletion	1.09	0.69 , 1.71		1.98	0.54 , 7.32	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.01	1.00	- , -	<0.01
	Class I mismatch	1.77	1.14 , 2.77		5.22	2.28 , 11.97	
	Class II mismatch	1.19	0.65 , 2.17		1.11	0.38 , 3.24	
	Class I and II mismatch	2.78	1.41 , 5.47		59.23	7.97 , 439.99	
GVHD	no acute GvHD	1.00	- , -	0.12	1.00	- , -	0.19
	acute GVHD	1.36	0.92 , 2.01		1.81	0.75 , 4.36	
	no chronic GvHD	1.00	- , -	0.16	1.00	- , -	0.91
	chronic GvHD	1.40	0.87 , 2.23		1.05	0.47 , 2.32	

HLA matching (for HLA-A, -B, -C, -DRB1 and -DQB1 loci) was the only variable in the model that was estimated to have a significant effect on disease free survival in the univariable analysis (p = 0.01, table 4-10). A patient transplanted with a class II mismatch only was estimated to have a 19% increased risk of death or relapse compared to a patient who had a matched transplant (HR = 1.19; 95% CI 0.65, 2.17) , a patient transplanted with a class I mismatch only was estimated to have a 77% greater risk of death or relapse (HR = 1.77; 95% CI 1.14, 2.77) and a patient transplanted with an HLA class I and a class II mismatch was estimated to be 2.8 times more likely to die or relapse (HR = 2.78; 95% CI 1.41, 5.47).

The estimated probability of disease free survival at 1 year, calculated using Kaplan-Meier methods, for patients with HLA matched transplants was 34% (95% CI, 22-46%). For HLA class II mismatched transplants it was 24% (95% CI, 3-44%), for HLA class I mismatched transplants was 14% (95% CI, 4-25%) and those with transplants mismatched at both a class I and a class II locus had all died or relapsed by one year (last event 287 days).

Multivariable analysis of the effect of the different factors on disease free survival showed that HLA matching was independently associated with disease free survival (p = <0.01), as were the disease (p = <0.01) and gender of donor and recipient (p = 0.02, table 4-10).

Patient who were mismatched with their donor for both a class I and a class II locus had the greatest risk of death or relapse compared with patients who were HLA matched with their donor (HR = 59.23; 95% CI 7.97, 439.99), patients with a class I mismatch only had over 5 times the risk of death or relapse (HR = 5.22; 95% CI 2.28, 11.97) and those with a class II mismatch only had an 11% increased risk (HR = 1.11; 95% CI 0.38, 3.24).

Patients with AML had a similar risk of dying or relapse as those with CML (HR = 1.07; 95% CI 0.48, 2.42), patients with other malignant diseases had an 82% reduced risk compared with CML patients (HR = 0.18; 95% CI 0.05, 0.63) and ALL patients had a 92% reduced risk (HR = 0.08; 95% CI 0.02, 0.41).

Female patients with female donors had twice the risk of death or relapse when compared with male patients transplanted with stem cells from male donors (HR = 2.07; 95% CI 0.66, 6.51). Transplants with a gender mismatch seemed to do better than those that were of the same sex, female patients with male donors had a 58% reduced risk of death or relapse (HR = 0.42; 95% CI 0.17, 1.02) compared to male patients with male donors, and male patients with female donors had a 69% reduced risk (HR = 0.31; 05% CI 0.12, 0.81).

Transplant related mortality

Univariable and multivariable analysis was carried out using Cox Regression to investigate the effect of all variables on the transplant related mortality of the patients in the study (table 4-11).

Table 4-11. Analysis of variables that may affect transplant related mortality. Cox regression was used in univariable and multivariable analysis to find which factors had an independent effect on transplant related mortality.

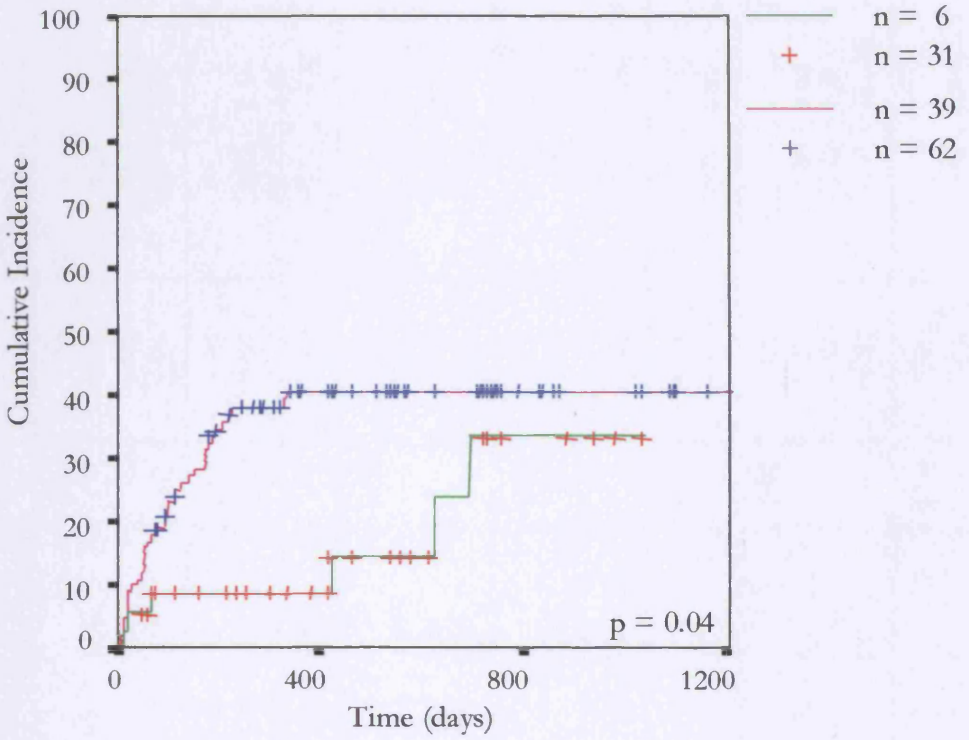
Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	0.11	1.00	- , -	0.07
	AML	2.43	1.14 , 5.15		12.91	1.98 , 83.40	
	ALL	1.88	0.89 , 3.98		1.04	0.04 , 27.14	
	malignant	1.37	0.46 , 4.05		6.68	0.82 , 54.23	
	non-malignant	0.45	0.06 , 3.38		0.00	0.00 , 0.00	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.05	1.00	- , -	0.58
	seropositive/seropositive	2.34	0.94 , 5.82		1.70	0.26 , 11.01	
	seropositive/seronegative	1.79	0.77 , 4.13		3.56	0.56 , 22.59	
	seronegative/seropositive	3.08	1.33 , 7.16		2.02	0.26 , 15.83	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.61	1.00	- , -	0.94
	<=36 years/<= 27 years	0.61	0.25 , 1.47		0.76	0.05 , 10.78	
	>36 years/<= 27 years	1.10	0.50 , 2.41		1.25	0.18 , 8.47	
	<=36 years/>27 years	0.88	0.40 , 1.94		1.47	0.19 , 11.57	
Gender (donor/patient)	male/male	1.00	- , -	0.75	1.00	- , -	0.56
	female/female	0.90	0.36 , 2.26		2.72	0.36 , 20.61	
	male/female	0.58	0.58 , 2.60		0.45	0.08 , 2.53	
	female/male	0.66	0.66 , 3.10		0.59	0.08 , 8.54	
T cell depletion	no T-cell depletion	1.00	- , -	0.05	1.00	- , -	0.92
	T-cell depletion	2.39	1.01 , 5.65		1.15	0.07 , 18.11	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.39	1.00	- , -	0.01
	Class I mismatch	1.43	0.72 , 2.83		14.72	2.54 , 85.31	
	Class II mismatch	1.44	0.59 , 3.50		4.79	0.85 , 27.07	
	Class I and II mismatch	2.34	0.85 , 6.42		210.61	5.51 , 8055.58	
GVHD	no acute GvHD	1.00	- , -	0.70	1.00	- , -	0.01
	acute GVHD	1.13	0.62 , 2.01		9.47	1.69 , 53.16	
	no chronic GvHD	1.00	- , -	0.08	1.00	- , -	0.08
	chronic GvHD	0.42	0.19 , 1.10		0.25	0.05 , 1.19	

Univariable analysis shows CMV status (p = 0.049) and T cell depletion (p = 0.047, table 4-11) were associated with increased risk of transplant related mortality. A CMV seropositive patient with a seronegative donor was estimated to be 3 times at risk of transplant related mortality as a seronegative patient with a seronegative donor (HR = 3.08; 95% CI 1.33, 7.16), and a seropositive patient with a seropositive donor was 2.3 times as likely to be at risk of transplant related mortality (HR = 2.34; 95% CI 0.94, 5.82).

The estimated risk of transplant related mortality at 1 year for a CMV seropositive donor and patient was 46% (95% CI, 19-73%), a seronegative patient with a seropositive donor was 48% (95% CI, 25-71%), a seropositive patient with a seronegative donor was 60% (95% CI, 35-85%) and a seronegative patient and donor was 27% (95% CI, 14-40%).

Univariable analysis shows a patient with a T cell depleted transplant had a 2.4 times greater estimated risk of transplant related mortality than a patient who received a T cell replete transplant (HR = 2.39; 95% CI 1.01, 5.65, table 4-11). Figure 4-5 shows the cumulative incidence of transplant related mortality for T cell replete transplants at 1 year was 9% (95% CI, 0-18%) and for T cell depleted transplants it was 41% (95% CI, 31-51%).

Figure 4-5. Kaplan-Meier analysis of the effect of T cell depletion on the risk of transplant related mortality. The risk of transplant related mortality of a patient who underwent a T cell replete transplant (green, censored events in red), was compared with that of a patient who underwent a T cell depleted transplant (pink, censored events in blue).



In multivariable analysis, increased risk of transplant related mortality was closely associated with HLA matching of donor and patient (p = 0.01, table 4-8) and incidence of acute GvHD (p = 0.01).

The greatest risk of transplant related mortality was seen for patients mismatched with their donors at both an HLA class I and a class II locus. These patients were estimated to have an increased risk of 210.6 times compared with patients who underwent an HLA matched transplant (HR = 210.61; 95% CI 5.51, 8055.58). Patients with a class I mismatch only had a 14.7 times increased risk of transplant related mortality compared with matched patients (HR = 14.72; 95% CI 2.54, 85.31), and patients with only a class II mismatch had 4.8 times increased risk (HR = 4.79; 95% CI 0.85, 27.07).

The incidence of acute GvHD was independently associated with transplant related mortality, with a 9.5 times increased risk compared to patients who did not develop acute GvHD (HR 9.47; 95% CI 1.69, 53.16).

Relapse incidence

Univariable and multivariable analysis was carried out to investigate the effect of all variables on the incidence of relapse of the patients in the study (table 4-12).

Table 4-12. Analysis of variables that may affect the incidence of disease relapse. Cox regression was used in univariable and multivariable analysis to find which factors had an independent effect on relapse incidence.

		Univariable Result			Multivariable Result		
Factor		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
Disease	CML	1.00	- , -	<0.01	1.00	- , -	<0.01
	AML	0.50	0.24 , 1.06		0.58	0.21 , 1.56	
	ALL	0.24	0.10 , 0.57		0.05	0.01 , 0.36	
	malignant	0.23	0.07 , 0.74		0.04	0.01 , 0.38	
CMV status (donor/patient)	seronegative/seronegative	1.00	- , -	0.56	1.00	- , -	0.29
	seropositive/seropositive	1.19	0.49 , 2.91		0.84	0.28 , 2.54	
	seropositive/seronegative	0.52	0.18 , 1.48		0.65	0.17 , 2.44	
	seronegative/seropositive	1.20	1.20 , 3.47		3.41	0.81 , 14.28	
Age (donor/patient)	>36 years/> 27 years	1.00	- , -	0.84	1.00	- , -	0.54
	<=36 years/<= 27 years	0.87	0.43 , 1.76		1.42	0.33 , 6.06	
	>36 years/<= 27 years	0.93	0.47 , 1.83		2.50	0.73 , 8.57	
	<=36 years/>27 years	1.17	0.63 , 2.18		1.25	0.43 , 3.59	
Gender (donor/patient)	male/male	1.00	- , -	0.89	1.00	- , -	0.02
	female/female	1.01	0.51 , 1.97		3.05	0.88 , 10.54	
	male/female	0.85	0.46 , 1.57		0.49	0.17 , 1.38	
	female/male	0.79	0.38 , 1.61		0.28	0.09 , 0.92	
T cell depletion	no T-cell depletion	1.00	- , -	0.22	1.00	- , -	0.45
	T-cell depletion	0.73	0.44 , 1.21		1.78	0.39 , 8.00	
HLA-A, -B, -C, -DRB1, -DQB1 matching	matched	1.00	- , -	0.02	1.00	- , -	<0.01
	Class I mismatch	1.90	1.08 , 3.34		3.33	1.30 , 8.52	
	Class II mismatch	1.50	0.74 , 3.02		1.17	0.32 , 4.22	
	Class I and II mismatch	3.52	1.50 , 8.27		68.00	6.07 , 761.88	
GVHD	no acute GvHD	1.00	- , -	0.08	1.00	- , -	0.62
	acute GVHD	1.56	0.95 , 2.54		1.31	0.45 , 3.81	
	no chronic GvHD	1.00	- , -	0.01	1.00	- , -	0.72
	chronic GvHD	2.02	1.16 , 3.50		1.20	0.45 , 3.20	

Table 4-12 shows that disease (p = <0.01), HLA matching (p = 0.02) and the presence of chronic GvHD (p = 0.01) were associated with the risk of disease relapse. Of the 68 patients who had recurrence of their original disease, 28 received a donor lymphocyte infusion. Univariable analysis showed CML patients had the greatest estimated probability of disease relapse (table 4-12), twice that of AML patients (HR = 0.50; 95% CI 0.24, 1.06). Patients with ALL and those with other malignant diseases had a >75% reduced risk of relapse compared to CML patients (HR = 0.24; 95% CI 0.10, 0.57 and HR = 0.23; 95% CI 0.07, 0.74, respectively).

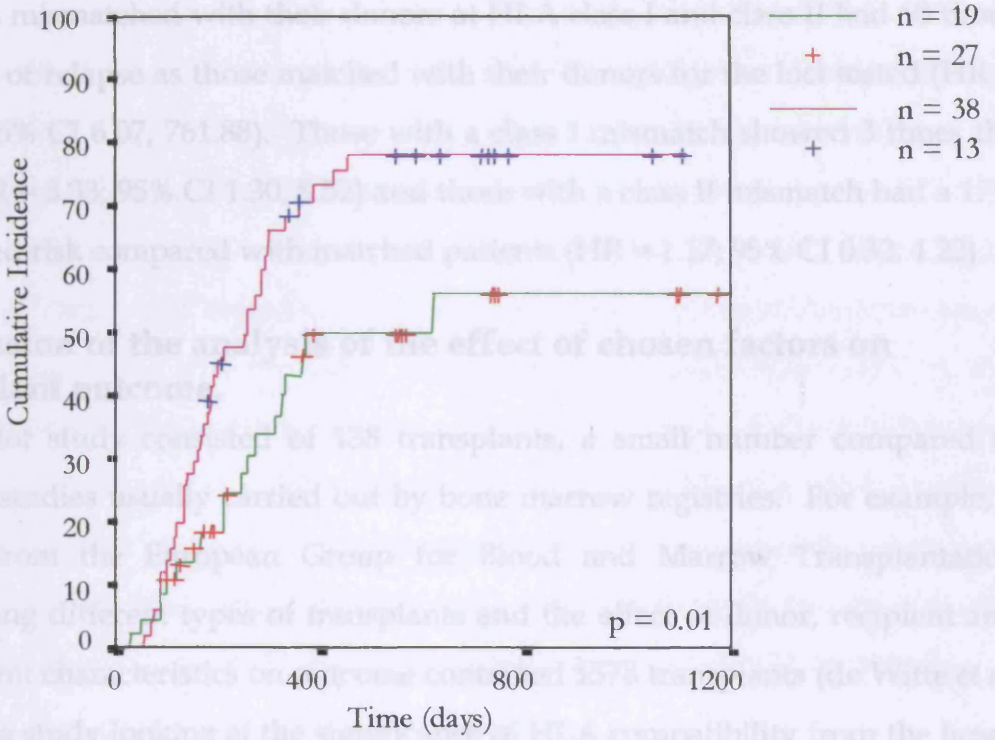
The 1 year cumulative incidence of relapse for patients with CML was 76% (95% CI, 65-87%), AML was 70% (95% CI, 42-98%), ALL was 39% (95% CI, 14-63%) and other malignant disease was 40% (95% CI, 1-59%). Two cases of acute phase CML and all 3 cases of CML in blast crisis relapsed within 6 months of transplant, and the cumulative incidence of relapse for CML in chronic phase at 1 year was 75% (95% CI, 63-87%, p = <0.01, data not shown).

Patients with an HLA class I and a class II mismatch had a 3.5 times greater risk of disease relapse compared to those matched with their donor for HLA-A, -B, -C, -DRB1 and -DQB1 (HR = 3.52; 95% CI 1.50, 8.27). Those with a class II mismatch only had a 50% greater risk of relapse (HR = 1.50; 95% CI 0.74, 3.02) and a class I mismatch only meant the patient had a 1.9 times greater risk of relapse (HR = 1.9; 95% CI 1.08, 3.34).

The 1 year cumulative incidence of relapse, as calculated with Kaplan-Meier methods, for patients transplanted with stem cells from an HLA matched donor was 51% (95% CI, 36-65%), with a class I mismatch was 74% (95% CI, 57-91%), class II mismatch was 73% (95% CI, 51-95%) and with a mismatch at HLA class I and class II was 81% (95% CI, 49-100%, last event occurred at 287 days).

Patients who developed chronic GvHD were estimated to have twice the risk of relapse as those who did not have chronic GvHD (HR = 2.02; 95% CI 1.16, 3.50). Figure 4-6 shows the effect of having chronic GvHD on the cumulative incidence of relapse.

Figure 4-6. Kaplan-Meier analysis of the effect of developing chronic GvHD on the risk of disease relapse. The relapse incidence of patients who did not develop chronic GvHD (green, censored events in red) was compared with that for patients who had developed chronic GvHD (pink, censored events in blue).



The 1 year cumulative incidence of relapse was 46% (95% CI, 29-63%) for patients who had not developed chronic GvHD and 71% (95% CI, 54-88%) for patients who had developed chronic GvHD (figure 4-6).

Multivariate analysis shows that disease (p = <0.01, table 4-12), gender matching between donor and patient (p = 0.02) and HLA matching (p = <0.01), were all independently associated with the risk of relapse.

AML patients had a 42% reduced risk of relapse compared with CML patients (HR = 0.58; 95% CI 0.21, 1.56), with ALL patients and those with other malignant diseases showing a 95% reduced risk of relapse (HR = 0.05; 95% CI 0.01, 0.36 and HR = 0.04; 95% CI 0.01, 0.38 respectively).

Female patients transplanted with stem cells from female donors had over 3 times the risk of relapse compared with male patients transplanted from male

donors (HR = 3.05; 95% CI 0.88, 10.54). Female patients with male donors had a 51% reduced risk (HR = 0.49; 95% CI 0.17, 1.38) and male patients with female donors had a 72% reduced risk (HR = 0.28; 95% CI 0.09, 0.92).

Patients mismatched with their donors at HLA class I and class II had 68 times the risk of relapse as those matched with their donors for the loci tested (HR = 68.00; 95% CI 6.07, 761.88). Those with a class I mismatch showed 3 times the risk (HR = 3.33; 95% CI 1.30, 8.52) and those with a class II mismatch had a 17% increased risk compared with matched patients (HR = 1.17; 95% CI 0.32, 4.22).

Discussion of the analysis of the effect of chosen factors on transplant outcome.

This pilot study consisted of 138 transplants, a small number compared to similar studies usually carried out by bone marrow registries. For example, a study from the European Group for Blood and Marrow Transplantation examining different types of transplants and the effect of donor, recipient and transplant characteristics on outcome contained 1378 transplants (de Witte *et al.* 2000a), a study looking at the significance of HLA compatibility from the Japan Marrow Donor Program included 1298 transplants (Morishima *et al.* 2002) and a study of factors determining long term survival by the International Bone Marrow Transplant Registry included 6691 transplants (Socié *et al.* 1999).

The analyses presented were carried out in order to give a model for the type of analysis that can be done on the data collected in this ongoing investigation. The results presented here could be used in sample size calculations to find the number of transplants required to give a definitive study. The results from larger studies may then be used in the attempt to improve the outcome of unrelated stem cell transplants through informed donor selection. In this section, the results of the preliminary analysis are discussed and it is understood, that due to the small number of transplants studied, some data may be not be precise as determined by the wide confidence intervals presented in some cases.

HLA matching

HLA matching for the HLA-A, -B, -C, -DRB1 and -DQB1 loci was

independently associated with reduced risk of relapse (table 4-12), increased disease free survival (table 4-10), reduced risk of transplant related mortality (table 4-11) and increased overall survival (table 4-9). In all cases, the worst outcome was associated with a mismatch at both class I and class II loci, which agrees with the results of a similar study by Petersdorf *et al* (Petersdorf *et al.* 1998), followed by a mismatch at class I only, which has also been cited as a risk factor for reduced survival (Sasazuki *et al.* 1998). Patients matched for HLA-A, -B, -C, -DRB1 and -DQB1 had the least risk of all outcomes, also found in the study by Petersdorf (Petersdorf *et al.* 1998). Interestingly, the mismatches found were not associated with the risk of developing acute and chronic GvHD in this study, whereas HLA mismatches are a known risk factor for the development of acute and chronic GvHD (Petersdorf *et al.* 1999; Sasazuki *et al.* 1998; Szydlo *et al.* 1997). As seen in the next chapter, many of the HLA mismatches seen were known pre-transplant and this may have affected the way the patient and transplant were conditioned, to reduce a potential graft-versus-host response.

Disease

Disease type was found to be independently associated with risk of relapse (table 4-12), disease free survival (table 4-10) and overall survival (table 4-9). CML patients had the greatest risk of disease relapse, followed by AML patients. However, AML patients had the greatest risk of dying from relapse as reflected in the overall survival and disease free survival analyses. AML patients were twice as likely to die as those with CML in univariable analysis, and ten times at risk when entered into the multivariable model. This may be due to 15 of the 22 AML patients being at a late stage of disease (2^{nd} complete remission) or relapsed at the time of transplant, which has shown to result in reduced overall survival (Greinix *et al.* 2002). This could also be due to CML patients being given a donor lymphocyte infusion to rescue them from disease relapse (Bacigalupo *et al.* 1997; Dazzi *et al.* 2000). In univariable analysis, disease was associated with increased risk of developing acute GvHD, with CML patients having the greatest risk.

Multivariable analysis was carried out including only patients with CML in chronic phase, to correct for any influence of disease type or stage. However, none of the analyses performed gave a significant result, most likely due to the small number of patients in the analysis (n = 65).

CMV status

CMV status of the patient and donor was only found to be associated with outcome in univariable analyses and no independent association was seen. The greatest risk of developing chronic GvHD was seen in the group where both donor and recipient were CMV negative (table 4-6). In other studies, a seronegative donor with a seronegative patient has been associated with a lower rate of chronic GvHD (Kollman *et al.* 2001). In the groups where CMV was present, seropositive patients with seronegative donors had the greatest risk of developing chronic GvHD, followed by seropositive patients with seropositive donors, indicating the positive CMV status of the patient to be more important with respect to the development of chronic GvHD compared with that of the donor. CMV seropositive patients were twice as likely to die as seronegative patients, regardless of the CMV status of the donor. A low level of overall survival of CMV positive patients, independent of donor CMV status has been reported (Broers *et al.* 2000), thought to be due to a high level of transplant related mortality in these patients. More recent investigations, however, indicate a CMV seropositive donor may be beneficial for a CMV seropositive patient in the hope of a transfer of specific immunity against CMV infection (Ljungman *et al.* 2003).

Age

Interestingly, although increased donor and recipient age have been shown to adversely affect outcome in various registry studies (Greinix *et al.* 2002; Kollman *et al.* 2001; Remberger *et al.* 2002; Socié *et al.* 1999), age of donor and recipient did not appear to be a risk factor for any of the outcomes tested in this study. This may have been because the cohort studied was relatively young (median patient age 29 years and median donor age 37 years).

Gender

The gender of the donor and recipient was independently associated with relapse (table 4-12) and disease free survival (table 4-10). Female patients with female donors had the greatest risk of relapse and the lowest risk of disease free survival followed by male patients with male donors. This may be due to a lack of graft-versus-leukaemia response directed at the H-Y antigen (Gratwohl *et al.* 2001). The group with the lowest risk were male patients with female donors. As previously mentioned, it has been observed that female recipients of male bone marrow have a higher risk of graft rejection than when transplanted with bone marrow from a female donor (Voogt *et al.* 1990). A similar effect has also been seen in the greater risk of developing GvHD in male recipients of female grafts and female recipients of male grafts have lower disease free survival (Barrett *et al.* 1989).

T cell depletion

In univariable analysis, patients who received T cell depleted transplants had a greater risk of transplant related mortality than those who had a T cell replete transplant (table 4-11). The greatest difference in cause of death between T cell depleted and T cell replete transplant groups was death from infection. Twenty-three percent of patients died from infection in the T cell depleted group, compared with 8% in the T cell replete group. The three patients who died from regimen related toxicity were all in the T cell depleted transplant group. Due to delayed immune reconstitution seen after T cell depleted stem cell transplantation, patients remain susceptible to opportunistic infections (Novitzky and Rouskova 2001).

GvHD

The presence of acute GvHD was independently associated with reduced overall survival. It was also associated with increased risk of transplant related mortality (table 4-11) in univariable analysis. The only factor to be independently associated with the development of chronic GvHD was the presence of acute GvHD (table 4-8). This result was expected and has been shown in many larger studies of both related and unrelated allogeneic bone

marrow transplants (Atkinson *et al.* 1990; Castro-Malaspina *et al.* 2002; Ochs *et al.* 1994; Remberger *et al.* 2002).

The absence of chronic GvHD was independently associated with reduced overall survival (table 4-9), and in univariable analysis, the presence of chronic GvHD was associated with increase risk of disease relapse (table 4-12). In other studies it has been shown that patients with chronic GvHD have a lower incidence of relapse and better disease free survival than do those without chronic GvHD (Horowitz *et al.* 1990; Weiden *et al.* 1979). However, the presence of moderate or severe chronic GvHD also implies an increased immunosuppressive state leading to a higher risk of infection and reduced function of the affected organs (Remberger *et al.* 2002). In our study, the presence of chronic GvHD was not significantly associated with better disease free survival and patients with chronic GvHD were twice as likely to relapse as those without (table 4-12), in univariable analysis. One explanation could be that the immunosuppressive state mentioned may be affecting the graft versus leukaemia effect. In conclusion, further study is required with larger numbers of patients to find if the effects seen here are true.

Chapter 5

RSCA analysis and the effects of HLA allele compatibility

Reference strand mediated conformation analysis was used to identify HLA allele mismatches between donor and patient for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 in 138 bone marrow transplant pairs. Any results discrepant with previously established methodologies were identified and the effects of HLA compatibility for all six loci were analysed, focusing on the role of HLA-DPB1 as a transplantation antigen.

RSCA analysis of all transplant pairs

DNA samples from both patient and donor were amplified by PCR and subjected to RSCA as described (Chapter 2). Mismatches were identified by running patient and donor separately in two lanes of the gel and mixed in a third lane. HLA types were assigned based on the position of the band in the gel, once alignment had been carried out as described. All HLA typing was carried out blind to the original typing results. Results are shown in table 5-1.

Table 5-1. RSCA typing results. Mismatches are highlighted in yellow, with turquoise for those homozygous versus heterozygous loci matched in a GvH direction.

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	5	2902	3001	1302	4501	0602	-	0401	0701	0301	0201/02	02012	-
Donor	6	2902	3001	1302	4501	0602	-	0401	0701	0301	0201/02	0401	1701
Patient	15	0201/09	2402	4001	5701	0303/04	0602	0701	1302	03032	0604	0401	0301
Donor	16	0201/09	2402	4001	5701	0303/04	0602	0701	1302	03032	0604	0401	02012
Patient	17	0101	2402	0801	1501	0303	0701/05/06	0401	1501	0302	0602	11011	4601
Donor	18	0101	2402	0801	1501	0303	0701/05/06	0401	1501	0302	0602	1901	4601
Patient	33	0101	0201/09	0702	-	0702	-	1302	1501	0602	0604	0401	0601
Donor	34	0101	0201/09	0702	-	0702	-	1302	1501	0602	0604	0301	0401
Patient	35	0205	-	1501	5001	0303/04	0602	0701	1301	0201/02	0603	1401	1301
Donor	36	0201/09	0205	1501	5001	0303/04	0602	0701	1301	0201/02	0603	0402	0301
Patient	37	3301	3001	1302	1402	0602	0802	0101	1501	0501	0601	0401	0402
Donor	38	3301	3201	1302	1402	0602	0802	0101	1502	0501	0601	0301	02012
Patient	39	0201/09	0205	27052	4001	0102	0302	0401	1501	0301	0601	0401	-
Donor	40	0201/09	-	27052	4001	0102	0302	0401	1501	0302	0601	0401	0301
Patient	43	0101	3001	0801	1302	0602	0701/05/06	0301	0701	0201/02	-	0401	0402
Donor	44	0101	3001	0801	1302	0602	0701/05/06	0301	0701	0201/02	-	0401	1301
Patient	47	0101	-	0801	5701	0602	0701/05/06	0301	0701	0303	0201	0401	1301
Donor	48	0101	-	0801	5701	0602	0701/05/06	0302	0701	0303	0201*	0402	2001
Patient	55	0101	2902	0801	4403	0701/05/06	1601/02	0301	0701	0201/02	-	0401	11011
Donor	56	0101	2902	0801	4403	0701/05/06	1601/02	0301	0701	0201/02	-	0401	11011
Patient	71	0201/09	2902	1401	44031	0802	0501	0701	-	0201/02	-	0401	11011
Donor	72	0201/09	2902	1401	44031	0802	0501	0701	-	0201/02	-	0301	0401
Patient	73	0201/09	0302	0702	1501	0303	0702	1101	1501	0301	0602	0401	-
Donor	74	0201/09	0302	0702	1501	0202	0702	1101	1501	0301	0602	02012	0401
Patient	75	0101	-	5701	-	0602	-	0701	-	0303	-	0401	-
Donor	76	0101	-	5701	-	0602	-	0701	-	0303	-	0401	-
Patient	79	2902	6801	44031	5201	1202	1501	0701	1502	0201	0601	0401	-
Donor	80	3301	6801	44032	5201	1202	1501	0701	1502	0201	0601	0401	1301

* unusual linkage - expect DRB1*0302-DQB1*0402

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	87	0101	2601	0801	1401	0701/05/06	0802	0301	0701	0201/02	-	0402	-
Donor	88	0101	2601	0801	1401	0701/05/06	0802	0301	0701	0201/02	-	01011	02012
Patient	89	0101	0201/09	0702	0801	0701/05/06	0702	0301	0404	0201/02	0302/05	02012	0401
Donor	90	0101	0205	0702	0801	0701/05/06	0702	0301	0404	0201/02	0302/05	0301	0501
Patient	93	0201/09	2402	0702	1501	0702	0304	0301	1102	0201/02	0301	02012	-
Donor	94	0201/09	2301	0702	1501	0702	0304	0301	1102	0602	0301	02012	0401
Patient	101	0201/09	1101	0801	52011	0701/05/06	1202	0301	1502	0201/02	0601	11011	-
Donor	102	0201/09	1101	0801	52011	0701/05/06	1202	0301	1502	0602	0601	0401	11011
Patient	103	0301	2402	1501	3503	0303/4	0401	0101	1301	0501	0603	02012	11011
Donor	104	0301	2402	1501	3503	0303/4	0401	0102	1301	0501	0603	1301	1901
Patient	111	0102	2902	44031	5701	0602	1601	0701	-	0201/02	0303	11011	2301
Donor	112	0102	2902	44031	5701	0602	1601	0701	-	0201/02	0303	2301	-
Patient	113	0101	2901	0801	1302	0602	0701/05/06	0301	1301	0201	0603	01011	0402
Donor	114	0101	0201/09	0801	1302	0602	0701/05/06	0301	1301	0201	0603	0402	-
Patient	117	0201/09	-	4001	4402	0302/04	0501	0101	0401	0301	0501	2301	2801
Donor	118	0201/09	-	4001	4402	0302/04	0501	0101	0401	0301	0501	2301	-
Patient	119	0201/09	0301	0702	3502	0401	0702	0101	1101	0301	0501	0401	2301
Donor	120	0201/09	0301	0702	3502	0401	1505	0101	1101	0301	0501	0401	2301
Patient	123	0201/09	1101	0801	4402	0202#	0702	0301	-	0201/02	-	0401	2301
Donor	124	0201/09	1101	0801	4402	0501	0702	0301	-	0201/02	-	01011	11011
Patient	125	0101	0201/09	1801	27052	02012	0701/05/06	0101	1301	0501	0603	1301	3801
Donor	126	0101	0201/09	1801	27052	02012	1203	0101	1301	0501	0603	1702	2301
Patient	131	0101	68012	0801	51011	0701/05/06	1402	0301	0701	0201/02	-	0601	1401
Donor	132	0101	68012	0801	51011	0701/05/06	1502	0301	0701	0201/02	-	0401	11011
Patient	133	0201/09	2501	1801	4402	0501	1202/03	03011	0401	0201	0301	0401	0402
Donor	134	0201/09	2501	1801	4402	0501	1202/03	03011	0401	0201	0301	0401	0402
Patient	135	0201/09	2402	1402	4402	0202	0501	0102	0401	0301	0501	0401	0901
Donor	136	0201/09	2402	1402	4402	0501	0802/03	0102	0401	0301	0501	02012	0401

#unusual B-C association - expect B*4405 - Cw*0202

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	141	0201/09	1101	4402	5101	0302/04^	1502	0402	1301	0603	0302	0301	0401
Donor	142	0201/09	1101	4005	5101	0302/04	1502	0402	1301	0603	0302	0301	0401
Patient	143	0201/09	0301	0702	4402	0501	0702	0401	1501	0301	0602	0401	1401
Donor	144	0201/09	0301	0702	4402	0501	0702	0401	1501	0301	0602	0401	1901
Patient	153	0201/09	2402	0702	4402	0501	0702	1501	0901	03032	0602	0301	0401
Donor	154	0201/09	2402	0702	4402	0501	0702	1501	0901	03032	0602	0301	0401
Patient	155	2301	2402	0702	44031	0401	0702	0701	1501	0202	0602	0301	0401
Donor	156	2301	-	0702	44031	0401	0702	0701	1501	0202	0602	0401	02012
Patient	157	0201/09	-	0702	-	0702	-	0407	1501	0301	0602	02012	0401
Donor	158	0201/09	-	0702	-	0702	-	0407	1501	0301	0602	0401	-
Patient	163	2402	6901	1402	3508	0401	0802/03	0403	0701	0201/02	0302	02012	0401
Donor	164	2402	0201/09	1402	3508	0401	0802/03	0403	0701	0201/02	0302	02012	-
Patient	175	0201/09	3002	4402	1302	0501	0602	0701	-	0201/02	-	0401	-
Donor	176	0201/09	3001	4403£	1302	0501	0602	0701	-	0201/02	-	0401	-
Patient	181	1101	-	0702	4402	0102	0702	0401/16	1501	0301	0602	0401	-
Donor	182	1101	-	0702	4402	0102	0702	0401/16	1501	0301	0602	0401	-
Patient	189	0201/09	68011	0802	1801	0701/05/06	-	0301	0404	0201/02	0302	0401	2801
Donor	190	0101	68012	0802	1801	0701/05/06	0501	0301	0404	0201/02	0302	0401	-
Patient	201	0101	0201/09	4402	3502	0401	0501	0101	1501	0501	0602	0401	0402
Donor	202	0101	0201/09	4402	3502	0401	0501	0101	1501	0501	0602	0401	0402
Patient	203	0101	0301	44031	5701	0602	1601	0701	-	03032	0201/02	01011	0301
Donor	204	0101	0301	44031	5701	0602	1601	0701	-	03032	0201/02	02012	-
Patient	207	0101	-	0801	-	0701/05/06	-	0701	-	0201/02	0303	01011	11011
Donor	208	0101	-	0801	-	0701/05/06	-	0701	0301	0201/02	0303	01011	01011
Patient	209	0201/09	2301	3801	4402	0501	1203	0401	1301	0301	0603	0401	2101
Donor	210	0201/09	2301	3801	4402	0501	1203	0401	1301	0301	0603	0401	2101
Patient	211	0101	0201/09	0702	0801	0701/05/06	0702	0101	0301	0201	0501	0401	-
Donor	212	0101	0201/09	0702	0801	0701/05/06	0702	0101	0301	0201	0501	01011	0601

^unusual B-C association - expect B*4005 - Cw*0302

£unusual B-C association - expect B*4402 - Cw*0501

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	215	0201/09	-	0702	1501	0304	0702	0301	1501	0201/02	0602	1301	2001
Donor	216	0201/09	-	0702	1501	0304	0702	0301	1502~	0201/02	0602	0301	0401
Patient	217	0301	1101	0702	51011	0702	1502	0701	1501	0201/02	0602	02012	0401
Donor	218	0301	1101	0702	51011	0702	1502	0701	1501	0201/02	0602	0401	4601
Patient	221	0101	0201/09	1401	4001	0303/04	0801	0701	1302	0201/02	0604/08	0301	0401
Donor	222	0101	0201/09	1401	4001	0303/04	0801	0701	1302	0201/02	0604/08	0402	1401
Patient	223	0201/09	1101	3501	4402	0401	0501	0401	1301	0301	0603/07	0301	1501
Donor	224	0201/09	1101	3501	4402	0401	0501	0407	1301	0301	0603/07	0301	0901
Patient	233	0201/09	68011	3503	4001	0304	1203	0401	0801	0301	0402	0401	0901
Donor	234	0201/09	-	3503	4001	0304	0401	0401	0801	0301	0402	0401	02012
Patient	239	0102	0201/09	4402	5001	0501	0602	0401	0701	0202/02	0301	0301	0401
Donor	240	0101	0201/09	4402	5001	0501	0602	0401	0701	0202/02	0301	0301	0401
Patient	241	0102	2402	0801	44031	0701/05/06	0401	0701	0301/05	0201/02	-	0301	0401
Donor	242	0102	2402	0801	44031	0701/05/06	1601	0701	0301/05	0201/02	-	0401	0601
Patient	253	0201/09	2402/03	0702	39062	0702	0702	0404	0801	0302	0402	0401	1001
Donor	254	0201/09	2402/03	0702	39062	0702	0702	0404	0801	0302	0402	0402	-
Patient	257	0201/09	-	4402	4901	0501	0701/05/06	0103	0401/16	0301	-	0301	0401
Donor	258	0201/09	-	4402	4901	0501	0701/05/06	0101/04"	0401/16	0301	-	0401	-
Patient	259	0101	0301	0801	4001	0202	0701/05/06	0301	0701	0201/02	-	0301	02012
Donor	260	0101	0301	0801	4001	0302	0701/05/06	0301	0701	0201/02	-	02012	0401
Patient	263	0101	2501	4402	-	0501	-	0101	0401	0301	0501	0301	2001
Donor	264	0101	2501	4402	-	0501	-	0101	0401	0302	0501	0401	0401
Patient	267	2301	-	44031	4901	04011	0701/05/06	0701	1101	0201/02	0301	0301	0401
Donor	268	2301	-	44031	4901	16011	0701/05/06	0701	1101	0201/02	0301	0401	11011
Patient	269	0101	2902	0801	44031	0701/05/06	1601/02	0301	0701	0201/02	-	01011	0202
Donor	270	0101	2902	0801	44031	0701/05/06	1601/02	0301	0701	0201/02	-	0401	11011
Patient	273	2402	3201	3503/13	51012	0401	1601/02	1501	1101	0301	0602	0401	-
Donor	274	2402	3201	3503/13	51012	0401	1601/02	1501	1101	0301	0602	1401	1701

~Unusual DRB1 - DQB1 association - expect DRB1*1501 - DQB1*0602

"unusual DRB1-DQB1 association - expect DRB1*0101 - DQB1*0501

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	277	0101	0301	0702	5701	0602	0702	0701	1401	0301	03032	0202	0401
Donor	278	0101	0301	0702	5701	0602	0702	0701	1401	03032	05031	01011	1001
Patient	295	0301	3201	0702	44031	0501	0702	0101	1101	0301	0501	1701	2701
Donor	296	0301	3201	0702	44031	0501	0702	0101	1101	0301	0501	0401	1701
Patient	313	0201/09	-	1501	4001	0401	-	0101	0801	0401/02	0501	0401	0402
Donor	314	0201/09	-	1501	4001	0202	-	0101	0801	0401/02	0501	02012	02012
Patient	317	0201/09	-	1501	4402	0304	0501	0401	-	0301	0302	0401	2001
Donor	318	0201/09	-	1501	4402	0304	0501	0401	-	0301	0302	0401	02012
Patient	319	3001	6802	1302	1402	0602	0802/04	0701	1303	0201/02	0301	2101	1401
Donor	320	3001	6802	1302	1402	0602	0802/04	0701	1303	0201/02	0301	2101	1401
Patient	321	0201/09	-	1302	51011	0602	1502	0401	1101	0301	0302	0401	-
Donor	322	0201/09	-	1302	51011	0602	1502	0401	1101	0301	0302	0401	-
Patient	333	0201/09	2402	5101	3501	0401	02021	1101	1104	0301	-	0401	0402
Donor	334	0201/09	2402	5101	3501	0401	1502	1101	1104	0301	-	0401	-
Patient	337	0201/09	31012	4001	5001	0303	0602	0404	0701	0201/02	0302	0401	0601
Donor	338	0205	31012	4001	5001	0303	0602	0404	0701	0201/02	0302	0401	0601
Patient	341	0205	1101	0702	1501	0401	0702	0401	-	0301	0302	0401	-
Donor	342	0201/09	1101	0702	1501	0401	0702	0401	-	0301	0302	0401	2301
Patient	349	0101	2902	0801	44031	0701/05/06	1601	03011	0701	0201/02	-	0401	01011
Donor	350	0101	2902	0801	44031	0701/05/06	1601	03011	0701	0201/02	-	11011	01011
Patient	351	0201	2902	3501	44031	0401	0501	0701	1201	0201/02	0301	0401	0402
Donor	352	0201/09	2902	3503	44031	0401	0501	0701	1201	0201/02	0301	0401	11011
Patient	355	6801	0302	0703	44031	0702	1601	1501	0301	0201/02	0602	0401	-
Donor	356	6802	0302	0702	44031	0702	1601	1501	0301	0201/02	0602	0301	0401
Patient	365	0102	1101	0702	0801	0702	0701/05/06	1501	-	0602	0603	0401	-
Donor	366	0102	1101	0702	0801	0702	0701/05/06	1501	-	0602	-	0401	-
Patient	367	0101	0201/09	0801	4001	0302/04	0701/05/061/06	0301	1302	0201/02	0604/08	0301	0401
Donor	368	0101	0201/09	0801	4001	0302/04	0701/05/061/06	0301	1302	0201/02	0604/08	0301	0401

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	381	0205	0301	0801	5001	0602	0701/05/06	0701	0301	0201/02	0602	0401	02012
Donor	382	0205	0301	0801	5001	0602	0701/05/06	0701	0301	0202/02	-	01011	1701
Patient	385	2402	0201/09	0702	3501	0401	0702	0101	01021	0501	-	2801	-
Donor	386	2402	0201/09	0702	3501	0401	0702	0101	01021	0501	-	0401	2801
Patient	387	0101	6801	0702	3501	04011	0702	0401	1501	0302	0602	0402	02012
Donor	388	0101	6802	0702	3501	04011	0702	0401	1501	0301	0602	0401	-
Patient	389	2402	-	0702	1501	0303	0702	0101	1301	0501	0603	0402	3801
Donor	390	2402	-	0702	1501	0303	0602	0101	1301	0504	0603	0301	0401
Patient	393	2601	-	3503	3801	0401	1203	0402	-	0302	-	0401	-
Donor	394	2601	-	3503	3801	0401	1203	0402	-	0302	-	0401	-
Patient	407	0201/09	31012	4402	2705	0202	0501	0401	1301	0301	0603	0401	-
Donor	408	0201/09	31012	4402	2705	0202	0501	0401	1301	0301	0603	0401	-
Patient	411	0201/09	2902	1302	4501	0602	-	0401	1501	0301	0602	0401	0901
Donor	412	0201/09	2902	1302	4501	0602	-	0401	1501	0301	0602	0401	0601
Patient	427	0101	0201/09	0702	0801	0701/05/06	0702	0301	1501	0201/02	0602	0601	-
Donor	428	0101	0201/09	0702	0801	0701/05/06	0702	0301	1501	0201/02	0602	0401	-
Patient	447	0201/09	2402	2705	5001	0202	0602	0701	1101	0201/02	0301	02012	2701
Donor	448	0205	2402	2705	5001	0202	0602	0701	1101	0201/02	0301	02012	2701
Patient	449	0201/09	2901	0705	5701	0602	1502	0701	-	03032	0201/02	0601	1401
Donor	450	0201	2901	0705	5701	0602	1502	0701	-	03032	0201/02	0202	2801
Patient	455	0101	-	0801	5701	0602	0701/05/061/06	0301	0701	0201	0303	0401	1301
Donor	456	0101	-	0801	5701	0602	0701/05/061/06	0301	0701	0201	0303	0401	0301
Patient	475	0201/09	-	1501	4402	0304	0501	0401	1501	0302	0602	0401	-
Donor	476	0201/09	-	1501	4402	0304	0501	0401	1501	0302	0602	0401	-
Patient	509	0301	2601	0702	3801	0702	1203	1301	1501	0602	0603	0402	0401
Donor	510	0301	2601	0702	3801	0702	1203	1301	1501	0602	0603	0401	3801
Patient	519	68011	2402	0801	39062	0702	0701/05/06	0301	0401	0201/02	0301	0401	0402
Donor	520	68011	2501	0801	39013	0701/05/06	1203	0301	0401	0201/02	0301	0401	11011

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	535	1101	0302	0702	3501	0401	0704[$]	1201	1501	0301	0601/02	0401	-
Donor	536	1101	0301	0702	3501	0401	0702	1201	1501	0301	0601/02	0401	1301
Patient	545	0101	-	0801	1501	0303	0701/05/06	0301	1301	0603	0201/02	0401	0402
Donor	546	0101	-	0801	1501	0303	0701/05/06	0301	1301	0603	0201/02	0401	01011
Patient	549	0101	-	0801	-	0701/05/06	-	0301	1201	0201	0603	0401	02012
Donor	550	0101	-	0801	-	0701/05/06	-	0301	1201	0201	0301	0401	1401
Patient	551	0301	-	0702	1801	0701/05/06/03	0702	09012	1501	0602	0303	0401	02012
Donor	552	0301	-	0702	1801	0701/05/06	0702	09012	1501	0602	0303	02012	-
Patient	557	0102	0301	0702	4402	0704	0702	0101	1501	0501	0602	0401	-
Donor	558	0102	0301	0702	4402	1602	0702	0101	1501	0501	0602	0402	0202
Patient	563	0101	0301	0702	3501	0401	0702	0101	0103	0301	0501	0401	0402
Donor	564	0101	0301	0702	3503	0401	0702	0101	0103	0301	0501	0401	0402
Patient	577	0101	0301	0801	1402	0701/05/06/06	0802	0301	1302	0609	0201	0401	0501
Donor	578	0101	-	0801	1402	0701/05/06/06	0802	0301	1302	0609	0201	0402	01011
Patient	579	0201/09	68011	1401	4402	0704	0802	0101/04	0701	0501	0201	0402	0202
Donor	580	0201/09	68011	1401	4402	0704	0802	0101/04	0701	0501	0201	0402	-
Patient	583	0201/09	0101	0801	0702	0701/05/06	0702	0301	1502[~]	0201/02	0602	0401	02012
Donor	584	0201/09	0101	0804	0702	0701/05/06	0702	0301	1501	0201/02	0602	0402	1401
Patient	585	0101	0301	27052	1401	0202	0802	1401	0701	0201/02	05031	0402	0301
Donor	586	0101	0301	27052	4001	0304	0102	1401	0701	0201/02	05031	0401	1501
Patient	589	2301	3301	44031	5801	0602	1601	0701	-	0201/02	-	0402	02012
Donor	590	2301	2902	44031	5802	0602	1601	0701	-	0201/02	-	1101	0402
Patient	619	0301	-	0702	3501	0401	0702	0101	0102	0504	0501	0401	0402
Donor	620	0301	-	0702	3501	0401	0702	0101	0102	0504	0501	0401	02012
Patient	637	0102	0201/09	4002	4402	0302/04	0501	0404	1101	0301	0302	0401	-
Donor	638	0102	0201/09	4002	4402	0302/04	0501	0404	1101	0301	0302	0401	1601
Patient	653	0301	3001	1302	3503	04011	0602	0701	07	0201	0303	0402	1701
Donor	654	0301	3001	1302	3503	04011	0602	0701	0701	0201	0303	0402	11011

[$]unusual B-C association - expect B*0702 - Cw*0702 [~]Unusual DRB1 - DQB1 association - expect DRB1*1501 - DQB1*0602

Table 5-1 continued...

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	667	0101	0201/09	0702	5701	0602	0702	0301	0701	0201/02	0303	0401	02012
Donor	668	0101	0201/09	0702	5701	0602	0702	0301	0701	0201/02	0303	0901	2301
Patient	681	0101	-	0801	-	0701/05/061	-	0301	-	0201/02	-	01011	-
Donor	682	0101	-	0801	-	0701/05/061	-	0301	-	0201/02	-	02012	-
Patient	687	0101	2402	0801	1801	0701/05/061/06	-	1104	0301/05	0201/02	0301	0401	0402
Donor	688	0101	2402	0801	1801	0701/05/061/06	-	1104	0301/05	0201/02	0301	11011	11011
Patient	1129	0201/09	-	4006	4402	0702	1203	0401	1501	0302	0602	0401	4601
Donor	1130	0201/09	-	4006	4402	0702	1203	0401	1501	0301	0602	0401	02012
Patient	1131	0201/09	-	0702	2705	0702	1203	1302	0405	0301	0604	0401	-
Donor	1132	0201/09	-	0702	4102	1701	-	1302	0404	0302	0604	0401	-
Patient	1133	0201/09	0301	0702	39062	0702	-	0101	1501	0501	0602	0401	0301
Donor	1134	0201/09	0301	0702	39062	0702	-	0101	1501	0501	0602	0401	0601
Patient	1135	0201/09	6802	51011	5301	04011	1502	1302	0403	0302	0604	0301	06011
Donor	1136	0201/09	6802	51011	5301	04011	1502	1302	0405	0302	0604	0401	0402
Patient	1137	0301	2501	0702	4402	0501	0702	1501	-	0602	-	0401	0402
Donor	1138	0301	2501	0702	4402	0501	0702	1501	-	0602	-	0401	-
Patient	1139	0101	2402	0702	0801	0701/05/061	0702	1501	0103	0501	0602	0401	-
Donor	1140	0101	2402	0702	0801	0701/05/061	0702	1501	0101	0501	0602	0401	-
Patient	1141	0102	-	4402	-	0501	-	0101	0404	0302	0501	0402	0301
Donor	1142	0102	-	4402	-	0501	-	0101	0404	0302	0501	0402	0301
Patient	1145	2501	2902	44031	1501	[z]0701/05/061	1601	0701	1401	0201/02	05031	0202	-
Donor	1146	2501	2902	44031	0801	0701/05/061	1601	0701	1401	0201/02	05031	0301	0501
Patient	1147	0201/09	6601	4102	5701	1701	0602	1303	1601	0301	0502	0401	1001
Donor	1148	0201/09	6601	4102	-	0501	-	1303	1601	0301	0502	0401	02012
Patient	1149	0101	2402	0801	4002	02022	0701/05/061	0301	0701	0201/02	-	11011	-
Donor	1150	0101	2402	0801	4002	02022	0701/05/061	0301	0701	0201/02	-	11011	0401
Patient	1151	0201/09	2601	0702	0801	0701/05/061	0702	0301	1501	0201/02	0602	0401	-
Donor	1152	0201/09	2601	0702	0801	0701/05/061	0702	0301	1501	0201/02	0602	0401	-

[z]unusual B - C association - expect B*0801 - Cw*0701

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	1153	0101	31012	0801	1524	0302	0701/05/061	0403	1302	0302	0604	0401	0402
Donor	1154	0101	31012	0801	1501	0302	0701/05/061	0401	1302	0302	0604	0401	-
Patient	1155	0201/09	0301	0702	1402	0802	0702	0102	1501/2	0501	0601	0401	-
Donor	1156	0201/09	0301	0702	1402	0802	0702	0102	1501/2	0501	0601	0401	1901
Patient	1157	0201/09	0202	4402	5801	04011	0701/05/061	0401	0301	0201	0301	0401	0402
Donor	1158	0201	0202	4402	5801	0701/05/061	0302	0401	0301	0201	0301	0401	-
Patient	1159	0301	2301	0702	44031	04011	0702	0301	0701	0201/02	03032	0301	02012
Donor	1160	0301	2301	0702	4402	04011	0702	0301	0701	0201/02	03032	02012	1301
Patient	1161	2301	2902	1501	44031	0303	1601	1501/2	0701	0201/02	0602	0401	0301
Donor	1162	2301	2902	1501	44031	0304	04011	1501/2	0701	0201/02	0602	0401	0402
Patient	1163	0101	0201/09	0702	0801	0701/05/061	0702	0301	1501	0201/02	0602	0401	0501
Donor	1164	0101	0201/09	0702	0801	0701/05/061	0702	0301	1502	0201/02	0602	0401	0601
Patient	1165	0201/09	2402	0702	4402	0702	0501	1101	1401	0301	05031	0401	0402
Donor	1166	0201/09	2402	0702	44031	0702	1601	1101	1401	0301	05031	0202	-
Patient	1167	0201/09	2501	0801	5703	0701/05/061	-	0301	1303	0201	0301	0401	2001
Donor	1168	0201/09	2501	0801	5701	0701/05/061	-	0301	1303	0201	0301	01011	2801
Patient	1169	0201	0301	0702	4402	0501	0702	1501	-	0602	-	0401	-
Donor	1170	0201	0301	0702	4402	0501	0702	1501	-	0602	-	0401	0901
Patient	1171	1101	0101	0702	0801	0701/05/061	0702	0301	1501	0201/02	0602	1701	-
Donor	1172	1101	0101	0702	0801	0701/05/061	0702	0301	1501	0201/02	0602	0401	0301
Patient	1173	0201	31012	4402	-	0501	-	1501	1201	0301	0602	0401	-
Donor	1174	0201	31012	4402	-	0501	-	1501	1201	0301	0602	0401	0402
Patient	1175	0101	0301	0801	3505	04011	0701/05/061	0404	0301	0201	0302	0401	0601
Donor	1176	0101	0301	0801	4001	0304	0701/05/062	0404	0301	0201	0302	0301	02012
Patient	1177	0201	3201	15	52011	0303	12022	0401	1502	0302	0601	0401	0402
Donor	1178	0201	3201	1501	52011	04011	12022	0401	1502	0302	0601	0401	-
Patient	1179	2902	3301	44031	1402	0102	0701/05/061	0102	0701	0201/02	0501	0401	1401
Donor	1180[a]	0201	0301	4001	5601	0302/04	-	0102	0701	0201/02	0501	02012	1001

[a]Retrospective analysis indicates that this sample may not have been from donor 1180

Table 5-1 continued…

		HLA-A		HLA-B		HLA-C		HLA-DRB1		HLA-DQB1		HLA-DPB1	
Patient	1181	0201	-	0702	4402	0702	1601/2	0404	1301	0302	0603	02012	0601
Donor	1182	0201	-	0702	4402	0702	1601/2	0404	1301	0302	0603	0401	1902
Patient	1183	0301	3201	3501	52011	04011	12022	0407	1502	0301	0601	0402	0301
Donor	1184	0301	3201	3501	52011	04011	12022	0403	1502	0302	0601	0401	02012
Patient	1185	0101	1101	1801	52011	1203	12022	1502	0701	0201/02	0301	0401	02012
Donor	1186	0101	1101	1801	52011	12022	0202	1502	0701	0201/02	0301	0401	01011
Patient	1187	0101	2501	1801	3501	1203	0401	1101	-	0602	0301	0401	02012
Donor	1188	0101	2501	1801	3501	1203	0401	1104	-	0301	-	0401	02012
Patient	1189	1101	0302	27052	3501	0102	04011	0101	1101	0301	0501	0402	-
Donor	1190	1101	0301	27052	3501	0102	04011	0101	1101	0301	0501	02012	-
Patient	1191	0201	-	27052	1801	0501	0202	0101	-	0501	-	0401	-
Donor	1192	0201	-	27052	1801	0102	0701/05/061	0101	-	0501	-	0401	0601
Patient	1193	0201/09	3201	0702	1524	0701/05/061	-	0101	1202	0501	0301	0401	0601
Donor	1194	0201/09	-	0702	1501	0701/05/061	-	0101	1201	0501	0301	0402	-
Patient	1195	0201	3201	52011	5001	0602	12022	1104	0701	0201/02	0301	0401	-
Donor	1196	0201	3201	52011	5001	0501	12022	1101	0701	0201/02	0301	0402	02012
Patient	1197	0101	3301	2702	1402	0202	-	0102	0403	0302	0501	11011	0401
Donor	1198	0101	0205	27052	1402	0102	0802	0101	0403	0301	0504	01011	0601
Patient	1199	0101	0201/09	0801	-	0701/05/061	-	0301	-	0201/02	-	01011	0601
Donor	1200	0101	0201/09	0801	-	0701/05/061	-	0301	-	0201/02	-	0401	-
Patient	1201	1101	0201	1501	5501	0303	0304	0401	1401	0302	0503	0401	0301
Donor	1202	1101	0201	1501	5501	0303	0304	0401	1401	0302	0503	0401	-
Patient	1203	0101	-	0801	4001	0302	0701	0401	0301	0201/02	0302	02012	2301
Donor	1204	0101	0102	0801	4002	0302	0701	0401	0301	0201/02	0302	0401	-

1185 rare B - C association - expect B*1801 - Cw*1203, 0701, 0501, 0202

The total number of pairs with a mismatch for HLA-A was 27 (2 considered matched in the GVHD direction), HLA-B was 22 (with no mismatches considered matched in the GVH direction), HLA-C was 30 (with 1 considered matched in the GVHD direction), HLA-DRB1 was 19 (with 1 considered matched in the GVHD direction), HLA-DQB1 was 15 (with no mismatches considered matched in the GVH direction) and for HLA-DPB1, 112 pairs had a mismatch (with 15 of these considered matched in the GVHD direction).

RSCA discrepant results

Where HLA typing was available by another method, the results were compared with those for RSCA. Any difference in type can be seen in table 5-2.

Table 5-2. Comparison of RSCA assigned types with original typing

Sample	Locus	RSCA Typing	Original Typing
1180	HLA-A	0201/09	29
189	HLA-A	0201/09	31
1198	HLA-A	0205	33
1180	HLA-A	0301	33
113	HLA-A	2901	2
1193	HLA-A	3201	2
1194	HLA-B	1501	63
1145	HLA-B	1501	8
1131	HLA-B	2705	41
1175	HLA-B	3505	60
1180	HLA-B	4001	44
1180	HLA-B	5601	65
1148	HLA-B	4402	57
233	HLA-C	1203	0401/02
104	HLA-DRB1	0102	0101
257	HLA-DRB1	0103	0101
48	HLA-DRB1	0302	0301
224	HLA-DRB1	0407	04
223	HLA-DRB1	0401	0407
207	HLA-DRB1	0701	0301
1187	HLA-DRB1	1101	1104
1188	HLA-DRB1	1104	1101
1193	HLA-DRB1	1202	1203
38	HLA-DRB1	1502	1501
216	HLA-DRB1	1502	1501
583	HLA-DRB1	1502	1501
1164	HLA-DRB1	1502	1501
1183	HLA-DRB1	1502	1501
1184	HLA-DRB1	1502	1501
277	HLA-DQB1	0301	5
1198	HLA-DQB1	0301	8
390	HLA-DQB1	0504	0501
94	HLA-DQB1	0602	0201/02
102	HLA-DQB1	0602	0201/02
381	HLA-DQB1	0602	2
1187	HLA-DQB1	0602	7
549	HLA-DQB1	0603	0301
114	HLA-DQB1	0603	0602

Mismatches were determined by running the donor and patient samples in adjacent lanes using RSCA, and a mixture of the patient and donor hybridisation products in a third lane. If an additional peak was seen in the third lane, the donor and recipient were mismatched at that HLA locus. These mismatches were used for the analysis of outcome later and in chapter 4.

The positions of the peaks in the gel, after correction, were used to assign HLA types based on the values of known samples run before. Cases where the RSCA type differed from the original typing can be seen in table 5-2. The HLA-A, -B, -DRB1 and -DQB1 type was available for all samples (patient and donor, n = 276), with the HLA-C type available for 53 samples from original typing. The total number of allele + antigen types known for all loci was 2314 (276 x 2 alleles/antigens per locus for HLA-A, -B, -DRB1 and -DQB1 + 53 x 2 HLA-C alleles/antigens). From table 5-2 it can be seen the number of alleles called by RSCA that were different from the original typing totalled 38. Of the total, 1.6% of alleles typed were miscalled.

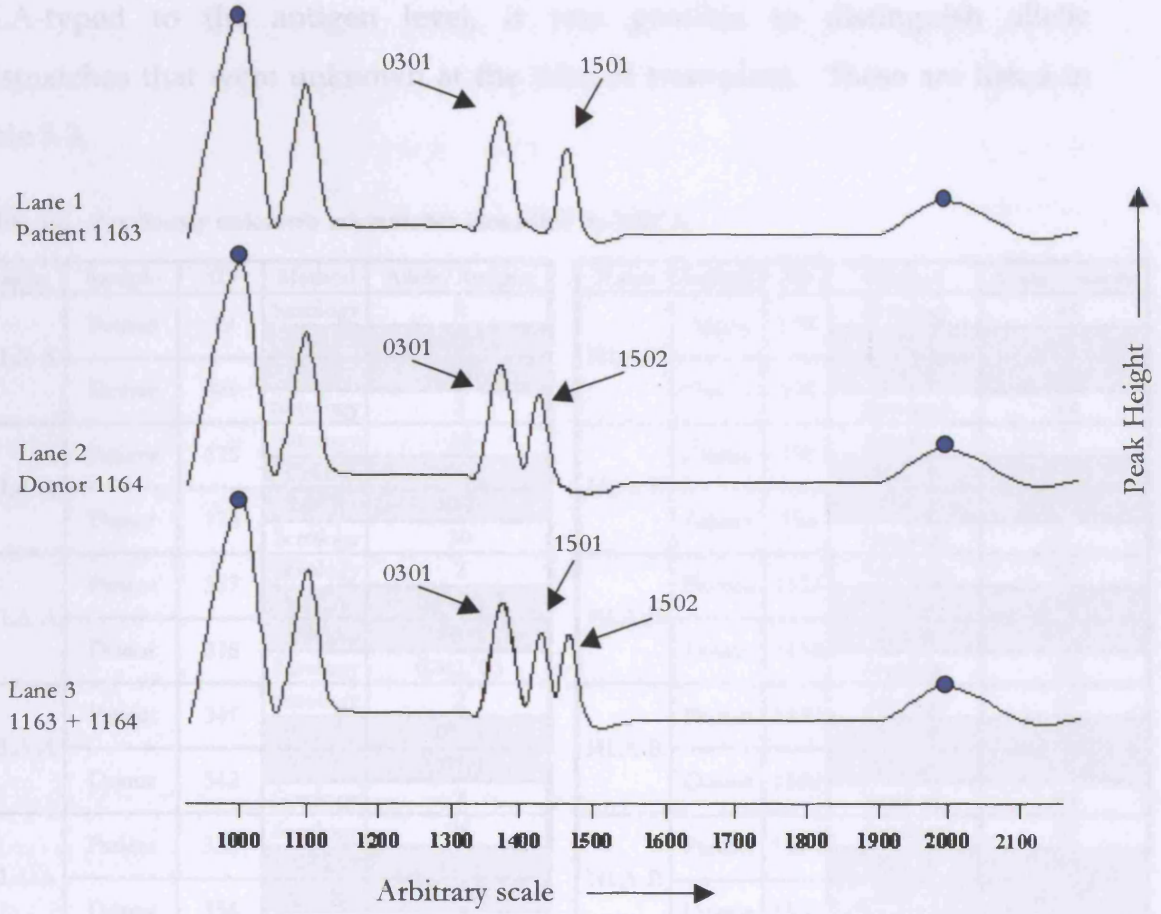
These differences would ideally have been investigated further using a third method, for example, a sequence based typing technique. Unfortunately, as all the RSCA analysis was carried out in a blinded manner, any difference with the original type was only identified at the end of the study. This meant that due to time constraints the additional analysis was not carried out. This extra step has been built into the continuation of the project and is being carried out in conjunction with the team continually developing the RSCA method.

The reason for these miscalls may have been that the peak seen was for an allele other than that called, but an example of that allele had not yet been run on RSCA and therefore, not assigned a value. The nearest known value was assigned to the peak and used in table 5-1. The RSCA method was validated, and the allele positions assigned using DNA samples from B-LCL samples that had been well classified in the 12th International Histocompatibility Workshop (Marsh *et al.* 1998). The main difference seen between the B-LCL hybridisation products and those from donors and patients was the width of the RSCA peak. In some samples the RSCA peak was wider than had been seen previously and

the tip of the peak had to be assigned manually, in order for the software to automatically allocate a numerical value. The manual assignment of peak tip position may have lead to a miss call of allele.

The most common example of RSCA calling a different type to the original method was for DRB1*1502 where the original type had been called DRB1*1501. DRB1*1501 ran at position 1444.3 ± 0.2 with FLR DRB1*1001 and DRB1*1502 ran at position 1433.7 ± 0.3. DRB1*1501 ran at position 1542.4 ± 0.2 with FLR DRB1*08021 and DRB1*1502 ran at position 1529.8 ± 0.5. From these values we can assume that the peak for DRB1*1501 cannot easily be confused with DRB1*1502. This is especially clear in cases such as patient 1163 (DRB1*0301, 1501) with donor 1164 (DRB1*0301, 1502), where both a DRB1*1501 and DRB1*1502 are present, as three peaks were present in the third lane (figure 5-1).

Figure 5-1. Cartoon example of the RSCA trace of patient 1163 (DRB1*0301, 1501) with donor 1164 (DRB1*0301, 1502). Lane 1 shows the RSCA trace for patient 1163, lane 2 shows the RSCA trace for donor 1164 and lane 3 shows the trace of the two hybridisation products mixed. Peaks highlighted in blue represent the bottom and top markers used to align the gel with arbitrary values of 1000 and 2000 respectively.



Mismatches identified by RSCA

As seen in figure 5-1, a mismatch in the sequence of two samples is easily identifiable using the RSCA method and mixing the patient and donor hybridisation products. Because many of the samples studied had only been HLA-typed to the antigen level, it was possible to distinguish allelic mismatches that were unknown at the time of transplant. These are listed in table 5-3.

Table 5-3. Previously unknown mismatches identified by RSCA.

Locus	Sample	ID	Method	Allele/Antigen
HLA-A	Patient	39	Serology	2
			RSCA	0205
	Donor	40	RSCA	0201
			Serology	2
HLA-A	Patient	175	Serology	30
			RSCA	3002
	Donor	176	RSCA	3001
			Serology	30
HLA-A	Patient	337	Serology	2
			RSCA	0201/09
	Donor	338	RSCA	0205
			Serology	0202/05
HLA-A	Patient	341	Serology	2
			RSCA	0205
	Donor	342	RSCA	0201/09
			Serology	2
HLA-A	Patient	355	Serology	28
			RSCA	6801
	Donor	356	RSCA	6802
			Serology	28
HLA-A	Patient	447	Serology	2
			RSCA	0201/09
	Donor	448	RSCA	0205
			Serology	2
HLA-A	Patient	535	Serology	3
			RSCA	0302
	Donor	536	RSCA	0301
			Serology	3
HLA-A	Patient	1189	Serology	3
			RSCA	0302
	Donor	1190	RSCA	0301
			Serology	3

Locus	Sample	ID	Method	Allele/Antigen
HLA-B	Patient	175	Serology	44
			RSCA	4402
	Donor	176	RSCA	4403
			Serology	44
HLA-B	Patient	355	Serology	7
			RSCA	0703
	Donor	356	RSCA	0702
			Serology	7
HLA-B	Patient	1153	Serology	62
			RSCA	1524
	Donor	1154	RSCA	1501
			Serology	62
HLA-B	Patient	1159	Serology	44
			RSCA	44031
	Donor	1160	RSCA	4402
			Serology	44
HLA-B	Patient	1165	Serology	44
			RSCA	4402
	Donor	1166	RSCA	44031
			Serology	44
HLA-B	Patient	1167	Serology	57
			RSCA	5703
	Donor	1168	RSCA	5701
			Serology	57
HLA-B	Patient	1177	Serology	62
			RSCA	15
	Donor	1178	RSCA	1501
			Serology	62
HLA-B	Patient	1193	Serology	62
			RSCA	1524
	Donor	1194	RSCA	1501
			Serology	62

Of the 138 donor/patient sample pairs that were originally typed to the antigen level, 14 had allelic mismatches detected by RSCA. Six pairs were mismatched

for HLA-A, 6 for HLA-B and 2 for both HLA-A and HLA-B (table 5-3).

The patient, donor and transplant characteristics including typing for HLA-DPB1 are shown in table 5-4. Once HLA-DPB1 typing was included in matching for HLA class I, the number of samples in the HLA matched group reduced from 60 to 15, and the number in the class I mismatch group from 40 to 7 (tables 4-2 and 5-4). Forty-five of the samples in the HLA-A, -B, -C, DRB1, -DQB1 matched group in the previous chapter had a mismatch for HLA-DPB1, meaning these transplants were now in the class II mismatch group. Forty-one of the HLA-A, -B, -C matched transplants from the previous chapter had now moved to the class I and class II mismatch group as they had an HLA-DPB1 mismatch. As both the matched and class I mismatch groups were now relatively small, they were combined into a single group for the statistical calculations in this chapter, in an attempt to increase the power of the analyses. The exclusion of those pairs with HLA-DP mismatches from this group resulted in a difference in the distribution of disease type, meaning the HLA matched/class I mismatch group now contained no patients with ALL or other malignant disease, and only one patient with non-malignant disease. Based on the results seen in the previous chapter, this may be likely to bias the analyses for this group, increasing the risk of developing acute GvHD, increasing the risk of reduced overall and disease free survival and increasing the risk of relapse. Also, no seropositive patients who received stem cells from a seronegative donor were now included in this group. The exclusion of these patients may mean this group is biased towards an increased risk of developing chronic GvHD, but a reduced risk of transplant related mortality. The effect of HLA matching, including HLA DPB1, on the development of acute and chronic GvHD, overall survival, disease free survival, transplant related mortality and relapse incidence was analysed by regression analysis to find if the inclusion for HLA-DPB1 matching made a difference to the effects seen with HLA matching excluding HLA-DP shown in the previous chapter. The factors included in multivariable analyses were as follows; type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender,

whether transplant was T-cell depleted or not, the occurrence of acute and chronic GvHD and HLA matching status.

Table 5-4. Demographics and transplant characteristics, with HLA typing including HLA-DPB1

	Total	Match (HLA-A, -B, -C, DRB1, -DQB1 & -DPB1)	Class I mismatch only (HLA-A, -B & -C)	Class II mismatch only (HLA-DRB1, -DQB1 & -DPB1)	Mismatch at class I and II (HLA-A, -B or -C & HLA-DRB1, -DQB1 or -DPB1)
Number of transplants	138	15	7	64	52
Disease					
CML	72	13	4	30	25
AML	23	2	2	12	6
ALL	24	0	0	11	13
Other malignant disease	12	0	0	9	3
Other non-malignant disease	8	0	1	2	5
Age (donor/patient)					
<=36 years/<= 27 years	30	1	1	14	14
<=36 years/>27 years	38	5	0	21	12
>36 years/<= 27 years	32	3	2	13	14
>36 years/> 27 years	38	6	4	16	12
CMV status# (donor/patient)					
seronegative/seronegative	51	10	1	24	16
seronegative/seropositive	15	0	0	7	8
seropositive/seronegative	19	2	3	9	5
seropositive/seropositive	14	2	2	5	5
Gender (donor/patient)					
male/male	60	6	3	30	21
male/female	31	2	1	17	11
female/male	25	4	2	7	12
female/female	22	3	1	10	8
T cell depletion					
T cell depleted	101	12	5	49	35
T cell replete	37	3	2	15	17

Effect of HLA matching including HLA-DPB1 on transplant outcome

Univariable and multivariable analysis was carried out as described previously (Chapter 2), with any HLA-DPB1 mismatches included with all other mismatches for HLA class II (HLA-DRB1 and -DQB1), and HLA class I mismatches grouped with matched transplants. This chapter presents the results for the HLA typing variable, as the association between the other factors and the outcome measures were presented in the previous section. The effect of the new definition of HLA matching in the multivariable models did not substantially alter the results shown in Chapter 4.

Graft versus host disease

Univariable and multivariable analysis, using binary logistic regression, was carried out to assess the effect of HLA matching for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 on the development of acute GvHD (table 5-5).

As can be seen in table 5-5, HLA matching for all loci was not significantly associated with the development of acute GvHD in univariable or multivariable analysis. Although there was no significant relationship between HLA matching and acute GvHD, after adjusting for other factors, there was some evidence that those with a class II mismatch could have as much as 2.5 times chance of developing acute GvHD (OR = 2.50; 95% CI 0.67, 9.30; p = 0.26). There was less evidence that having a mismatch at class I and II increased the risk of developing acute GvHD, as the odds ratio was 1.03 (95% CI 0.26, 4.03; p = 0.26), so the estimated of increase in risk is 3%, when compared with that for an HLA class I mismatch alone, or a matched patient.

Univariable and multivariable analysis, using binary logistic regression, was also carried out to assess the effect of HLA matching for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 on the development of chronic GvHD (table 5-6).

In both univariable and multivariable analysis, the odds of developing chronic GvHD with a class II mismatch alone were over 3 times that of patients who were matched or had a class I mismatch with their donor (OR = 3.18; 95% CI

1.16, 8.67 and OR = 3.32; 95% CI 0.55, 20.09 respectively). However, these findings were not significant at the 5% level ($p = 0.06$ and $p = 0.37$).

Table 5-5. Univariable and multivariable analysis of the effect of HLA matching for all loci on the development of acute GvHD.

Factor		Odds Ratio	95% Confidence Interval	p	Odds Ratio	95% Confidence Interval	p
All HLA loci	matched or class I mismatch	1.00	- , -	0.65	1.00	- , -	0.26
	Class II mismatch	1.43	0.61 , 3.33		2.50	0.67 , 9.30	
	Class I and II mismatch	1.08	0.44 , 2.63		1.03	0.26 , 4.03	

Table 5-6. Univariable and multivariable analysis of the effect of HLA matching for all loci on the development of chronic GvHD.

		Univariable Result			Multivariable Result		
Factor		Odds Ratio	95% Confidence Interval	p	Odds Ratio	95% Confidence Interval	p
All HLA loci	matched or class I mismatch	1.00	- , -	0.06	1.00	- , -	0.37
	Class II mismatch	3.18	1.16 , 8.67		3.32	0.55 , 20.09	
	Class I and II mismatch	2.92	1.01 , 8.49		1.20	0.19 , 7.57	

The above analyses were adjusted for type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender, whether transplant was T-cell depleted or not and the occurrence of acute and chronic GvHD.

Overall survival

Univariable and multivariable analysis was carried out to investigate the effect of HLA matching, including HLA-DPB1, on the overall survival of the patients in the study (table 5-7).

HLA matching for all loci was not associated with reduced overall survival when subjected to univariable analysis. In a multivariable model, compared to patients who had a class I mismatch or were matched with their donor, those with a class I and class II mismatch had a 2.4 increased risk of death (HR = 2.37; 95% CI 0.64, 8.69; p=0.20) and those with a class II mismatch had a 45% reduced risk of dying (HR = 0.55; 95% CI 0.13, 2.32; p=0.41). However, the global p-value for HLA matching was significant at the 5% level (p=0.02).

Table 5-7. Univariable and multivariable analysis of the effect of HLA matching, including HLA-DPB1, on overall survival

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
All HLA loci	matched or class I mismatch	1.00	- , -	0.41	1.00	- , -	0.02
	Class II mismatch	1.10	0.54 , 2.26		0.55	0.13 , 2.32	
	Class I and II mismatch	1.49	0.72 , 3.06		2.37	0.64 , 8.69	

Table 5-8. Univariable and multivariable analysis of the effect of HLA matching, including HLA-DPB1, on disease free survival.

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
HLA all loci	matched or class I mismatch	1.00	- , -	<0.01	1.00	- , -	<0.01
	Class II mismatch	0.54	0.32 , 0.93		0.21	0.08 , 0.56	
	Class I and II mismatch	1.17	0.69 , 2.00		1.58	0.63 , 3.98	

The above analyses were adjusted for type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender, whether transplant was T-cell depleted or not and the occurrence of acute and chronic GvHD

Disease free survival

Table 5-8 shows the results of univariable and multivariable analysis carried out to investigate the effect of HLA matching, including HLA-DPB1, on the disease free survival of the patients in the study. Both univariable and multivariable analysis showed HLA matching for all loci to be significantly associated with disease free survival at the 5% level ($p = <0.01$).

Univariable analysis showed patients who only had a class II mismatch with their donor had a 46% reduced risk of relapse or death compared with those mismatched for class I or matched (HR = 0.54; 95% CI 0.32, 0.93). Patients mismatched for class I and class II had an increased risk of 17% (HR = 1.17; 95% CI 0.69, 2.00).

The relationship remained in multivariable analysis, with patients who only had a class II mismatch with their donor having a 79% reduced risk of relapse or death compared with those mismatched for class I or matched (HR = 0.21; 95% CI 0.08, 0.56). Patients mismatched for class I and class II had an increased risk of 58% (HR = 1.17; 95% CI 0.63, 3.98).

Transplant related mortality

Univariable and multivariable analysis was carried out to investigate the effect of HLA matching, including HLA-DPB1, on the transplant related mortality of the patients in the study (table 5-9).

Table 5-9. Univariable and multivariable analysis of the effect of HLA matching, including HLA-DPB1, on transplant related mortality

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
All HLA loci	matched or class I mismatch	1.00	- , -	0.81	1.00	- , -	0.27
	Class II mismatch	1.04	0.44 , 2.46		0.37	0.06 , 2.22	
	Class I and II mismatch	1.25	0.52 , 2.99		1.05	0.19 , 5.88	

Table 5-10. Univariable and multivariable analysis of the effect of HLA matching, including HLA-DPB1, on relapse incidence.

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
All HLA loci	matched or class I mismatch	1.00	- , -	0.002	1.00	- , -	<0.01
	Class II mismatch	0.49	0.25 , 0.96		0.25	0.08 , 0.77	
	Class I and II mismatch	1.29	0.67 , 2.48		1.85	0.63 , 5.43	

The above analyses were adjusted for type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender, whether transplant was T-cell depleted or not and the occurrence of acute and chronic GvHD.

Table 5-9 shows that HLA matching for all loci was not significantly associated with transplant related mortality. However, the Hazard Ratio for patients with a class II mismatch indicates there may be an association between a class II mismatch and decreased risk of transplant related mortality compared with the other groups assessed (HR = 0.37; 95% CI 0.06, 2.22).

Relapse incidence

Univariable and multivariable analysis was carried out to investigate the effect of HLA matching, including HLA-DPB1, on the relapse incidence of the patients in the study (table 5-10).

HLA matching for all loci was found to have a significant effect on the incidence of relapse in both univariable (p = <0.01) and multivariable (p = <0.01) analysis (table 5-10). Univariable analysis showed patient mismatched for class I and II with their donor had a 29% increased risk of relapse compared to those with a class I mismatch and matched patients (HR = 1.29; 95% CI 0.67, 2.48), whereas patients with a class II mismatch had a 51% reduced risk of relapse (HR = 0.49; 95% CI 0.25, 0.96). Multivariable analysis showed mismatching for class II alone gave a more reduced risk of relapse (HR = 0.25, 95% CI 0.08, 0.77), with 26 out of the 62 patients in this group relapsing.

Discussion - including HLA-DP compared with excluding HLA-DP

Independent of whether HLA-DPB1 matching was (as shown in this chapter) or was not included (as shown in Chapter 4), the relationship between outcome and HLA matching in most scenarios appeared to remain the same. There was no significant relationship between HLA matching and GvHD when HLA-DP matching was excluded or included (tables 4-4, 4-6, 4-8, 5-5 and 5-6). The greatest risk of reduced overall survival was associated with a mismatch at HLA class I and class II (table 4-9). A similar association was seen for disease free survival (tables 4-10 and 5-8) and increased relapse incidence (tables 4-12 and 5-10). Both when HLA-DP matching was taken into account, and when it was not, the most unfavourable outcome in each of these cases was with an

HLA mismatch at both class I and class II. The only outcome measure upon which the additional locus matching changed the association, was transplant related mortality. When HLA matching was excluded, a mismatch at class I and class II was independently associated with an increased risk of transplant related mortality (table 4-11), however, the inclusion of HLA-DPB1 matching meant no significant association was seen at the 5% level (table 5-9).

When HLA-DP matching was excluded, it was possible to compare any HLA mismatches to the HLA-A, -B -C, -DRB1, -DQB1 matched group. For all multivariable analyses where a significant association was found, the most favourable outcome was associated with the HLA matched group, followed by a class II mismatch only, and then a class I mismatch only (tables 4-9, 4-10, 4-11 and 4-12). When HLA-DP matching was included, and it was necessary to group the matched and class I mismatch only groups, the most favourable outcome became associated with a class II mismatch alone in cases where a significant relationship was found (tables 5-7, 5-8 and 5-10). However, when matched transplants and those mismatched for class I were grouped, it can be hypothesised that the unfavourable effect of a class I mismatch masked the favourable effect of matching for all loci, making it appear that a class II mismatch was more favourable.

Multivariable analysis shows that an HLA class I and II mismatch gives the least favourable result with respect to overall survival, disease free survival, and relapse incidence, independent of the presence of an HLA-DP match or mismatch. In the analysis of an effect on the risk of transplant related mortality, a class I and II mismatch gave the least favourable result when HLA-DPB1 matching was excluded, but when included, no significant effect was seen.

These results indicate that the hierachy of mismatches in this study is as follows;

HLA class I and class II > HLA class I alone > HLA class II alone > HLA matched

The effect of an HLA-DPB1 mismatch alone

To confirm the hypothesis that HLA-DP matching did not contribute to the effect on transplant outcome, a group with an HLA-DPB1 mismatch alone (n = 45), was compared with matched transplants (n = 15). No significant association was seen with an HLA-DPB1 mismatch alone and the development of acute or chronic GvHD, overall survival or transplant related mortality (data not shown). A study by Petersdorf *et al*, in patients matched for HLA-A, -B, -DRB1 and -DQB1 showed no association between an HLA-DPB1 mismatch and increased risk of acute GvHD (Petersdorf *et al.* 1993a). However, a more recent study, by Varney *et al*, has shown a significant association with matching for HLA-DPB1 and reduced risk of developing acute GvHD (Varney *et al.* 1999).

When HLA-DPB1 matching alone was analysed to find if there was an association with outcome a significant effect was seen with disease free survival and relapse incidence. Table 5-11 shows the result of univariable and multivariable analysis of the effect of an HLA-DPB1 mismatch on disease free survival.

Univariable analysis showed a patient mismatched with their donor for only HLA-DPB1 had a 50% reduce chance of death or relapse compared to those matched with their donor for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 (HR = 0.50; 95% CI 0.26, 0.94, p = 0.03, table 5-11). Multivariable analysis showed an HLA-DPB1 mismatch gave a 96% reduced risk of death or relapse (HR = 0.04; 95% CI 0.00, 0.39, p = 0.01).

Table 5-11. The effect of an HLA-DPB1 mismatch on disease free survival

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
HLA all loci	matched for all loci	1.00	- , -	0.03	1.00	- , -	0.01
	mismatch for HLA-DPB1	0.50	0.26 , 0.94		0.04	0.00 , 0.39	

Table 5-12. The effect of an HLA-DPB1 mismatch on relapse incidence

Factor		Univariable Result			Multivariable Result		
		Hazard Ratio	95% Confidence Interval	p	Hazard Ratio	95% Confidence Interval	p
HLA-DPB1	matched for all loci	1.00	- , -	0.003	1.00	- , -	0.01
	mismatch for HLA-DPB1	0.31	0.14 , 0.67		0.02	0.00 , 0.31	

The above analyses were adjusted for type of disease, donor CMV status, patient CMV status, donor age, patient age, donor gender, patient gender, whether transplant was T-cell depleted or not and the occurrence of acute and chronic GvHD.

The effect of an HLA-DPB1 mismatch alone on relapse incidence is shown in table 5-12. An HLA-DPB1 mismatch was significantly associated with reduced risk of relapse when compared to patients matched for all HLA loci with their donor ($p = <0.01$). A patient with an HLA-DPB1 mismatch had a 69% reduced risk of relapse compared to a patient matched with their donor for all loci (HR = 0.31; 95% CI 0.14, 0.67). When subjected to multivariable analysis, an HLA-DPB1 mismatch was independently associated with reduced risk of relapse ($p = 0.01$). A patient with an HLA-DPB1 mismatch only had a 98% reduced risk of relapse compared with a patient matched with their donor for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 (HR = 0.02; 95% CI 0.00, 0.31). This reduction in risk of relapse with a mismatch for HLA-DPB1 may be due to the recognition of HLA-DP antigens expressed on leukaemic blasts, by HLA-DP-specific T cells, as has been previously described (Ibisch *et al.* 1999).
When HLA-DP matching was analysed without correction for other HLA mismatches, no significant association was seen with any of the outcomes tested (data not shown). This further supports the theory that an HLA-DPB1 mismatch does not have the same level of effect as a mismatch at any of the other loci tested. However, based on the results shown in table 5-11 and 5-12, it can be postulated that a mismatch for HLA-DPB1 may protect from relapse and therefore lead to improved disease free survival, in patients matched for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1. In conclusion, to show a true effect of an HLA-DPB1 mismatch on transplant outcome, a greater number of transplant pairs need to be analysed. Ideally, in a patient group who have the same disease and status, with equal numbers in the matched and mismatched groups.

Chapter 6

Minor histocompatibility antigen matching in unrelated bone marrow transplantation

Introduction

Minor histocompatibility antigens (mHAg) are peptides which, when bound to MHC molecules, are recognised by T cells causing an allogeneic response. This phenomenon was discovered by Snell and co-workers, when tumours were transplanted between MHC identical individuals and were rejected less aggressively than those mismatched for MHC antigens (Counce *et al.* 1956; Snell 1948). Few mHAgs have been described, and can be peptides encoded by genes on sex chromosomes, or encoded by autosomal or mitochondrial DNA. For example, the H-Y peptides encoded by genes on the Y chromosome (Vogt *et al.* 2000; Wang *et al.* 1995). Or non-sex-linked mHAgs encoded by the polymorphic genes of adhesion molecules (Behar *et al.* 1996; Maruya *et al.* 1998), although the effect of mismatches between these on transplant outcome has been controversial (Nichols *et al.* 1996). The presence of a further set of non-Y-linked mHAgs has been described by Goulmy *et al*, and called HA-1, HA-2, HA-3, HA-4 and HA-5 (Van Els *et al.* 1992). HA-1, HA-2 and HA-3 were found to occur at a frequency of 69-95% in a healthy population, while HA-4 and HA-5 were only present in 7-16% of individuals tested (Van Els *et al.* 1992).

HA-1 is restricted by HLA-A2, and has two allelic forms, $HA\text{-}1^{H}$ which is presented by HLA-A2 and $HA\text{-}1^{R}$, which is not presented by HLA-A2, therefore $HA\text{-}1^{R}$ homozygous individuals are said to be HA-1 negative. Expression of

HA-1 and HA-2 is limited to the cells of the haematopoietic lineage, whereas H-Y, HA-3 and HA-4 have been found ubiquitously expressed (de Bueger *et al.* 1992). The frequency of HA-1 alleles in the normal Caucasian population has been reported as approximately 40% HA-1^H and 60% HA-1^R (Tseng *et al.* 1998). On our cohort of patients and donors we found 38% HA-1^H alleles and 62% HA-1^R alleles. Observations in HLA identical sibling transplants have shown that a HA-1 mismatch between HLA-A*0201 positive donors and recipients is significantly associated with the development of acute GvHD ($p = 0.02$) (Goulmy *et al.* 1996). This association has not yet been explored in the unrelated bone marrow transplant setting, and for this reason we chose to observe the effect of an HA-1 on the outcome of transplant in our cohort which contained 57 HLA-A*0201 donors and recipients.

The effect of HA-1 mismatch on transplant outcome

DNA from 57 recipients and donors from HLA-A*0201 positive unrelated bone marrow transplant pairs was amplified in a PCR reaction using HA-1^H and HA-1^R specific primers to discover whether each individual was positive or negative for HA-1 (Wilke *et al.* 1998). A positive individual was either homozygous or heterozygous for HA-1^H, whereas a negative individual was homozygous for HA-1^R (den Haan *et al.* 1998). The results of this typing are shown in figure 6-1.

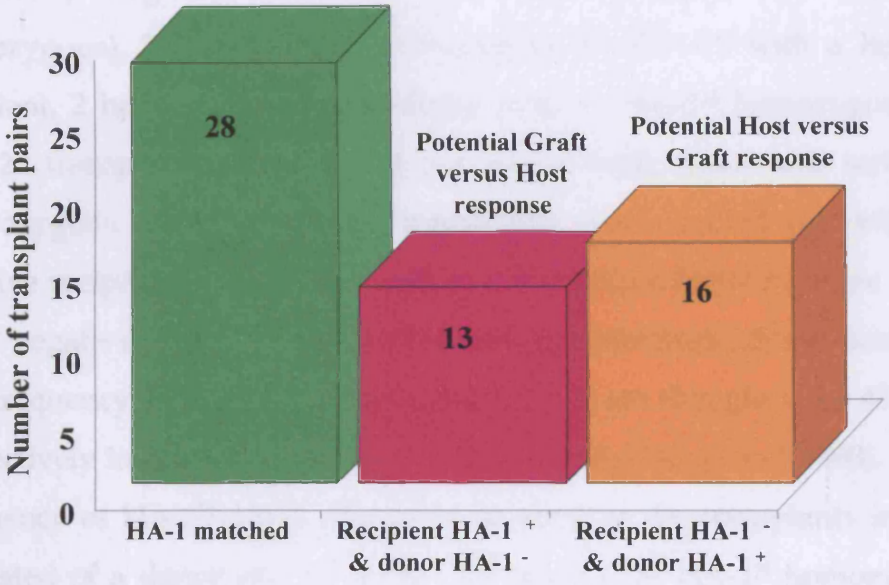


Figure 6-1. HA-1 typing of 57 HLA-A*0201 positive transplant recipients and their donors. 28 patients received a HA-1 matched transplant (green). 13 HA-1 positive patients received a HA-1 negative transplant and 16 HA-1 negative patients received a HA-1 positive transplant. HA-1 typing was carried out using a sequence specific PCR kit (Robbins Scientific) (Wilke *et al.* 1998).

Twenty-eight individuals were matched for their HA-1 status. Of these, 3 were completely negative for HA-1 (i.e. both recipient and donor $HA\text{-}1^R$ homozygous), 3 had a donor homozygous for $HA\text{-}1^H$ with a heterozygous recipient, 2 had a heterozygous donor with an $HA\text{-}1^H$ homozygous recipient and 20 transplants were carried out where both donor and recipient were heterozygous. The remaining transplants were carried out with a HA-1 positive recipient transplanted with marrow from a HA-1 negative donor, or a HA-1 negative recipient transplanted with marrow from a HA-1 positive donor. The frequency of the $HA\text{-}1^H$ and $HA\text{-}1^R$ alleles are thought to be 40% and 60% respectively in HLA-A*0201 positive individuals (Tseng *et al.* 1998). This lower frequency of $HA\text{-}1^H$ could explain why none of the transplants in our study consisted of a donor and recipient who were both $HA\text{-}1^H$ homozygous. The mismatches in HA-1 status were correlated with overall survival, the risk of developing acute and chronic GvHD (where grade II or higher indicated the presence of acute GvHD), and the risk of relapse post transplant. HA-1 negative (HA-1R homozygous) donor/recipient and their donor/recipient being HA-1 positive defined a HA-1 mismatch. On transplanting a HA-1 negative recipient with marrow from a HA-1 positive donor, a possible outcome would be a host-versus-graft reaction, whereas when transplanting a HA-1 positive recipient with HA-1 negative marrow, a possible outcome would be GvHD.

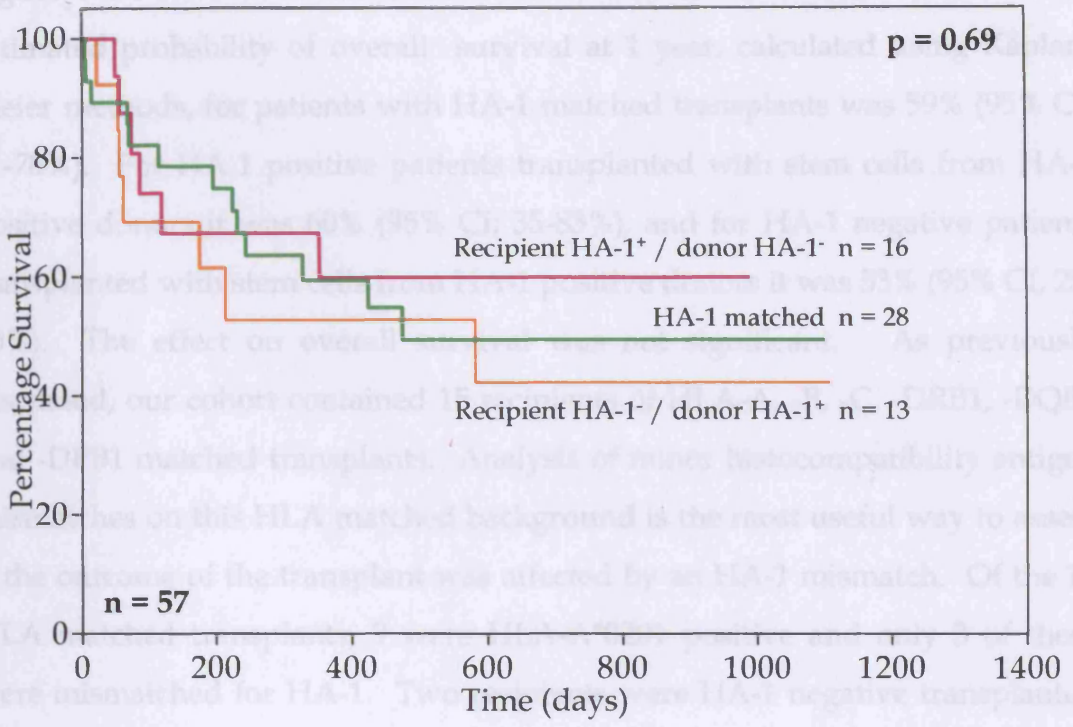


Figure 6-2. The effect of HA-1 mismatch on survival. A Kaplan Meier survival plot of percentage survival against time in days. HA-1 matched transplant recipients (green) compared with those who were positive for HA-1 transplanted with an HA-1 negative donor (pink), or those who were negative for HA-1 transplanted with an HA-1 positive donor (orange). Significance was calculated using the Log Rank Statistic in a pair wise comparison with recipients whose transplants were matched for HA-1 (green).

The effect of HA-1 matching on overall survival

Figure 6-2 shows the effect of an HA-1 mismatch on overall survival. The estimated probability of overall survival at 1 year, calculated using Kaplan-Meier methods, for patients with HA-1 matched transplants was 59% (95% CI, 41-78%). For HA-1 positive patients transplanted with stem cells from HA-1 positive donors it was 60% (95% CI, 35-83%), and for HA-1 negative patients transplanted with stem cells from HA-1 positive donors it was 53% (95% CI, 25-80%). The effect on overall survival was not significant. As previously discussed, our cohort contained 15 recipients of HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 matched transplants. Analysis of minor histocompatibility antigen mismatches on this HLA matched background is the most useful way to assess if the outcome of the transplant was affected by an HA-1 mismatch. Of the 15 HLA matched transplants, 9 were HLA-A*0201 positive and only 3 of these were mismatched for HA-1. Two recipients were HA-1 negative transplanted with bone marrow from an HA-1 positive donor, and 1 transplant was carried out with an HA-1 positive recipient and an HA-1 negative donor. At the time of analysis, one of the 2 HA-1 negative recipients was alive (634 days post transplant) and the other had died 22 days post transplant from infection and veno-occlusive disease (VOD). The HA-1 positive recipient of an HA-1 negative transplant was also alive (834 days post transplant).

An effect of HA-1 mismatching on the development of GvHD has been observed post HLA identical sibling transplant (Goulmy *et al.* 1996; Tseng *et al.* 1999). We assessed the effect of an HA-1 mismatch on the risk of developing acute and chronic GvHD (where grade II or higher indicated the presence of acute GvHD), the results of which can be seen in figure 6-3.

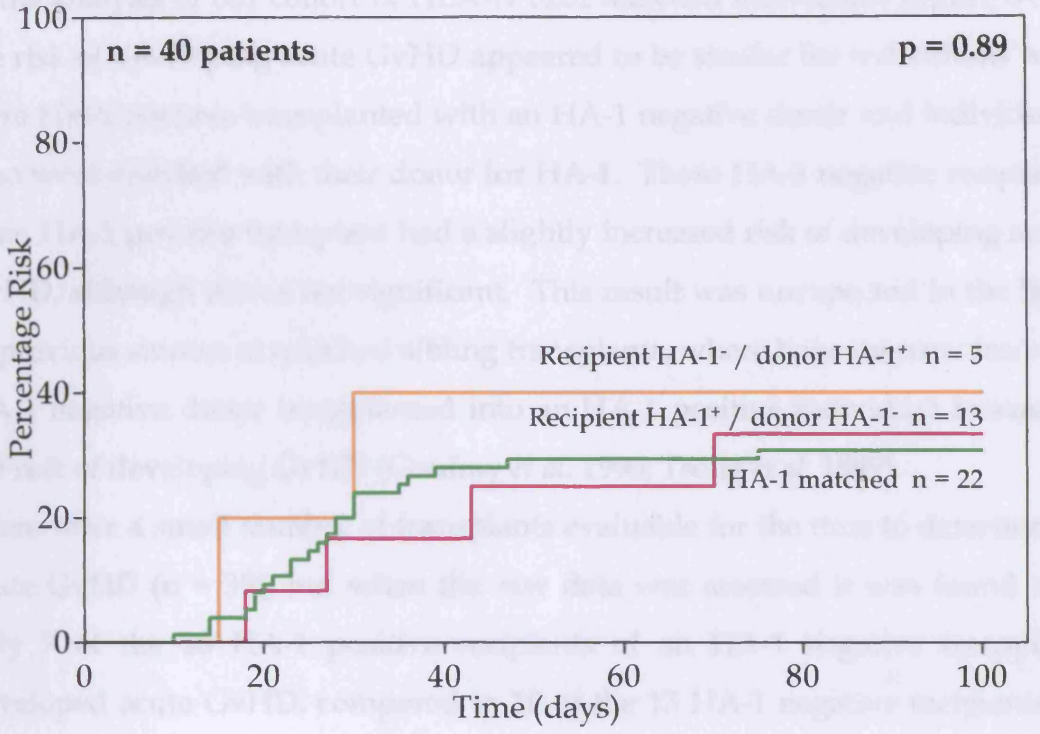


Figure 6-3a. The effect of HA-1 mismatch on the risk of developing acute GvHD. A Kaplan Meier hazard plot of cumulative hazard against time in days. HA-1 matched transplant recipients (green) compared with those who were positive for HA-1 transplanted with an HA-1 negative donor (pink), or those who were negative for HA-1 transplanted with an HA-1 positive donor (orange). Significance was calculated using the Log Rank Statistic in a pair wise comparison with recipients whose transplants were matched for HA-1 (green).

Effect of HA-1 matching on the development of GvHD

In the analysis of our cohort of HLA-A*0201 matched individuals (figure 6-3a), the risk of developing acute GvHD appeared to be similar for individuals who were HA-1 positive transplanted with an HA-1 negative donor and individuals who were matched with their donor for HA-1. Those HA-1 negative recipients of an HA-1 positive transplant had a slightly increased risk of developing acute GvHD, although it was not significant. This result was unexpected in the light of previous studies in matched sibling transplants, where bone marrow from an HA-1 negative donor transplanted into an HA-1 positive individual increased the risk of developing GvHD (Goulmy *et al.* 1996; Tseng *et al.* 1999).

There were a small number of transplants evaluable for the time to detection of acute GvHD (n = 39), but when the raw data was assessed it was found that only 7 of the 16 HA-1 positive recipients of an HA-1 negative transplant developed acute GvHD, compared to 10 of the 13 HA-1 negative recipients of an HA-1 positive transplant. In the HA-1 matched group, 15 of 28 individuals developed acute GvHD. None of the 3 HA-1 matched, HLA matched, HLA-A*0201 transplant recipients were reported to have developed acute GvHD. This data implies that an HA-1 mismatch may not confer an independent increase in the risk of developing acute GvHD after unrelated bone marrow transplantation, although it must be stressed that this analysis was carried out on transplants with some known HLA mismatches, and there were only three individuals without known HLA mismatches available for analysis.

The effect of an HA-1 mismatch on the risk of developing chronic GvHD was also assessed in the individuals (figure 6-3b).

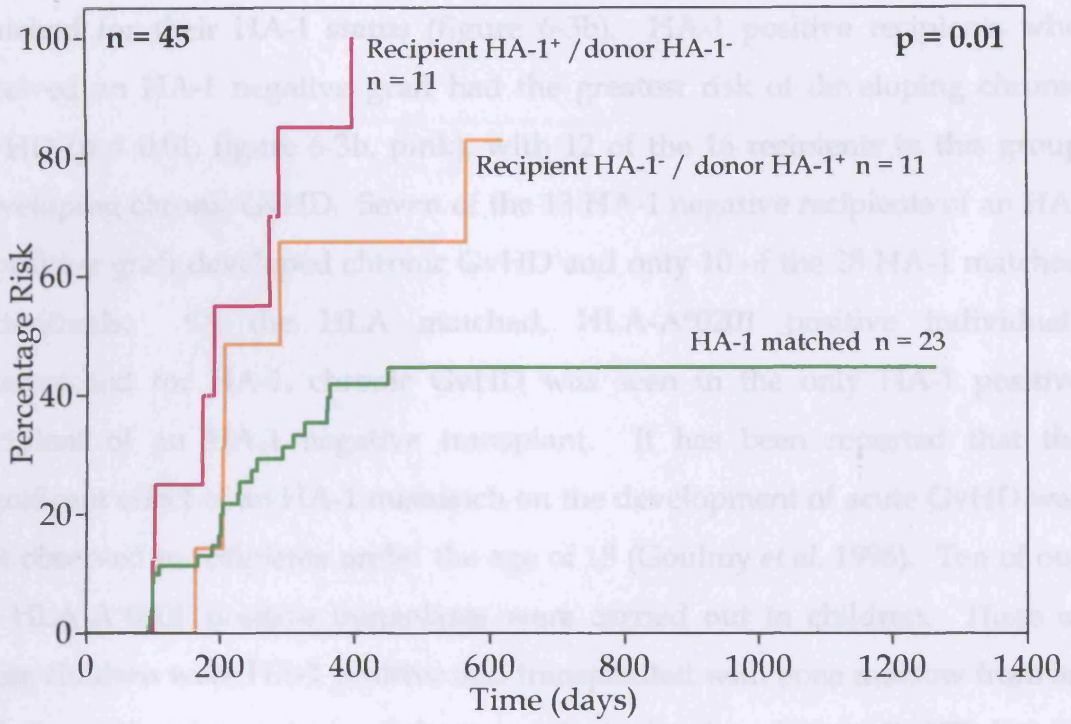


Figure 6-3b. The effect of HA-1 mismatch on the risk of developing chronic GvHD. A Kaplan Meier hazard plot of cumulative hazard against time in days. HA-1 matched transplant recipients (green) compared with those who were positive for HA-1 transplanted with an HA-1 negative donor (pink), or those who were negative for HA-1 transplanted with an HA-1 positive donor (orange). Significance was calculated using the Log Rank Statistic in a pair wise comparison with recipients whose transplants were matched for HA-1 (green).

In this cohort of HLA-A*0201 positive transplants, the effect of a mismatch for HA-1 in either direction was seen to be significant compared with individuals matched for their HA-1 status (figure 6-3b). HA-1 positive recipients who received an HA-1 negative graft had the greatest risk of developing chronic GvHD ($p < 0.01$, figure 6-3b, pink), with 12 of the 16 recipients in this group developing chronic GvHD. Seven of the 13 HA-1 negative recipients of an HA-1 positive graft developed chronic GvHD and only 10 of the 28 HA-1 matched individuals. Of the HLA matched, HLA-A*0201 positive individuals mismatched for HA-1, chronic GvHD was seen in the only HA-1 positive recipient of an HA-1 negative transplant. It has been reported that the significant effect of an HA-1 mismatch on the development of acute GvHD was not observed in recipients under the age of 18 (Goulmy *et al.* 1996). Ten of our 57 HLA-A*0201 positive transplants were carried out in children. Three of these children were HA-1 positive and transplanted with bone marrow from an HA-1 negative donor, none of these recipients developed acute GvHD, but all developed chronic GvHD. This may suggest that the effect of an HA-1 mismatch in child recipients or generally in unrelated bone marrow transplantation, may elicit a chronic GvHD response as opposed to the acute GvHD seen in HLA identical sibling transplantation.

A graft-versus-host response has been associated with a graft-versus-leukaemia effect (Gratwohl *et al.* 1995). It is also been shown that HA-1-specific CTLs can lyse leukaemic cells expressing HLA-A*0201 restricted HA-1 antigens (Falkenburg *et al.* 1991; Mutis *et al.* 1999b). If this were true in our cohort, we would expect to see a reduced risk of relapse in HA-1 positive recipients of an HA-1 negative graft. The effect of an HA-1 mismatch on the risk of disease relapse is shown in figure 6-4.

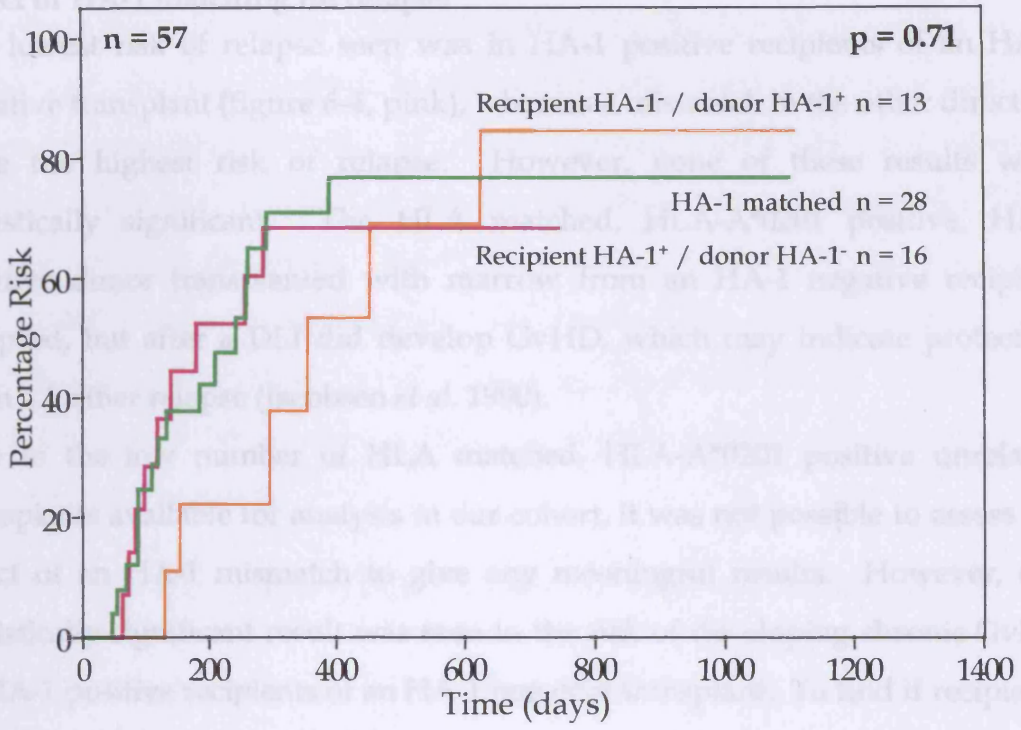


Figure 6-4. The effect of HA-1 mismatch on the risk of relapse. A Kaplan Meier hazard plot of cumulative hazard against time in days. HA-1 matched transplant recipients (green) compared with those who were positive for HA-1 transplanted with an HA-1 negative donor (pink), or those who were negative for HA-1 transplanted with an HA-1 positive donor (orange). Significance was calculated using the Log Rank Statistic in a pair wise comparison with recipients whose transplants were matched for HA-1 (green).

Effect of HA-1 matching on relapse

The lowest risk of relapse seen was in HA-1 positive recipients of an HA-1 negative transplant (figure 6-4, pink), whereas a mismatch in the other direction gave the highest risk of relapse. However, none of these results were statistically significant. The HLA matched, HLA-A*0201 positive, HA-1 positive donor transplanted with marrow from an HA-1 negative recipient relapsed, but after a DLI did develop GvHD, which may indicate protection from a further relapse (Jacobsen *et al.* 1990).

Due to the low number of HLA matched, HLA-A*0201 positive unrelated transplants available for analysis in our cohort, it was not possible to assess the effect of an HA-1 mismatch to give any meaningful results. However, one statistically significant result was seen in the risk of developing chronic GvHD in HA-1 positive recipients of an HA-1 negative transplant. To find if recipients of HA-1 mismatched transplants from unrelated donors had HA-1 specific T cells present post transplant, as has been seen in HLA identical sibling transplants (Goulmy *et al.* 1996; Van Els *et al.* 1992), we collected post transplant blood samples from such recipients and tested for HA-1-specific CTLs using HLA-A*0201:HA-1 tetramers.

Detection of HA-1 specific T cells in bone marrow transplant recipients

In order to assess whether HA-1 antigens were being recognised in an unrelated transplant situation we collected PBMCs post transplant from HLA-A*0201-positive recipients of unrelated transplants from the Hammersmith Hospital, London. These PBMCs were stained with anti-CD8 and anti-CD4 monoclonal antibodies, and HLA-A*0201:HA-1 tetramers we had previously made (described in Chapter 2), in order to detect the presence of any donor-derived HA-1-specific T cells using flow cytometry.

The tetramers were formed from the extracellular domains of HLA-A*0201 molecules bound to the HA-1 peptide and human β_2-microglobulin. The exon encoding the transmembrane region of the HLA-A*0201 molecule was replaced with a C-terminal sequence coding for a biotinylation target. This complex was

biotinylated and then tetramerised with Phycoerythrin-labelled Streptavidin. The newly formed tetramers had been tested for fidelity at each stage of the formation procedure, however, HA-1-specific T-cells were not readily available to use as a positive control. Therefore, methods had to be undertaken to produce these cells in order to test the tetramers. Two protocols for T-cell stimulation were followed. Firstly, a mixed lymphocyte culture/reaction (MLR/MLC) between individuals who were HLA-A*0201 positive, but mismatched for their HA-1 status. Secondly, stimulation of HA-1 negative or HA-1 positive PBMCs with autologous peptide-pulsed mononuclear cells (described in Chapter 2).

Mixed lymphocyte culture for the formation of HA-1-specific T cells

Blood from two healthy HLA-A*0201 positive donors was available for use in the mixed lymphocyte culture, AML ($HA\text{-}1^-$) and RH ($HA\text{-}1^+$), and one pre-transplant $HA\text{-}1^-$ donor with an $HA\text{-}1^+$ recipient, where PBMCs were stored in liquid nitrogen. Briefly, PBMCs were mixed at a ration of 1:1 ($HA\text{-}1^+$ or $HA\text{-}1^-$ responder: $HA\text{-}1^-$ or $HA\text{-}1^+$ irradiated stimulator) and cultured for 7 days at 37°C in 5% CO_2. One million irradiated stimulator cells were then added, with 20 units/ml IL-2. At day 12, one million cells were removed from each culture and stained with anti-CD8-FITC and anti-CD3-PE monoclonal antibodies (Beckton Dickinson) and the HLA-A*0201:HA-1 tetramer. These cells were then analysed using a FACscan flow cytometer and the results analysed with CELLQuest software (Beckton Dickinson). The results of this staining can be seen in figure 6-5.

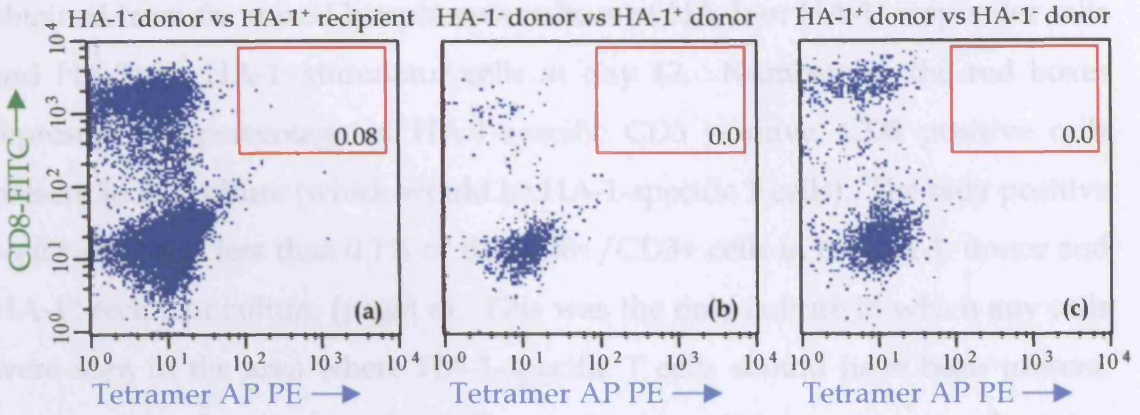


Figure 6-5. HLA-A*0201:HA-1^{H}:PE tetramer staining of PBMCs from MLR experiments. MLRs were carried out with PBMCs from an HA-1 positive donor against their HA-1 negative recipient, an HA-1 positive donor against an HA-1 negative donor and an HA-1 positive donor against an HA-1 negative donor. The cells remaining after 12 days of culture were stained with anti-CD3 and anti-CD8 (y axis) monoclonal antibodies (Beckton Dickinson) and HLA-A*0201:HA-1^{H}:PE tetramer (x axis, panels a, b and c respectively). Numbers in the red squares represent the percentage of tetramer positive T cells (which also gave a positive result when stained with the CD3-PerCP and CD8-FITC specific antibodies)

Figure 6-5 represents the results of tetramer staining of the live $CD3^+$ cells obtained from the mixed lymphocyte culture of HA-1^- or HA-1^+ responder cells and HA-1^+ or HA-1^- stimulator cells at day 12. Numbers in the red boxes represent the percentage of HA-1-specific CD3 positive, CD8 positive cells present in the culture (which would be HA-1-specific T cells). The only positive result seen was less than 0.1% of the CD8+/CD3+ cells in the HA-1^- donor and HA-1^+ recipient culture (panel a). This was the only culture in which any cells were seen in the area where HA-1-specific T cells should have been present. Interestingly, the number of T cells seen in this culture was greater than that seen for those from the healthy donors, who were known to have many more HLA mismatches than in the case of the recipient and donor (figure 6-5, panels b and c). The tetramer positive cells seen in panel a, however, are probably not HA-1-specific, $CD3^+/CD8^+$ T cells as there are also $CD8^-$ cells present in the area below the red box, indicating non-specific background staining. It was thought that this method of HA-1-specific T cell production had too many inconsistencies between responder and stimulator to produce T cells specific for a minor histocompatibility antigen, especially as there were already major histocompatibility differences present.

Induction of HA-1-specific T cells using synthetic peptide-pulsed APCs

Mutis *et al* have published a protocol for the ex-vivo induction of HA-1-specific CTLs (Mutis *et al.* 1999b). The main problem with this protocol is the requirement for large numbers of autologous PBMCs on a regular basis. Therefore, for the production of the first round of antigen presentation, a EUCAPS protocol for the production of dendritic cells from peripheral blood, was used (see Chapter 2). These antigen presenting cells (APCs) from the HA-1^- and HA-1^+ healthy donors (AML and RH respectively) were pulsed with synthetically produced HA-1 (10μg/ml), and mixed at a ratio of 1:10 APC to responder cells. The cells were cultured in RPMI with 10% autologous plasma and 10U/ml IL-2 at 37°C 5% CO_2 for 5 days. 10U/ml IL-2 was added at day 5 and at day 7, peptide-pulsed adherant monocytes were added at a responder : stimulator ratio of 3:1. Twenty-four hours after each stimulation, 10U/ml IL-2

was added. One million cells were removed from each culture for testing with the HLA-A*0201:HA-1^{H}:PE tetramer as before, two days before each restimulation. A representative result of these stainings can be seen in figure 6-6.

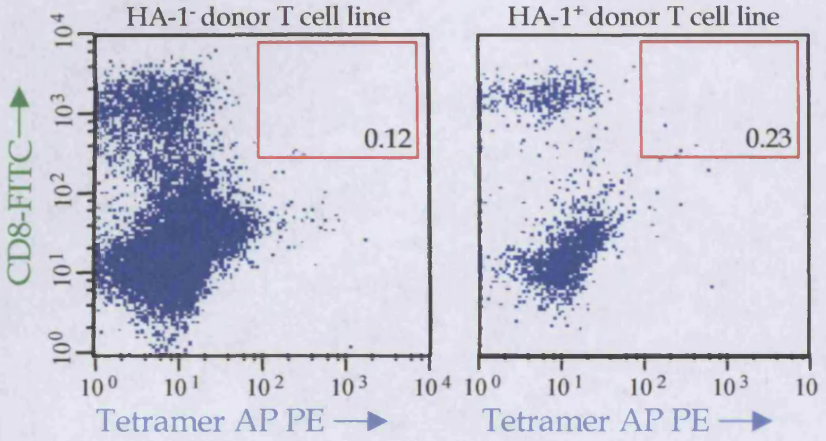


Figure 6-6. HLA-A*0201:HA-1^{H}: PE tetramer staining of T cells stimulated with HA-1^{H} peptide-pulsed mononuclear cells. Following the published protocol (Mutis *et al.* 1999b), HA-1 negative (a) or positive (b) PBMCs were stimulated with autologous peptide-pulsed adherant cells. The resulting T lymphocytes were stained with anti-CD3-PerCP and anti-CD8-FITC (y-axis) monoclonal antibodies (Beckton Dickinson) and HLA-A*0201:HA-1^{H}:PE tetramer (x-axis). Numbers in the red squares represent the percentage of tetramer positive T cells (which also gave a positive result when stained with the CD3-PerCP and CD8-FITC specific antibodies)

As seen in figure 6-6 panel a, the population of CD3+/CD8+ cells available at the time of analysis was greater for the HA-1$^-$ donor than had been seen in the previous experiment (figure 6-5, panel b). Also, the number of tetramer positive cells was greater in both individuals, however, the presence of CD8- cells was still seen in the are below the red box.

The *ex-vivo* stimulation was not a successful way of inducing HA-1-specific T cells. It was decided that the tetramer should be tested on some post unrelated bone marrow transplant samples from HA-1$^+$ recipients who had been transplanted with HA-1$^-$ donors, where a greater percentage of HA-1-specific T cells may be present.

Analysis of post transplant PBMCs from HA-1$^+$ recipients of HA-1$^-$ bone marrow

Whilst using *in vitro* experiments in an attempt to find if my tetramer was working, Geraldine Aubert, at the Anthony Nolan Research Institute, had also produced a HLA-A*0201:HA-1 tetramer following the same protocol (Altman *et al.* 1996). Geraldine had previously prepared three HLA-A*0201 tetramers containing different peptides from the CMV surface glycoprotein, pp65 (AE42, AE44 and AE45), which are also recognised in an HLA-A*0201 restricted manner (Meyers *et al.* 1986; Webster *et al.* 1993), and which have been shown to stain CMV-specific T cells (Aubert *et al.* 2000). All tetramers described were produced using the same protocol (Altman *et al.* 1996). An HLA-A*0201:HA-1^H:PE tetramer was also kindly provided by Professor Els Goulmy, who had previously described its use in the detection of HA-1-specific T cells in post transplant PBMC populations from HA-1$^+$ recipients of HLA identical sibling HA-1$^-$ bone marrow transplants (Mutis *et al.* 1999a). These tetramers and their abbreviated names are represented in Table 6-1.

Table 6-1. Description of tetramers used in the analysis of HA-1-specific T cells. Tetramers were kindly provided by Geraldine Aubert (Anthony Nolan Research Institute, UK) and Professor Els Goulmy (Department of Immunohaematology and Blood Bank, Leiden University Hospital, The Netherlands).

Tetramer	Originator	Components	Specificity
APHA-1	Andrea Pay	HLA-A*0201:HA-1^{H}:PE	HLA-A*0201:HA-1^{H}
GAHA-1	Geraldine Aubert	HLA-A*0201:HA-1^{H}:PE	HLA-A*0201:HA-1^{H}
MLHA-1	Els Goulmy	HLA-A*0201:HA-1^{H}:PE	HLA-A*0201:HA-1^{H}
GACMV	Geraldine Aubert	HLA-A*0201:AE42:PE	HLA-A*0201:CMV pp65

Staining of post transplant PBMC samples from two unrelated and one sibling recipient post HA-1 mismatched transplant was carried out with all three tetramers and a representation of the results is shown in figure 6-7.

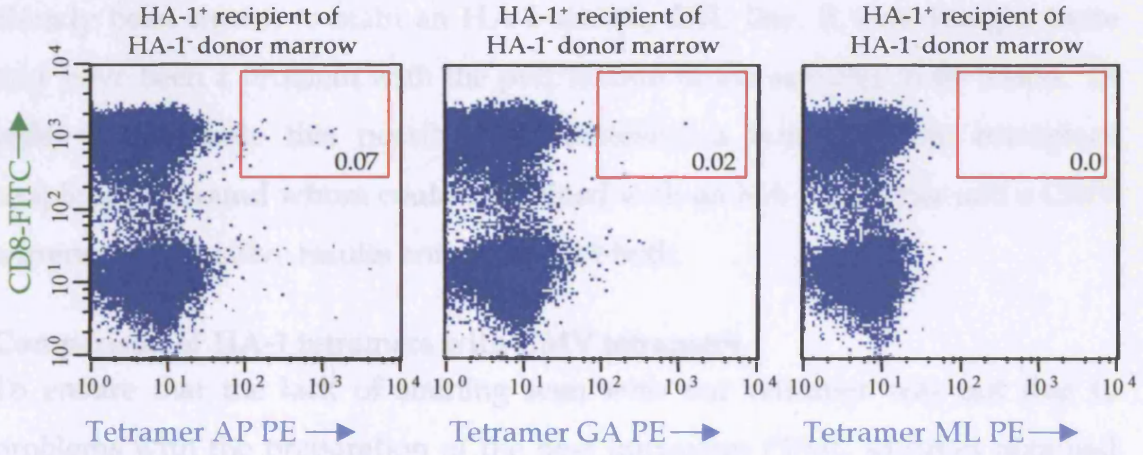


Figure 6-7. HLA-A*0201:HA-1^{H}:PE tetramer staining of PBMCs from an HA-1^{+} patient transplanted with an HA-1^{-} donor. PBMCs from an HA-1 positive recipient of HA-1 negative unrelated donor transplant stained with anti-CD3-PerCP and anti-CD8-FITC (y axis) monoclonal antibodies (Beckton Dickinson) HLA-A*0201:HA-1^{H}:PE tetramers made by myself (AP, panel a), Geraldine Aubert (GA, panel b) and the originators of the technique from Leiden, Holland (ML, panel c). Numbers in the red squares represent the percentage of tetramer positive T cells (which also gave a positive result when stained with the CD3 and CD8 specific antibodies)

The first panel (a) in figure 6-7 shows staining of a post transplant PBMC sample from an HA-1$^+$ recipient of an unrelated HLA-A*0201 matched HA-1$^-$ transplant with the APHA-1 tetramer. Panel b shows the same sample stained with the GAHA-1 tetramer and panel (c) shows the same sample stained with the MLHA-1. The MLHA-1 tetramer has been shown to stain HA-1-specific CTLs produced in the same laboratory (Mutis *et al.* 1999a; Mutis *et al.* 1999b). It can be seen from figure 6-6 panel (c) that there were no cells present in the sample which were stained by the MLHA-1 tetramer. In panels (a) and (b) it can be seen that a small percentage of cells were stained by the two tetramers made in our laboratory (APHA-1 and GAHA-1), but the number of CD8$^+$ and CD8$^-$ cells seen inside and below the red box respectively are approximately equal, indicating that the staining seen is not specific. The MLHA-1 tetramer shows no such staining below the red box, indicating there was no background staining seen. Due to the lack of staining seen with the tetramer that had already been shown to stain an HA-1-specific CTL line, it was thought there may have been a problem with the preparation of the samples to be tested. In order to eliminate this possible inconsistency, a bone marrow transplant recipient was found whom could be stained with an HA-1 tetramer and a CMV tetramer and positive results anticipated for both.

Comparison of HA-1 tetramers with CMV tetramers

To ensure that the lack of staining seen with our tetramer was not due to problems with the preparation of the post transplant PBMC samples obtained from HA-1$^+$ recipients of HA-1$^-$ bone marrow grafts, the same sample was tested with APHA-1 and GAHA-1 tetramers and the GACMV tetramer. This sample was from an HA-1$^+$, CMV$^+$ recipient of an HA-1$^-$ bone marrow transplant who had reactivated CMV viraemia (MY). The sample was stained with the APHA-1, GAHA-1 and GACMV tetramers. I myself, to ensure each process was carried out in the same manner, carried out sample collection; preparation; stainings and analyses simultaneously. The results of these stainings can be seen in figure 6-8.

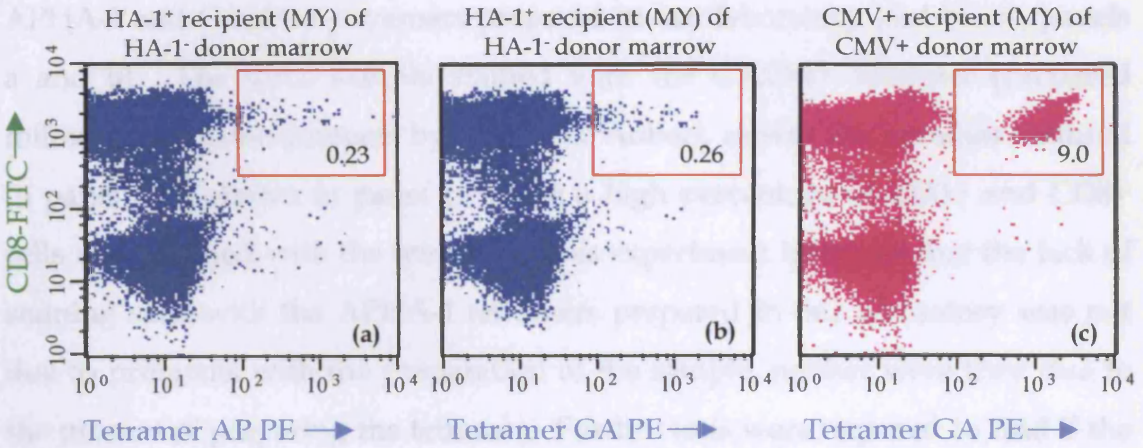


Figure 6-8. HLA-A*0201:HA-1^{H}:PE and HLA-A*0201:CMV peptide AE42:PE tetramer staining of PBMCs from an HA-1^{+} patient transplanted with an HA-1^{-} donor who was also CMV positive. PBMCs from an HA-1 positive, CMV positive recipient of HA-1 negative unrelated donor transplant stained with anti-CD3 and anti-CD8 (y axis) monoclonal antibodies (Beckton Dickinson) HLA-A*0201:HA-1^{H}:PE tetramers made by myself (AP, panel a) and Geraldine Aubert (GA, panel b) . The same cells were also stained with another tetramer made by Geraldine Aubert following exactly the same protocol, but with a peptide from CMV pp65, AE42 (panel c). Numbers in the red squares represent the percentage of tetramer positive T cells (which also gave a positive result when stained with the CD3-PerCP and CD8-FITC specific antibodies)

In figure 6-8 it can be seen that the PBMC sample from the HA-1$^+$, CMV$^+$ recipient of an HA-1$^-$ transplant showed a comparatively high staining with the APHA-1 and GAHA-1 tetramers prepared in our laboratory (red boxes, panels a and b). The same sample stained with the GACMV tetramer (prepared following the same protocol by Geraldine Aubert, as was the tetramer featured in panel b) is shown in panel c, where a high percentage of CD3+ and CD8+ cells were stained with the tetramer. This experiment indicates that the lack of staining seen with the APHA-1 tetramers prepared in our laboratory was not due to problems with the preparation of the sample, neither were they due to the process of preparing the tetramer. Further tests were required to find if the APHA-1 tetramers could stain HA-1-specific T cells.

Testing of HA-1 tetramers with the HA-1-specific CTL line

The final quality control currently available to us for the tetramers produced in our laboratory was to test them against the HA-1-specific CTL line produced in The Netherlands (Mutis *et al.* 1999a; Mutis *et al.* 1999b). We sent a sample of the tetramers on dry ice to Dr Tuna Mutis in the Netherlands for testing with their CTL line in conjunction with their MLHA-1 tetramer. The results of this staining can be seen in figure 6-9.

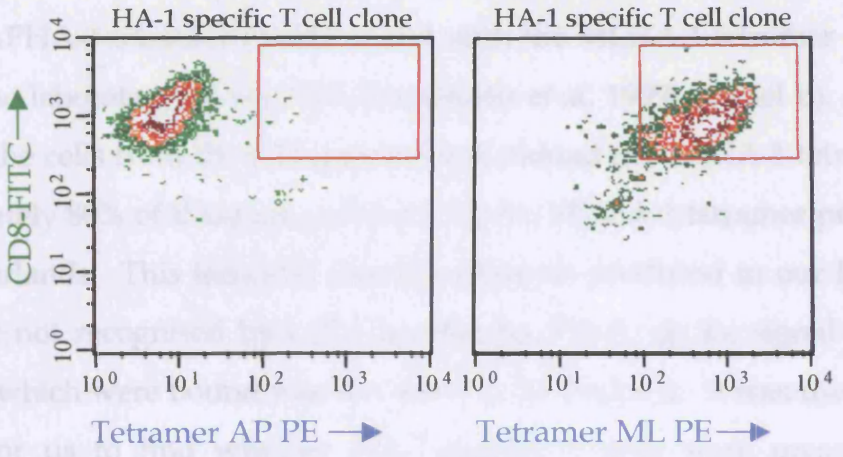


Figure 6-9. HLA-A*0201:HA-1^{H}:PE tetramer staining of an HA-1-specific cytotoxic T cell clone. HLA-A*0201:HA-1^{H}:PE tetramers (AP, panel a) were sent to The Netherlands for testing against the only known HA-1-specific cytotoxic T cell line available, and the results compared with those for a similar tetramer prepared in the same institute (ML, panel b). Numbers in the red squares represent the percentage of tetramer positive T cells (which also gave a positive result when stained with the CD3-PerCP and CD8-FITC specific antibodies)

Figure 6-9 shows staining of an HA-1-specific T cell line (Mutis *et al.* 1999b), with the APHA-1 tetramer (panel a) and with the MLHA-1 tetramer produced in the same laboratory as the CTL line (Mutis *et al.* 1999a)(panel b). It can be seen that the cells from the CTL line are not stained the APHA-1 tetramer, but approximately 80% of them are stained with the MLHA-1 tetramer prepared in The Netherlands. This indicates that the tetramer produced in our laboratory was either not recognised by CTLs specific for HA-1, or the signal from any tetramers which were bound was too weak to be detected. It was therefore not possible for us to find whether HA-1-specific T cells were present in the peripheral blood of HA-1$^+$ recipients of HA-1$^-$ unrelated bone marrow transplants.

Discussion of the effect of an HA-1 mismatch

Due to the small number of HLA-A*0201 positive, HLA matched transplants available for study in our cohort of donors and recipients, it was not possible to analyse HA-1 matching on an HLA matched background to get meaningful results. We therefore carried out the majority of our analyses on HLA-A*0201 matched transplants with known HLA mismatches (n = 57). No statistically significant difference was seen between HA-1 matched and HA-1 mismatched transplants when overall survival, risk of developing acute GvHD and risk of relapsed were analysed (figures 6-2, 6-3a and 6-4). However, on the analysis of the effect of HA-1 matching on the risk of developing chronic GvHD, a significantly increased risk of HA-1 mismatching was seen when compared with HA-1 matched transplants (figure 6-3b). This effect has not been reported post sibling, HA-1 mismatched transplant, however, a significant association with the risk of developing acute GvHD has been seen when an HA-1$^+$ individuals were transplanted with marrow from an HA-1$^-$ sibling donor (Tseng *et al.* 1999). It must be recognised, however, that the individuals who had an HA-1 mismatched transplant who had chronic GvHD could also have had a class II mismatch, which has already been shown to be a significant risk factor for the development of chronic GvHD (figure 6-3b). Twenty-nine of the 57 transplants analysed were mismatched for HA-1 with 19 of these 29

developing chronic GvHD. 89% of the HA-1 mismatched transplants where chronic GvHD was reported also had a mismatch at HLA class II, along with 74% of the HA-1 matched transplants. This shows that conclusions cannot be drawn from this data set with respect to the effect of an HA-1 mismatch on the development of chronic GvHD in unrelated bone marrow transplantation.

As these anaylses gave inconclusive results, it was decided to try and detect the presence of HA-1-specific T cells in HA-1$^+$ recipients of HA-1$^-$ transplants, the presence of which has been reported in the HLA identical sibling transplant situation (Goulmy *et al.* 1996). Tetramers were formed as described from monomers of HLA-A*0201 heavy chain, β_2m light chain and synthetic HA-1^H peptide, biotinylated and bound together with PE-labelled Steptavidin (see Chapter 2 for full description protocol) (Altman *et al.* 1996). Although during the preparation of these reagents, all stages were tested successfully (Chapter 2), there were no HA-1-specfic T cells available for testing the completed reagent. Attempts were therefore made in order to induce these specific T cells in vitro, using mixed lymphocyte reactions and autologous stimulation with peptide-pulsed APCs. However, on staining the few cells recovered from these experiments with tetramer, anti-CD8-FITC and anti-CD3-PerCP, no tetramer-positive cells were seen (figures 6-5 and 6-6).

It was then decided, due to the lack of appropriate positive controls, that the tetramers should be tested directly on post transplant PBMC samples from HA-1$^+$ recipients of HA-1$^-$ transplants. Another scientist at the Anthony Nolan Research Institute, Geraldine Aubert, more experienced than myself in the preparation of tetramers, also prepared an HLA-A*0201:HA-1^H:PE tetramer in the same manner, for comparison. When both these tetramers were tested on a relevant recipient sample, no clear positive result could be seen with either tetramer (figure 6-7 panels a and b). These two tetramers were also tested on the same samples alongside an HLA-A*0201:HA-1^H:PE tetramer prepared in The Netherlands, that had previously shown positive staining with an HA-1-specific CTL line prepared in the same laboratory (Mutis *et al.* 1999a; Mutis *et al.* 1999b). No difference was found in the staining of any of the samples between

the tetramers prepared in out laboratory and those provided by the laboratory in The Netherlands figure 6-7). The common denominator in all these experiments was the preparation of the samples. To test whether this was the problem, a relevant sample was prepared in the same manner for staining with the HA-1 tetramers and an HLA-A*0201:CMV peptide AE42 tetramer. Staining the PBMCs from this HA-1$^+$, CMV$^+$ recipient of an HA-1$^-$ transplant showed staining with the CMV tetramer, but not with the HA-1 tetramers (figure 6-8). Finally, aliquots of the HA-1 tetramers prepared in our laboratory were sent to The Netherlands to be tested for staining of the HA-1-specific CTL line induced in their laboratory. Staining was carried out simultaneously with our tetramer and their tetramer. Our tetramer did not show positive staining with the HA-1-specific CTL line (figure 6-9).

Due to the positive results seen in the preparation of the HLA-A*0201:HA-1^H:PE tetramer (Chapter 2), and the similar staining patterns seen with a similar tetramer prepared by another, more experienced scientist in the laboratory, it is not thought that the negative results seen are due to problems in the preparation of the reagent. The main drawback of the formation of these reagents is the lack of positive controls for testing their quality. In attempting to induce HA-1-specific T cells for quality control purposes, it was found that lack of HLA-A*0201 positive HA-1$^-$ donors was a problem. The protocol described for the *ex vivo* formation of HA-1-specific CTLs required large numbers of PBMCs on a regular basis which were difficult to acquire (Mutis *et al.* 1999b). Due to the small number of cells available originally, and the very few APCs likely to be dendritic cells formed, it was impossible to carry out detection methods to prove their phenotype. Ideally we would have used anti-CD3, anti-CD14, anti-CD16 and anti-DR monoclonal antibodies to characterise these antigen-presenting cells likely to be dendritic cells, which should have been negative for CD3, CD14, CD16 and CD19 and positive for HLA-DR. The cells ultimately pulsed with peptide were originally adherant, and had a "spidery" appearance. But there were not enough produced to carry out the confirmatory phenotyping and to also use them as APCs for the induction of

HA-1-specific T cells. After the initial stimulation with these HA-1 peptide-pulsed antigen-presenting cells, further stimulations were carried out with peptide-pulsed autologous adherant monocytes as APCs. These cells were described as an option for use in this technique, although in the previous study they were found not to be as efficient dendritic cells in the induction of HA-1-specific T cells (Mutis *et al.* 1999b). However, fewer autologous PBMCs were required for the stimulation using monocytes, therefore these were the APC of choice. After three rounds of stimulation with peptide-pulsed adherant monocytes, all the cells remaining had to be used in the tetramer analysis. To try and induce HA-1-specific T cells using fewer donor PBMCs, a mixed lymphocyte culture method was also attempted, but the number of cells recovered for analysis were few, and it was presumed that the HLA difference between the PBMCs of the individuals used led to the induction of T cells against major histocompatibility loci instead. In order for either of these techniques to give a sufficient number of HA-1-specific T cells to from a CTL line which could be used as a positive control for the HA-1 tetramers, an unlimited supply of HLA-A*0201$^+$ HA-1$^-$ and HA-1$^+$ HLA identical PBMCs would be required, and presently those resources do not exist at the Anthony Nolan Research Institute.

To test the hypothesis that any T cells produced from the mixed lymphocyte cultures were HLA-specific and not HA-1-specific, it would have been desirable to have carried out a cytotoxicity test with the targets radiolabelled with ^{53}Chromium. However, the number of cells recovered were only sufficient for testing the HA-1 tetramers.

When our tetramers were finally tested on the HA-1-specific CTL line, and the tetramers prepared in The Netherlands were tested on the recipient samples, no positive results were seen. The unlikely explanation is that the tetramers from The Netherlands are only specific for the CTL line and our tetramers are not. This is proven to be untrue, however, as the HA-1 tetramer from The Netherlands has shown positive results with recipients samples (Mutis *et al.* 1999a). Another explanation could be that the tetramers lose activity during

transport, either by loss of the Phycoerythrin signal, or the tetramer or monomers falling apart due to changes in temperature or salt concentration due to evaporation. Variations could also be due to differences between the staining and storage techniques in the laboratories. Our tetramers were stored at 4°C for four months before testing was carried out in The Netherlands, whereas they keep their tetramers at -70°C with 10% glycerol. It should however be noted that tetramers made with different peptides in our laboratory have been stored for longer periods than this, and have still been found to give the same results as those prepared freshly.

Experiments using these reagents are continuing, and the next step will be to concentrate the remaining tetramer and attempt to stain the HA-1-specific CTL line. I have been invited to go to The Netherlands and prepare the same tetramers using our HLA-A*0201 heavy chain, our β_2m, our synthetic peptide and our PE-labelled Streptavidin in their laboratory. Both tetramers can then be tested on patient samples and the HA-1-specific CTL line in situ to avoid all the previously mentioned factors that could affect the efficiency of the tetrameric complex.

In conclusion, the detection of HA-1-specific T cells in individuals with acute or chronic GvHD post transplant would be a useful tool. It will give an indication of whether it is important to match for the HA-1 antigens in the selection of an unrelated donor in the future.

Chapter 7

Conclusions and Discussion

A database of clinical information was established, and a bank of PBMCs, plasma and DNA was collected for retrospective analysis of the effect of patient, donor and transplant characteristics on the outcome of bone marrow transplants from donors provided by the Anthony Nolan Register. A cohort of 138 transplants were selected for analysis in a pilot study and the level of matching for HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 alleles was determined using RSCA. HLA matching, type of disease, CMV status of donor and patient, age of donor and patient, gender of donor and patient and the T cell depletion status of the transplant were included in univariable and multivariable analysis. The effect of these variables on the development of acute and chronic GvHD, overall survival, disease free survival and transplant related mortality was assessed. The effect of having acute and chronic GvHD was also adjusted for, where relevant. Factors found to be independently associated with reduced overall survival included a diagnosis of AML or other malignancy as compared to CML, a male patient receiving stem cells from a female donor, a mismatch for HLA class I and class II alleles and the development of acute GvHD. The risk of reduced disease free survival was associated with the patient having CML or AML, stem cells transplanted from a same sex donor and an HLA mismatch at class I and II, or only class I. A mismatch for HLA class I and II or class I alone were also associated with an increased risk of transplant related mortality, as was having acute GvHD. CML patients had an increased risk of relapse, as did those with an HLA class I and II mismatch. Interestingly, HLA mismatches were not associated with the development of acute or chronic GvHD, which

may have been a reflection of the small number of mismatches found for HLA-A, -B, -C, -DRB1 and -DQB1. The inclusion of HLA-DPB1 matching did not appear to alter the effects seen when other mismatches were present. However, matching at the allele level for all loci gave an increased risk of relapse and reduced disease free survival compared with patients mismatched with their donor a for HLA-DPB1 only. These results indicate that a mismatch for HLA-DPB1 alone may protect from relapse. No significant association was seen in the same group with the risk of developing GvHD or increased risk of transplant related mortality. Although the sample number was small for the analyses required, in the continuation of the project more samples will be available for analysis and the information from this pilot study can be used as a basis for bigger, more informative investigation.

Sample size estimation

The data produced from this pilot study, in some cases, gave statistically significant results at 5% level. However, in many cases, the confidence intervals seen were wide, indicating a lack of precision in the analysis. Also, some of the outcomes were unexpected, for example, the lack of an effect of HLA mismatch on the development of acute and chronic GvHD. These unexpected or imprecise results may be a reflection of the small number of samples included in the study. Other influences may be the inclusion of transplants performed at many different centres with different protocols and patient groups.

To answer the question "does an HLA allele mismatch increase the risk of developing acute GvHD". When matched for all HLA loci, 54% of patients, developed acute GvHD. It can be anticipated that a mismatch for an HLA class II allele will increase the risk to 61% (as the HR = 1.13, table 4-4). What sample size would be required in order to detect such an effect with 80% power at a 5% level of statistical significance?

In order to calculate the number of patients required in each group (m) for a definitive study, where the true effect of HLA matching on the development of acute GvHD may be seen, a power calculation was carried out, using the

formula:

$$m = 2 \times \frac{[z_{(1-\alpha/2)} + z_{(1-\beta)}]^2}{\Delta^2}$$

First, the standardised difference, Δ, was calculated. In the case of two proportions, p_1 (0.61 or 61%) and p_2 (0.54 or 54%),

$$\Delta = \frac{p_1 - p_2}{\sqrt{\bar{p} \times (1-\bar{p})}} \quad \text{where} \quad \bar{p} = \frac{(p_1 + p_2)}{2} \qquad \frac{(0.61 + 0.54)}{2} = 0.56$$

$$\Delta = \frac{0.61 - 0.54}{\sqrt{0.61 \times (1-0.61)}} = \frac{0.07}{0.49} = 0.143$$

Using the values for a significance level of 5%, $z_{(1-a/2)} = 1.96$, and a power of 80%, $z_{(1-b)} = 0.8416$,

$$m = \frac{2 \times [1.96 + 0.8416]^2}{0.143^2} = 767.7$$

This means we require 768 transplant pairs in each group to assess the effect of a mismatch at HLA class II on the development of acute GvHD. Other examples were; the effect on the development of acute GvHD, which would require 107 transplant pairs in each group, the effect of an HLA-DPB1 mismatch alone on overall survival, which would require 148 transplant pairs in each group, and the effect of an HLA-DPB1 mismatch alone on the risk of transplant related mortality, which would require 246 samples in each group.

Development of the project for future studies

Many difficulties were encountered and overcome during the study. Some resolutions were put in place and have already been discussed in chapter 3.

Further development of the project is required for the analysis of a greater number of transplant pairs. Detailed in this section are some procedures and resources that may be required in order to improve and develop the project to analyse a larger number of transplants.

Degraded samples

One of the main problems found was the poor quality of PBMCs and DNA obtained from samples that had a long transit time. This was overcome, to some extent, with the use of transport medium and the introduction of a courier service to some locations. To improve the quality of DNA further, the use of a commercially available stabilisation product could be used, for example PAXgene™ Blood DNA stabilisation tubes (PreAnalytiX, QIAGEN Ltd., West Sussex, UK), which contain a reagent which it is claimed maintains the integrity of genomic DNA in whole blood at room temperature for 14 days. Another option may be to use buccal swabs as a source of DNA, however, this may require the re-optimisation of the PCR for lower quantities of DNA and increased monitoring of potential cross-contamination. The quality of the PBMCs may also be improved with the use of a BD™ Vacutainer™ Cell Preparation Tube™ (BD UK Limited, Oxford, UK) for blood collection. The tube contains density gradient fluid and a gel plug, with a Sodium Heparin anticoagulant. The whole blood sample can be taken directly into the tube, mixed by inversion and then centrifuged, separating the red blood cells and neutrophils from the lymphocytes, monocytes and plasma with the gel plug. The lymphocytes and monocytes can be resuspended in the plasma for transport of the sample, without the threat of contamination from the products of red cell lysis.

Database maintenance

Validation of data

A significant amount of time was taken validating the FileMaker Pro clinical database manually, with electronic validation of data fields carried out at the time of analysis in SPSS. A new database should be created with the same

fields, adding the validation processes listed in table 3-1, then the data re-entered. This would allow automatic validation of all current clinical data and all the data entered from now onwards. This would mean all data could be directly exported, in a spreadsheet format, into SPSS for analysis without extra validation. Also, any fields added later could be included in the validation on entry into the database.

Clinical data collection forms

At the time clinical data analysis for this pilot study, many gaps were found in the clinical data for the patients involved. New Clinical data request forms (figure 3-9) were send out to the Transplant Coordinators in an attempt to collect as much missing data as possible. In retrospect, the main challenge to obtaining clean data was the design of the forms (figures 3-5, 3-6, 3-7 and 3-9), these allowed the entry of free text in every field, meaning interpretation was required for entry into the final database. This could be avoided by the introduction of a form including only check boxes for each field. For example:

Disease - Leukaemia	**CML**	☒
	AML	☐
	ALL	☐
	CLL	☐

Ideally, electronic forms could be used, that could be completed at the transplant centre in real time, on a secure website, with password entry. The forms could be interfaced with the database. This would avoid the large amount of paperwork required, and any errors due to illegibility of writing or interpretation of information. An electronic calendar could be associated with each record to alert the coordinator by email when an update was due. An electronic link could be created to the Anthony Nolan database, SOLAR, which contains donor HLA typing information, avoiding double entry of data.

Time constraints

A lot of time was taken building and distributing sample collection kits to the transplant centres, and a lot of reagents and consumables wasted if the patient did not agree to give a sample. This could be overcome by obtaining early

consent from the patient, therefore allowing sample collection kits to be sent only to those patients who had agreed to give a sample. Outsourcing the building and distribution of the sample collection kits may also save time.

Personnel

One of the major difficulties found throughout the project was trying to obtain clinical information. It would be a great advantage to have a clinician involved in the project full time, to liaise with the doctors and transplant coordinators and also, if necessary, to obtain the required information from the patient records. Access to patient records is confidential only to clinically qualified professionals. A practicing bone marrow transplant clinician could also oversee the fields in the database, updating them when new conditioning regimens or GvHD prophylaxis were introduced and advise on the analyses carried out. The intervention of a clinician would also help with political conflicts in some cases.

If the project were to be expanded, to include all transplants carried out with Anthony Nolan donors, and not only those in the UK, it would be advantageous to employ a project manager. The project manager would be responsible for liaison with the harvest and transplant centres, organise kit and sample transit, and work with the technical team.

In order to carry out sample processing and experimental work simultaneously, a technician and a clinical scientist would be required. The technician could process the samples and control the storage of the plasma, PBMCs and DNA. The clinical scientist would be responsible for the PCR and RSCA running and gel analysis.

A data manager would also be required to maintain the database and carry out statistical calculations.

In the past, many studies have been carried out to try and understand the complexity and effect on transplant oucome of many different factors. So far, no single study has been able to make a recommendation with regard to HLA matching due to unclear relationships, or lack of typing resolution for some

loci. Only global organisations, such as the International Histocompatibility Workshop, may be able to gather enough information to assess the effect of individual loci by collecting data from many centres aound the world. In conclusion, this study has provided a useful starting point for a large-scale analysis. This study will ultimately contribute to the understanding of the variable factors that influence transplant outcome and will allow optimization of donor selection for different patient types at the Anthony Nolan Trust. For example, should HLA-DPB1 matching be included in the selection of donors, and is it more important to match for HLA class I alleles compared to HLA class II.

Appendix I - Primers used in PCR amplification

Target	Orientaion	Name	Sequence (5' – 3')
HLA-A	Sense	5AIn 1-46	GAAACSGCCTCTGYGGGGAGAAGCAA
HLA-A	Antisense	3AIn 3-66	TGTTGGTCCCAATTGTCTCCCCTC
HLA-A	Antisense	3A36L	TGTTGGTCCCAATTGTCTCCCCTCCTTGTGGGAGGC
HLA-B	Sense	3BIn 1-57	GGGAGGAGCGAGGGGACCSCAG
HLA-B	Antisense	3Bin 3-12	GGAGGCCATCCCCGGCGACCTAT
HLA-B	Antisense	3BLong	GGAGGCCATCCCCGGCGACCTCAMGGA
HLA-C	Sense	5CIn 1-61	AGCGAGGKGCCCGCCCGGCGA
HLA-C	Antisense	3BCIn 3-12	GGAGATGGGGAAGGCTCCCCACT
HLA-C	Antisense	3CLongS	GGAGATGGGGAAGGCTCCCCACTGCC
HLA-DRB1	Sense	DRB101 5'	CCGGATCCTTCGTGTCCCCACAGCACG
HLA-DRB1	Antisense	DRB101 3'	CGCCCCGCGCCGCGCT
HLA-DRB1	Sense	DRB102 5'	TTCCTGTGGCAGCCTAAGAGG
HLA-DRB1	Antisense	DRB102 3'	CCGCCCGCGGCCATGCT
HLA-DQB1	Sense	DQBAMP-A	CATGTGCTACTTCACCAACGG
HLA-DQB1	Antisense	DQBAMP-B	CTGGTAGTTGTGTCTGCACAC
HLA-DPB1	Sense	DPBAMP-A	GAGAGTGGCGCCTCCGCTCAT
HLA-DPB1	Antisense	DPBAMP-B	GCCGGCCCAAAGCCCTCACTC
MICA exon 3	Sense	MICAEX35'	TGGGGGAGGGCCAGGGAGGCGTAC
MICA exon 3	Antisense	MICAEX33'	CGATGTGCCAACAGGAAATGCCTT
MICA exon 4	Sense	MICAEX45'	CAGACTTGCAGGTCAGGGGTCCCG
MICA exon 4	Antisense	MICAEX43'	CAATGACTCTGAAGCACCAGCACT

M = A or C Y = C or T
S = G or C K = G or T

Appendix II - Letter to Consultants inviting them participate

Letter 1

Transplant Centre

THE ANTHONY NOLAN
BONE MARROW TRUST

Where leukaemia meets its match

The Royal Free Hospital

[redacted]

Date

Dear *Consultant*

The Anthony Nolan Research Institute is currently embarking on a study investigating the importance of HLA matching in unrelated bone marrow transplants. Recent advances in molecular typing have made it possible to obtain very high resolution tissue types, but this in turn makes it even more difficult to find a "perfectly" matched donor. As you will be well aware, this is already a very difficult task and often only a partially matched donor is available.

In the past these techniques were not available and consequently many transplants were performed with seemingly perfectly matched donors which nowadays would not hold up to scrutiny. Many of these transplants though have very successful outcomes.

The purpose of this study is firstly to use all available techniques to tissue type patient/donor pairs to the highest possible resolution, and secondly, by following the outcome of transplant we would then be able to correlate specific mismatches with a good or bad transplant outcome. In the future this will enable us to advise which donor should be chosen in the event that no fully compatible donor is available and there is a choice of partially matched donors.

The work involved would require a small patient sample (50mls from adults or whatever volume is appropriate from children - even as little as 5mls would be gratefully received) and regular updates on the progress of the transplant, and in this your help would be essential. Once a transplant date has been set we would be in contact and send universals containing preservative-free heparin and EDTA and a pre paid box, to try and minimise your workload in this study. In return we will endeavour to keep you informed of any results concerning your patients.

Please could you complete the enclosed consent form and return it so I have a record that your transplant centre will be prepared to participate. Please call me if you have any queries ([redacted]). Thank you very much.

Yours sincerely

Andrea Pay

Appendix III - Study proposal sent to the Ethics Committee

Proposal for study on donor/patient samples

Our group at the Anthony Nolan Research Institute has developed a novel technique which can be used for high resolution HLA class I and class II typing and to verify 'HLA-matched' patient and donor pairs in bone marrow transplantation. This technique, which we have named: *Double Strand Conformation Analysis* (DCSA) involves fluorescently labelling a locus specific reference sense strand and hybridising with locus specific amplified DNA from patient and donor. The duplexes formed are separated by polyacrylamide gel electrophoresis. Results are quickly obtained using an automated sequencing instrument. Comparison of positions of donor duplexes with patient duplex enables the identification of best matched donor. This technique is being assessed here at the Anthony Nolan Research Institute for use in the final stage analysis of donor and patient before transplant. We believe this is the most accurate and rapid way to match donors and patient DNA samples at the allelic level. It is a DNA method analogous to the isoeletric focusing technique used in protein separation and is ideal for resolving allelic differences not found by molecular typing methods.

We propose to analyse samples from every Anthony Nolan donor/recipient pair which are transplanted in the UK using the DCSA technique to determine the degree of class I and class II matching, and to identify mismatches between donor and patient. Any samples that we detect as being mismatched will be retyped using either a high resolution molecular typing technique developed in our laboratory known as *Universal Recombinant Site Targeting Oligonucleotide Procedure* (URSTOP) or Sequence Based Typing (SBT).

Once the transplant follow up data has been acquired and more is known about the outcome of the transplant, it will be possible to formulate future experiments to analyse other factors as well as MHC which affect Bone Marrow Transplant success.

Appendix IV - Consent form

Protocol Nº1

To: Potential bone marrow donors and recipients

From: Dr J Alejandro Madrigal
Research Director
Anthony Nolan Research Institute

THE ANTHONY NOLAN
BONE MARROW TRUST

Where leukaemia meets its match

The Royal Free Hospital

Consent Form

Re: HLA matching of Anthony Nolan donors

The Anthony Nolan Research Institute is currently embarking on a study investigating the importance of HLA matching in unrelated bone marrow transplants. Recent advances in molecular typing have made it possible to obtain very high resolution tissue types, but this in turn makes it even more difficult to find a "perfectly"matched donor. As you will be well aware, this is already a very difficult task and often only a partially matched donor is available.

In the past these techniques were not available and consequently many transplants were performed with seemingly perfectly matched donors which nowadays would not hold up to scrutiny. Many of these transplants though, have very successful outcomes.

The purpose of this study is firstly to use all available techniques to tissue type patient/donor pairs to the highest possible resolution, and secondly, by following the outcome of transplant we would then be able to correlate specific mismatches with a successful transplant outcome. In the future this will enable us to advise which donor should be chosen in the event that no fully compatable donor is available and there is a choice of partially matched donors.

The work involved would require a blood sample. **The DNA extracted from this sample will only be used for matching studies in our laboratory.**

I am willing to take part in this study and agree to provide the samples requested, I have read and understand the details of the study.

Health Care Witness	Patient or Bone Marrow Donor
Signed.....	Signed.....
Name.....	Name.....
Position.....	Hospital.....

Appendix V - Harvest Centres used by the Anthony Nolan Trust

London Clinic
20 Devonshire Place
London
W1N 2DH

Royal Free Hospital
Pond Street
Hampstead
London
NW3 2QG

University College Hospital
25 Grafton Way
London
WC1E

Abbreviations

ABMDR	Australian Bone Marrow Donor Registry
ALL	Acute Lymphocytic Leukaemia
AML	Acute Myeloid Leukaemia
ANBMT	Anthony Nolan Bone Marrow Trust
APC	Antigen presenting cell
B-LCL	B-lymphocyte cell line
BMT	Bone marrow transplant
CD	Cluster of differentiation
CLIP	Class II associated invariant chain peptide
CML	Chronic Myeloid Leukaemia
CY	Cyclophosphamide
CMV	Cytomegalovirus
^{51}Cr	Chromium-51 radioactive isotope
CR	Complete Remission
CTL	Cytotoxic T-lymphocyte
CTLp	Cytotoxic T-lymphocyte precursor
DIG	Di-Digoxygenin
DLI	Donor lymphocyte infusion
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylene diamine tetra acetic acid
EUCAPS	European Union Concerted Action Program for Peptide Sensitisation
FCS	Foetal Calf Serum
FLR	Fluorescent labelled reference
FPLC	Fast performance liquid chromatography

GvHD	Graft-versus-host disease
GvL	Graft-versus-leukaemia
HIV	Human Immunodeficiency virus
HL-A	Human Leukocyte Locus A
HLA	Human Leukocyte Antigen
HR	Hazard Ratio
HTL	Helper T-lymphocyte
HTLp	Helper T-lymphocyte precursor
HVR	Hypervariable region
IFN-α	Interferon-alpha
Ig	Immunoglobulin
IHWS	International Histocompatibility Workshop
KIR	Killer immunoglobulin-like receptor
LCMV	Lymphocytic choriomeningitis virus
MDS	Myelodysplastic syndrome
mHag	Minor Histocompatibility Antigen
MHC	Major Histocompatibility Complex
MIC	MHC class I chain related gene
MLC	Mixed Lymphocyte Culture
MLR	Mixed Lymphocyte Reaction
MIIC	MHC class II compartment
NK	Natural Killer
NHL	Non-Hodgkins Lymphoma
OD	Optical density
OR	Odds Ratio
PAGE	Polyacrylamide gel electropheresis
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PLT	Primed Lymphocyte Test
RCLB	Red cell lysis buffer
RNA	Ribonucleic Acid
RSCA	Reference strand mediated conformation analysis
SBT	Sequence based typing
SCID	Severe Combined Immunodeficiency
SDS	Sodium dodecyl sulphate

SPSS	Statistical computer package
SSO	Sequence specific oligotyping
SSP	Sequence specific primers/PCR
TBE	Tris buffered EDTA
TBI	Total body irradiation

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