Bone marrow transplantation in HLA closely-matched donor/recipient pairs: study of the histoincompatibilities detected before and after transplantation and their significance in Graft-versus-Host disease

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Thèse No 2811

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FACULTE DES SCIENCES

Doctorat ès sciences
mention biologique

Thèse de *Mademoiselle Nathalie R U F E R*

intitulée :

"BONE MARROW TRANSPLANTATION IN HLA CLOSELY-MATCHED DONOR/RECIPIENT PAIRS : study of the histoincompatibilities detected before and after transplantation and their significance in Graft-versus-Host Disease."

La Faculté des Sciences, sur le préavis de Messieurs M. JEANNET, professeur associé et directeur de thèse (Faculté de Médecine - Unité immunologie de transplantation, Hôpital cantonal universitaire), A. LANGANEY, professeur ordinaire et répondant de la Faculté des Sciences (Département d'anthropologie et d'écologie), B. CHAPUIS, professeur adjoint (Faculté de Médecine - Unité d'hématologie, Hôpital cantonal universitaire) et E. GOULMY, docteur (Department of immunohematology and bloodbank - Leiden, England), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 2 avril 1996

Thèse - 2811 -

Le Doyen, Eric DOELKER

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

Nombre d'exemplaires à livrer par colis séparé à la Faculté : - 7 -

Ce travail a été réalisé à l'Unité d'Immunologie de Transplantation de la Faculté de Médecine. J'aimerais tout d'abord remercier le Professeur Michel Jeannet pour m'avoir accueillie au sein de son unité, me donnant ainsi l'opportunité d'y effectuer ce travail de thèse.

Je remercie le Professeur André Langaney d'avoir accepté la responsabilité de ce travail vis-à-vis de la Faculté des Sciences, ainsi que le Professeur Bernard Chapuis et le Dr. Els Goulmy d'avoir accepté de participer au jury de thèse, malgré leurs nombreuses sollicitations.

Je tiens à remercier très spécialement le Dr. Eddy Roosnek pour sa disponibilité et ses excellents conseils tout au long de ce travail; il a su au cours de ces quatres années me guider et me captiver à travers les multiples dédales et recoins de l'immunologie... et de la science en général... un grand merci.

J'exprime ma reconnaissance au Professeur Bernard Chapuis, aux Dr. Claudine Helg et Michel Starobinski pour leur précieuse collaboration médicale, et pour m'avoir permis de découvrir le monde secret des "isolettes".

Je remercie le Dr. Jean-Marie Tiercy pour sa gentillesse et ses conseils avisés dans le domaine de la biologie moléculaire et du système HLA.

Je tiens également à remercier tous les membres de l'Unité et du Laboratoire National de Référence pour leur aide technique, leur compétence et leur gentillesse; merci à Alexandre, Claudine, Christine, Etienne, Gilles, Marta, Myriam, Véréna et Véronique.

Un remerciement particulier à mes collaborateurs et amis du 4ème, dont la gentillesse et le soutien me furent extrêmement précieux: namasté Florence, Narges, Sophie, Xiaowen, Solange, Béatrice, Reto, Christophe, Joëlle et Andréa.

Enfin, je remercie Flo, Sylvain, Alexia, Michou, Anne-Sophie, Fred, Andréas, Sylvie et Isabelle pour leur patience et leur précieuse amitié.

A Romain, Yannick, Rose-Marie
Pierre-Jean, Wolfgang, Dounya
Adrian, Elianne, José, Pascal
Steve... et tous les autres...

An meine Eltern.

CONTENTS

RESUME

La greffe de moelle osseuse

La transplantation de moelle osseuse représente depuis une vingtaine d'années un traitement efficace pour soigner les patients atteints de tumeurs hématologiques malignes (leucémie myéloide chronique ou aiguë et leucémie lymphoblastique) ou de déficits immunitaires sévères (anémie aplastique et SCID). Les doses de radio- et chimiothérapies intenses reçues par le patient comportent une toxicité majeure à l'égard de toutes les cellules du système hématopoïétique: les cellules cancéreuses, mais également les lignées cellulaires saines. Dès lors, le patient se trouvant en aplasie médullaire, il est indispensable de restaurer un nouveau système. Actuellement, la greffe de moelle est une thérapie de choix, puisqu'elle subvient à cette nécessité.

Le donneur de moelle potentiel

Le succès de cette thérapie dépend de nombreux facteurs, dont principalement celui du choix du donneur de moelle volontaire. Malgré l'avènement des immunosuppresseurs tels que la cyclosporine, le meilleur donneur potentiel reste celui que l'on trouve au sein même de la famille du patient, parmi ses frères et soeurs. En effet, une compatibilité maximale au niveau des antigènes du Complexe Majeur d'Histocompatibilité (nommé HLA chez l'homme) entre le patient et son donneur, est exigée lors d'une greffe de moelle. Puisque les gènes codant pour le CMH sont localisés sur un seul chromosome, cette opportunité se retrouve chez 25 % des membres d'une même fratrie. Malheureusement, la faible proportion d'enfants par famille limite la probabilité de trouver un tel donneur; il faut alors se tourner vers les registres de donneurs de moelle volontaires.

Cependant, ces donneurs non apparentés sont souvent beaucoup moins compatibles au niveau des molécules HLA, et une greffe de moelle s'accompagne alors de complications pouvant engendrer une mortalité accrue chez les patients greffés.

Les complications liées à la greffe de moelle

Outre la susceptibilité augmentée du patient envers les agents infectieux, une des complications majeures survenant après transplantation est la maladie Greffe-contre-Hôte (GvH). La maladie GvH se manifeste par une atteinte destructrice des épithéliums de la peau, du système gastro-intestinal et du foie. Celle-ci est la conséquence de la reconnaissance des tissus de l'hôte par les cellules immunocompétentes ou lymphocytes T du donneur qui ont été transfusées avec la moelle lors de la greffe. Ces lymphocytes sont activés suite à une interaction avec les cellules du patient exprimant des molécules HLA incompatibles avec le donneur, et peuvent ainsi initier une maladie GvH. L'incidence et la sévérité de cette maladie GvH peuvent être diminuées par la déplétion des cellules T de la moelle transfusée et par des traitements immunosuppresseurs. Cependant, une des meilleures préventions reste la compatibilité totale entre l'hôte et son donneur.

Les molécules du CMH

Les gènes du système HLA codent pour des glycoprotéines de surface membranaire et sont répartis en deux groupes; (a) les antigènes de classe I nommés HLA-A, -B et -C et (b) les antigènes de classe II nommés HLA-DR, -DQ et -DP.

Une des caractéristiques principales de ces gènes est leur extraordinaire complexité et diversité dans la population. En effet, plus de 450 molécules distinctes ont pu, à ce jour, être décrites chez l'homme.

Les molécules HLA ont un rôle majeur et unique au sein du système immunitaire d'un organisme. En effet, ces protéines sont impliquées dans de nombreux aspects de la reconnaissance immune, en présentant aux lymphocytes T, les fragments peptidiques viraux d'une cellule infectée.

Ces antigènes peuvent aussi endosser une autre fonction dans la situation exceptionnelle que représente la greffe de moelle osseuse; celle d'être reconnus comme "étrangers" par des lymphocytes T cytotoxiques.
Il existe deux cas distincts pouvant engendrer une telle réponse alloréactive. Dans le premier cas, l'incompatibilité se trouve située sur les molécules HLA elles-mêmes, et l'alloréactivité manifestée par les lymphocytes T du donneur est dirigée contre ces molécules et peut être mesurée in vitro dans des tests cellulaires de cytotoxicité.
Dans le second cas, seul le fragment peptidique présent dans la gouttière de la molécule HLA est incompatible. Cette incompatibilité est liée à l'existence d'un polymorphisme de certaines protéines dans la population. Dès lors, si le patient exprime un peptide dont l'allèle est absent chez le donneur, les lymphocytes T du donneur pourront potentiellement réagir contre cet épitope et également induire une maladie GvH. Dans les cas où les antigènes HLA sont identiques entre deux individus, les produits peptidiques incompatibles sont appelés les *antigènes mineurs d'histocompatibilité*. Il n'y a actuellement que peu de moyens de prédire, avant une greffe de moelle, la présence ou non d'une incompatibilité au niveau de ces antigènes dans une combinaison receveur/donneur. Il existe, cependant, une seule exception; l'antigène mineur dont le(s) gène(s) est/sont codé(s) sur le chromosome Y, et donc exclusivement présent(s) dans les cellules masculines.

Le typage des molécules HLA

La présence d'incompatibilités HLA entre deux individus est détectée à l'aide de plusieurs techniques.
La sérologie est l'une des plus anciennes méthodes de typage utilisées. Celle-ci est basée sur la reconnaissance de la structure tertiaire des molécules HLA par des anticorps. La sérologie représente un outil simple et rapide pour caractériser ces antigènes ainsi que la distribution des différents haplotypes parmi les membres d'une famille. Toutefois, le polymorphisme du système HLA est tel que la sérologie est souvent incapable d'une fine distinction entre deux variants proches, ce qui empêche un typage précis des combinaisons patient-donneur *non apparentés.*

Il est donc indispensable, pour typer ces individus, d'utiliser une technologie de pointe, telle que les tests cellulaires et les techniques de biologie moléculaire. Ainsi, grâce à l'utilisation de sondes spécifiques de chaque allèle HLA (ou oligotyping), l'identification de tous les variants des molécules de classe II est devenue réalité. En revanche, cette technique ne se prête encore que très peu à la discrimination des antigènes de classe I. Ainsi, il a fallu élaborer un test fonctionnel permettant de caractériser précisement ces différences, invisibles aux yeux de la sérologie.

Ce test, nommé "cytotoxic T lymphocyte precursor frequency test" ou CTLpf permet d'évaluer in vitro la proportion de lymphocytes T cytotoxiques du donneur pouvant potentiellement reconnaître et détruire les tissus du patient. Il est basé sur les principes de dilutions-limites, sur l'expansion de lymphocytes T cytotoxiques précurseurs et la détection de leur activité cytolytique.

La conjugaison des techniques classiques (sérologie) et des techniques de haute résolution (oligotyping, tests cellulaires) permet un typage complet et précis de chacune des molécules HLA de classe I et de classe II présente et exprimée chez un individu.

La définition du donneur "HLA-ABDR identique" non apparenté

Ces techniques sophistiquées ne sont pas indispensables pour la caractérisation des antigènes HLA présents chez les couples patient/donneur intra-familiaux. En effet, la sérologie est suffisante pour discriminer les quatres haplotypes exprimés entre le patient et le reste de sa famille. De plus, deux individus apparentés et compatibles par la sérologie partagent les mêmes variants HLA, puisqu'ils héritent des mêmes haplotypes.

En revanche, les techniques classiques sont incapables de discriminer précisement tous les variants HLA de classe I et II présents dans les combinaisons non apparentées. Ainsi, ces combinaisons bien que surnommées "HLA-ABDR identiques" peuvent encore présenter certaines incompatibilités aux niveaux de sous-types de ces antigènes. De plus, d'autres incompatibilités non détectées (HLA-Cw, -DQ et -DP) pourraient également apparaître chez ces individus.

L'augmentation de l'incidence de complications telles que la maladie GvH observée lors des transplantations entre paires non apparentées pourrait être expliquée par la présence d'un certain nombre d'incompatibilités non révélées par la sérologie.

Estimation du degré d'incompatibilité HLA dans les paires "ABDR-identiques"

Nous nous sommes donc intéressés, dans la première partie de ce travail, à évaluer la réalité de la définition du donneur non apparenté HLA "ABDR-identique". Les questions posées sont les suivantes:
Quel est le degré d'incompatibilité HLA dans les paires définies comme compatibles par la sérologie ? Quel est le seuil de sensibilité de cette technique ?

Nous avons analysé 131 combinaisons de patients/donneurs non apparentés en comparant différentes techniques de typisation : la sérologie, l'oligotyping, le séquençage de certains allèles de classe I, le test cellulaire CTLpf ainsi que l'analyse spécifique de clones T cytotoxiques.

Cette étude a démontré que l'union de ces différentes technologies de pointe a permis l'identification précise et rigoureuse de nombreuses histo-incompatibilités. Ainsi, sur l'ensemble de ces combinaisons, seuls 27 % des couples de patient-donneur sont réellement HLA-ABCDR identiques. Parmi les 73 % restants, 22 %, 21 % et 30 % respectivement, sont incompatibles au niveau d'un variant HLA de classe I, classe II ou classe I+II.

En parallèle, quatres antigènes HLA de classe I ont été étudiés plus en détails dans le but d'analyser la proportion de certains variants au sein de la population. Les antigènes HLA-A2, -B35, -B44 et -B41 sont subdivisés en de nombreux variants fréquents, tous capables de provoquer une réponse immune. Dès lors, ces molécules représentent un danger potentiel dans l'alloreconnaissance par les lymphocytes T du donneur après une greffe de moelle.

Ces travaux apportent plusieurs éléments nouveaux dans la problématique de la sélection d'un donneur de moelle:

1) L'oligotyping représente sans nul doute, une technique extrêmement prometteuse pour le typage des antigènes HLA de classe I. Il était donc nécessaire d'établir des sondes spécifiques pour les antigènes fréquemment exprimés dans une population. Actuellement, l'oligotyping se substitue de plus en plus aux techniques de culture cellulaire, "plus lourdes". Cependant, l'un des avantages du test CTLpf reste son potentiel de détection d'allèles HLA rares ou encore inconnus. C'est ainsi que nous avons identifié et séquencé un nouvel allèle de l'antigène HLA-B41.

2) Jusqu'à récemment de nombreuses recherches ciblées sur l'analyse de l'impact de ces histo-incompatibilités dans la survie des patients, étaient limitées par l'usage exclusif de techniques classiques de typage HLA. Ainsi, de nombreuses incompatibilités non pu être révélées lors de ces travaux, et les tentatives pour établir une corrélation entre survie et incidence de maladie GvH après transplantation se sont soldées par des échecs. A présent, les techniques combinées de biologie moléculaire et cellulaire représentent un formidable outil pour entreprendre de nouvelles études.

Evaluation de l'impact de la compatibilité HLA sur la survie des patients greffés

Quarante-quatre combinaisons patient/donneur *non apparentés* ont été inclus dans cette étude clinique. Ces individus répondaient tous au critère "HLA-ABDR identique" défini par les techniques de sérologie et un certain nombre d'incompatibilités HLA non détectées par ces techniques ont pu être identifiées par les techniques de haute résolution.

Notre étude a démontré que les patients qui avaient été transplantés avec la moelle provenant de donneurs HLA-définis comme identiques par ces outils performants, avaient une meilleure survie que ceux greffés à partir de donneurs moins compatibles. Ainsi, le pourcentage de survie de ces patients après trois années se situe à 61, contre 13 seulement pour les patients dont la moelle avait été prélevée chez des individus HLA non identiques.

En conclusion, malgré le nombre relativement restreint de cas étudiés, la différence entre ces deux groupes de patients, sur la survie à long terme, est conséquente. C'est pourquoi les incompatibilités HLA subsistantes entre donneur et receveur peuvent influencer l'incidence de maladie GvH.

Evaluation du rôle respectif des antigènes majeurs et mineurs dans la transplantation de moelle osseuse

L'identification d'un donneur de moelle non apparenté, compatible au niveau de chacun des antigènes HLA (A, B, C, DR, DQ et DP) semble, cependant, utopique. Ainsi, par exemple, le pourcentage de recombinaison entre HLA-DQ et -DP est tel que la plupart des couples donneur/receveur non apparentés sont incompatibles au niveau du locus HLA-DP. Cet éloignement des gènes se traduit en effet par une perte de liaison. D'autre part, l'extrême polymorphisme du système HLA entrave également la sélection d'un donneur compatible.

Finalement, une des question-clefs de ces études est de déterminer s'il existe ou non des incompatibilités HLA tolérées par le système immunitaire du donneur après la greffe. Les molécules HLA-Cw et -DP, dont les rôles semblent secondaires lors de réactions immunologiques, sont évidemment, les premiers candidats potentiels impliqués.

La seconde partie de cette thèse a été focalisée sur l'identification des histo-incompatibilités détectées *après* transplantation de moelle osseuse. Nous avons étudié l'importance relative de certaines incompatibilités localisées au niveau de variants HLA, de l'antigène HLA-Cw et des antigènes mineurs, chez cinq patients greffés présentant des signes cliniques de maladie GvH.

Dans un premier temps, nous avons réalisé par les techniques de haute résolution, le typage complet des molécules HLA de chacun de ces individus. Trois combinaisons présentaient effectivement des différences HLA de classe I, au niveau de deux allèles HLA-A et d'un allèle HLA-C. En revanche, les deux autres paires ont été déclarées HLA-identiques. Dans un second temps, la reconnaissance de ces antigènes durant la maladie GvH a pu être évaluée par l'analyse de clones T cytotoxiques.

Ces recherches confirment l'importance des histo-incompatibilités HLA dans l'apparition de la maladie GvH et le rôle primordial des techniques performantes de typage HLA dans l'identification de ces incompatibilités. Ainsi, les différences HLA détectées avant transplantation sont les cibles reconnues par les lymphocytes T du donneur après la greffe. La nature des incompatibilités HLA reconnues est également instructive; s'il était de notoriété publique qu'une différence au niveau d'un allèle HLA pouvait engendrer une réponse alloréactive, en revanche, ces études montrent qu'un variant HLA et une incompatibilité HLA-C produisent aussi une forte réaction immune. Finalement, l'évaluation du rôle joué par les antigènes mineurs, qui fut jusqu'à présent négligée dans les transplantations entre individus *non apparentés*, a permis de redéfinir leur importance dans la greffe de moelle osseuse. En effet, dans les deux seules combinaisons HLA-compatibles, la réponse immune était exclusivement dirigée contre des antigènes mineurs, dont cinq nouvelles formes ont pu être caractérisées.

Conclusion

Les résultats présentés dans cette thèse permettent une meilleure compréhension des paramètres influençant la survie des patients après transplantation de moelle osseuse.

L'étude de l'impact de chaque incompatibilité majeure et/ou mineure sur le développement de la maladie GvH n'a pu être réalisée que grâce à l'utilisation de techniques performantes dans le typage des molécules HLA. Finalement, ces études apportent une connaissance et une optique nouvelle sur la problématique cruciale que représente la sélection d'un donneur de moelle osseuse.

List of Abbreviations

ABDR	HLA-A, -B and -DR
Ags	Antigens
APCs	Antigen presenting cells
BMT	Bone marrow transplantation
β2-M	β2-microglobulin
CLIP	Class II-associated Ii peptide
CTLpf	Cytotoxic T cell precursor frequency test
D/Rs	Donor/recipient pair
EBV	Epstein Barr Virus
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
GvHD	Graft-versus-Host Disease
HLA	Human leucocyte antigens
HPLC	High-performance liquid chromatography
HS	Human serum
H-Y	Male specific minor antigen
IL-2	Interleukine-2
MLC	Mixed lymphocyte culture test
MHC	Major Histocompatibility Complex
mHC	Minor Histocompatibility Complex
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
TcR	T cell receptor
Th	Helper T lymphocyte
T cell	Cytotoxic T lymphocyte
TRM	Transplant Related Mortality

Chapter 1

INTRODUCTION

1. The immune system

1.1 Introduction

The highly elaborated immune system of vertebrates is able to eliminate various types of foreign pathogens. It is now well established that three major cell types are required for those immune responses. Two of them are lymphocytes that represent the antigenic specific part: first, B cells that mature in the bone-marrow and that produce antibodies or immunoglobulins (Igs). Secondly, T cells that after being educated in the thymus, exhibit a heterogeneous set of functions such as providing help to B cells and specific killing of virus-infected cells. Macrophages constitute the third cell type; their function is to ingest pathogens or soluble proteins and to present fractions of these antigens to T lymphocytes in the context of proteins called the Major Histocompatibility Complex (MHC).

B and T lymphocytes express receptors on the cell surface with high affinity for antigens which are designated (surface-) Ig receptors for B cells, and T cell Receptors (TcR) for T cells, respectively. Ig and TcR receptors are products of somatically rearranged genes giving rise to the huge diversity of B and T cell receptors that are clonally expressed. Therefore, each lymphocyte carries exclusively one single antigenic specificity. It has been speculated that the total amount of lymphocytes is able to recognize more than 10^8 distinct antigens.

The main difference in the recognition of the antigenic determinant (or epitope) between these two kind of receptors is the following: whereas the TcR recognizes short antigenic fragments of 9 to 20 amino acid of length presented by the MHC proteins, the IgRs interact directly with the three-dimensional structure of the antigen. Therefore, when a pathogen invades the organism,

different immune responses depending on the nature of the involved agent can be mounted in order to eliminate it specifically. On one side, antibodies secreted by the B lymphocytes will interact with extra-cellular pathogens such as bacteria or worms which are found for example in the blood. This will activate other cell types (eosinophiles, neutrophiles, etc.) with a destructive capacity. On the other side, virus and intra-cellular protozoa's which can escape those humoral responses, will be presented to cytotoxic T lymphocytes (Tc) in small peptidic fragments by MHC molecules. Upon activation, these Tc identify the infected cells and destroy them selectively. Finally, T helper cells (Th) provide specific help for B cells and for Tc by secreting lymphokines, the hormones of the immune system (Figure 1).

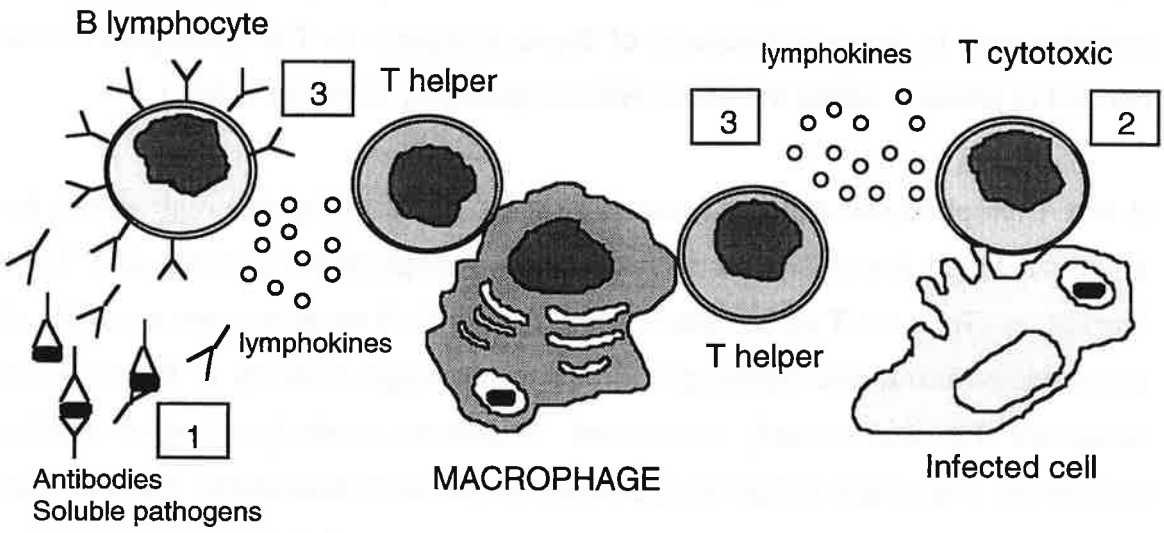


Figure 1: B and T lymphocyte effector functions against extra and intra-cellular pathogens. 1 = B cell secreted antibodies 2 = cytotoxic T cell response 3 = helper T cell response.

The immune system has an enormous destructive capacity by means of these lymphocytes and other non described effector cells. If such a system is not precisely regulated and controlled, this can result into the destruction of healthy tissues with severe pathologic consequences. Such disregulations are found in hypersensitivity or allergic reactions, in autoimmune diseases like diabetes mellitus (type I) or rheumatoid arthritis. Furthermore, this efficient capacity to destroy invaders is also responsible for the rejection of bone-marrow, kidney, heart, and other organ transplants.

1.2 The Major Histocompatibility Complex: a central molecule in immune interactions

The MHC molecules were identified for the first time in the early 40's by specialists of organ transplantations (1,2). They described a polymorphic antigenic locus localized on chromosome 17 in mice which was essential for tumor rejections and called it the H-2 locus (H = histocompatibility). Many years later, the human equivalent was discovered by Dausset on chromosome 6 and the encoded genes were named Human Leukocyte Antigens or HLA (3-6). There are two structurally distinct, but related families of MHC molecules: class I and class II molecules. One particular characteristic is their extraordinary diversity in the population. In humans, over 450 structurally distinct class I and class II MHC molecules have been described to date (7). Because in a single individual six loci are expressed codominantly, two unrelated individuals will seldomly share the same HLA molecules.

In humans, the MHC class I molecules are divided into three independent loci: HLA-A, -B, and -C are non-covalently linked heterodimers consisting of a heavy chain (44 kD) and a non-MHC-encoded protein of 12 kD, the β2- microglobulin or β2-M (8-10). The class I heavy chain is composed of an extracellular domain, a transmembrane region and a cytoplasmic tail (Figure 2) (11). The extracellular portion is divided into three domains of respectively 90, 92 and 92 amino acids:

α1, α2, α3 (12). Most amino acid substitutions between the products of different alleles are found in the α1 and α2 domains (13).

The HLA class II genes encode for HLA-DR, -DQ and -DP molecules respectively and differ from class I molecules by the fact that they are composed of two-chain surface glycoproteins, the α- and β-chains (33 and 48 kD respectively). These chains can be divided into two extracellular domains α1 α2 and β1 β2, a transmembrane region and a cytoplasmic tail (Figure 2) (14-17). As for MHC class I products, the external part of the molecule, α1 and in particular β1 domain contains most of the polymorphic residues.

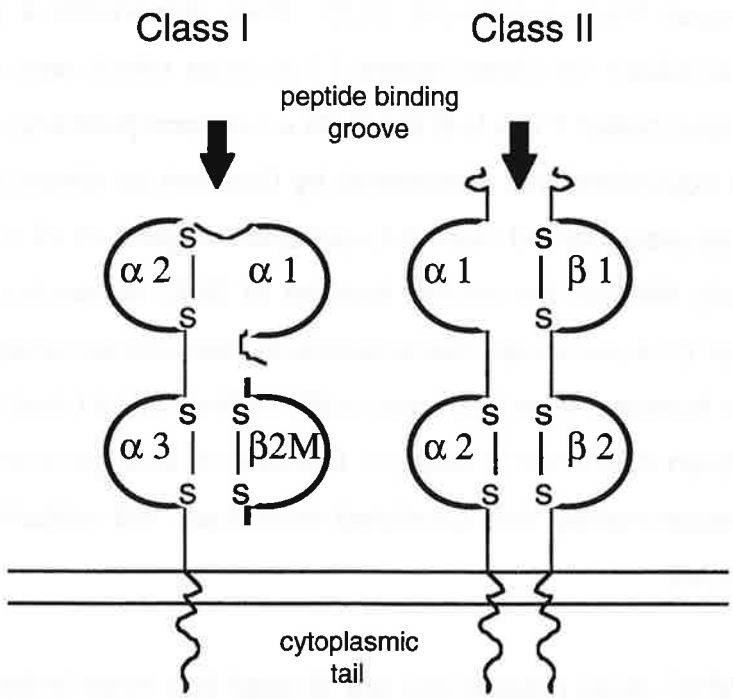
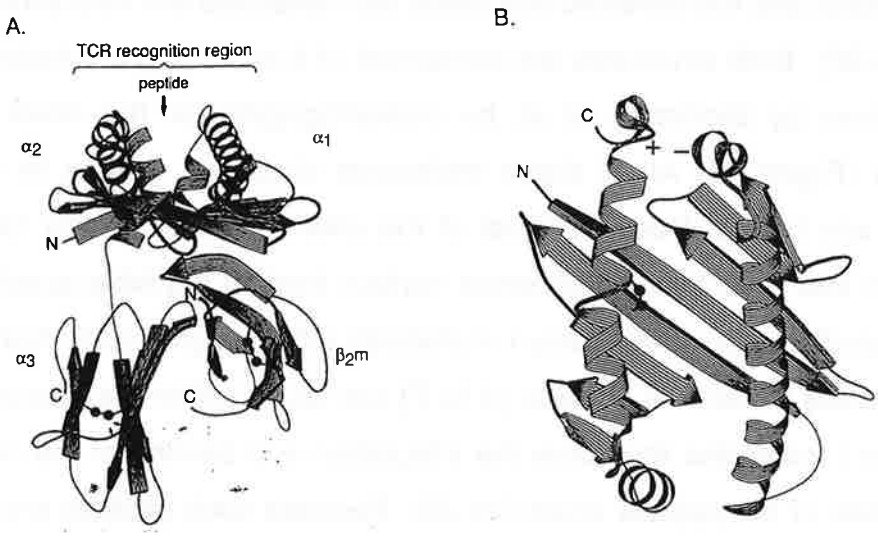


Figure 2 : The structure of MHC class I and class II molecules

Crystallographic analysis of the three-dimensional structures of class I and class II molecules has revealed that these two molecules are very similar to each other (18-25). Both structures are composed of β-strands and α-helices as has been shown by Bjorkman et al. by crystallography for the class I HLA-A2 molecule (Figure 3). All of these molecules display a groove in which the peptides are bound. Because most of the allelic differences are found to be located in this cleft, this will generate various binding peptides specificity. The peptide binding site of the class I molecules differs significantly from the one found in class II. Several pockets (A to F) are found within the groove of those HLA class I molecules that allow the integration and binding of the eight to ten amino-acids of the peptide chain (26-28). Because such pockets are absent in the class II cleft, the majority of the interactions will occur in the center of the groove and the bound peptide. Therefore, the class II associated peptides will be longer and more heterogeneous in size than class I associated peptides. A further explanation comes from the fact that both ends of the class I peptide binding site are closed which is not the case for the class II sites (29).

Common peptide structural features based on the analysis of the sequence of naturally processed peptides purified from different class I molecules could be identified (reviewed in 26, 27-28). Peptides isolated from several class I molecules showed a strong selection at position two and five. Furthermore, because the A and F pockets stabilize the amino- and the carboxyl termini respectively, major motifs for MHC associated peptides have been identified in these carboxyl terminal residues.

Figure 3: Three dimensional structure of the HLA class I molecule. a) side view b) top view of the antigen binding groove (taken from Bjorkman and Parham, Annu Rev Biochem. 1990; 59:253).



One essential function of MHC molecules is the ability of binding a large number of different peptides. This could be demonstrated by electrospray ionization and mass spectrometric analysis. More than 2000 peptides were estimated to be associated with class I molecules HLA-A*0201 and -B7 and 90 % of these peptides were present in the range of 100 to 1000 complexes per cell (30,31). Since most of the cells express 10^5-10^6 MHC molecules on their cell surface, the total number of different peptides presented could be over 10'000. Finally, it has been estimated that one T cell directed against a particular epitope needs at least 100-500 of such peptide-MHC complexes to be activated (32-36). However, the impact on the recognition of such various and abundant peptides by the T lymphocytes on one antigen-presenting cell is far from being understood.

The first experiments, in the early 1970's, demonstrated that virus- and minor histocompatibility-specific T cells recognize the foreign antigen on cells that express self-MHC molecules (37,38). T cell recognition is therefore "MHC-restricted" and subsequent transfection experiments showed that the transfection of one single TcR is able to bind and recognize the MHC molecule and its processed peptide (39).

In the following years, many immunologists have tried to understand how the T lymphocytes could actually recognize the foreign peptide and the MHC molecule simultaneously and how these epitopes were generated through the various intracellular pathways.

Class I and class II MHC molecules trigger two distinct immune responses by activating two subsets of T lymphocytes and by presenting two different categories of antigens. Class I molecules usually present peptides derived from endogenously synthesized proteins such as a virus to cytotoxic T lymphocytes bearing the CD8 cell-surface glycoprotein (40,41). On the other hand, class II molecules present peptides derived from exogenously proteins to CD4-bearing helper T lymphocytes (42,43) (see Figure 4).

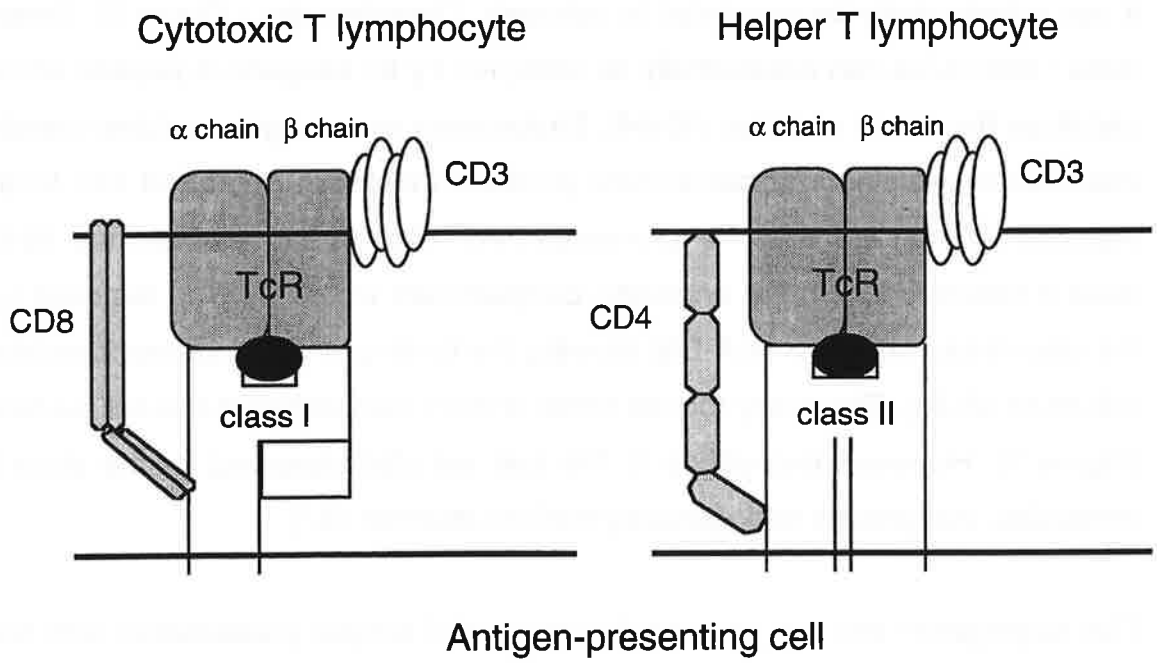


Figure 4: Structures involved in the trimolecular recognition of antigen
a)Tc-peptide-MHC class I and b) Th-peptide-MHC class II

The distinction in the presentation of peptides by class I and class II MHC molecules is thought to result from the segregation of two distinct intracellular pathways (44,45). Briefly, the class I MHC pathway starts when newly synthesized viral and cellular proteins are degraded in the cytosol by an enzymatic complex called the proteasome. The obtained peptides are exported to the endoplasmic reticulum (ER) through the peptide transport molecules Tap-1 and Tap-2 (46,47). In the ER, the transported peptides will bind to the newly synthesized heterodimeric class I heavy chain/ β–2M complex (41,48). These small products do not bind to the MHC class II α β chain-heterodimer due to the fact that this molecule is already occupied by a molecule named the invariant chain or Ii (49,50). Indeed, the invariant chain is degraded during transport from the Golgi to the endosomes into a fragment called class II-associated Ii peptide or CLIP thereby preventing the binding of class I endogenous peptides (51). The resulting class I heterotrimer migrates through the Golgi to the cell surface where it can subsequently be presented to cytotoxic T lymphocytes (Figure 5). Empty class I molecules can occasionally be occupied by an exogenous peptide which stabilizes the whole complex (52-54). Endosomes or endocytic vesicles contain internalized proteins and cell-surface proteins which are degraded into small peptides through the action of proteases (44). Those peptides meet the MHC class II heterodimers in the endocytic compartment where CLIP is removed by the class II-like structure HLA-DM allowing the binding of the antigenic peptides (48,49,51,55,56). The newly formed trimer is then transported to the cell surface (Figure 5). However, exceptions to this rule are also observed for the class II molecules, that present endogenously derived peptides (57).

This segregation into two separated pathways of antigen-presentation with two different subsets of T lymphocytes involved, illustrates a fundamental mechanism of the immune system. Indeed, class I MHC molecules are found on almost every nucleated cell, whereas class II molecules have a more limited distribution and are present on immune cells such as B lymphocytes, dendritic cells, thymic epithelial cells and macrophages.

Pathways of Antigen Processing and Presentation

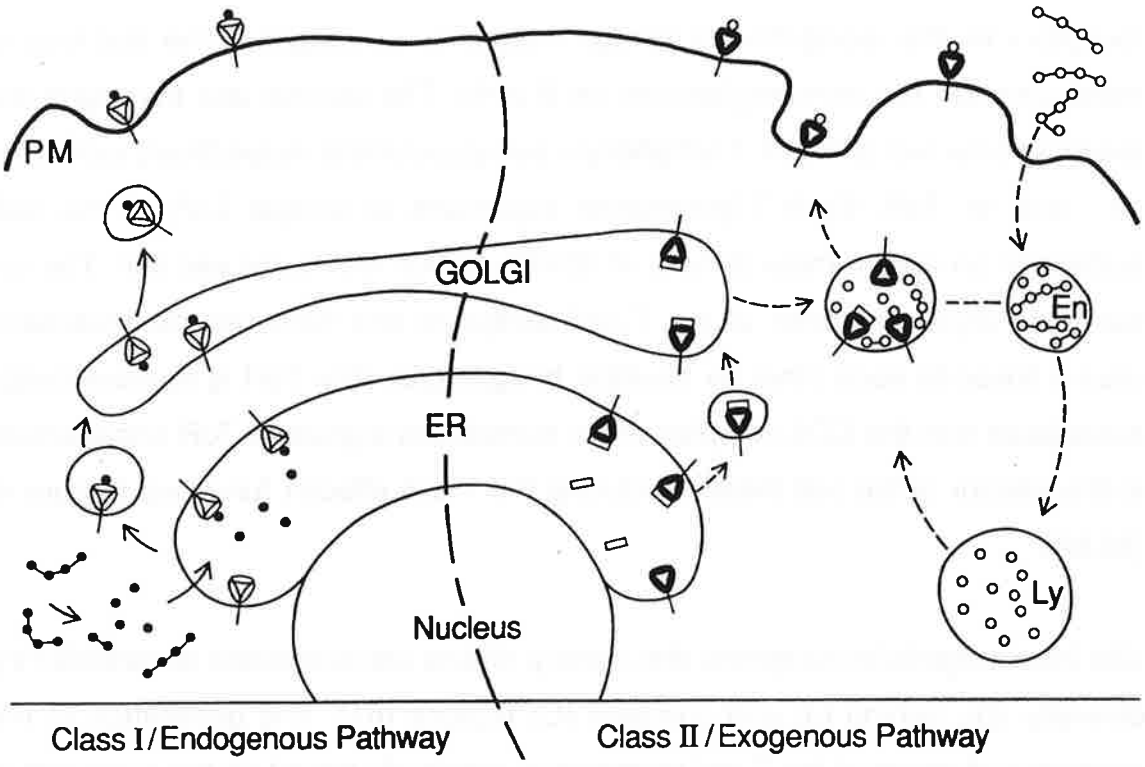


Figure 5: The endogenous and exogenous antigen-presenting pathways. Endogenous proteins (●-●-●), exogenous proteins (○-○-○), MHC class I (open triangles), MHC class II (filled triangles), ER = endoplasmic reticulum, Ly = lysosomal compartment, En = endosomal compartment. (Taken from Bjorkman and Parham, Annu Rev Bioch. 1990; 59:253).

Therefore, class I-restricted T cytotoxic lymphocytes are able to recognize every viral-infected cell in the whole organism whereas evolution created MHC class II molecules to allow specific interactions with B and T cytotoxic cells by helper T lymphocytes. Furthermore this dichotomy prevents that class II^+ cells which present antigens without being infected by a virus, to be killed by cytotoxic T lymphocytes, whereas a cell that present antigens via class I MHC molecules is destroyed.

1.3 Self and non-self recognition

The immune system possesses three elaborated system of extracellular receptors for the recognition of foreign molecules or antigens. The first type of molecules are the immunoglobulins on B cells. The second and third type are expressed by two different T lymphocyte sub-populations respectively called the αβ - and γδ -TcR. Each T lymphocyte expresses an unique TcR on the cell-surface at an approximate density of 20'000-40'000 molecules per cell. The αβ-subtypes represent most of the T cell receptors and form two heterodimeric chains linked to each other by disulfide bridges (58). The TcR is non-covalently associated with the CD3 complex which transduces signals of TcR engagement to the interior of the cell thereby inducing the T cell effector functions (Figure 4) (59,60).

Like immunoglobulin receptors, the α and β chains are composed of variable (V), diversity (D), joining (J) and constant (C) regions (61). The generation of the enormous diversity of the T cell receptors is principally based on the presence of multiple copies of V, D and J genes, on random gene rearrangement and on junctional and insertional diversity (62-65). Therefore each individual with more than 10^8 of T lymphocytes possesses such a TcR diversity that every foreign antigens should be recognized. However, if TcRs are randomly created, the TcRs found on mature T lymphocytes will also recognize self-molecules. What are the mechanisms leading to the deletion of the self directed TcRs ?

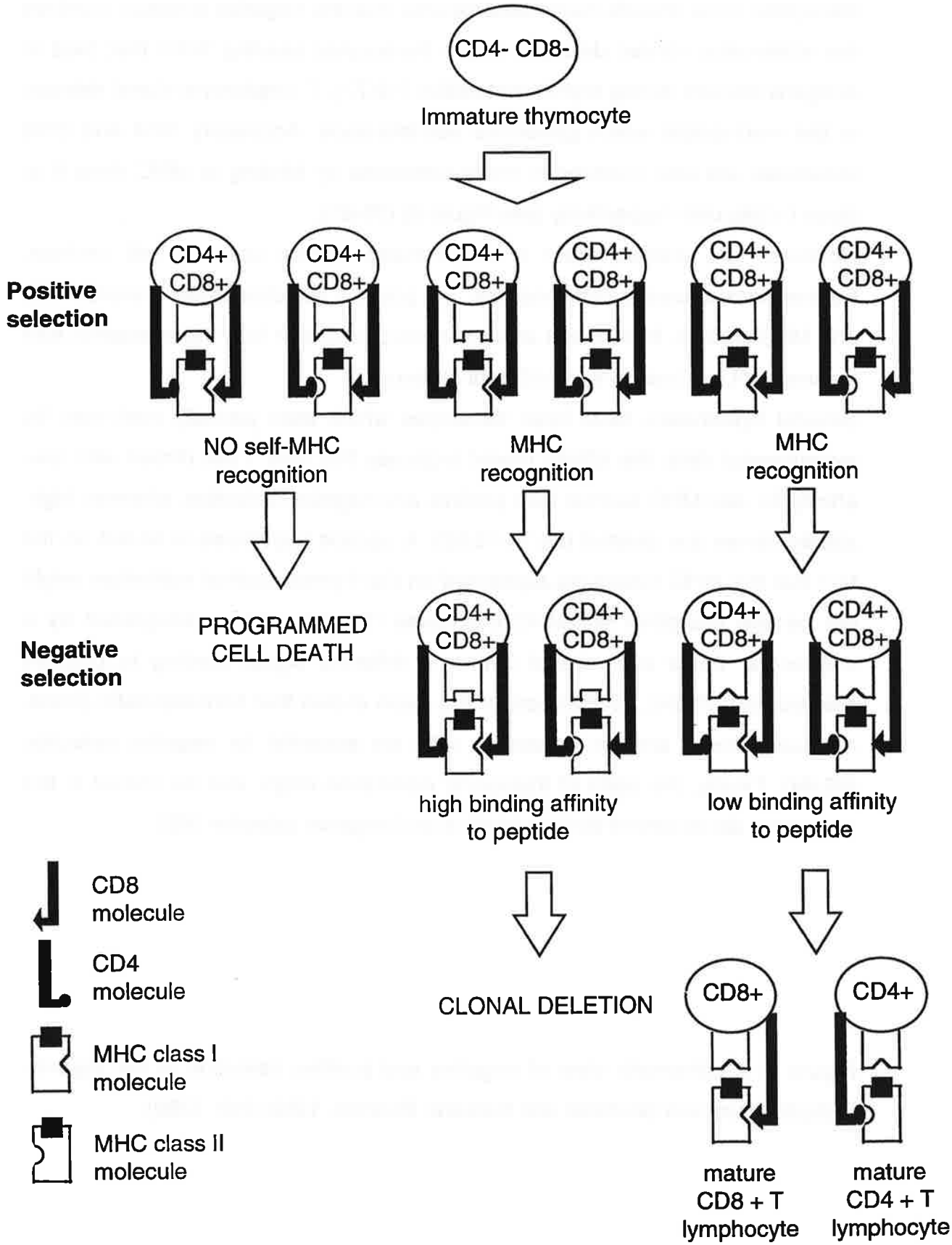
The specificity of the T cell compartment is determined during it's development and education in the thymus. The immature T cells or thymocytes have to carry out a multi-step process in order to become mature CD8-cytotoxic or CD4-helper T lymphocytes. The first step is the creation of the extensive TcR diversity mentioned above. This repertoire will then be modified by two processes, i.e. positive and negative selection (66). Positive selection allows the maturation of thymocytes bearing receptors which recognize foreign antigen in the context of *self* MHC molecules (67-71). Several experiments on superantigens and

transgenic mice models have demonstrated that the negative selection involves the elimination (clonal deletion) of the thymocytes bearing TcRs that bind to antigens present during thymic maturation (72-77). T lymphocyte clonal deletion is the mechanism which generates self-tolerance. Accessory CD4 and CD8 molecules are also involved in those selections by binding to MHC class II or class I molecules respectively (see Figure 6) (78-81).

However, the precise order in which these events occur is still unclear. Furthermore, because both negative and positive selection need a similar TcR and MHC contact, there is still an unresolved paradox on how the interaction with a same TcR can lead to such different responses.

Several hypotheses have been developed which were partially confirmed by experimental data; the affinity model proposes that only T cell clones with low-affinity for self-MHC survive both positive and negative selection, whereas high-affinity clones are deleted (65,74-75,82). A second hypothesis is based on the fact that the MHC molecules expressed on the thymus cortical epithelium might not be like the other self-MHC molecules (75, 83). When recognized by a thymocyte, those cells would deliver a different signal leading to positive selection (69,75,84). Furthermore, it has been shown that hematopoietic (bone-marrow derived) antigen presenting cells are essential for negative selection (69,84). Finally, the state of thymocyte maturation might also be crucial in the sequential development through positive and negative selection (85).

Figure 6: A schematic view of negative and positive selection in the thymus. (Adapted from von Boehmer and Kisielow. Science. 1990; 248: 1369).


CD4- CD8-
Immature thymocyte
Positive selection
CD4+ CD8+
NO self-MHC recognition
MHC recognition
MHC recognition
PROGRAMMED CELL DEATH
Negative selection
high binding affinity to peptide
low binding affinity to peptide
CLONAL DELETION
CD8+
CD4+
mature CD8 + T lymphocyte
mature CD4 + T lymphocyte
CD8 molecule
CD4 molecule
MHC class I molecule
MHC class II molecule

Although probably most of the T cells are educated in the thymus through these processes, clonal selection is unable to explain how the immune system learns to be tolerant against self molecules which are not expressed in the thymus. Therefore, other mechanisms found outside the thymus must somehow inhibit, inactivate or destroy auto-reactive mature T lymphocytes. These extra-thymic mechanisms are called peripheral tolerance. Many reports have shown that in some circumstances T lymphocytes become inactivated or anergized, whereas in others, they are clonally deleted on encounter of self proteins (85-92). T cell anergy is referred to a state in which the T cell survives but is functionally inactive. To date, no clear experiments provide further explanations on the respective role and mechanism of these different extra-thymic processes that induce T cell tolerance.

However, it is accepted that the activation of a T lymphocyte must proceed through two distinct signals; the first one, corresponds to the binding of the αβ TcR to the MHC molecule, whereas the second one might be induced by the co-binding of CD28 and B7 molecules and/or other accessory molecules (89,93-95). The first signal in the absence of the second one leads to T lymphocyte inactivation or anergy. The second signal is antigen non-specific and results in T cell activation, when CD28 present on those T cells interacts with the B7 molecules expressed on professional antigen-presenting cells (Figure 7) (96). Furthermore, the role of the CD28-B7 cosignal could be demonstrated in tumor models, in which the transfection of the B7 gene on B7-negative melanoma cell lines induced a specific immune recognition by cytotoxic T lymphocytes of these cells followed by tumor rejection (97-98). However, recent studies on CD28 knock-out mice, showed that these mice develop normal immune responses to viral infections in vivo (99). These results and others (100,101) suggest that CD28 might not be as essential as it was thought to be at the beginning and that costimulatory signals are partially provided by other accessory surface molecules.

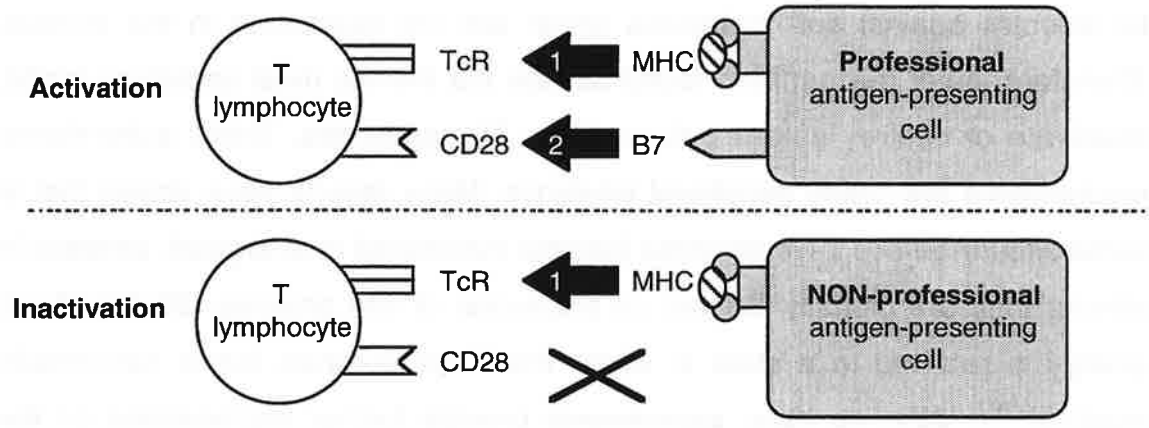


Figure 7: The two-signal model; costimulation of signal 1 and 2 is necessary for T cell activation. (Schema adapted from Lanzavecchia A. Identifying strategies for immune intervention. Science. 1994; 260:937).

In a normal immune system, the intra and extra-thymically educated T lymphocytes should not recognize nor destroy the cells of their own body. Unfortunately, in some cases, the system may slip and uncontrolled T or B lymphocytes may react against self tissues leading to autoimmune diseases. Furthermore, such a vigorous T cell activation will also be found during transplantations and can result in the rejection of the transplanted organ.
It is only by studying the various mechanisms controlling the extra-thymic T cell tolerance that a better understanding and prevention of autoimmune and alloreactive responses might become a reality one day.

2. BMT: a subtle balance between success and failure

2.1 Introduction

Since the first marrow transplants in mice performed in the early 1950's (102,103), many developments in HLA typing, in immunosuppression and in cryobiological techniques have led to an exponential increase in the numbers of human allogeneic bone marrow transplantations (BMT). At present, BMT has been accepted as an effective treatment for hematological malignancies like leukemias and for inborn disorders of the immune system (104,105).

Because the intensive chemo- and radio-therapy used for treatment of cancer are highly toxic to the hematopoietic system, a BMT will have to restore a new system after the destruction of host residual cancer and immune cells. Allogeneic BMT is performed by transferring marrow cells from one individual to another. After intravenous infusion into the patient, the donor hematopoietic stem cells colonize the marrow which has become pancytopenic following the multiple treatments against the malignant cells and the preconditioning. After a few weeks, hematological recovery starts with the emergence of granulocytes and leukocytes. In general, the patient peripheral blood contains nearly normal amount of leukocytes, erythrocytes and platelets within 100 days. However, the immune functions of those patients only return to normal after 1 to 2 years following transplantation (106). Furthermore, bone marrow transplantation differs from the solid organ transplants by the fact that the patient's immune system itself is removed and replaced by donor cells. Because donor immune cells transfused with the marrow will recognize the patient tissues if MHC incompatible, a very high HLA identity between the potential donor and the patient is necessary.

2.2 Potential bone marrow donors

The best choice as an allogeneic bone marrow donor is an HLA-genotypically identical sibling. Since the genes encoding for the HLA molecules are located on one chromosome, any two siblings have 25 % of chance to share the same HLA haplotypes. Due to the small average size of western families (two children per family), such HLA-matched donors are only found for about 25 to 30 % of the patients (107,108). For the 70 % of patients who lack such donors, two other types of donors might be considered.

Firstly, one might select a related donor matched only partially, who shares one of the MHC haplotypes with the patient (109). Secondly, an unrelated HLA-phenotypically matched bone marrow donor can be selected from the national registries of bone marrow donor volunteers (108,110,111). Due to the extremely large HLA polymorphism in the population, such a registry must contain several million donors to provide a reasonable chance to find a compatible donor (112,114). Fortunately, certain HLA phenotypes or haplotypes are found in linkage desequilibrium among the population, thereby, increasing the probability that two unrelated Caucasians[1] for example, share the same antigens (113).

2.3 Bone marrow transplantation: success rates and complications

The application of BMT for the treatment of hematopoietic diseases has gone through two stages in history. The first stage occurred in the 1950's, at a time that only little was known about the MHC complex, preconditioning treatments, transfusion technology of platelets and granulocytes, antibiotics and immunosuppression. As a consequence, many attempts in human bone marrow transplantation were unsuccessful.

It is the research on animal models which provided twenty years later, the technology and the enthusiasm for a restart of human bone marrow transplants. Since then, the number of allogeneic BMT performed worldwide has increased

[1] Although caucasian is not the correct definition in the anthropological sense, in HLA genetic it is used to describe Homosapiens sapiens of european origin.

exponentially and at present several thousands of patients have been transplanted (105,107).

Many reports from different transplantation centers show that three years survival following allogeneic transplantation is approximately 40 to 50 %. However, comparison of success rates is complex and involves multiple variables (Figure 8). First, the type of disease, the patient's condition at the time of transplantation or the patient's age are crucial factors. Therefore, transplantation of very young patients in early stages of disease results in much better survival. Secondly, HLA compatibilities or mismatches between the patient and his donor, the type of donor (related or unrelated) also represent factors associated with disease-free survival after transplantation (114).

Several reports have evaluated the impact on the clinical outcome of 0, 1, 2 or more HLA-mismatches between a patient and his marrow donor. Whereas, some of the investigators have demonstrated that survival with marrow incompatible for one HLA locus was not significantly different from survival with marrow from HLA phenotypically-identical donors (108,109,114-117), more recent and precise studies have shown, that each HLA mismatch might be crucial for clinical outcome, and that the choice of unrelated donors should be based on high resolution HLA typing technology (see 2.4 HLA typing techniques) (117a-120).

Finally, in vitro or in vivo marrow manipulations such as depletion of T lymphocytes also represent an important factor modulating the transplanted patient 's survival curves.

The severe marrow aplasia caused by immunosuppression following the first weeks after transplantation and the addition of pretransplant factors that will negatively influence survival provide a high risk for the development of post-transplant complications. One of the most important post-transplant complication with associated mortality is Graft-versus-Host Disease (GvHD). Besides this, as shown in Figure 8, other complications such as graft-rejections, infections or the recurrence of the disease might also occur (107).

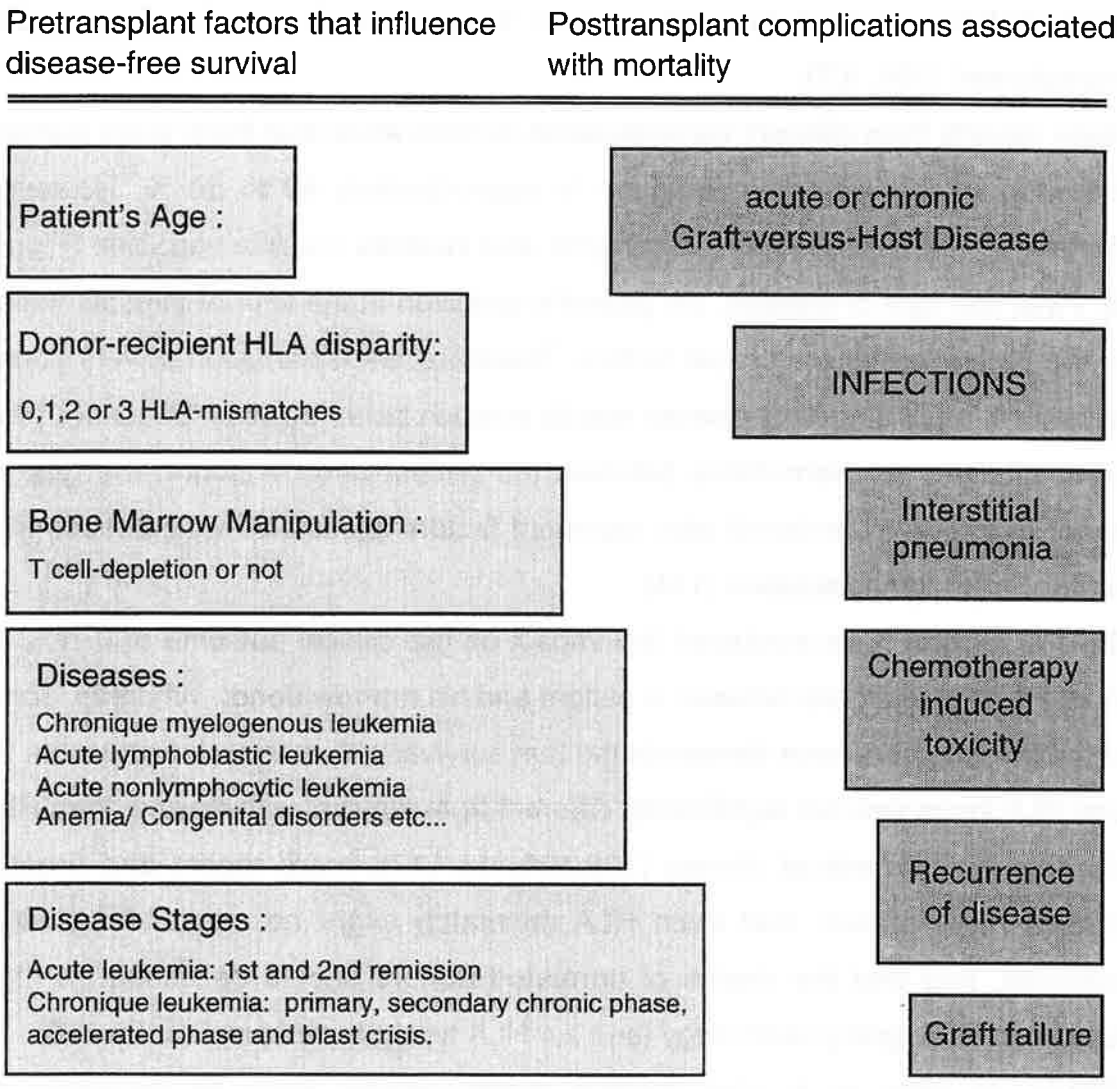


Figure 8: Pretransplant factors that influence disease-free survival and post-transplant complications associated with mortality.
(Adapted from Kernan NA, et al. Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med. 1993; 328:593).

Furthermore, pulmonary complications usually induced by cytomegalovirus are also common causes of death after allogeneic BMT. The administration of prophylactic antibiotics significantly reduces the incidence of potentially lethal complications (121,122).

GvHD was first described in early experiments in allogeneic marrow transplantations in mice (123). Although the animals recovered normally from marrow aplasia and lethal irradiation, they subsequently died of a secondary disease that was later designated GvHD. This disease affects multiple organs and is principally associated with destruction of the skin epithelium, the gastrointestinal system and the liver (124). Furthermore, the treatment of GvHD by immuno-suppressive agents can lead to profound immunodeficiencies and thereby to a high susceptibility to infections.

GvHD can be divided into two clinical entities: acute disease which occurs during the first 3 months following transplantation and chronic disease developing more than 100 days after transplantation. Although the chronic form shares certain clinical features with the acute disease, this form seems however, more likely to affect elderly patients and finally lead to autoimmune complications.

Graft-versus-Host reaction is the result of specific recognition of the host's tissues by the donor T cells in the graft. Many studies in mice and in human have now provided strong evidence that the T lymphocytes transfused with the marrow recognize incompatible MHC molecules expressed by the recipient's tissues and can initiate GvHD (125). By coinjections of purified T-cell subsets into transplanted mice, Korngold and Sprent have shown that both CD8+ and CD4+ T lymphocytes directed against MHC class I and class II incompatibilities respectively, were able to induce lethal GvHD. Therefore, MHC incompatibilities between a bone marrow donor and a patient represent the most important factor of risk for the induction of GvHD.

However, other immune cells such as macrophages and natural killer (NK) cells were also found to be involved in the development of the pathology (126). These findings led Ferrara and Deeg to suggest a two phase model (Figure 9).

First, the T cells transfused with the marrow induce the afferent phase by recognizing alloantigens and releasing lymphokines. Secondly, these lymphokines activate other cell lines like NK cells which mediate and destroy host tissues.

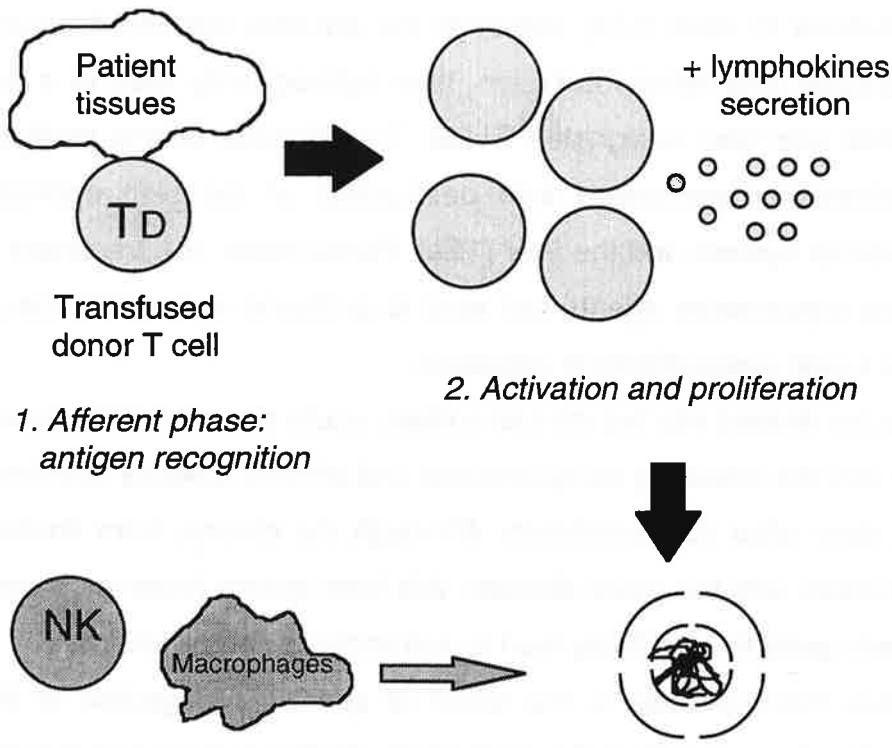


Figure 9: The two-phase model of GvHD. (Adapted from Ferrara and Deeg. Graft-versus-Host Disease. N Engl J Med. 1991; 324:667).

Other groups give even more weight to this model by attributing a central role to the lymphokine release and see GvHD as a result of a "cytokine storm" (127). They think that damage to host tissues during chemotherapy, radiotherapy and infections may release inflammatory cytokines such as tumor necrosis factor α and interleukine-1.

The cumulative action of both released cytokines and MHC incompatibilities between the host versus donor tissues will then induce proliferation of the transfused donor T lymphocytes and the production of interleukine-2 (IL-2). This may result finally into a cytokine cascade which activates other cells such as macrophages or NK cells.

The use of immunosuppressive drugs such as cyclosporine and methotrexate, or the removal of T cells from the graft efficiently prevents the occurrence of post-transplant GvHD (128,129). Unfortunately the depletion of donor T cells from the marrow is associated with a higher rate of graft failure and incidence of recurrent disease in particular for patients suffering of chronic myeloïd leukemia (129,130). Therefore, several authors have suggested that some T cells in the marrow are important in the elimination of residual leukemic cells and might induce the so-called graft-versus-leukemia effect (131). However, it is still quite unclear whether graft-versus-host disease and graft-versus-leukemia are mediated by the same (host-specific) or by separate (leukemia-specific) donor T cell populations (132-135).

2.4 HLA matching: classical and high resolution typing technology

Transplantation with bone marrow from other than a genotypically HLA identical donor has become extensively used, however, it is associated with an increased incidence of GvHD. The higher degree of incompatibility in unrelated combinations is likely to be primarily caused by disparities in the MHC.

Currently, several methods are available that determine the different HLA alleles expressed by the patient and his potential donor with different resolution.

One of the oldest and most widely used method of tissue typing is based on the recognition of the determinants specific for a given HLA allele by a set of different antibodies (136). Such serological techniques represent a very useful and quick

tool for the characterization of the different HLA A, B and DR molecules. By this method, the various haplotypes shared by each member of a family are precisely characterized and a genotypically HLA-identical donor within a family can easily be identified since identical siblings inherite the same haplotypes. Therefore, many centers have been, or are still exclusively focused on this classical typing technique. However, serology is unable to identify each HLA variant precisely, and the HLA typing of unrelated individuals requires therefore much more precise technology such as cellular assays and molecular biology techniques. This is outlined in Figure 10; HLA serotyping of a patient and his potential *unrelated* donor did not reveal the presence of several HLA subtype mismatches. Thereby, an HLA analysis exclusively based on serology would have missed several HLA incompatibilities in this pair, whereas more complete analysis would have detected those mismatched molecules.

During the last ten years, molecular biology has developed significantly and at present it can be used as a very efficient tool for precise HLA typing. DNA sequencing of the MHC genes has revealed that the polymorphism at each HLA locus is far greater than the one that can be discerned by serology. At present, over 450 HLA class I A/B/C and class II DR/DQ/DP alleles have been sequenced (7). The characterization of all these sequences has allowed the synthesis of specific oligonucleotide probes (oligotyping) which define every single HLA allele precisely (137,138). However, the oligotyping techniques for HLA class I have not been developed to the same extent as for class II molecules. Furthermore, because the probes are exclusively applicable on known gene sequences, new or rare HLA variants still present in unrelated individuals will be missed.

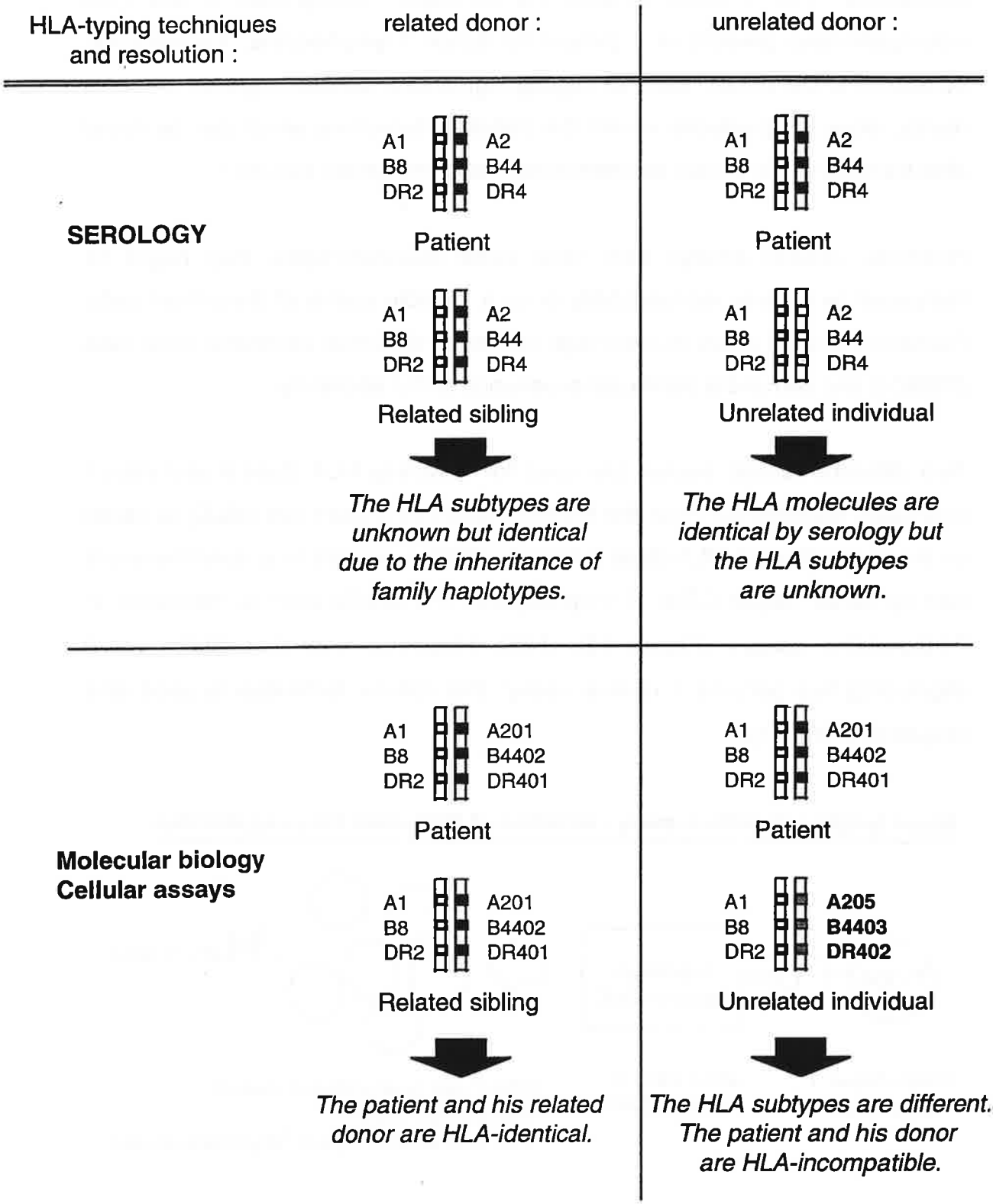


Figure 10: An example illustrating HLA typing resolution level of the classical serotyping technique and the efficient oligotyping and cellular assays.

Finally, cellular assays represent another strategy for typing of various individuals. This method reflects the functional recognition of the HLA incompatibilities present in a patient by donor T lymphocytes. Furthermore, compared to the others, sero- or oligotyping, cellular assays might be closer to reality, since it reproduces in vitro the cellular interactions which can be found after transplantation in vivo between donor cells and patient tissues.

However, cellular assays also have some disadvantages: they might be hampered by a lower reproducibility or by a variable quality of the patient cells. Furthermore, such assay do need high quantities of human peripheral blood cells (PBMCs) and demand a significant experience of the laboratory.

Two different cellular assays are used for matching HLA class II and class I molecules respectively. First, the mixed lymphocyte culture test (MLC) is based on the recognition of HLA class II incompatibilities present in a donor/recipient pair by donor helper CD4+ T lymphocytes. The proliferation is measured in ^{3}H-thymidine assays (Figure 11) (139). However, now that HLA class II oligotyping has become a routine assay, this cellular technique is used less frequently (140,141).

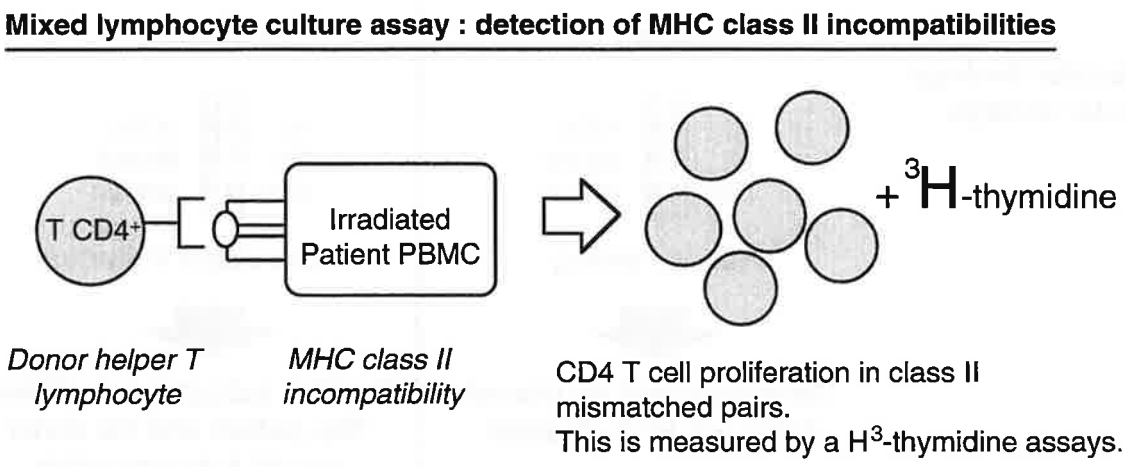


Figure 11: Mixed lymphocyte culture assay: detection of class II mismatches.

In addition, other studies have been focused on the detection of anti-host IL-2 producing cells. Some reports have showed that the detection of high frequency of those IL-2 producing cells may predict the occurrence of severe GvH disease (142-144), however, the use of this cellular assay remains limited to research laboratories.

The second cellular technique is the cytotoxic T lymphocyte precursor frequency test (CTLpf) which allows the detection of MHC class I incompatibilities. This assay is based on limiting dilution systems, on the expansion of cytotoxic T cell precursors and on the detection of their cytolytic activity (145,146). Briefly, irradiated patient cells are incubated during 10 days with limiting numbers of donor T lymphocytes. During this differentiation phase, a precise quantity of IL-2 normally provided in vivo by T helper cells, is added to the culture plates. After 10 days, the cytotoxic activity of the differentiated donor T cells is measured against ^{51}Cr-labeled patient cells in a ^{51}Cr release assay and the frequency of donor cytotoxic T precursor lymphocytes is calculated (Figure 12) (147).

Because oligotyping for HLA class I alleles has been developed only recently and only a few oligoprobes for frequent HLA alleles are available, the cytotoxic T lymphocytes precursor test (CTLpf) provides for the moment an alternative and efficient typing technique for the detection of HLA subtype incompatibilities (148). This cytotoxic technique described by Kaminski et al (147), has been adapted in our lab and used extensively for this thesis. A complete protocol is provided in Appendix 1.

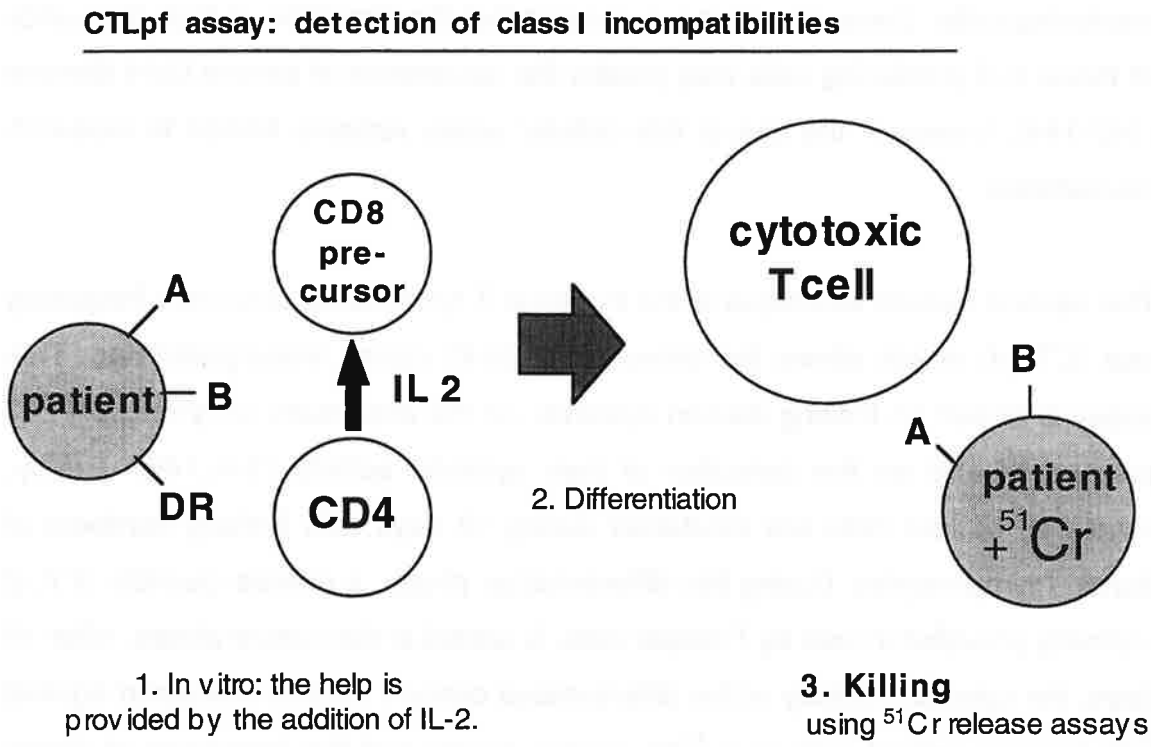


Figure 12: CTLpf test detects the HLA class I incompatibilities present in donor/recipient pairs.

3. The molecular basis of allorecognition

3.1 Introduction

As described in the first part of this introduction, T lymphocytes mediate specific immune responses against foreign pathogens after being triggered through the trimolecular complex: MHC-peptide-TcR. Because, peripheral T lymphocytes have been educated in the thymus to recognize self-MHC molecules plus antigen, such an interaction is called MHC-restricted (38). However, a second kind of MHC / T cell interaction has been described twenty-five years ago which has puzzled many immunologists since then. The first observations defining this phenomenon were detected studying the transplantation of incompatible tissues and rejections (2). This T cell immune response to foreign histocompatibility molecules was defined as *allorecognition or alloreactivity* (Figure 13).

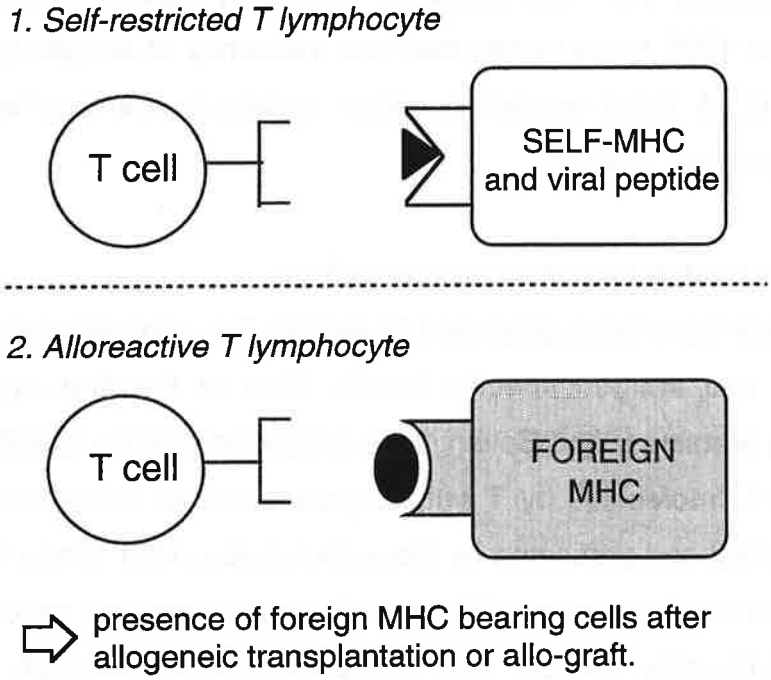


Figure 13: self-MHC restricted and allo-reactive immune recognition.

Since many patients and their unrelated donors are found to be HLA incompatible, allorecognition is an important phenomenon after BMT. Furthermore, as mentioned in the previous part, GvHD after transplantation is mediated by alloreactive donor T lymphocytes co-infused with the bone marrow inoculum. Therefore, a better understanding of the mechanisms triggering allorecognition could help to develop strategies to avoid such post-transplant complications.

3.2 Allorecognition: a paradox in the immune system

Two unusual features characterize alloreactive immune responses. Since alloreactive T lymphocytes recognize allogeneic MHC molecules in a non restricted fashion, a first question arises: how do these T cells recognize foreign antigens in such a non-restricted MHC context ? Secondly, why does such an extraordinary high frequency of T lymphocytes participate in the allogeneic response ? Indeed, the T cell precursor frequency against a MHC alloantigen may be 100 to 1000 times higher than the frequency of antigen-specific T cells (145,149-151). A third crucial question comes forward: what is actually recognized by those T cells ?

3.2.1 Different models to explain allorecognition:

Several models have been proposed to explain the apparent paradox between alloreactive- and antigen specific-T cells. One of the first hypothesis was developed by Matzinger and Bevan. They proposed that the specific recognition of foreign MHC molecules by T lymphocytes could be influenced by the self-structures carried and presented by those molecules (152). Since firstly, a single MHC molecule presents many different cellular peptides and secondly, different T cell receptors may interact with those MHC + X, the high frequency of alloreactivity is a consequence of the diversity of these multiple "binary complexes" recognized.

An alternative hypothesis based on low and high T cell receptor binding affinities has been proposed by Bevan (153). Unlike conventional foreign antigens which are presented by low numbers of MHC molecules, each allo-MHC antigen contains foreign sequences. Thereby, the number of foreign epitopes which is potentially presented to the T cells is far more abundant and the activation of many T lymphocytes with *low* affinity receptors may be possible. In such a model, the peptides are not of central importance, due to the fact that the alloreactive T lymphocytes recognize the allogeneic molecule it-self. Furthermore, because a very high proportion of T cells would share these low affinity binding features, this hypothesis provides a potential explanation for the activation of 1 to 10 % of the T cell repertoire.

Although many studies in mice and in human have tried to understand how and by which mechanism alloreactivity occurs, it is still unclear which of the different models prevails. Furthermore, one should keep in mind that although several studies demonstrate that each of the different described model may exist, some of them may actually not be representative of a true *in vivo* alloresponse due to very specific and particular experimental conditions.

3.2.2 Are alloreactive T cells part of the antigen-specific repertoire ?

The current technology for typing the various T cell receptor Vβ chains has confirmed that a large number of receptors are used in response to alloantigens (154-155). Furthermore, there is strong evidence that alloreactive T cells belong actually to the antigen-specific repertoire. Several murine and human T cell clones were found with specificity for both self-MHC + antigen and allogeneic MHC molecules (156-159). For instance, Burrows et al. showed that cytotoxic T cell clones specific for an Epstein-Barr virus epitope presented by HLA-B8 could also recognize the HLA-B*4402 alloantigen (160). Furthermore, the use of the same T cell receptor Vβ gene segment was observed in both alloreactivity and antigen recognition (157,161). Thereby, allorecognition may be the result of

cross-reactive responses between MHC-restricted specific T cell clones and foreign MHC molecules.

3.2.3 The role of endogenous peptides in MHC allorecognition

At present, it has been accepted that all T cell recognition, including self-restricted and alloreactive MHC responses, involves both the MHC molecule and its associated peptide ligand. The first experiments that suggested that peptides bound by MHC molecules were involved in allorecognition came from MHC class II studies (162,163). The identification of an alloreactive class II T cell clone "specific" for an albumin-epitope by Panina-Bordignon et al. demonstrated for the first time that a self-peptide was central in alloreactive responses (164). Furthermore, other more recent studies have also demonstrated that alloreactive mouse cytotoxic T cell recognize peptides bound to the MHC class I molecules (165-167).

A number of different natural peptides recognized by different alloreactive T cell clones has been isolated. For example, Udaka et al. showed that a MHC class I alloreactive T clone recognized peptides derived from an endogenously self-protein: the mouse 2-oxoglutarate dehydrogenase (168).

The observations that cells taken from different mice tissues were unable to stimulate the alloreactive T cell clones in spite of the fact that they expressed the appropriate allo-MHC molecules, led to the concept that peptide involvement in allorecognition might be partially *tissue-specific*. Indeed, this could be explained by the fact that each cell synthesizes common and tissue-specific peptides. Kappler and Marrack have shown for instance, that T cell hybridomas could recognize murine MHC class II allo-antigens on B cells but not on macrophages or fibroblasts expressing the same antigens (169). As a consequence, these results suggested that class II molecules were necessary but not sufficient to account for allorecognition, and emphasized again the central role of peptides in alloreactivity.

Finally, studies on mutant cell lines such as T2 and RMA-S that are defective in processing and transporting of endogenous peptides clearly demonstrated that

most of the alloreactive T cell clones could not recognize empty MHC molecules (166-167).

Because the other MHC alleles present within the same allogeneic cells are also endogenously derived proteins, some of these proteins could be presented as peptides by allogeneic MHC molecules. Indeed, it has clearly been demonstrated in mice and in human that different MHC class I or class II molecules were recognized not only as intact molecules but also as peptides presented by other MHC molecules (reviewed in 170, 171-175).

Figure 14 summarizes the various structures which can be recognized by alloreactive T cell clones based on the different hypothetical allorecognition models and the subsequent experimental observations. The definition of an alloreactive molecule is in general used to define a foreign MHC it-self or an allogeneic derived peptide bound either to another allogeneic- or to a self-MHC molecule. These allogeneic peptides can be derived a) from MHC class I or class II molecules, b) from tissue-specific proteins, or c) from other intracellular polymorphic proteins called minor antigens (see 3.4 minor histocompatibility antigens).

3.3 Indirect allorecognition

From the studies described above, one can conclude that an alloresponse involves a *direct* interaction between the T cells and the allogeneic MHC molecules (+ an allogeneic or self peptide). Furthermore, it has been demonstrated that such interaction represents most of the alloreactive responses found in vivo. However, several in vitro studies could reveal the existence of another alloreactive pathway called *indirect allorecognition*.

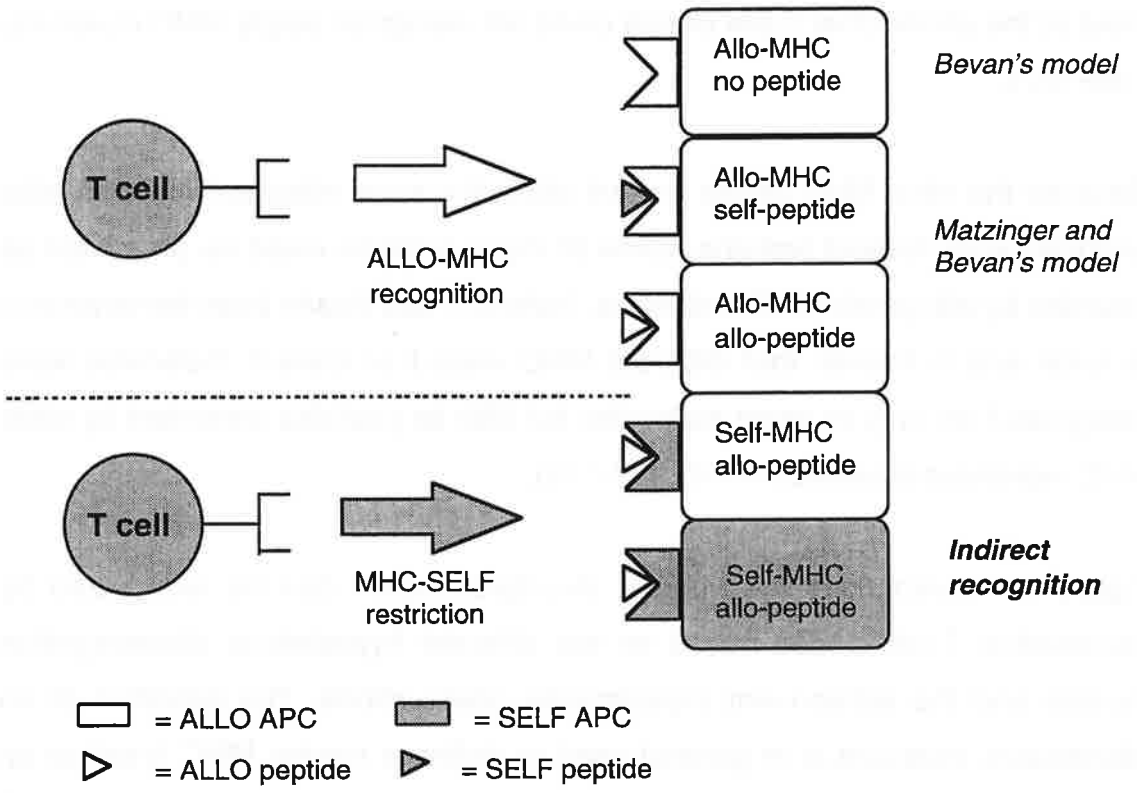


Figure 14: The different models and target structures of allorecognition

This second pathway is based on the observations that foreign MHC or other alloantigens can also be processed and presented in association with self-MHC molecules on *host* antigen presenting cells (APCs) (Figure 14). This pathway is called "indirect allorecognition" since the allogeneic molecule is recognized by T cells in a MHC restricted fashion after being internalized and processed by host APC's (176-178). Furthermore, it has been speculated that such a mechanism might provide an alternative explanation for chronic rejection of kidney allografts (179-182). However, it is still not clear whether such a pathway may be relevant for bone marrow transplantation and represent or not a physiologic event in vivo.

3.4 Minor histocompatibility antigens

In the 1940's, GD Snell discovered two histocompatibility systems, responsible for tumor graft rejection: the first one is the Major Histocompatibility Complex while the second one is called the minor Histocompatibility Complex or mHC (2). The name of *minor* was assigned to these newly discovered antigens because they were characterized by weaker immunological rejection responses as compared to those elicited by the MHC antigens. However, since then, many experiments have showed that these antigens do form transplantation barriers after HLA-identical bone-marrow transplantation and can lead to graft-rejection or GvHD.

More than 40 minor histocompatibility loci were identified by skin or graft-rejections on the Snell's and Bailey's congenic mouse strains (reviewed in 183). In human, minor antigens have been also isolated following in vivo priming after marrow transplantation and identified by T lymphocyte functional assays. Whereas many minor loci could be identified in the mice model, only a very few antigens were isolated in man, and the localization of those genes remains unknown, except for the male-specific H-Y minor antigen. Indeed, the best defined mH antigen is the H-Y minor antigen encoded on the long arm of the Y chromosome (184). Beside this one, other minors called HA-1 to HA-5 were identified by Goulmy et al. in human HLA-identical bone marrow transplants (185).

3.4.1 What is the nature of minor H antigens ?

Based on several results of the last five years, it has become clear that minor antigens are small peptides derived from endogenously synthesized polymorphic proteins which are presented by the MHC molecules to T lymphocytes. The following experimental observations have led to this assumption.

Firstly, responses against minor H antigens are strictly T cell-mediated and MHC class I or class II-*restricted* (183, 186). Secondly, like immune responses against nominal antigens such as viral peptides, the anti-minor H antigen response is also characterized by a low frequency of cytotoxic T cell precursors. Thirdly,

Rammensee et al. have demonstrated that naturally peptides bound to the MHC molecules can be recognized by MHC class I-restricted cytotoxic T lymphocytes (167). These peptides were isolated from MHC class I and class II molecules by acid elution and subsequently separated by reverse phase high-performance liquid chromatography (HPLC) fractionation. By incubating Tap1/2 mutant target cells that express the same MHC-restricted molecule and minor antigen specific T cell clones in the presence of those purified peptides, specific fractions containing minor antigen peptides were identified. Through those experiments, the crucial role of peptides in anti-minor antigen responses became accepted (187-189).

Finally, four minor antigens derived from intracellular proteins have been identified and their amino-acid sequence could be determined. One of them is the polymorphic part of a mitochondrial gene, ND1, while another one is the myxovirus resistance gene, Mx-1 (190,191). The sequence of the male-specific H-Y minor antigen in the human and in the mice have also been identified (192,193). Both H-Y minor H peptides were found to derived from SMCY, a conserved protein encoded on the Y chromosome. Furthermore, the minor histocompatibility HA-2 antigen isolated from bone marrow post-transplanted patients was found to belong to the myosin family (194). Taken together, the actual view on the nature of minor antigens is the one represented in Figure 15.

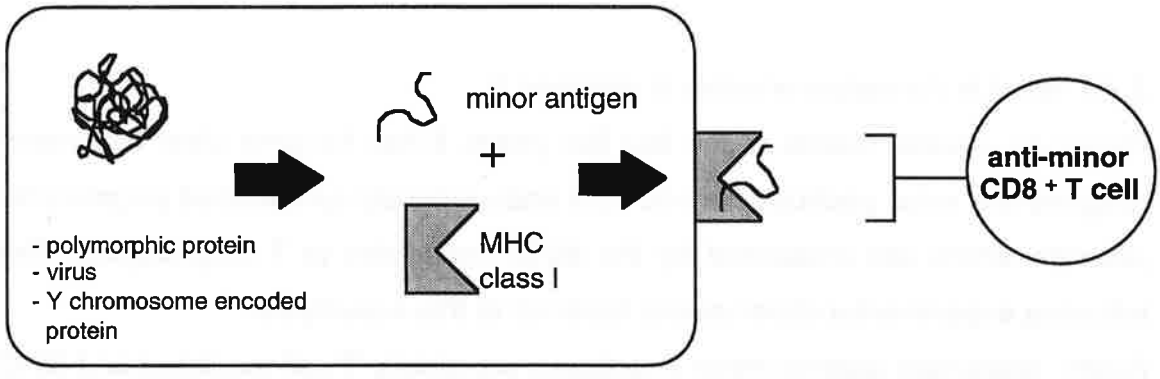


Figure 15: Minor antigen peptides derive from endogenously polymorphic proteins and are presented to T lymphocytes by MHC molecules.

3.4.2 The role of minor antigens in bone-marrow transplantation

Minor antigens have been shown to form significant transplantation barriers, since those polymorphic allogeneic peptides can be presented by self-MHC molecules and induce alloreactive responses (Figure 14). Such an alloreactive response is found when a patient expresses the allele "A" of a polymorphic protein which is not expressed by the donor's tissues. The best understood example is illustrated with the male-specific H-Y minor antigen. Indeed, several studies have shown that a gender-mismatch in HLA-identical donor/recipient pairs correlates with an increased incidence of post-transplant complications (195,196).

Apart from the H-Y antigen, other minor H antigens have been studied for their phenotype frequencies inside the population and their association with the incidence of GvHD. Like the results found in the murine model (197), the frequency of the human mH alleles may vary between low and high. For instance HA-1, HA-2, HA-3, HA-4 and HA-5 have the following phenotype frequencies; 69, 95, 88, 16 and 7 % respectively (185). An intermediate frequency such as 69 % for MiHA-1 has been shown to be associated with higher incidence of GvHD (197'). Indeed, the probability of finding any two individuals sharing the same particular minor antigen is lower when the phenotype frequency of that minor is found in about 40 to 60 % instead of 95 % of the population (Figure 16). Furthermore, by application of fundamental population genetics, PJ. Martin demonstrated that the disparity for minor antigens is greater between HLA-identical unrelated individuals than between HLA-identical siblings (198). Figure 16a shows the probability of disparity among unrelated and related individuals following Hardy-Weinberg equilibrium at a single biallelic locus when alleles "A" and "a" are *codominant* and are both presented by the MHC molecules. The dangerous situation or minor antigen disparity is defined as the presence of an allele in the patient and not in the donor (in the GvHD sense). However, such a model is valid only when the peptides derived from both alleles share the same affinities to the MHC molecules in order to be codominantly expressed.

Figure 16b shows the probability of disparity among related and unrelated individuals when one of the alleles is always expressed whereas the second one is not. Although the probabilities of mH disparities represent here half of the values found by Martin (Figure 16a), the disparity for minor antigens is still greater between unrelated individuals than between two siblings. A more complete description of the Hardy-Weinberg equilibrium and the derivations of the formulas are found in Appendix 2.

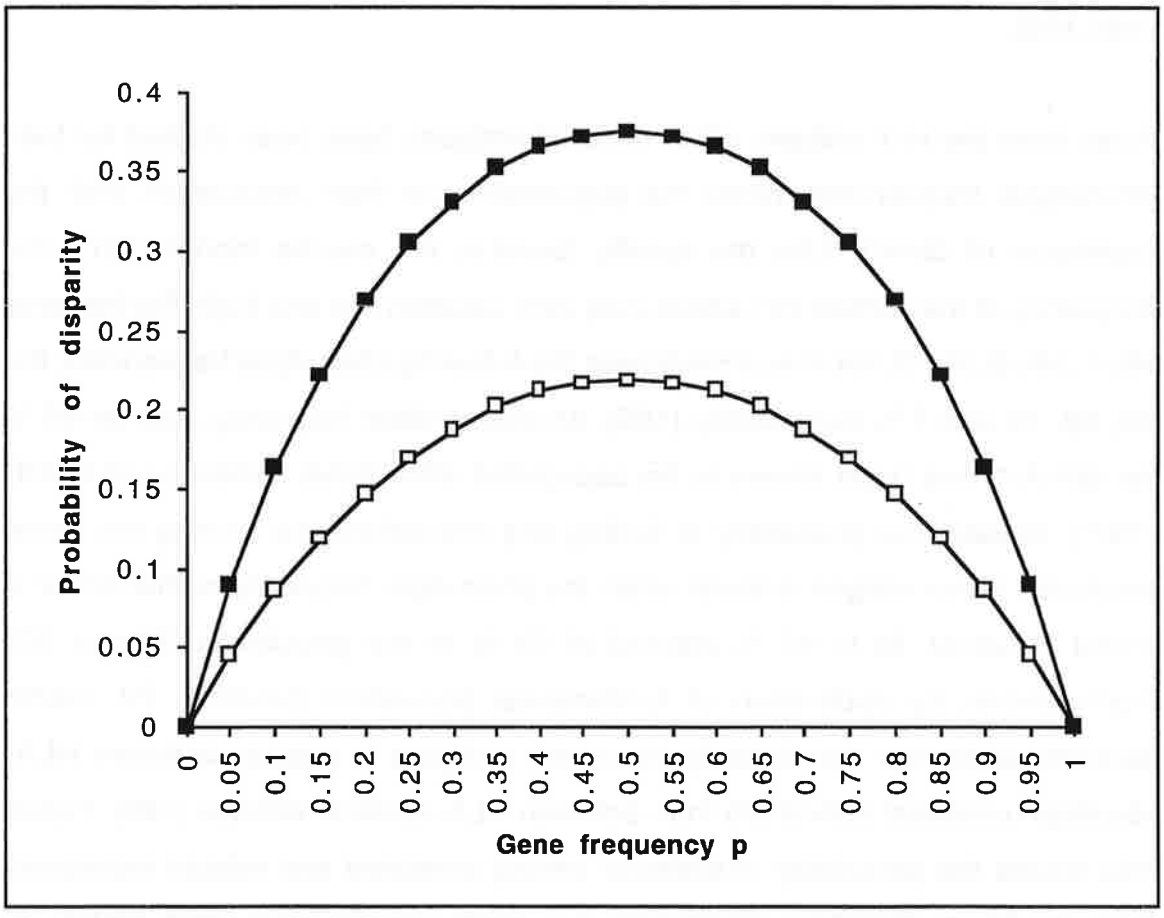


Figure 16a: Probabilities of minor H antigen disparity for a single biallelic codominant locus in any two related (□) and unrelated (■) individuals. (Adapted from PJ. Martin. Bone Marrow Transpl. 1991; 8:217).

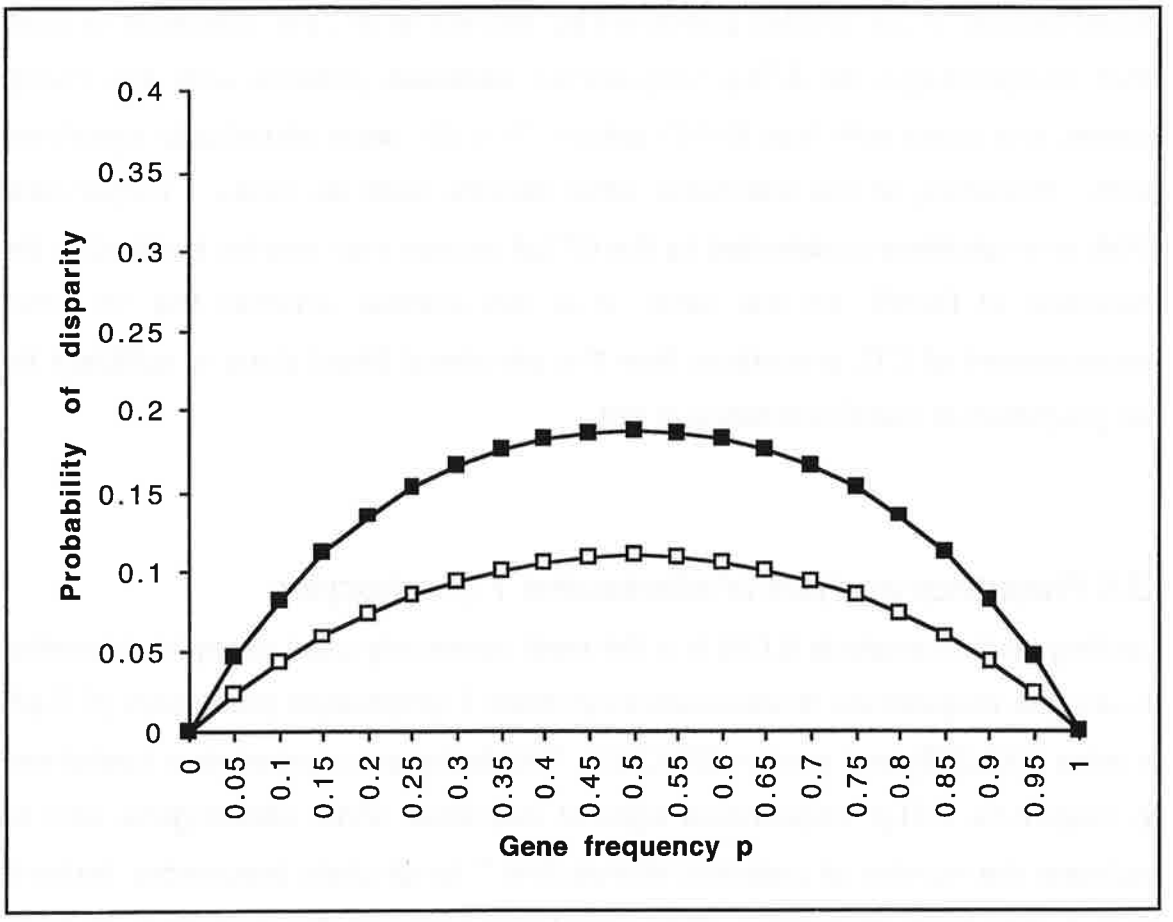


Figure 16b: Probabilities of minor H antigen disparity for a single biallelic locus in which one allele is always expressed whereas the other one never, in any two related (□) and unrelated (■) individuals.

The precise role of minor antigen disparity in the occurrence of GvHD or graft-rejection remains unclear. Firstly, there is quite a bit of evidence that cytotoxic T lymphocytes are able to induce GvHD across minor H barriers (199,200). However, more recent studies in the mice model and in the human demonstrated that the frequency of recipient-reactive CTLp in MHC-identical siblings after bone-marrow transplant is not correlated with the occurrence of GvHD (201,202). De Bueger et al. reported that high frequencies of mH antigen specific CTLp could be detected in the blood of each patient with or without GvHD.

Nevertheless, in the studies published by Irschick et al., the difference in post-BMT recipient-specific CTLp frequencies between patients with low GvHD grades and those with high GvHD grades (II to III), were statistically significant (203). Therefore, at the one hand, other factors, such as CD4+ T helper cells (204) and cytokines undetected by the CTLpf assays may also be involved in the induction of GvHD, on the other, it is still unclear whether the "in vitro" measurement of CTL precursors from the peripheral blood alone is sufficient for the prediction of GvHD incidence or not.

3.5 Frequency analysis of alloreactive T lymphocytes

Limiting dilution analysis (LDA) is a the most commonly used assay to determine in vitro the frequencies of alloreactive cytotoxic T lymphocyte precursors (CTLpf) in mice (145,205) and in man (206,207). This technique represents a useful tool to determine CTLp frequencies against individual MHC alloantigens and to estimate the number of potential alloreactive T lymphocyte precursors. Indeed, four studies have reported a clear correlation between the frequency of host-reactive cytotoxic T lymphocytes and the incidence of severe GvHD after unrelated bone marrow transplantation (117a-120).

Furthermore, limiting dilution assays represent also a way to assess the impact of MHC incompatibilities on the success or the failure of transplantations. For instance, it has been shown that HLA matching in organ transplants is not an absolute necessity and that antigens such as HLA-B are perhaps more important for graft-survival (208) than other mismatches that might be considered as acceptable (209,210). This is definitively not the case in bone-marrow transplantations, since a more complete HLA identity between the bone marrow donor and the patient is required in order to avoid GvHD. However, it is still unclear whether less immunogenic antigens such as class I HLA-Cw- or class II HLA-DP molecules also play a crucial role on the clinical outcome or if they should be considered as permissible incompatible antigens.

Finally, *after* bone-marrow transplantation, limiting-dilution assays provide a useful tool for the detection of minor histocompatibility antigens specific T cells. Indeed, *in vitro* detection of anti-minor specific T lymphocyte responses in human PBMCs requires in general *in vivo* priming. Those particular features can be met following BMT, during pregnancy or after multiple blood transfusions (211). Although, many studies are focused in the elaboration of a more sensitive LDA test for the generation of a primary anti-minor T cell response, it is still impossible to detect and predict the occurrence of such response in a donor/ recipient pair before BMT.

3.6 Immunodominance

Most of the peptides that can bind to a particular MHC molecule have been shown to share structural primary motifs (212,213). The definition of these motifs is quite broad, since several peptides may bind to one MHC molecule. This allows the MHC antigens expressed in a given individual to bind a large number of different peptides necessary to ensure the T cell immune response. However, a phenomenon based on the fact that B or T lymphocytes usually respond only to a few *dominant* peptide epitopes presented by MHC-molecules has been described. Such phenomenon called immunodominance is defined by the fact that, it is the dominance of a given epitope that prevents another one to be recognized. A dominant peptide may compete and prevent the presentation of other peptides either at the level of peptide binding to the MHC molecules or mostly through the interactions with the T cell receptor. Several factors thereby may affect immunodominance and are listed in Table 1. A T cell determinant is formed by an agretope (= the MHC binding site) and an epitope (= TcR binding site). (Adapted from Sercarz et al. Annu Rev Immunol. 1993; 11:729).

Table 1: Factors affecting immunodominance

at the peptide level	at the TcR recognition level
The existence of enzymatic processing sites giving rise to agretopes.	The existence of a T cell determinant: presence of an agretope and epitope.
The peptides transport system: Tap 1/2.	The T repertoire specific for a given determinant.
The competition for binding the same or a different MHC molecule.	The affinity of these T cell receptors for the MHC complex.
The affinity for the binding site of the MHC molecule.	The number of MHC / determinant complexes present at the cell surface.

Many studies using particular mice or in vitro models have demonstrated that immunodominance could take place at the peptide binding level, however, more realistic models predict that such phenomenon should actually be based at the TcR recognition level (reviewed in 214).

Immunodominant responses have also been observed following immunization of mice against multiple minor antigens. Indeed, several reports showed that in mice challenged by multiple minor H antigens, cytotoxic T cells preferentially responded to a few immunodominant minor antigens (215-217). For instance, Wettstein reported that the CTL responses against autosomal minor histocompatibility antigens dominated the CTL responses against the male H-Y antigen (217). However, since only very few minor antigens have been characterized in the human, such immunodominant responses could only be suggested by Goulmy et al. (185).

4. Aim of the study

The main objective of the studies described in this thesis was first, to define the histoincompatibilities detected in unrelated donor/recipient pairs *before* bone marrow transplantation, and secondly, to analyze in those patients their relevance *after* transplantation during severe Graft-versus-Host Disease.

Because HLA genotypically-identical siblings can only be found for less than 1/3 of the patients, alternative bone marrow transplantation from "HLA-identical" unrelated donor has been extensively developed. Unfortunately, transplantation with bone marrow from phenotypically matched donors is associated with an increased incidence of Graft-versus-Host Disease. Such donors are less compatible than HLA-identical siblings also when they seem to be HLA-matched. This increased incompatibility is mainly caused by flaws in classical serotyping techniques that do not discriminate the different HLA-variants that occur in unrelated individuals. Furthermore, most of the laboratories are focused on HLA-A, -B and -DR matching only, other antigens such as HLA-C, -DQ and -DP might still be incompatible.

The first part of this thesis was aimed to assess the degree of HLA mismatches in 131 unrelated donor/recipient combinations who were "ABDR-matched" by classical serotyping techniques. This study was performed by using the following high resolution typing techniques: isoelectrofocusing, oligotyping, primary cytotoxic T lymphocytes assays (CTLpf) and specific T cell clones ***(Chapter 2).*** Furthermore, two frequent HLA class I antigens (B44 and B35) have been studied in more details to characterize in several "ABDR-identical" D/R pairs, the occurrence of HLA-variant incompatibilities ***(Chapter 3).***

One of the main advantage of the CTLpf assay compared to oligotyping is his ability for the detection of new or rare HLA variants. Thereby, we described here the identification by specific cytotoxic T cell clones of a new HLA-B41 subtype and its complete nucleotide sequence ***(Chapter 4).***
Finally, to assess the importance of the HLA mismatches detected in those unrelated "ABDR-identical " pairs on the clinical outcome, 44 transplanted patients were analyzed for their survival after transplantation ***(Chapter 5).***

The second part of this thesis is focused on the identification of the histoincompatibilities found after bone marrow transplantation during severe Graft-versus-Host Disease. Two crucial factors are mainly involved in the occurrence of such post-transplant complications; HLA incompatibilities and minor histocompatibility antigens mismatches. Therefore, to study the relative importance of incompatible HLA-serotypes, HLA-subtypes, HLA-Cw and of minor antigens in the onset of GvHD, five patients with various major or no detectable incompatibilities and with GvHD ≥ 2 have been analyzed ***(Chapter 6).***
The following chapter describes in more detail some aspects and characteristics of the various alloreactive responses detected after transplantation in those five patients. Furthermore, the seven identified minor histocompatibility antigen patterns are here precisely analyzed and described ***(Chapter 7).***

Finally, ***Chapter 8*** gives a general discussion on the various histo-incompatibilities detected before and after bone marrow transplantation and on their potential implication in post-transplant complications such as GvHD.

5. References

1. Gorer PA. The antigenic basis of tumour transplantation. J Path Bac. 1938; 47:231
2. Snell GD. Methods for the study of histocompatibility genes. J Genet. 1948; 49:87
3. Dausset J. Leuco-agglutinins IV Leucoagglutinins and blood transfusions. VoxSang. 1954; 4:190.
4. Van Rood JJ, Eernisse JG, Van Leeuwen A. Leukocyte antibodies in sera from pregnant women. Nature. 1958; 181:1735.
5. Breuning MH, Van De Berg-Loonen P, Bernini LF, et al. Localization of HLA on the short arm of chromosome 6. Hum Genet. 1977; 37:131.
6. Hardy DA, Bell JI,Long EO, Lindsten T, McDewitt HO. Mapping of the class II region of the human histocompatibility complex by pulsed-field gel electrophoresis. Nature. 1986; 323:453.
7. Bodmer JG, Marsh SGE, Albert ED, Bodmer WF, et al. Nomenclature for factors of the HLA system, 1995. Tissue antigens. 1995; 46:1.
8. Cresswell P, Turner MJ, Strominger JL. Papain-solubilized HL-A antigens from cultured human lymphocytes contain two peptide fragments. Pro. Natl. Acad. Sci. USA. 1973; 70:1603.
9. Lopez de Castro JA, Barbosa JA, Krangel JA, Biro PA, Strominger JL. Structural analysis of the functional sites of class I HLA antigens. Imm Rev. 1985; 85:149.
10. Goodfellow PN, Jones EA, Van Heyningen V, Solomon E, Bobrow M. The β2-microglobulin gene is on chromosome 15 and not in the HL-A region. Nature. 1975; 254:267.
11. Springer TA, Strominger JL. Detergent-soluble HLA antigens contain a hydrophobic region at the COOH-terminus and a penultimate hydrophobic region. Pro Natl Acad Sci USA. 1976; 73:2481.
12. Orr HT, Lopez de Castro JA, Parham P, Ploegh HL, Strominger JL. Comparison of amino acid sequences of two human histocompatibility antigens, HLA-A2 and HLA-B7: Location of putative alloantigenic sites. Pro Natl Acad Sci USA. 1979; 76:4395.
13. Parham P, Lomen CE, Lawlor DA, Ways JP, Holmes N, et al. Nature of polymorphism in HLA-A, -B, and -C molecules. Pro Natl Acad Sci USA. 1988; 85:4005.
14. Shackelford DA, Strominger JL. Demonstration of structural polymorphism among HLA-DR light chains by two-dimensional gel electroforesis. J Exp Med. 1980; 151:144.
15. Charron DJ, McDevitt HO. Characterization of HLA-D-region antigens by two-dimensional gel-electrophoresis. J Exp Med. 1980; 152: 18 s.

16. Kaufman JF, Andersen RL, Strominger JL. HLA-DR antigens have polymorphic light chains and invariant heavy chains as assessed by lysine-containing tryptic peptide analysis. J Exp Med. 1980; 152: 37 s.
17. Shackelford DA, Kaufman JF, Korman AJ, Strominger JL. HLA-DR antigens : structure, separation of subpopulations , gene cloning and function. Imm Rev. 1982; 66:133.
18. Bjorkmann PJ, Saper MA, Samraouri B, Bennet WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. Nature. 1987; 329:506.
19. Bjorkmann PJ, Saper MA, Samraouri B, Bennet WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature. 1987; 329:512.
20. Garrett TPJ, Saper MA, Bjorkmann PJ, Strominger JL, Wiley DC. Specificity pockets for the side chains of peptide antigens in HLA-Aw68. Nature. 1989; 342:692.
21. Saper MA, Bjorkmann PJ, Wiley DC. Refined structure of the human histocompatibility antigen HLA-A2 at 2.6 A resolution. J Mol Biol. 1991; 219:277.
22. Fremont DH, Matsumura M, Stura EA, Peterson PA, Wilson IA. Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. Science. 1992; 257:919.
23. Zhang W, Young ACM, Imarai M, Nathenson SG, Sacchettini JC. Crystal structure of the major histocompatibility complex class I H-2Kb molecule containing a single viral peptide: implications for peptide binding and T cell receptor recognition. Proc Natl Acad Sci USA. 1992; 89:8403.
24. Madden DR, Gorga JC, Strominger JL,Wiley DC. The three-dimensional structure of HLA-B27 at 2.1 A resolution suggest a general mechanism for tight peptide binding to MHC. Cell. 1992; 70:1035.
25. Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC. The three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature. 1993; 364:33.
26. Engelhard VH. Structure of peptides associated with class I and class II MHC molecules. Annu Rev Immunol. 1994; 12:181.
27. Falk K, Rötzschke O, Stevanovic S, Jung G, Rammensee H-G. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature. 1991; 351:290.
28. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. Science. 1992; 255:1261.
29. Rudensky AY, Preston-Hurlburt P, Hong S-C, Barlow A, Janeway CA. Sequence analysis of peptides bound to MHC class II molecules. Nature. 1991; 353:622.
30. Huezko EL, Bodnar WM, Benjamin D, Sakaguchi K, Zhu NZ, Shabanowitz J, et al. Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. J Immunol. 1993; 151:2572.

31. Engelhard VH, Appella E, Benjamin DC, Bodnar WM, Cox AL, Hunt DF, et al. Mass spectrometric analysis of peptides associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and identification of structural features that determine binding. Chem Immunol. 1993; 57:39.

32. Demotz S, Grey HM, Sette A. The minimal number of class II MHC-antigen complexes needed for T cell activation. Science. 1990; 249:1028.

33. Rötzschke O, Falk K, Stevanovic S, Jung G, Walden P, Rammensee HG. Exact prediction of a natural T cell epitope. Eur J Immunol. 1991; 21(11):2891.

34. Falk K, Rötzschke O, Deres K, Metzger J, Jung G, Rammensee HG. Identification of naturally processed viral nonapeptides allows their quantification in infected cells and suggests an allele-specific T cell epitope forecast. J Exp Med. 1991; 174:425.

35. Christnick ER, Luscher MA, Barber BH, Williams DB. Peptide binding to class I MHC on living cells and quantification of complexes required for CTL lysis. Nature. 1991; 352:67.

36. Wallny HJ, Deres K, Faath S, Jung G, Van Pel A, Boon T, Rammensee HG. Identification and quantification of a naturally presented peptide as recognized by cytotoxic T lymphocytes specific for an immunogenic tumor variant. Int Immunol. 1992; 4:1085.

37. Rosenthal AS, Shevach EM. Function of macrophages in antigen recognition by guinea pig T lymphocytes. I. Requirement for histocompatible macrophages and lymphocytes. J Exp Med. 1973; 138:1194.

38. Zinkernagel RM, Doherty PC. Restriction of in vitro T cell mediated cytotoxicity in lymphocytic chorionmeningitis within a syngeneic or allogeneic system. Nature. 1974; 248:701.

39. Dembic Z, Haas W, Weiss S, Mc-Cubrey J, et al. Transfer of specificity by murine alpha and beta T cell receptor genes. Nature. 1986; 320:232.

40. Parnes JR. Molecular biology and function of CD4 and CD8. Adv Immunol. 1989; 44:265.

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

42. Teh HS, Kisielow P, Scott B, Kishi H, Uematsu Y, et al. Thymic major histocompatibility complex antigens and the alpha beta T cell receptor determine the CD4/CD8 phenotype of T cells. Nature. 1988; 335:229.

43. Unanue ER, Allen PM. The basis for the immunoregulatory role of macrophages and other accessory cells. Science. 1987; 236:551.

44. Germain RN. The ins and the outs of antigen processing and presentation. Nature. 1986; 322:687.

45. Bevan MJ. Antigen recognition. Class discrimination in the world of immunology (news). Nature. 1987; 325:192.

46. Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A. Sequences encoded in the class II region of the MHC related to the 'ABC' superfamily of transporters. Nature. 1990; 348:741.

47. Spies T, Bresnahan M, Bahram S, et al. A gene in the human major histocompatibility complex class II region controlling the class I antigen presentation pathway. Nature. 1990; 348:744.

48. Braciale TJ, Braciale VL. Antigen presentation: structural themes and functional variations. Immunol Today. 1991; 12(4):124.

49. Elliott WL, Stille CJ, Thomas LJ, Humphreys RE. An hypothesis on the binding of an amphipathic, helical sequence in Ii to the desetope of class II antigens. J Immunol. 1987; 138:2949.

50. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature. 1990; 345:615.

51. Benham A, Tulp A, Neefjes JJ. Synthesis and assembly of MHC-peptide complexes. Immunol Today. 1995; 16:359.

52. Ljunggren H-G, Stam NJ, Öhlén C, et al. Empty MHC class I molecules come out in the cold. Nature. 1990; 346:476.

53. Rock KL, Gamble S, Rothstein L. Presentation of exogenous antigen with class I major histocompatibility complex molecules. Science. 1990; 249:918.

54. Moore MW, Carbone FR, Bevan MJ. Introduction of soluble protein into the class I pathway of antigen processing and presentation. Cell. 1988; 54:777.

55. Neefjes JJ, Stollorz V, Peters PJ, Geuze HJ, Ploegh HL. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. Cell. 1990; 61:171.

56. Neefjes JJ, Monburg F. Cell biology of antigen presentation. Curr Opin Immunol. 1993; 5:27.

57. Nuchtern JG, Biddison WE, Klausner RD. Class II MHC molecules can use the endogenously pathway of antigen presentation. Nature. 1990; 343: 74.

58. Meuer SC, Fiztgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved on antige-specific human T cell function. J Exp Med. 1983; 157:705.

59. Oettgen HC, Terhost C, Cantley LC, Rosoff PM. Stimulation of the T3-T cell receptor complex induces a membrane-potential-sensitive calcium influx. Cell. 1985; 40:583.

60. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, Stobo J. The role of the T3/antigen receptor complex in T-cell activation. Annu Rev Immunol 1986; 4:593.

61. Davis MM, Bjorkmann PJ. T-cell antigen receptor genes and T-cell recognition. Nature. 1988; 334:395.

62. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature. 1984; 308:145.

63. Arden B, Klotz J, Siu G, Hood L. Diversity and structure of genes of the alpha family of mouse T-cell antigen receptor. Nature. 1985; 316:783.

64. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol. 1986; 4:529.

65. Fowlkes BJ, Pardoll DM. Molecular and cellular events of T cell development. Adv Immunol. 1989; 44:207.

66. Von Boehmer H. The developmental biology of T lymphocytes. Annu Rev Immunol. 1988; 6:309.

67. Zinkernagel RM, Callahan GN, Althage A, Cooper S, Klein PA, Klein J. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition ? J Exp Med. 1978; 147:882.

68. Bevan MJ, Fink PJ. The influence of thymus H-2 antigens on the specificity of maturing killer and helper cells. Immunol Rev. 1978; 42:3.

69. Lo D, Sprent J. Identity of cells that imprint H-2 restricted T cell specificity in the thymus. Nature. 1986; 319:672.

70. Scott B, Blüthmann H, The HS, Von Boehmer H. The generation of mature T cells requires interaction of the αβ T-cell receptor with major histocompatibility antigens. Nature. 1989; 338:591.

71. Kisielow P, The HS, Blüthmann H, Von Boehmer H. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature. 1988; 335:730.

72. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. Cell. 1987; 49:273.

73. Pullen AM, Marrack P, Kappler JW. The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens. Nature. 1988; 335:796.

74. Sprent J, Webb SR. Function and specificity of T cells subsets in the mouse. Adv Immunol. 1987; 41:39.

75. Blackmann M, Kappler J, Marrack P. The role of the T cell receptor in positive and negative selection of developing T cells. Science. 1990; 248:1335.

76. Von Boehmer H, Kisielow P. Self-Nonself Discrimination by T cells. Science. 1990; 248:1369.

77. Von Boehmer H. Developmental biology of T cells in T cell-receptor transgenic mice. Annu Rev Immunol. 1990; 8:531.

78. Doyle C, Strominger JL. Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature. 1987; 330:256.

79. Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. Cell-cell adhesion mediated by CD8 and MHC class I molecules. Nature. 1988; 336:79.

80. Teh H-S, Garvin AM, Forbush KA, Carlow DA, Davis CB, Littman DR, Perlmutter RM. Participation of CD4 coreceptor molecules in T-cell repertoire selection. Nature. 1991; 349:241.

81. Ingold AL, Landel C, Knall C, Evans GA, Potter TA. Co-engagement of CD8 with the T cell receptor is required for the negative selection. Nature. 1991; 352:721.

82. Nikolic-Zugic J. Phenotypic and functional stages in the intrathymic development of αβ T cells. Immunol Today. 1991; 12(2):65.

83. Murphy DB, et al. A novel MHC class II epitope expressed in thymic medulla but not cortex . Nature. 1989; 338:765.

84. Marrack P, Lo D, Brinster R, et al. The effect of thymus environment on T cell development and tolerance. Cell. 1988; 53:627.

85 . Finkel TH, et al. The thymus has two functionally distinct populations of immature alpha beta + T cells: one population is deleted by ligation of alpha beta TCR. Cell. 1989; 58:1047.

86. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion vs functional clonal inactivation. Annu Rev Immunol. 1989; 7: 445.

87. Jenkins MK, Schwartz RH. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. J Exp Med. 1987; 165:302.

88. Lamb JR, Skidmore BJ, Green N, Chiller JM, Feldmann M. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. J Exp Med. 1983; 157:1434.

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

90. Jones LA, Chin T, Longo DL, Kruisbeek AM. Peripheral clonal elimination of functional T cells. Science. 1990; 250:1726.

91. Rocha B, Von Boehmer H. Peripheral selection of the T cell repertoire. Science. 1991; 251:1225.

92. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells : clonal elimination as a consequence of immunity. Cell. 1990; 63:1249.

93. Bretscher P, Cohn M. A theory of self-nonself discrimination. Science. 1970; 169:1042.

94. Bretscher P. The two-signal model of lymphocyte activation twenty-one years later. Immunol Today. 1992; 13:74.

95. June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. Immunol Today. 1994; 15(7):321.

96. Liu Y, Linsley PS. Costimulation of T cell growth. Curr Opin Immunol. 1992; 4:265.

97. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science. 1993; 259:368.

98. Chen L, Ashe S, Brady WA, et al. Costimulation of antitumor immunity by the B7 counter receptor for the T lymphocyte molecules CD28 and CTLA-4. Cell. 1992; 71:1093.

99. Shahinian A, Pfeffer K, Lee KP, Kundig TM, et al. Differential T cell costimulatory requirements in CD28-deficient mice. Science. 1990; 261:609.

100. Sperling AI, Green JM, Mosley RL, Smith PL, DiPaolo RJ, Klein JR, Bluestone JA, Thompson CB. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. J Exp Med. 1995; 182:139.

101. Linsley PS. Distinct roles for CD28 and cytotoxic T lymphocyte-associated molecule-4 receptors during T cell activation ?. J Exp Med. 1995; 182:289.

102. Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst. 1951; 12:197.

103. Congdon CC, Lorenz E. Humoral factor in irradiation protection: Modification of lethal irradiation injury in mice by injection of rat bone marrow. Am J Physiol. 1954; 176:298.

104. Bortin MM, Rimm AA. Increasing utilization of bone marrow transplantation. Transplantation. 1986; 42:229.

105. Martin PJ, Hansen JA, Storb R, Thomas ED. Human marrow transplantation: an immunological perspective. Adv Immunol. 1987; 40:379.

106. Blume KG, Petz LD. Clinical bone marrow transplantation. Churchill Livingstone Inc. 1983; 1:1.

107. Armitage JO. Bone marrow transplantation. N Engl J Med. 1994; 330:827.

108. Beatty PG, Anasetti C, Hansen JA, Longton GM, et al. Marrow transplantation from unrelated donors for treatment of hematologic malignancies : effect of mismatching of one HLA locus. Blood. 1993; 81:249.

109. Beatty PG, Clift RA, Mickelson EM, Nisperos BB, et al. Marrow transplantation from related donors other than HLA-identical siblings. N Eng J Med. 1985; 313:765.

110. Hansen JA, Clift RA, Thomas ED, Buckner CD, Storb R, Giblett ER. Transplantation of marrow from an unrelated donor to a patient with acute leukemia. N Engl J Med. 1980; 303:565.

111. Mc Glave PB, Beatty PG, Ash R, Hows JM. Therapy of chronic myelogenous leukemia with unrelated donor bone marrow transplantation: results in 102 cases. Blood. 1990; 75:1728.

112. Mc Cullough J, Hansen JA, Perkins H, Stroncek D, Bartsch G. Establishment of national bone marrow donor registry. In Gale RP, Champlin RE (eds): Bone marrow transplantation: current controversies. New York. NY. Liss. 1989; p641.

113. Beatty PG, Dahlberg S, Mickelson EM, et al. Probability of finding HLA-matched unrelated marrow donors. Transplantation. 1988; 45:714.

114. Kernan NA, Bartsch G, Ash RC, Beatty PG, et al. Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med. 1993; 328:593.

115. Anasetti C, Amos D, Beatty PG, Appelbaum FR, et al. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. N Engl J Med. 1989; 320:197.

116. Hows JM, Yin JL, Marsh J, et al. Histocompatible unrelated volunteer donors compared with HLA nonidentical family donors in marrow transplantation for aplastic anemia and leukemia. Blood. 1986; 68:1322.

117. Anasetti C, Beatty PG, Storb R, et al. Effect of HLA incompatibility on Graft-versus-Host Disease, relapse and survival after marrow transplantation for patients with leukemia or lymphoma. Human Immunology. 1990; 29:79-81.

117a. Kaminski E, Hows J, Man S, Brookes P, et al. Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor marrow transplantation. Transplantation. 1989; 48:608.

118. Roosnek E, Hogendijk S, Zawadynski S, Speiser D, Tiercy J-M, et al. The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. Transplantation. 1993; 56:691.

119. Spencer A, Brookes PA, Kaminski E, Hows JM, et al. Cytotoxic T lymphocyte precursor frequency analyses in bone marrow transplantation with volunteer unrelated donors: Value in donor selection. Transplantation. 1995; 59:1302.

120. Petersdorf EW, Longton GM, Anasetti C, Martin PJ, et al. The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. Blood. 1995; 86:1606.

121. Meyers JD, Reed EC, Shepp DH, et al. Acyclovir for prevention of cytomegalovirus infection and disease after allogeneic marrow transplantation. N Engl J Med. 1988; 318:70.

122. Goodrich JM, Mori M, Gleaves CA, et al. Early treatment with ganciclovir to prevent cytomegalovirus disease after allogeneic bone marrow transplantation. N Engl J Med. 1991; 325:1601.

123. Van Bekkum DW, De Vries MJ. Radiations chimaeras. London: Logos Press. 1967.

124. Thomas ED, Storb R, Clift RA, et al. Bone marrow transplantation. N Engl J Med. 1975; 292: 832-43, 895-902.

125. Korngold R, Sprent J. T cell subsets in graft-versus-host disease. In: Burakoff SJ, Deeg HJ, Ferrara J, Atkinson K, eds. Graft-versus-Host Disease: immunology, pathophysiology, and treatment. New York: Marcel Dekker, 1990: 30.

126. Ferrara JL, Guillen FJ, van Dijken PJ, et al. Evidence that large granular lymphocytes of donor origin mediate acute graft-versus-host disease. Transplantation. 1989; 47:50.

127. Vogelsang GB, Hess AD. Graft-versus-Host Disease : new directions for a persistent problem. Blood. 1994; 84:2061.

128. Storb R, Deeg HJ, Pepe M, et al. Methotrexate and cyclosporine versus cyclosporine alone for prophylaxis of graft-versus-host disease in patients given HLA-identical marrow grafts for leukemia: long-term follow-up of a controlled trial. Blood. 1989; 73:1729.

129. Mitsuyasu RT, Champlin RE, Gale RP, et al. Treatment of donor bone marrow with monoclonal anti-T-cell antibody and complement for the prevention of graft-versus-host disease: a prospective, randomized, double-blind trial. Ann Intern Med. 1986; 105:20.

130. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. Blood. 1990; 75:555.

131. Weiden PL, Flournoy N, Thomas ED, et al. Antileukemic effect of graft-versus-host disease in human recipients of allogeneic-marrow grafts. N Engl J Med. 1979; 300:1068.

132. Falkenburg JHF, Goselink HM, van der Harst D, et al. Growth inhibition of clonogenic leukemic precursor cells by minor histocompatibility antigen-specific cytotoxic T lymphocytes. J Exp Med. 1991; 174:27.

133. Faber LM, van Luxemburg-Heijs SAP, Willemze R, et al. Generation of leukemia-reactive cytotoxic T lymphocyte clones from the HLA-identical bone marrow donor of a patient with leukemia. J Exp Med. 1992; 76:1283.

134. Van Lochen E, De Gast B, Goulmy E. In vitro separation of host specific graft-versus-host and graft-versus-leukemia cytotoxic T cell activities. Bone Marrow Transplant. 1992; 10:181.

135. Van der Harst D, Goulmy E, Falkenburg JHF, et al. Recognition of minor histocompatibility antigens on lymphocytic and myeloid leukemic cells by cytotoxic T cell clones. Blood. 1994; 83:1060.

136. Terasaki P, McClelland J. Microdroplet assay of human serum cytotoxins. Nature. 1964; 204:998.

137. Tiercy J-M, Morel C, Freidel AC, Zwahlen F, Gebuhrer L, et al. Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. Proc Natl Acad Sci USA. 1991; 88:7121.

138. Tiercy J-M, Grundshober C, Jeannet M, Mach B. A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific olignucleotide probes. In: Handbook of HLA typing techniques, Hui KMB, ed Boca Raton, CRC Press Inc. 1993; 117.

139. Grosse-Wilde M, Doxiadis I, Brant H. Definition of HLA-D with HTC. In E.D.Albert, M.P Braur et R.W Moyr (eds). Histocompatibility Testing. 1984, p249 Springer-Verlag Berlin, Heidelberg.

140. Baxter-Lowe LA, Eckels DD, Ash R, Casper J, Hunter JB, Gorski J. The predictive value of HLA-DR oligotyping for MLC responses. Transplantation. 1992; 53:1352.

141. Termijtelen A, Ehrlich HA, Braun LA, Verduyn W, Drabbels JJM, et al. Oligonucleotide typing is a perfect tool to identify antigens stimulatory in the mixed lymphocyte culture. Hum Immunol. 1991; 31:241.

142. Theobald M, Nierle T, Bunjes D, Arnold R, Heimpel H. Host-specific interleukin-2 secreting donor T-cell precursors as predictors of acute graft-versus-host disease in bone marrow transplantation between HLA-identical siblings. N Engl J Med. 1992; 327:1613.

143. Schwarer A, Jiang Y, Brookes P, et al. Frequency of antirecipient alloreactive helper T-cell precursors in donor blood in graft-vs-host disease after HLA-identical siblings bone marrow transplantation. Lancet. 1993; I:203.

144. Nierle T, Bunjes D, Arnold R, Heimpell H, Theobald M. Quantification assessment of post transplant host-specific interleukin-2 secreting T-helper cell precursors in patients with and without graft-versus-host after allogeneic HLA-identical sibling bone marrow transplants. Blood. 1993; 81:841.

145. Fisher-Lindahl K, Wilson DB. Histocompatibility antigen-activated cytotoxic T lymphocytes. II Estimates of the frequency and specificity of precursors. J Exp Med. 1977; 145:508.

146. Kabelitz D, Herzog WR, Zanker B, Wagner H. Human cytotoxic T lymphocytes: I. Limiting-dilution analysis of alloreactive cytotoxic T-lymphocyte precursor frequencies. Scand J Immunol. 1985; 22:329-35.

147. Kaminski E, Sharrock C, Hows J, Ritter M, Arthur C, McKinnon S, Batchelor JR. Frequency analysis of cytotoxic T precursors-possible relevance to HLA-matched unrelated donor bone marrow transplantation. Bone Marrow Transplant. 1988; 149-165.

148. Rufer N, Tiercy J-M, Speiser D, Helg C, et al. Characterization of pretransplant CTL activity in "perfectly matched" unrelated bone marrow donor/recipient combinations. Bone Marrow Transplant. 1993; 11 suppl.1:20-23.

149. Wilson DB, Blyth JL, Nowell PC. Quantitative studies on the mixed lymphocyte interaction in rats. J Exp Med. 1968; 128:1157.

150. Ford WL, Atkins RC. The proportion of lymphocytes capable of recognizing strong transplantation antigens in vivo. Adv Exp Biol Med. 1973; 29:255.

151. Widmer MB, MacDonald HR. Cytolytic T lymphocyte precursors reactive against mutant K^b alloantigens are as frequent as those reactive against a whole foreign haplotype. J Immunol. 1980; 124: 48.

152. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens. Cell Immunol. 1977; 29:1.

153. Bevan MJ. High determinant density may explain the phenomenon of alloreactivity. Immunol Today. 1984; 5(5):128.

154. Garman R, Ko JL, Vulpe C, Raulet D. T-cell receptor variable gene usage in T cell population. Proc Natl Acad Sci USA. 1986; 83:3987.

155. Bill J, Yague J, Appel VB, White J, et al. Molecular genetic analysis of 278 I-A^{bm12}-reactive T cells. J Exp Med. 1989; 169:115.

156. Portoles P, Rojo JM, Janeway CA. Asymmetry in the recognition of antigen : self class II MHC and non-self class II MHC molecules by the same T-cell receptor. J Mol Cell Immunol. 1989; 4:129.

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

158. Sredni B, Schwatz RH. All reactivity of an antigen specific T cell clone. Nature. 1980; 287:855.

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

160. Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein-Barr virus CTL epitope: implications for Graft-versus-Host Disease. J Exp Med. 1994; 179:1155.

161. Rupp F, Acha-Orbea H, Hengartner H, et al. Identical Vb T-cell receptor genes used in alloreactive cytotoxic and antigen plus I-A specific helper T cells. Nature. 1985; 315:425.

162. Eckels DD, Gorski J, Rothbard J, Lamb JR. Peptide-mediated modulation of T cell allorecognition. Proc Natl Acad Sci USA. 1988; 85:8191.

163. Demotz S, Sette A, Sakaguchi K, Buchner R, et al. Self peptide requirement for class II major histocompatibility complex allorecognition. Proc Natl Acad Sci USA. 1991; 88:8730.

164. Panina-Bordignon P, Corradin G, Roosnek E, Sette A, Lanzavecchia A. Recognition by class II alloreactive T cells of processed determinants from human serum proteins. Science. 1991; 252:1548.

165. Heath WR, Kane KP, Mescher MF, Sherman LA. Peptide-dependent recognition of H-$2K^b$ by alloreactive cytotoxic T lymphocytes. Nature. 1989; 341:749.

166. Heath WR, Kane KP, Mescher MF, Sherman LA. Alloreactive T cells discriminate among a diverse set of endogenous peptides. Proc Natl Acad Sci USA. 1991; 88:5101.

167. Rötzschke O, Falk K, Faath S, Rammensee HG. On the nature of peptides involved in T cell alloreactivity. J Exp Med. 1991; 174:1059.

168. Udaka K, Tsomides TJ, Walden P, Fukusen N, Eisen HN. A ubiquitous protein is the source of naturally occurring peptides that are recognized by a $CD8^+$ T-cell clone. Proc Natl Acad Sci USA. 1993; 90:11272.

169. Marrack P, Kappler J. T cells can distinguish between allogeneic major histocompatibility complex products on different cell types. Nature. 1988; 332:840.

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

171. Maryanski JL, Pala P, Corradin G, Jordan BR, Cerottini JC. H-2 restricted cytolytic T cells specific for HLA can recognize a synthetic HLA peptide. Nature. 1986; 324:578.

172. De Koster HS, Anderson DE, Termitjelen A. T cells sensitized to synthetic HLA-DR3 peptide give evidence of continuous presentation of denatured HLA-DR3 molecules by HLA-DP. J Exp Med. 1989; 169:1191.

173. Chen BP, Madrigal JA, Parham P. Cytotoxic T cell recognition of an endogenous class I HLA peptide by a class II HLA molecule. J Exp Med. 1990; 172:779.

174. Liu Z, Sun YK, Xi YP, Harris P, Suciu-Foca N. T cell recognition of self-human histocompatibility leukocyte antigens (HLA)-DR peptides in context of syngeneic HLA-DR molecules. J Exp Med. 1992; 175:1663.

175. Harris PE, Zhuoru L, Sucio-Foca N. MHC class II binding of peptides derived from HLA-DR1. J Immunol. 1992; 148:2169.

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

177. Watschinger B, Gallon L, Carpenter CB, Sayegh MH. Mechanisms of allorecognition: in vivo primed T-cells recognize major histocompatibility complex polymorphisms presented as peptides by responder antigen-presenting cells. Transplantation. 1994; 57:572.

178. Parker KE, Dalchau R, Fowler JV, et al. Stimulation of CD4 T lymphocytes by allogeneic MHC peptides presented on autologous antigen presenting cells. Transplantation. 1992; 53:918.

179. Sherwood RA, Brent L, Rayfield LS. Presentation of alloantigens by host cells. Eur J Immunol. 1986; 16:569.

180. Fangmann J, Dalchau R, , Fabre JW. Rejection of skin allografts by indirect allorecognition of donor class I major histocompatibility complex peptides. J Exp Med. 1992; 175:1521.

181. Auchincloss HJ, Lee R, Shea S, et al. The role of "indirect" recognition in initiating rejection of skin grafts from major histocompatibility complex class II-deficient mice. Proc Natl Acad Sci. 1993; 90:3373.

182. Benichou G, Takizawa AP, Olson AC, McMillan M, Sercarz EE. Donor major histocompatibility complex (MHC) peptides are presented by recipient MHC molecules during graft rejection. J Exp Med. 1992; 175:305.

183. Wettstein PJ. Minor histocompatibility loci. In: Litwin S ed. Human immunogenetics. New York: John Wiley, 1986.

184. Simpson E, Chandler P, Goulmy E, Disteche CM, et al. Separation of the genetic loci for the H-Y and the testis determination on the human Y-chromosome. Nature. 1987; 326:876.

185. Van Els CACM, D'Amaro JD, Pool J, et al. Immunogenetics of human minor histocompatibility antigens: their polymorphism and immunodominance. Immunogenetics. 1992; 35:161.

186. Goulmy E, Termijtelen A, Bradley BA, van Rood JJ. Y-antigen killing by T cells of women is restricted by HLA. Nature. 1977; 266:544.

187. Falk K, Rötzschke O, Rammensee HG. Cellular peptide composition governed by major histocompatibility complex class I molecules. Nature. 1990; 348:248.

188. Rötzschke O, Falk K, Wallny HJ, Faath S, Rammensee HG. Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. Science. 1990; 249:283.

189. Wallny HJ, Rammensee HG. Identification of classical minor histocompatibility antigen as cell-derived peptide. Nature. 1990; 343:275.

190. Loveland BE, Fisher Lindahl K. Definition and expression of minor histocompatibility antigens. In: Mc Cluskey J ed. Antigen processing and presentation. London: CRC press. 1991; 173.

191. Speiser DE, Zürcher T, Ramseier H, Hengartner H, et al. Nuclear myxovirus-resistance protein Mx is a minor histocompatibility antigen. Proc Natl Acad Sci USA. 1990; 87:2021.

192. Scott DM, Ehrmann IE, Ellis PS, Bishop CE, et al. Identification of a mouse male-specific transplantation antigen, H-Y. Nature. 1995; 376:695.

193. Wang W, Meadows LR, den Haan JMM, Sherman NE, et al. Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. Science. 1995; 269:1588.

194. den Haan JMM, Sherman NE, Blokland E, et al. Identification of a graft-versus-host disease-associated human minor histocompatibility antigen. Science. 1995; 268:1476.

195. Advisory Committee of IBMTR. Report from the International Bone Marrow Transplant Registry. Bone Marrow Transplant. 1989; 4:221.

196. Atkinson K, Farrell C, Chapman G, Downs K, et al. Female donors increase the risk of acute graft-vs-host disease: effect of donor and parity and analysis of cell subpopulations in the donor marrow inoculum. Br J Haematol. 1986; 63:231.

197. Rammensee HG, Klein J. Polymorphism of minor histocompatibility genes in wild mice. Immunogenetics. 1983; 17:637.

197'. Goulmy E, Schipper R, Pool J, Blokland E, et al. Influence of minor histocompatibility antigen mismatches on the development of graft-versus-host-disease after bone marrow transplantation from HLA identical donors. N Engl J Med. in press.

198. Martin PJ. Increased disparity for minor histocompatibility antigens as a potential cause of increased GvHD risk in marrow transplantation from unrelated donors compared with related donors. Bone Marrow Transplant. 1991; 8:217.

199. Korngold R, Sprent J. Variable capacity of L3T4+ T cells to cause lethal GvHD across minor histocompatibility barriers in mice. J Exp Med. 1987; 165:1552.

200. Tsoi MS, Storb R, Santos E, Thomas ED. Anti-host cytotoxic cells in patients with acute graft-versus-host disease after HLA-identical marrow grafting. Transplant Proc. 1983; 15:1484.

201. Fontaine P, Langlais J, Perrault C. Evaluation of in vitro CTL assays as a predictive test for the occurrence of graft vs host disease. Immunogenetics. 1991; 34:222.

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

203. Irschick EU, Hladik F, Niederwieser D, Nussbaumer W, et al. Studies of the mechanism of tolerance or Graft-versus-Host Disease in allogeneic bone marrow recipients at the level of cytotoxic T-cell precursor frequencies. Blood. 1992; 79:1622.

204. Van Els CACM. Bakker A, Zwinderman AH, et al. Effector mechanisms in GvHD in response to minor histocompatibility antigens. II: Evidence for a possible involvement of proliferative T cells. Transplantation. 1990; 50:67.

205. Teh HS, Harley E, Phillips RA, Miller RG. Quantitative studies on the precursors of cytotoxic lymphocytes. J Immunol. 1977; 118:1049.

206. Bryan DJ, Pinto CE, Fuller TC. Direct estimation of the frequency of human cytotoxic T lymphocytes and their precursors following in vitro allosensitization. Hum Immunol. 1983; 6:31.

207. Moretta A, Pantaleo G, Moretta L, Mingari MC, Cerottini JC. Quantification assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloreactivated peripheral blood T cell populations. J Exp Med. 1983; 158:571.

208. Takiff H, Cook DJ, Himaya NS, Mickey MR, Terasaki PI. Dominant effect of histocompatibility on ten-year kidney transplant survival. Transplantation. 1988; 45:410.

209. Matas AJ, Tellis VA, Quinn T, et al. Short- and long-term graft survival with 0 antigen-matched first cadaver renal transplants: A single institution study. Clin Transp. 1989; 3:22.

210. Hickey DP, Lutins JA, Lopatin WB. Lack of effect of DR matching on five-year renal allograft and patient survival with cyclosporine. Clin Transpl. 1989; 3:83.

211. Goulmy E. Minor histocompatibility antigens and their role in transplantation. In: Morris PJ, Tilney NL ed. Transplantation reviews. Philadelphia: WB Saunders Co. 1988: 29.

212. Delisi C, Berzofski J. T-cell antigenic sites tend to be amphipathic structures. Pro Natl Acad Sci USA. 1985; 82:7048.

213. Rothbard JB, Taylor WR. A sequence pattern common to T cell epitopes. EMBO. 1988; 7:93.

214. Sercarz EE, Lehmann PV, Ametani A, et al. Dominance and crypticity of T cell antigenic determinants. Annu Rev Immunol. 1993; 11:729.

215. Li Y, Poirier G, Neth O, et al. Few peptides dominate cytotoxic T lymphocyte responses to single and multiple minor histocompatibility antigens. International Immunol. 1993; 5:1003.

216. Korngold R, Wettstein PJ. Immunodominance in the graft-versus-host disease T cell response to minor histocompatibility antigens. J Immunol. 1990; 145:4079.

217. Wettstein PJ. Immunodominance in the T cell response to multiple non-H-2 histocompatibility antigens. III. Single histocompatibility antigens dominate the male antigen. J Immunol. 1986; 137:2073.

Chapter 2

Histoincompatibilities in ABDR-matched unrelated donor recipient combinations

Bone Marrow Transplantation, (1995) 16, 641–646

Histoincompatibilities in ABDR-matched unrelated donor recipient combinations

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Summary:

To get an insight into the degree of major histocompatibility mismatches in donor/recipient (D/R) combinations who were 'ABDR-matched' by serology for class I and by oligotyping for DR1-14 (low resolution typing), we performed additional HLA testing using a combination of molecular, biochemical and cellular techniques. For class II we used extended oligotyping, discriminating all the common DRB1/B3/B5-subtypes. For class I (-subtypes) we used oligotyping (HLA-A2,-A3,-B35,-B41,-B44), sequencing (HLA-B35,-B41,-Cw16), isoelectrofocusing (IEF), primary cytotoxic T lymphocyte (CTL) assays and class I-subtype specific T cell clones. In addition, all combinations were serologically typed for HLA-C. This high resolution typing by the combination of techniques revealed numerous histoincompatibilities. Fifty-three per cent of all 'ABDR-matched' combinations tested ($n = 198$) appeared to be DR incompatible. Moreover, independent of the presence of a class II mismatch, 47% of the donors tested ($n = 131$) displayed pretransplant cytotoxic activity against the patient. This activity was found to be rigorously correlated with the presence of class I incompatibilities, predominantly HLA-A,-B subtypes and HLA-C. Thus, although the D/R pairs had been originally matched for AB including serological splits and by generic class II typing, only 28% of the pairs were in fact ABCDR identical. As many as 38% of the D/R pairs were mismatched for one, 14% for two, 13% for three and 6% for four A, B, C or DRB1 antigens. We conclude that the presence of such a high number of histoincompatibilities in a group of relatively well matched D/R pairs will severely hinder the analysis of the role of HLA in marrow transplantation and that conclusions from studies in which D/R pairs are matched by conventional typing must be interpreted with extreme caution.

Keywords: HLA; BMT; unrelated

Transplantation with marrow from an HLA-identical sibling is successfully used to treat a number of hematological malignancies and inborn errors of the immune system.[1,2] One of the main limitations to this therapy is the availability of a genotypically matched related donor that is only available for about 30% of the patients. For patients without such a donor, it has now become an option to find an 'ABDR-matched' donor in international registries. Such donors however, will be phenotypically matched only and appear to be less compatible, which is reflected by a higher rate of post-transplant complications such as GVHD.[3–6]

At present, it is not clear whether this incompatibility is a direct consequence of a mismatched allele of the MHC itself or whether it is a reflection of a higher rate of mismatched minor transplantation antigens.[7] In fact, a recent study has shown that patients who are 'ABDR-matched' with their unrelated donor are not at lower risk of GVHD than patients who are mismatched for one HLA antigen.[3] However, these results might be flawed because the 'one antigen mismatched' group might indeed not be so different from the group that is considered to be 'perfectly matched', because the latter can still be incompatible for a number of antigens undetected by routine typing as well.

An unrelated donor might be 'ABDR-matched' but nevertheless histoincompatible with a recipient for two different reasons. Firstly, matching is done on the basis of HLA-A, -B, and -DR antigens only and other antigens such as HLA-C, -DQ, and -DP might still be incompatible. The second reason why a patient can be histoincompatible with his unrelated donor lies in the limited resolution power of routine HLA typing techniques that were set up primarily for the matching of siblings. Because these routine techniques are not able to make out the large polymorphism of the human MHC, 'pefectly matched, HLA identical' unrelated donors will often be mismatched for antigens not discriminated by conventional typing. Such D/R pairs will also have an increased risk of being incompatible for the antigens not typed for, because other antigens such as HLA-C and -DQ will be in linkage disequilibrium with the incompatible HLA-A, -B or -DR antigens.[8,9]

At present, this problem is close to being resolved for DRB1 typing, as molecular techniques distinguishing most of the common alleles have become routine in the tissue typing laboratory.[10,11] Several studies have shown that many D/R pairs that were matched on the basis of serology, were in fact DR-incompatible.[12–14] For class I, the situation is less clear because precise methods such as DNA typing

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Received 4 March 1995; accepted 9 June 1995

are more cumbersome than for class II and an analysis of representative numbers of D/R pairs has not been done yet. It is however becoming apparent that the situation for class I might be quite similar as for class II and that serologically 'ABDR-matched' D/R pairs are more frequently AB-subtype mismatched than has been anticipated.[8,9,15–18] To get insight into the frequency and nature of mismatches in 'ABDR-matched' D/R pairs, we studied 131 combinations consisting of 73 patients with their unrelated potential bone marrow donors who had been recruited from international registries between 1990 and March 1994. All donors were selected on the basis of extended serology for AB and by oligotyping for DR 1-14.[11,12,19] In spite of the fact that these pairs were now matched for AB including serological splits, and by generic class II typing, the 'ABDR-matched' donors were frequently HLA-incompatible, strongly suggesting that such a group or groups that have been matched with lower stringency[3] are less suitable for assessing the influence of HLA on the outcome of a bone marrow transplantation.

Patients, materials and methods

Identification of potential donors

Blood samples of potential donors identified on the basis of AB(DR) serology were requested by the Swiss unrelated bone marrow donor registry[20] from international registries and ABDR-identity was verified by extended serotyping for HLA-A (10 large antigens, splits of A9, A10, A19, a total of 23 specificities), HLA-B (25 large antigens, splits of B5, B12, B15, B16, B17, B21, B22, B40, B70, a total of 38 specificities), and by HLA-oligotyping for DR1-14. Only donors who were matched for six ABDR antigens were considered for further study and selection.

High resolution class II typing

Oligotyping for HLA-DRB1/B3/B5 and -DQ, discriminating the vast majority of the HLA-DR and -DQ genes was performed as previously described.[10,11]

Class I oligotyping

Oligotyping for HLA-A2, -A3, and -B44 was performed as described before.[17] This method based on a PCR/SSO-oligotyping protocol is able to discriminate 10 HLA-A2, two HLA-A3 and two HLA-B44 subtypes.

Analysis of B35 subtype variants

After HLA-B group-specific PCR,[17] B*3501–3508 were discriminated by oligotyping using five probes (Gauchat *et al*, submitted). In some combinations partial HLA-B35 direct sequencing was done using a biotinylated 5′primer and a B-specific 3′ primer[21] with minor modifications (Gauchat *et al*, submitted). After separation of the biotinylated strand by streptavidin-coated Dynabeads M-280, B35 alleles were sequenced by the dideoxy-chain termination Sanger method (USB kit) over a segment comprising over 60 nucleotides of exon 3 (amino acids 94–116).

IEF

ID-isoelectrofocusing was performed as previously described.[22] In brief, PHA blasts (3×10^6) were radiolabelled with 100 μCi of ^{35}S-methionine. Precleared cell extracts were incubated with 3 μg of an anti-class I monoclonal antibody (W6/32). Immunoprecipitates were subjected to neuraminidase treatment and analyzed by ID-IEF.

HLA-C typing

Cw1–Cw8 was determined by serology. In some individuals, no HLA-C allele could be identified. This was due to either insufficient quality of the allo antisera used or to the expression of HLA-C specificities other than Cw1–Cw8. HLA-C*1601 was typed for by CTL clones.

Primary CTLp-test

Donor mononuclear cells were cultured in 200 μl 96-round bottom plates with irradiated recipient mononuclear cells as stimulators.[23] After 10 days of culture, the wells were tested against ^{51}Cr labelled, phytohemagglutinin-stimulated recipient T cell blasts.

Cloning of class I specific T cells

Cytotoxic T cell clones were established from the positive wells of the CTLp assay as described.[24] Specificity towards recipient cells was tested using autologous (donor) cells and third party cells that did not express HLA antigens in common as negative controls. After the identification of the fine specificity against a panel of cells expressing well-defined HLA-A, -B subtypes or HLA-C antigens, some of these clones were used as typing reagents.

Calculation of frequencies of ABCDR-mismatches

Because many of the combinations analyzed included patients for whom a search was going on and for whom these data would be used to identify the most compatible donor, the group analyzed is not entirely representative of a group of randomly selected 'ABDR-matched' D/R pairs. Frequencies were calculated after adjustment for the fact that CTLp's and subsequent analysis of the incompatibility have been done more frequently for donors who were preselected on the basis of DR-subtype compatibility, and analysis of class I has been performed more frequently for the CTLp-positive combinations than for the CTLp-negative ones.

Results

The majority of 'ABDR-matched' D/R pairs are ABCDR-incompatible

At present a large number of volunteer bone marrow donors are available through the international registries. These

donors have been (partially) HLA typed so that a first selection of potential donors can be done on the basis of the available HLA data. Because the number of donors is extremely high and only a minority will be recruited, most registries type their donors by low resolution serological methods only, and at this stage matching for A, B and DR does not warrant MHC compatibility. Therefore, before a final donor can be selected, blood samples of potential donors will have to be retyped with more precise HLA typing techniques. Figure 1 shows that when DR is typed by 'high resolution' oligotyping discriminating practically all known subtypes (82 DRB1, three DRB3, three DRB5),[11] only 47% of the 'ABDR-matched' D/R pairs appear to be DR-(DRB1/B3/B5) compatible. It should be noted that all the combinations studied in this report were 'ABDR-matched' on the basis of extended serotyping for HLA-A, -B (including 'splits') and by HLA-oligotyping for DR1-14. This first screening, referred to in this report as 'low resolution typing', had already excluded more than 20% of the potential donors, mostly on the basis of flaws in DR-serology.[19]

In the DR-matched group, very few pairs were matched for all three class II loci. Matching for DQ and DP reflected their respective strong (DQ) and weak (DP) linkage disequilibrium with DR.[25] Of the DRB1/B3/B5 compatible combinations studied (n = 59), more than 90% were matched for DQ. However, only two of the 26 pairs tested were matched for both DP alleles.

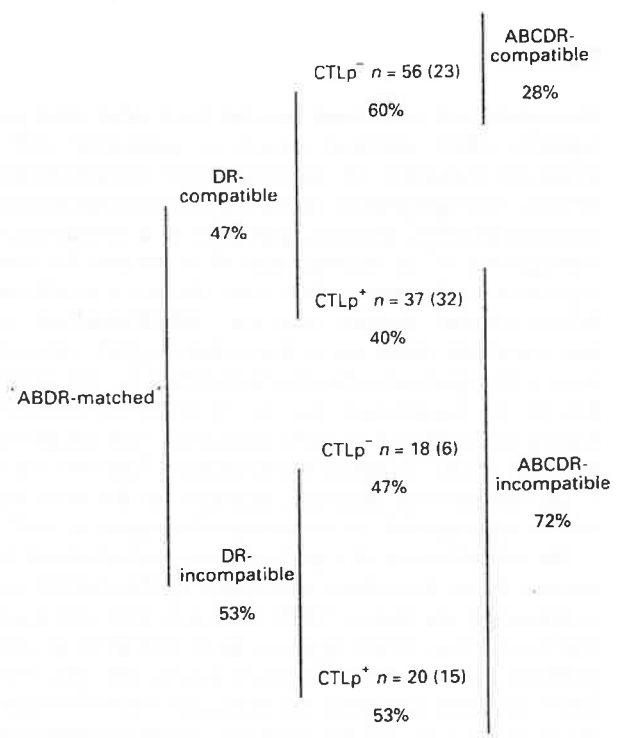

Figure 1 ABCDR compatibility as determined by high resolution DR typing and CTLp assays. The percentage of CTLp-positive combinations is calculated from the 131 combinations tested with both techniques. The percentage of DR-incompatible combinations is calculated from the pairs consisting of the patient with all of his 'ABDR-matched' donors that were recruited and that were not all tested in the CTLp assay (n = 61). The numbers in parentheses represent the combinations that were analyzed to characterize the incompatibility(-ies) recognized

Routine class I typing does not comprise HLA-C serology nor does it characterize class I variant subtypes. Therefore 'ABDR-matched' D/R pairs might not only be mismatched for DR subtypes, DQ or DP, but also for class I subtypes and/or HLA-C. Because these mismatches are efficiently detected by CTLp-assays,[8,9] our 'high resolution' typing procedures for the selection of unrelated donors includes the determination of CTLpf in the GVHD direction.[19] Figure 1 shows the results of 73 patients and their 131 'ABDR-matched' unrelated potential donors. Of the DR-subtype mismatched 'ABDR-matched' D/R pairs, 53% were CTLp-positive. Moreover, of the pairs who were matched for DR subtypes, 40% were CTLp-positive. This resulted in an overall compatibility of only 28%. Because CTLp-positivity is strongly correlated with class I incompatibilities (see below), these results should represent a precise estimate of the degree of ABCDR-incompatibility in 'ABDR-matched' D/R pairs.

Characterization of CTLp-positivity in 'ABDR-matched' D/R pairs

The CTLpf can only serve as a diagnostic assay for class I compatibility when it efficiently detects all class I mismatches without yielding false-positive results. To test this, we analyzed the class I mismatches in 47 CTLp-positive and in 29 CTLp-negative pairs. We used a combination of class I oligotyping (HLA-A2, -A3, -B35, -B44), sequencing (HLA-B35, -B41, -Cw16), IEF (HLA-A, -B), serology (HLA-C) and class I subtype specific CTLs (HLA-A, -B, -C).[8,9] Table 1 shows that these techniques were able to detect numerous class I mismatches in the combinations studied. It also shows that CTLp-positivity is rigorously correlated with the presence of class I mismatches and that only very few such incompatibilities were missed by the CTLp assay. Of a total of 65 class I incompatibilities detected, 61 occurred in the CTLp-positive combinations while only four occurred in CTLp-negative ones. Moreover, by cloning of the recipient-specific CTLs, we could show that in 45 of the 48 cases tested, the incompatible class I molecule was indeed the target recognized and that DR-subtype incompatibilities present both in the CTLp-positive and -negative group (Table 1), were not detected.[8,9,15,17] Therefore, DR-incompatible CTLp-positive D/R combi-

Table 1 Occurrence of HLA-A, -B, -C and DR-mismatches in CTLp-negative and CTLp-positive combinations

	HLA-A, -B[a]	*HLA-C*	*HLA-A, -B, -C*	*HLA-DR*
CTLp⁻	2 (29)[b]	2 (28)	4 (29)	18 (74)
CTLp⁺	33 (47)[c]	28 (42)[d]	61 (47)	20 (57)

[a]Subtypes by IEF (20 mismatches/61 combinations tested), oligotyping for HLA-A2 (7/54), oligotyping for HLA-B44 (2/35), oligotyping and sequencing for HLA-B35 (9/24) and sequencing for HLA-B41 (1/2).
[b]Expressed as no. disparate antigens (total no. combinations tested).
[c]CTL-specificity could be attributed to the incompatible subtype in 32 of the 33 combinations tested.
[d]In combinations that were HLA-A, -B compatible, HLA-C incompatible, CTL-specificity could be attributed to the incompatible HLA-C in 13 of the 15 cases tested

nations should be considered as being concomitantly mismatched for one or more class I antigens. These results emphasize two important points. Firstly, a CTLp assay may be used as a test to assess class I compatibility. Secondly, although the number of class I mismatches in combinations matched for class II at the molecular level might be lower than in 'ABDR-matched' D/R pairs, 40% of such combinations are still class I incompatible.

Occurrence of HLA-A, -B, -C and -DR incompatibilities in 'ABDR-matched' D/R pairs

Table 2 shows the frequency of HLA-A, -B, -C and -DR mismatches detected after high resolution DR-oligotyping and identification of the CTL-activity. DRB1 incompatibilities were primarily found for DR1 (14% of the pairs were mismatched), DR4 (46%), DR11 (25%) and DR13 (39%). CTL-activity could be attributed to various forms of class I incompatibilities. Apart from five cases that could not be discerned because no other target in our cell panel could be identified that was recognized by the recipient-specific CTLs, and three cases in which the CTLs were specific for the male antigen presented by HLA-A2 or unidentified minors presented by respectively HLA-A2 and -B60, all other activity was against class I molecules that had not been adequately discriminated by serology. The majority comprised subtypes of HLA-A2, -B7, -B35, -B41, -B44, -B51, and -B62. In four cases, the serological splits of HLA-A10 had been assessed incorrectly. Moreover, numerous serological blanks in donors thought to be homozygous were in fact due to flaws in serology. This was particularly frequent for HLA-C, but did hold true for other antigens as well. Of as few as six serologically homozygous HLA-B35 donors that we sequenced in the first place to identify the putative HLA-B35-subtype recognized by the CTLs, two were in fact HLA-B35/-B71 and HLA-B35/-B79 heterozygotes, respectively.

From Table 2, it is also apparent that, due to the linkage disequilibria between the respective MHC loci, D/R pairs are more frequently mismatched for more than one antigen than is to be expected on the basis of the frequency of the mismatched antigens themselves. This has important consequences for the appraisal of certain mismatches, and this should be taken into account when routine tissue typing procedures do not include subtyping for all loci. For instance, of the pairs mismatched for HLA-C (32%), 80% are mismatched for a subtype of HLA-A, -B or -DR as well. Thus, even when an HLA-C mismatch itself might be less harmful, a donor with such an incompatibility detected by routine typing methods might be less optimal due to undetected linked HLA incompatibilities. Furthermore, some class I-subtypes are in strong linkage disequilibrium with particular DR-subtypes and in such cases a mismatch at the DR locus detected by the routine typing procedure will be a strong indication for a class I mismatch. This tendency was especially striking for HLA-B35 and -B41 positive D/R pairs where DR-subtype incompatible meant being class I-subtype incompatible as well.[9,17,26]

In combination with the fact that numerous incompatibilities are not detected, such linkage disequilibria will constrain the analysis of the significance of HLA mismatches on bone marrow transplantation enormously, when only standard tissue typing techniques have been used. It is therefore obvious that such an analysis should only be done on the basis of tissue typing with the highest possible resolution.

Table 2 HLA incompatibilities in 'ABDR-matched' D/R pairs (n = 76)

	%[a]
2(AB) + DR[b] + C	6.0 (2)[c]
2(AB) + DR	1.9 (1)
(AB) + DR + C	11.2 (6)
(AB) + DR	5.6 (3)
(AB) + C	2.9 (5)
(AB)	7.0 (12)
DR + C	5.6 (3)
DR	20.9 (5)
C	5.9 (9)
Class I unident.	4.2 (5)
Class I restr. minor	1.8 (3)
Total	72.9 (54)
DR-compatible/ CTLp-negative	27.1 (22)

[a]Estimated frequency in a (random) group of 'ABDR-matched' D/R combinations (see Methods).
[b]DRB1-subtype mismatch with a linked DRB3/4/5 mismatch are scored as one single mismatch.
[c]Total no. of combinations analyzed for the respective class I and class I antigen(s)

Discussion

Transplantation with bone marrow from other than genotypically HLA identical donors is associated with an increased incidence of post-transplant complications.[3–6] Whereas in experimental animal models the contribution of mismatched MHC-antigens appears to be a pivotal one, the consequence of an incompatible HLA antigen for human marrow transplantation is less clear. In fact, it is still under debate whether patients who are 'ABDR-matched' with their unrelated donor are at lower risk of graft-versus-host disease than patients who are mismatched for one antigen.[3] One of the explanations for this nevertheless unexpected finding might be that in such studies the 'one antigen mismatched' group is indeed not so different from the one that is considered to be 'perfectly matched', as the latter might still be incompatible for a number of antigens as well.

The identification of a perfectly matched unrelated bone marrow donor is severely challenged by the extreme polymorphism of the human MHC. Not only will it be impossible to establish donor registries large enough to provide a perfectly matched donor for every patient, but also, once a donor has been identified, the techniques needed to type all 24 HLA antigens are too elaborate for most tissue typing laboratories. Therefore, histoincompatibilities are inevitable and the main task lies in determining which mismatches are the most acceptable ones.

Unfortunately, the published observations on the effect of HLA-mismatches on the outcome of a bone marrow transplant are not in agreement. When the marrow donor is an HLA-non-identical family member, significant effects

of one A-, B- or DR-mismatch on transplant-related mortality have been reported.[27–29] When the donor is not related, the one locus mismatch effect can be significant for the occurrence of GVHD and survival,[30] significant for the occurrence of GVHD with trends for survival,[5] significant for the occurrence of GVHD only,[6] or not significant at all.[3] These data might however be less controversial than they seem when one takes into account that the background of other (hidden) incompatibilities in these studies may vary substantially. It is obvious that the direct effect of one potentially additional mismatch will only become apparent when the donor and the recipient are sufficiently well matched for the other antigens. This tendency is clear from the studies mentioned above. It is in the multicenter study,[3] in which typing is performed by a number of different laboratories and matching is based on serology only, that no correlation between GVHD and one antigen mismatched is found. In contrast, when in the other studies the matching is backed up by more precise techniques such as IEF for class I homozygous individuals and Dw- or oligotyping for class II,[5,6] the influence of one locus mismatch becomes clear. It is therefore tempting to believe that by increasing the resolution of tissue typing even more, the effect of an HLA mismatch will become still more apparent. Our data show that this would be conceivable because in a group that is matched with such moderate stringency, numerous mismatches do remain undetected.

The key question of course is whether for the selection of an unrelated donor the tissue typing techniques should still be extended. On the one hand there are the technical problems that could slow down the selection procedure so that the possible beneficial effect of a more compatible donor is neutralized by the fact that the patient might have to be transplanted in a later stage of his disease. On the other hand, it remains still to be shown that a patient would indeed benefit from a transplantation with a more compatible donor. However, we think that the fact that there is still no clear answer to the question to what extent post-transplant complications are due to HLA mismatches is an argument valid enough to improve our tissue typing techniques. Then, even when this extended protocol would not identify a better donor, immune suppression regimens could be better accommodated to the degree of histoincompatibility so that some of the negative effects of a (too) strong conditioning could be circumvented.

How can this better matching be obtained in practice? Unfortunately, given the extreme large polymorphism of the human MHC, only sequencing of all 24 HLA antigens of two unrelated individuals would have a high enough resolution power to determine their degree of histocompatibility precisely. Clearly, methods should be less elaborate and be focused on antigens for which it still would be feasible to find a matched donor. Therefore, HLA-DP that is less strongly linked to the other HLA antigens[25] so that matching becomes almost impossible[19] or very rare subtypes of HLA-AB/DR should probably not be included in the protocol.

In this study, we have used a combination of high resolution oligotyping for class II and a cellular assay for class I. We think that at present, this is one of the most practical approaches to identify ABCDR-mismatches. This procedure discriminates most DR-alleles by a relatively simple oligotyping protocol[10–12] and, as we show in this paper, it does detect most of the class I incompatibilities that we were able to discriminate with the other techniques available. However, we do not think that such a protocol comprising a cellular assay will become the standard routine for the future, as long as the CTLp test detects class I incompatibilities without providing additional information about the biological relevance of such mismatches. We think that this is the case for HLA-A/B mismatches, because we found the straightforward correlation between class I incompatibility and CTLp-positivity for all the antigens for which the full resolution techniques were available. However, we cannot be sure that this also holds for HLA-C mismatches because the typing techniques used in this study, could have missed some mismatches that also failed to induce CTL activity.

At present, however, the merit of cellular techniques is clearly its potential to detect incompatibilities for which molecular techniques have not yet been developed. Whereas oligotyping techniques would detect only the antigens that the protocol had been set up for, CTLs would be activated by any incompatible class I molecule independent of whether the sequence would be known or not. This advantage is clearly shown in our study. During the analysis of the incompatibilities recognized by the CTLs we found two new class I subtypes (HLA-B41 and -B44). Furthermore, many serological blanks were unmasked by the CTLp assay. Therefore, the CTLp assay has given us the information as to which antigens should be oligotyped to cover a substantial part of class I subtypes that might be mismatched. At present, oligotyping for HLA-A2, -B35, -B44 subtypes are already routine procedures in our laboratory and protocols for HLA-A10, -B7, -B27 and -B41 are being set up. We think that even with such a limited set of specificities, a routine laboratory would be able to cover a relevant number of class I subtypes. In combination with oligotyping for DR, this routine high resolution typing would be able to screen a large number of unrelated D/R pairs. This would not only allow the selection of the most compatible donor but this should also facilitate a better appraisal of the importance of HLA mismatches for bone marrow transplantation.

Acknowledgements

We thank C Grundschober for Cw*1601 sequencing, A Moser and M Ossola for performing a part of the CTLp assays, M Bujan, V Elamly and C Gonet for the HLA serology, and B Kervaire and P Roux-Chabbey for HLA oligotyping. This work was supported in part by the Swiss National Science Foundation (31-33620.92, 31-36247.92 and 32-35641.92) and by the 'Ligue Genevoise contre le Cancer'. A Slavcev (on leave from the National HLA Center IKEM Praha) was supported by the 'Fondation de l'Europe des Sciences et des Cultures' (grant 01/1992).

References

1 Martin PJ, Hansen JA, Storb R, Thomas ED. Human marrow transplantation: an immunological perspective. *Adv Immunol* 1987; **40**: 379–438.

2 Gratwohl A, Hermans J. Bone marrow transplantation activity in Europe 1992: report from the European Group for Bone Marrow Transplantation (EBMT). *Bone Marrow Transplant* 1994; **13**: 5–10.

3 Kernan NA, Bartsch G, Ash R *et al*. Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. *New Engl J Med* 1993; **328**: 593–602.

4 McGlave P, Bartsch G, Anasetti C *et al*. Unrelated donor marrow transplantation therapy for chronic myelogenous leukemia: initial experience of the national marrow donor program. *Blood* 1993; **81**: 543–550.

5 Ash R, Casper JT, Chitambar CR *et al*. Successful allogeneic transplantation of T cell-depleted bone marrow from closely HLA-matched unrelated donors. *New Engl J Med* 1990; **322**: 485–494.

6 Beatty PG, Anasetti C, Hansen JA *et al*. Marrow transplantation from unrelated donors for treatment of hematologic malignancies: effect of mismatching for one HLA locus. *Blood* 1993; **81**: 249–253.

7 Beatty PG. Minor histocompatibility antigens. *Exp Hematol* 1993; **21**: 1514–1516.

8 Rufer N, Breur-Vriesendorp BS, Tiercy J-M *et al*. High-resolution histocompatibility testing of a group of 16 B44-positive, ABDR serologically matched unrelated donor-recipient pairs: analysis of serologically undisclosed incompatibilities by cellular techniques, isoelectrofocusing, and HLA oligotyping. *Hum Immunol* 1993; **38**: 235–239.

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

10 Tiercy J-M, Morel C, Freidel AC *et al*. Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. *Proc Natl Acad Sci USA* 1991; **88**: 7121–7125.

11 Tiercy J-M, Grundschober C, Jeannet M, Mach B. A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes. In: Hui KMB (ed). *Handbook of HLA Typing Techniques* CRC Press: Boca Raton, 1993, pp 117–147.

12 Tiercy J-M, Roosnek E, Speiser D *et al*. Replacement of HLA class II serology by the HLA-DR microtitre plate oligotyping assay: a one-year experience in unrelated bone marrow donor selection. *Br J Haematol* 1993; **85**: 417–418.

13 Baxter-Lowe LA, Eckels DD, Ash R *et al*. The predictive value of HLA-DR oligotyping for MLC responses. *Transplantation* 1992; **53**: 1352–1357.

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

15 Rufer N, Tiercy J-M, Speiser D *et al*. Characterization of pretransplant CTL activity in 'perfectly matched' unrelated bone marrow donor/recipient combinations. *Bone Marrow Transplant* 1993; **11** (suppl. 1): 20–23.

16 Breuning MH, Breur BS, Engelsma MY *et al*. Activation of cytotoxic T lymphocytes in HLA-A, -B and -C-identical responder–stimulator pairs. II. New subtypes of HLA-Bw35. *Tissue Antigens* 1984; **24**: 90–97.

17 Tiercy J-M, Djavad N, Rufer N *et al*. Oligotyping of HLA A2, A3, and B44 subtypes: detection of subtype-incompatibilities between patients and their serologically matched unrelated bone marrow donors. *Hum Immunol* 1994; **41**: 207–215.

18 Santamaria P, Reinsmoen NL, Lindstrom AL *et al*. Frequent HLA class I and DP sequence mismatches in serologically (HLA-A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA-identical unrelated bone marrow transplant pairs. *Blood* 1994; **83**: 280–287.

19 Speiser D, Tiercy J-M, Rufer N *et al*. Relation between the resolution of HLA-typing and the chance of finding an unrelated bone marrow donor. *Bone Marrow Transplant* 1994; **13**: 805–809.

20 Gratwohl A, Helg C, Chapuis B *et al*. Schweizer Register für unverwandte Knochenmarksspender: erste Resultate. *Schweiz Med Wochenschr* 1992; **122**: 1117–1122.

21 Zemmour J, Little AM, Schendel DJ, Parham P. The HLA-A,B 'negative' mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. *J Immunol* 1992; **148**: 1941–1948.

22 Neefjes JJ, Breur-Vriesendorp BS, van Seventer GA *et al*. An improved biochemical method for the analysis of HLA-class I antigens. Definition of new HLA-class I subtypes. *Hum Immunol* 1986; **16**: 169–181.

23 Roosnek E, Hogendijk S, Zawadynski S *et al*. The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. *Transplantation* 1993; **56**: 691–696.

24 Roosnek E, Lanzavecchia A. Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. *J Exp Med* 1989; **170**: 297–302.

25 Begovich AB, McClure GR, Suraj VC *et al*. Polymorphism, recombination, and linkage disequilibrium within the HLA class II region. *J Immunol* 1992; **148**: 249–258.

26 Rufer N, Roosnek E, Kressig R *et al*. Sequencing of a new HLA-B41 subtype (B*4102) that occurs with a high frequency in the Caucasoid population. *Immunogenetics* 1995; **41**: 333.

27 Beatty PG, Clift RA, Mickelson EM *et al*. Marrow transplantation from related donors other than HLA-identical siblings. *New Engl J Med* 1985; **313**: 765–771.

28 Anasetti C, Amos D, Beatty PG *et al*. Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. *New Engl J Med* 1989; **320**: 197–204.

29 Davies SM, Ramsay NKC, Haake RJ *et al*. Comparison of engraftment in recipients of matched sibling or unrelated donor marrow allografts. *Bone Marrow Transplant* 1994; **13**: 51–57.

30 Gajewski JL, Ho WG, Feig SA *et al*. Bone marrow transplantation using unrelated donors for patients with advanced leukemia or bone marrow failure. *Transplantation* 1990; **50**: 244–249.

Chapter 3

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1. High-resolution histocompatibility testing of a group of sixteen B44-positive, ABDR serologically matched unrelated donor-recipient pairs

2. HLA-B35-subtype mismatches in ABDR serologically matched unrelated donor-recipient pairs

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High-Resolution Histocompatibility Testing of a Group of Sixteen B44-Positive, ABDR Serologically Matched Unrelated Donor–Recipient Pairs

Analysis of Serologically Undisclosed Incompatibilities by Cellular Techniques, Isoelectrofocusing, and HLA Oligotyping

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ABSTRACT: We have characterized HLA incompatibilities in a group of 16 B44-positive patients who were serologically ABDR matched with their 23 (unrelated) potential bone marrow donors. After analysis with a combination of cellular techniques, IEF for HLA-A/B and oligotyping for class II and HLA-B44, 44% of the patients revealed one or more HLA incompatibility with at least one of their potential donors. CTL activity was detected in 12 of the 22 combinations tested. CTL incompatibility occurred more frequently in DR subtype-mismatched combinations, but CTL reactivity was always directed against class I. To characterize these incompatibilities between matched unrelated individuals, we analyzed the specificity of T-cell clones from seven primary CTL cultures. In three combinations, CTL reactivity was directed against a subtype of B44. In two combinations, the CTL reactivity was directed against a non-B44 class I subtype. In two of seven combinations, the CTLs recognized an antigen that, though unconditionally associated with B4403, was expressed by 60% of the B4403$^+$ cells only. Because all 12 of these B4403$^+$ targets recognized could be typed for one HLA-C allele only (Cw1–Cw8), we believe that this alloreactivity might be directed against a serologically undefined Cw antigen. *Human Immunology 38, 235–239 (1993)*

ABBREVIATIONS

CTL	cytotoxic T lymphocyte
CTLp	CTL precursor
D/R	donor–recipient pair
GvHD	graft-versus-host disease
IEF	isoelectric focusing
MHC	major histocompatibility complex
PCR	polymerase chain reaction

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Received (U) July 26, 1993; accepted September 1, 1993.

INTRODUCTION

ABDR serologically identical unrelated donor–recipient pairs (D/Rs) often appear to be less compatible than serologically identical D/Rs within a family. This higher degree of incompatibility is reflected by an increased incidence and severity of posttransplant complications such as graft-versus-host disease (GvHD) [1–3]. The most simple explanation for this phenomenon lies in

Human Immunology 38, 235–239 (1993)

0198-8859/93/$6.00

the relatively low resolution of a standard tissue-typing technique that, though adequate for the matching of siblings, is not able to make out the large polymorphism of the human major histocompatibility complex (MHC) in an unrelated population.

Incompatibilities between serologically matched unrelated D/Rs also become evident in in vitro cellular assays. Most incompatibilities in DR subtypes that are not differentiated by serology do cause positive mixed-lymphocyte reactions [4, 5]. Furthermore, donors often display cytotoxic activity against matched unrelated recipients [6–8] while they do not do so against related ones [6].

Because the presence of such cytotoxic precursor cells (CTLps) is a strong indication for posttransplant complications [6, 7], it is clear that the incompatibilities detected by CTLp assays are relevant ones. We therefore characterized these incompatibilities in a group of D/Rs without serologically detectable ABDR mismatches. Because a variant class I subtype would be the most likely candidate target structure recognized, we focused on HLA-B44, a class I antigen that is frequent and that is known to occur in several subtypes [9, 10].

MATERIALS AND METHODS

HLA matching of donor–recipient pairs. Unrelated donors were selected on the basis of HLA matching by serotyping for HLA-A, -B and by HLA oligotyping for HLA-DR1–14. Only $B44^+$ D/R combinations, compatible for the antigens mentioned above, were included in this study. After the first selection, the combinations were further typed by high-resolution oligotyping for DR/DQ [4] and serotyping for HLA Cw1–Cw8. In some of the individuals, no HLA-C allele could be identified.

Primary CTLp test. Limiting numbers of donor mononuclear cells (5×10^4–0.125×10^4) were cultured in 96 200-μl round-bottom plates with constant numbers (5×10^4) of irradiated (2500 rad) recipient mononuclear cells as stimulators. After 10 days of culture, the wells were tested against ^{51}Cr-labeled phytohemagglutinin-stimulated recipient T-cell blasts [7].

Cloning of patient-specific T cells. Cytotoxic T-cell clones were established from the positive wells of the CTLp assay as described [11]. Specificity toward recipient cells was tested using autologous (donor) cells and third-party cells that did not express HLA antigens in common as negative controls.

Oligotyping for B44. Exon-3 group-specific polymerase chain reaction (PCR) was performed on genomic DNA with a combination of a 5′ primer specific for the nucleotide sequence encoding Ile at codon 95 and an HLA-B locus-specific 3′ primer (Tiercy et al., submitted). PCR-amplified DNA was hybridized with the digoxygenin–ddUTP-labeled oligo probe D-156 (5′ CTCT-GTCCTGCTCCGCCAC 3′) specific for the nucleotide sequence encoding Asp at codon 156. Within the 18 B alleles selected by the PCR, this sequence is specific for HLA-B4401, -B4402, and -B3701 and thus enables differentiation of B4401/2 and B4403. All procedures for dot-blotting, hybridization, washing, and chemiluminiscent detection were done as described [12].

Isoelectrofocusing (IEF). One-dimensional (ID)-IEF was performed as previously described [13]. In brief, phytohemagglutinin blasts (3×10^6) were radiolabeled with 100 μCi of [^{35}S]methionine. Precleared cell extracts were incubated with 3 μg of an anti-class-I monoclonal antibody (W6/32). Immunoprecipitates were subjected to neuraminidase treatment and analyzed by ID-IEF.

RESULTS

Definition of an (ABDR)-matched pair. Whereas ABDR-matched siblings are most likely identical for all HLA antigens, this is not the case for unrelated D/Rs. Therefore, a group of ABDR-matched unrelated D/Rs is still very heterogeneous with respect to their degree of histocompatibility and the designation matched is a rather broad one. In this article, we use the term ABDR matched in the usual sense. Thus, ABDR-matched combinations are identical for the specificities that are differentiated by classic serology (A,B and DR1–14), but might still be incompatible for HLA-C, -DQ, -DP and for subtypes of HLA-A/B and -DR. Within this group, we differentiate DR(subtype)-incompatible and the DR-compatible ones that, due to the strong linkage disequilibrium between DR and DQ, are also DQ compatible (results not shown).

Incompatibilities in B44-positive ABDR-matched unrelated D/Rs. Table 1 shows the HLA typing of all $B44^+$ patients for whom we were able to identify an ABDR-matched bone marrow donor. Although all patients can be considered as ABDR matched in the classic sense (see above), a number of mismatches could be identified. Five of the D/Rs (p1–p5) were DR incompatible. Furthermore, a large number of HLA-C mismatches were found. Incompatibilities were also detected at the level of alloreactive cytotoxic T lymphocytes (CTLs). In 12 of 22 combinations tested, patient-specific CTL activity could be measured.

Variant subtypes of class I are the major targets for CTL recognition of ABDR-matched individuals. As the incom-

TABLE 1 HLA typing of B44+ ABDR-matched donor–recipient combinations

	A	B	C	DRB1	CTLp[a]
p1[b]	26,26	44,51	5	404,1501	
p1d	26	44,51	4[c]	404,16	+
p2	2,29	44,62	3	407,1501	
p2d1	2,29	44,62	3,7	404,1501	+
p2d2	2,29	44,62	3	401,1501	+
p3	2,2	44,62	3,6	1101,1302	
p3d1	2	44,62	3,5	1101,1301	+
p3d2	2	44,62	7	1101,1401	+
p3d3	2	44,62	NT[d]	11,1401	+
p3d4	2	44,62	NT	12,1301	+
p4	2,32	38,44	5	1301,1501	
p4d	2,32	38,44	NT	1301,15/16	–
p5[e]	2,2	44,60	3	401,1302	
p5d	2,2	44,60	3,5?	408,1302	–
p6	1,3	7,44	7	7,1501	
p6d1	1,3	7,44	4,7	7,1501	+
p6d2	1,3	7,44	7	7,1501	–
p7	2,3	38,44	3,4	7,1301	
p7d1	2,3	38,44		7,1301	+
p7d2	2,3	38,44		7,1301	+
p7d3	2,3	38,44	NT	7,1301	NT
p8	2,3	40,44	3,5	101,1302	
p8d	2,3	40,44	3,5	101,1302	+
p9	2,2	44,60	3,5	404,1501	
p9d	2,2	44,60	3,5	404,1501	+
p10	24,29	35,44		7,1401	
p10d	24,29	35,44		7,1401	–
p11	1,2	44,57	5,6	7,1301	
p11d	1,2	44,57	6	7,1301	–
p12	2,2	44,44	5	401,1301	
p12d	2	44	NT	401,1301	–
p13	2,29	44,51		7,1101	
p13d	2,29	44,51		7,1101	–
p14	23,24	44,60	3,4	7,1301	
p14d	23,24	44,60	3,4	7,1301	–
p15	1,2	8,44	5,7	301,801	
p15d	1,2	8,44	5,7	301,801	–
p16	3,23	27,44	NT	301,7	
p16d	3,23	27,44	NT	301,7	–

[a] CTLp range, $6/10^6$–$167/10^6$ (number of patient-specific CTLps per 10^6 donor mononuclear cells).

[b] p1, patient 1; p1d1, donor 1 of patient 1; etc.

[c] Incompatibilities are printed in bold.

[d] NT, not tested (blank, not identified).

[e] Haploidentical related D/R.

patibilities recognized by donor CTLs might be those responsible for GvHD, the specificity of these alloreactive T cells might provide information on the biologic significance of various mismatches. We therefore characterized these specificities by testing a number of T-cell clones from CTL-positive combinations against a panel of HLA-typed cells. Furthermore, as it became evident that none (!) of the clones were specific for either the HLA-DR or the HLA-C incompatibilities shown in Table 1, we typed the 23 individuals for subtypes of B44 either by IEF or by DNA typing with the oligo D-156 (Table 2). Three of the seven CTL-positive patients were B44 mismatched with one of their potential donors. Table 2 further shows the analysis of the specificity of the CTL clones derived from five positive cultures. In two (p1d → p1 and p2d2 → p2d1), the mismatched B4402 was also the incompatibility recognized as the CTL clones derived from these CTL cultures killed all $B4402^+$ targets without recognizing the $B4403^+$ ones. However, in the two combinations where B4403 was the mismatched B44 variant expressed by stimulator cells (p2d1 → p2d2 and p2d1 → p2), two different specificities were found. In one combination (p2d1 → p2d2), B4403 was the incompatibility recognized as the CTLs killed all of the $B4403^+$ targets. In the other combination (p2d1 → p2), however, the CTLs did not recognize B4403 directly as the clones recognized some, but not all, $B4403^+$ targets. Interestingly, the clones were able to distinguish between two B4403-identical but CTL-incompatible individuals (p6d1/p6, p7d1/p7, and p7d2/p7) but not between a B4403 CTL-compatible one (p6d2/p6).

The incompatibility recognized could therefore be the same as that recognized in the B4403-identical combination p6d1 → p6. This suggestion was further enforced when the clones raised in this combination differentiated the $B4403^+$ targets in the same manner. Although we were unable to identify the exact incompatibility recognized, further panel studies (Table 3) showed that whereas the anti-B4402 and the anti-B4403 clones lysed all their respective targets, the clones raised in p2d1 → p2 and p6d1 → p6 killed only targets that expressed B4403 and that could be typed for one HLA-C allele only. Because HLA-C serology is far from perfect, the alloreactivity in these two combinations might be directed against an HLA-C other than Cw1–Cw8 that is closely associated with B4403.

Of the other three combinations (p3d1-4 → p3, p8d → p8, and p9d → p9), we were able to identify the incompatibility only in p9d → p9. As reported before [8], all patient-specific CTL reactivity was directed against A205, a rare A2 variant expressed only by this patient. It is unquestionable, however, that also in the other two cases the clones recognize hidden class I incompatibilities as they are all of the CD8 phenotype and specific lysis can be inhibited by anti-class-I and not by anti-class-II antibodies (data not shown).

TABLE 2 B44 subtypes and specificity of the alloreactive CTLs

Targets	D-156[a] oligo	IEF	p1d[b] → p1	p2d2 → p2d1	p2d1 → p2d2	p2d1 → p2	p6d1 → p6
p1	4402	NT	+	+	−	NT	−
p1d	4403	NT	−	−	+	NT	−
p2	4403	44.1[c]	−	−	+	+	+
p2d1	4402	44.2	+	+	−	−	−
p2d2	4403	44.1	−	−	+	+	+
p3	4402	44.2	+	+	−	−	−
p3d1	4402	44.2	+	+	−	−	−
p3d2	4402	44.2	+	+	−	−	−
p3d3	4402	44.2	+	+	−	−	−
p3d4	4402	44.2	+	+	−	−	−
p6	4403	44.1	−	−	+	+	+
p6d1	4403	44.1	−	−	+	−	−
p6d2	4403	44.1	−	−	+	+	+
p7	4403	44.1	−	−	+	−	−
p7d1	4403	44.1	−	−	+	+	+
p7d2	4403	44.1	−	−	+	+	+
p7d3	4402	44.2	+	+	−	NT	−
p8	4402	NT	+	+	−	−	−
p8d	4402	NT	+	+	−	−	−
p9	4402	NT	+	+	−	−	−
p9d	4402	NT	+	+	−	−	−

[a] Although oligo D-156 hybridizes with both B4401 and 4402, all D-156-positive individuals in this study can be designated B4402 because they are all B44 identical by CTL analysis and thus must be B4402, which is far more frequent than B4401.

[b] CTL clones (2–5/combination) established from primary cultures: p1d → p1, donor of patient 1 against patient 1.

[c] IEF subtypes according to Fleischhauer et al. [10].

DISCUSSION

ABDR-matched unrelated bone marrow transplantation is associated with a higher incidence of GvHD than that observed in transplantations between HLA-identical siblings [1–3]. This obviously reflects the higher degree of histoincompatibility between donor and recipient, but a precise evaluation has remained difficult as our standard typing techniques do not identify all of the variants of HLA antigens that the immune system is able to discriminate.

In addition to the standard techniques based on serology or on DNA typing, histoincompatibility between donor and recipient can be evaluated by cellular techniques. Although these methods will never provide results as precise as those found by direct genomic HLA analysis, they will detect incompatibilities that the latter methods are not especially set up for. Not only will they detect possible additional differences outside of the DNA sequences selected to be analyzed, but they will also detect "allogeneity" caused by antigens other than those that we type for. Our data presented in this study clearly illustrate this point. Although the CTL assay was unable to characterize the histoincompatibility directly, it marked 12 of 22 B44$^+$ ABDR-matched D/Rs as incompatible. Furthermore, none of these incompatibilities would have been detected by routine typing techniques.

The further characterization of the incompatibilities recognized by the CTLs pointed out two important things. First, the results substantiate the capacity of a CTLp assay to predict GvHD [6, 7] because this test apparently detects serologically undetectable class I subtype incompatibilities that can be as detrimental as serologically detectable ones [14]. Second, together with the results of HLA-C and DR oligotyping, it shows that the number of histoincompatibilities in a group of unrelated ABDR-matched D/Rs is still significant. Moreover, it shows that, once donor and recipient are found to be incompatible for one antigen—and thus express antigens with a weaker linkage disequilibrium—the chance is great that they are incompatible for a second one. As in Table 1, in all AB-matched D/Rs tested in our laboratory (n = 70), we found that DR-mismatched donors are much more likely to display patient-specific class-I-restricted CTL activity than DR compatible donors (56% vs 27%). If this holds in general, one might argue

TABLE 3 Further characterization of CTLp incompatibility between B44+ individuals

Targets	Clones		
	anti-B4402[a]	anti-B4403[b]	anti-B4403 associated[c]
B4402			
Cw1-8,Cw1-8	13/13[d]	0/13	0/13
Cw1-8,Cwx	9/9	0/9	0/9
B4403			
Cw1-8,Cw1-8	0/6	6/6	0/6
Cw1-8,Cwx	0/13	13/13	12/13
B44-			
Cw1-8,Cw1-8	0/8	0/8	0/8
Cw1-8,Cwx	0/4	0/4	0/4

[a] p1d → p1 and p2d2 → p2d1.

[b] p2d1 → p2d2.

[c] p2d1 → p2 and p6d1 → p6.

[d] Targets lysed per targets tested.

that the negative effect of class II incompatibilities on the outcome of a bone marrow transplantation is partially caused by a (second) class I incompatibility and that single class II mismatches (that is, the CTL-negative ones) might be acceptable.

ACKNOWLEDGMENTS

This work was supported in part by the *Ligue Genevoise contre le Cancer*, by the *Fondation Dr. H. Dubois-Ferrière-Dinu-Lipatti*, and by the Swiss National Science Foundation (3100-33620.92). A.S.S. (on leave from the National HLA Center, IKEM, Prague) was supported by the *Fondation de l'Europe des Sciences et des Cultures* (grant 01/1992).

REFERENCES

1. Ash R, Casper JT, Chitambar CR, Hansen R, Bunin N, Truitt RL, Lawton C, Murray K, Hunter J, Baxter-Lowe LA, Gottschall JL, Oldham K, Anderson T, Camitta B, Menitove J: Successful allogeneic transplantation of T-cell-depleted bone marrow from closely HLA-matched unrelated donors. N Engl J Med 322:485, 1990.
2. Kernan NA, Bartsch G, Ash R, Beatty P, Champlin R, Filipovich A, Gajewski J, Hansen JA, Henslee-Downey J, McCullough J, McGlave P, Perkins HA, Phillips GL, Sanders J, Stoncek D, Thomas ED, Blume KG: Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med 328:593, 1993.
3. McGlave P, Bartsch G, Anasetti C, Ash R, Beatty P, Gajewski J, Kernan NA: Unrelated donor marrow transplantation therapy for chronic myelogenous leukemia: initial experience of the national marrow donor program. Blood 81:543, 1993.
4. Tiercy J-M, Morel C, Freidel AC, Zwahlen F, Gebuhrer L, Betuel H, Jeannet M, Mach B: Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. Proc Natl Acad Sci USA 88:7121, 1991.
5. Tiercy J-M, Roosnek E, Mach B, Jeannet M: Bone marrow transplantation with unrelated donors: HLA class II oligonucleotide typing as predictive of mixed lymphocyte culture reactivity. Transplant Proc 25:1243, 1993.
6. Kaminski E, Hows J, Man S, Brookes P, Mackinnon S, Hughes T, Avakian O, Goldman JM, Batchelor JR: Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantation. Transplantation 48:608, 1989.
7. Roosnek E, Hogendijk S, Zawadynski S, Speiser D, Tiercy J-M, Helg C, Chapuis B, Gratwohl A, Gmür J, Seger R, Jeannet M: The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. Transplantation 56:691, 1993.
8. Rufer N, Tiercy J-M, Speiser D, Helg C, Gratwohl A, Chapuis B, Jeannet M, Roosnek E: Characterization of pretransplant CTL activity in "perfectly matched" unrelated bone marrow donor/recipient combinations. Bone Marrow Transplant 11(Suppl 1):20, 1993.
9. Bodmer JG, Marsh SGE, Albert ED, Bodmer WF, Dupont B, Erlich HA, Mach B, Mayr WR, Parham P, Sasazuki T, Schreuder GMTh, Strominger JL, Svejgaard A, Terasaki PI: Nomenclature for factors of the HLA system, 1991. Tissue Antigens 39:161, 1992.
10. Fleischhauer K, Kernan NA, Dupont B, Yang SY: The two major subtypes of HLA-B44 differ for a single amino acid in codon 156. Tissue Antigens 37:133, 1991.
11. Roosnek E, Lanzavecchia A: Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. J Exp Med 170:297, 1989.
12. Tiercy J-M, Grundschober C, Jeannet M, Mach B: A comprehensive HLA-DRB, -DQB and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes. In Hui K, Bidwell J (eds): Handbook of HLA Tissue Typing Techniques. Boca Raton, FL, CRC, 1993, p 117.
13. De Waal LP, Mesman HJ, Engelfriet CP: Serological and biochemical identifications of HLA-Aw33 subtypes. Immunogenetics 26:211, 1987.
14. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY: Bone marrow–allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N Engl J Med 323:1818, 1990.

HLA-B35-Subtype Mismatches in ABDR Serologically Matched Unrelated Donor–Recipient Pairs

Nathalie Rufer, Birgitta Breur-Vriesendorp, Jean-Marie Tiercy, Dominique Gauchat-Feiss, Xiaowen Shi, Anthony Slavcev, Neubury Lardy, Bernard Chapuis, Alois Gratwohl, Daniel Speiser, Michel Jeannet, and Eddy Roosnek

ABSTRACT: We have characterized HLA incompatibilities in a group of 17 B35-positive patients who were ABDR matched (AB serology and oligotyping for DR 1–14) with their 28 (unrelated) potential bone marrow donors. High-resolution oligotyping for DR subtypes disclosed that nine combinations were in fact DR mismatched. Cytotoxic T-lymphocyte (CTL) activity was detected in nine combinations (32%). In the group matched for DR subtypes, three (16%) of 19 combinations were CTL positive. Patient-specific cytotoxic activity appeared to be directed against HLA C (two cases) or against a subtype of B35. In the group of DR-subtype-mismatched combinations, CTL activity was found in six (67%) of nine pairs. In all four cases that were studied in detail, however, CTL reactivity appeared to be directed against a variant subtype of B35. We have studied the B35 incompatibilities recognized in five different combinations by specificity analysis of the B35-specific CTLs and by partially sequencing of relevant segments of B35 exon 3. Preliminary data show that, within this relatively small Caucasoid group, at least five B35-variant subtypes could be distinguished. This would make B35 an antigen that will be frequently subtype mismatched, in particular when DR matching is done with low resolution (DR 1–14) only. *Human Immunology 41, 96–101 (1994)*

ABBREVIATIONS

CTL	cytotoxic T lymphocyte
CTLp	CTL precursor
D/R	donor–recipient
GvHD	graft-versus-host disease
PCR	polymerase chain reaction

INTRODUCTION

Conventional typing techniques that are usually satisfactory to determine whether a sibling donor–recipient (D/R) pair is histocompatible are less precise when an unrelated potential donor has to be tested. The occurrence of serologically indistinguishable HLA subtypes and/or unknown HLA polymorphism does complicate the analysis to an extent that a significant number of mismatches remain undetected. Not only does this prevent optimal donor selection, but this also hampers the analysis of the influence of HLA mismatches on the outcome of a transplantation as the degree of being mismatched of a given D/R combination will often have been incorrectly assessed. This problem is close to being resolved for DRB1 typing [1, 2], where molecular techniques that distinguish most of the alleles within a population have become routine in the tissue-typing laboratory.

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0198-8859/94/$7.00

Human Immunology 41, 96–101 (1994)

For class I, however, matching is still done by serology. At present, the consequences of the fact that this technique does not discriminate class I subtypes become evident. Indeed, serologically ABDR-matched D/R pairs appear to be more frequently AB-subtype mismatched than has been anticipated [3–5]. Because alloreactive T cells efficiently recognize these subtype mismatches, T-cell-mediated complications after transplantation, such as graft-versus-host disease (GvHD) and graft rejection, might be as hazardous as in patients undergoing transplantation with a donor mismatched for an antigen that is discriminated by serology [6–8].

Class-I-subtype mismatches between serologically matched unrelated D/Rs also activate cytotoxic T lymphocytes (CTLs) in vitro. Therefore, assays that estimate the frequency of patient-specific CTL precursors (CTLp) are effective tools for predicting the occurrence of GvHD [6, 8]. These techniques also enable mismatches between unrelated individuals to be identified directly by characterization of the specificity of the CTL clones [3, 5, 9]. We have used this method to study the incidence of subtype mismatches of HLA B35, an antigen that is known to be a composite of at least eight subtypes [10–14].

PATIENTS, MATERIALS AND METHODS

HLA matching of donor–recipient pairs. Unrelated donors were selected on the basis of HLA matching by serotyping for HLA-A and -B, and by HLA oligotyping for DR1–14. All B35-positive patients for whom an unrelated donor compatible for the antigens just mentioned had been recruited since January 1990 were included in this study. After the first selection, the combinations were further typed by high-resolution oligotyping for DR/DQ [1] and serotyping for Cw1–Cw8. In some of the individuals, no HLA-C allele could be identified.

Primary CTLp-test. Limiting numbers of donor mononuclear cells (5×10^4–0.125×10^4) were cultured in 200-μl 96 round-bottomed plates with constant numbers (5×10^4) of irradiated (2500 rad) recipient mononuclear cells as stimulators. After 10 days of culture, the wells were tested against ^{51}Cr-labeled, phytohemagglutinin-stimulated recipient T-cell blasts [8].

Cloning of patient-specific T cells. Cytotoxic T-cell clones were established from the positive wells of the CTLp assay as described [15]. Specificity toward recipient cells was tested using autologous (donor) cells and third-party cells that did not express HLA antigens in common as negative controls.

Genomic analysis of B35-subtype variants. Partial HLA-B35 direct sequencing was done after polymerase chain reaction (PCR) amplification of cDNA by using a biotinylated 5′ primer and a B-specific 3′ primer [13], with minor modifications (Gauchat et al. submitted). After separation of the biotinylated strand by streptavidin-coated M-280 Dynabeads, B35 alleles were sequenced by the dideoxy-chain termination Sanger method (USB kit) over a segment comprising over 60 nucleotides of exon 3 (amino acids 94–116). Discrimination of B*3501/B*3508 that differ for a single nucleotide at position 156 was done by oligotyping after B-specific exon-3 PCR [16] for the B*3508-specific nucleotide sequence encoding Arg156.

RESULTS

Definition of an (ABDR)-matched pair. The definition of a matched (unrelated) D/R pair depends on the resolution of the tissue-typing techniques applied. Even a frequently used designation such as ABDR matched is ill-defined as, for instance, some groups would classify D/Rs who differ for serologic HLA-AB splits as compatible [17], whereas others would not [18, 19]. Throughout this study, we will use the designation ABDR matched for combinations that are identical for the specificities that are discriminated by classic serology (A, B, and DR1–14), but might still be incompatible for HLA-C, -DQ, or -DP and for subtypes of HLA-A, -B, and -DR.

Histoincompatibilities in B35-positive ABDR-matched unrelated D/R pairs. Table 1 shows that by high-resolution typing techniques a number of incompatibilities can still be detected in the 28 ABDR-matched combinations studied. Five combinations were HLA-C incompatible. High-resolution oligotyping [2] revealed DR-subtype incompatibilities in nine combinations. Furthermore, patient-specific CTLs could be generated in three DR-subtype-compatible and in six DR-subtype-incompatible pairs. Analysis of the CTL specificity showed that in the DR-compatible combinations the CTLs recognized HLA-C (two cases) or a B35 variant. In the DR-subtype-incompatible pairs, all cytotoxicity was directed against B35 variants.

Evidence of the occurrence of at least five variant subtypes of B35 in the Caucasoid population. To characterize the fine specificity of the CTL clones in more detail, we selected 13 of the 28 combinations for further analysis. These selected combinations comprise seven of the nine CTLp-positive, four of the five HLA-C-incompatible, and four of the nine DR-subtype-incompatible combinations (Table 2). The (patient) B35 antigens inducing (donor) cytotoxic T-cell activity were analyzed by testing the B35-specific clones against a panel of B35-positive cells that were selected to express B35 molecules discriminated by

TABLE 1 CTLp positivity in 28 B35-positive D/R combinations and analysis of the mismatches recognized by the CTL-clones

	Patients[a]	D/Rs	DR = [b]	DR' subtype ≠
No B35$^+$ D/R	17	28	19	9
No (%) CTLp$^+$ D/R	6 (35%)	9 (32%)	3 (16%)	6 (67%)
Incompatibility recognized			B35 (1×) Cw7 (2×)	B35 (6×)

[a] Detailed HLA typing of the nine patients (13 D/R combinations) studied in detail are depicted in Table 2.

[b] ABDR-matched, DR-subtype-matched combinations.

[c] ABDR-matched, DR-subtype-mismatched combinations.

CTLs. Four of these cellularly defined B35 variants (A–D) have been described [9, 20]. Variants 2 (L-L) and A′ (PER and 4029) have isoelectric points identical to IEF-B35.2 and the most acidic IEF-B35* [21], respectively (data not shown), and therefore are distinct B35 subtypes.

The data in Table 3 show that from the five CTLp cultures with anti-B35 activity, CTL clones with six distinct specificities could be established. Donor-derived CTLs against patient 1 (d → p1) lysed the cells expressing the variants A′ and 2. Because the two specificities appeared to be associated with two distinct sets of T-cell clones, and none of the clones lysed the targets expressing the A–D variants, these clones were considered to be specific for A′ and 2. Consequently, the B35 serologically homozygous patient 1 (p1) was assumed to express A′ and 2 and the donor of patient 4 (p4d) A′. CTL reactivity of d → p2 was directed against C. Therefore, C could be assigned to p2 and p4d. CTL reactivity of d → p3 appeared to be directed against variants A and D. On the basis of the B35 sequences found, however (see below), p3 could be designated A univocally. In contrast, p4 and p5d, for whom we do not yet have conclusive sequence data, cannot be classified and are therefore designated D? (sequence unknown but A could be excluded) and A/D.

TABLE 2 HLA typing of B35$^+$ ABDR-matched donor–recipient combinations[a]

	A	B	C	DRB1	CTLp[b]
p1[c]	24,24	35,35	*4*[d]	*0411*,1101	
p1d	24	35	1,7	0401,1101	+
p2	11	55,35		*0402*,1501	
p2d	11	35,55		0407,1501	+
p3	2,3	35	NT[e]	8,*1303*	
p3d	2,3	35	NT	8,1302	+
p4	3,24	35		*1101*,1301	
p4d	3,24	35		1104,1301	+
p5	3,24	18,35	4,7	301,1501	
p5d1	3,24	18,35	4,5	301,1501	+
p5d2	3,24	18,35	4,5	301,1501	+
p5d3	3,24	18,35	4,5	301,1501	+
p6	3,32	22,35	3,4	0101,1401	
p6d	3,32	22,35	3,4	0101,1401	−
p7	11	13,35	4,6	0101,7	
p7d1	11	13,35	4,6	0101,7	−
p7d2	11	13,35	4,6	0101,7	−
p8	26,29	35,38	4	0101,1301	
p8d	26,29	35,38	4,7	0101,1301	−
p9	1,2	8,35	4,7	0101,0301	
p9d1	1,2	8,35	4,7	0101,0301	−
p9d2	1,2	8,35	4,7	0101,0301	−

[a] Results are shown only for the 13/28 ABDR-matched D/R combinations that were studied in detail.

[b] Range CTLp: $8/10^6$–$100/10^6$ (number of patient-specific CTLp per 10^6 donor mononuclear cells).

[c] p1, patient 1; p1d1, donor 1 of patient 1; etc.

[d] Incompatibilities are printed in ***bold italic***.

[e] NT, not tested (blank, not identified).

One other type of cross-reactive CTL clone was found in d → p4 that yielded two different types of activities. One clone appeared to be specific for A/D, confirming the expression of A on p3, the other for A/B/D. Finally, the CTL reactivity of d → p5 appeared to be specific for B, thereby assigning B to p1d, p2d, p4, p5, and to the four CTLp-negative combinations p6–9. To investigate whether the cellular patterns could be directly explained by the occurrence of different B35-subtype variants, we performed a partial genomic analysis of the five D/Rs known to comprise the five distinct cellularly defined B35 variants. We sequenced a segment of over 60 nucleotides of exon 3 to discriminate between B*3501/07/08, B*3502, B*3503, B*3504, B*3505, and B*3506, respectively. Furthermore, we performed PCR–sequence-specific oligonucleotide oligotyping of position 156 in exon 3 to discriminate B*3501 from B*3508. At present, we are able to identify the sequences of amino acids 94–116 characteristic for five published B35 subtypes. Assuming that these sequences indeed represent

TABLE 3 B35 subtypes and specificity of the alloreactive CTLs

Target	B35[a] (T cell)	d → p1[b] A′	d → p1[b] 2	d → p2 C	d → p3 A/D	d → p4 A/D	d → p4 A/B/D	d → p5 B	Subtype
GRO	B	−	−		−		+		
P-D	B	−	−	−	−	−	+	+	
WYT	B	−	−		−				
PET	D	−	−	−	+	+	+	−	
BRE	A	−	−		+		+	−	
ROO	A	−	−		+				
WOR	A	−	−		+				
L-L	2	−	+		−	−	−	−	
W-H	C	−	−	+	−	−	−	−	
KAM	C	−	−		−		−		
PER	A′	+	−			−	−	−	
4029	A′	+	−		−		−		
p1	A′ + 2	+	+		−	−	−	−	3502/3505
p1d	B	−	−		−	−	+	+	3501
p2	C	−	−	+		−	−	−	3508
p2d	B	−	−	−	−	−	+	+	3501
p3	A	−	−		+	+	+	−	3503
p3d	B	−	−		−	−	+	+	3501
p4	B + D?	−	−	−	+	+	+	+	3501/35x?
p4d	A′ + C	+	−	+	−	−	−	−	3502/3508
p5	B	−	−		−	−	+	+	3501
p5d	A/D	−	−		+	+	+	−	
p6	B	−	−		−		+	+	
p6d	B	−	−		−		+	+	
p7	B	−	−		−			+	
p7d	B	−	−		−			+	
p8	B	−	−		−		+	+	
p8d	B	−	−		−		+	+	
p9	B	−	−		−		+	+	
p9d1	B	−	−		−		+	+	
p9d2	B	−	−		−		+	+	

[a] As defined by CTLs that discriminate B35-positive individuals.

[b] CTL clones (2–5/combination) established from primary cultures (d → p1, patient 1 with his ABDR-compatible donor; etc.)

[c] Specificity of CTLs based on recognition of panel cells.

the known subtypes and not new subtypes identical for the stretch of nucleotides sequenced, we were able to assign the CTLp positivity of all five combinations to distinct incompatible B35 subtypes. The most frequent variant B in our panel was identified as B*3501, the dominant allele in Caucasoids [21, 22]. Variant A′ was identified as B*3502, variant A as B*3503, variant 2 as B*3505, and variant C as B*3508. Therefore, all CTLp activity could be attributed to the presence of incompatible B35 subtypes. Because all of these subtypes were present in a relatively small number of Caucasoid individuals, it is likely that ABDR-matched D/Rs will be frequently B35-subtype incompatible.

DISCUSSION

Bone marrow transplantation with donors other than HLA-identical siblings is associated with an increased incidence of GvHD and graft rejection [17–19, 23–25]. Although this certainly reflects a higher degree of incompatibility between donor and recipient, the precise effect of HLA mismatches has remained difficult to assess. While some studies show significant correlations between the presence of one antigen mismatch and the occurrence of GvHD [18, 19, 24] or graft rejection [26], others do not [17]. The reason for this might be that the effect of one mismatch is hidden by the effect of mismatches of HLA antigens for which D/Rs are supposed to be compatible. This is not unlikely, as our standard tissue-typing techniques do not identify all of the variants of HLA antigens that the immune system is able to recognize.

The imperfectness of standard serologic tissue-typing techniques is well documented for class II. Genomic typing techniques still reveal numerous DR-subtype incompatibilities in serologically DR-matched pairs [1,

27]. It now becomes evident that the same holds for class I. Cellular [3, 5, 8, 9] and molecular biological techniques [4, 16] still detect a significant heterogeneity in serologically apparent homogeneous class I antigens. Of course, due to linkage disequilibrium between the HLA antigens, the change of being mismatched for class I subtype varies with the degree of matching for the other loci. In fact, as we have reported previously [16], the chance of being mismatched for B44, for instance, is rather low when a D/R pair is AB/DR-subtype matched. It is only by accepting such DR-subtype mismatches that the chance of an additional class-I-subtype mismatch becomes significant. This effect seems to be even more substantial for B35. In 19 ABDR-matched D/R pairs who were also matched for DR subtypes, only one B35-subtype incompatibility was found. In the nine ABDR-matched D/R pairs who were DR-subtype mismatched, however, as many as six appeared to be class-I-subtype incompatible. In all four cases studied, they were identified as a B35-subtype variant. Therefore, while selecting unrelated donors by class I serology and oligotyping for class II, two types of DR-subtype-mismatched potential donors will be found. While some will be mismatched for the DR subtype only, others will be also class-I-subtype mismatched. It is clear that when a search has failed to identify a DR-matched donor and one would be willing to accept a DR-subtype mismatch, only the class-I-matched donor would be acceptable. It is in such circumstances that a high-resolution class I typing will be a prerequisite.

ACKNOWLEDGMENTS

The authors are grateful to Dr. D. Schendel for helpful discussion. This work was supported in part by the Swiss National Science Foundation (32-35641.92 and 31-36247.92) and by the *Ligue Genevoise contre le Cancer*. A.S. (on leave from the National HLA Center IKEM Praha) was supported by the *Fondation de l'Europe des Sciences et des Cultures* (grant 01/1992).

REFERENCES

1. Tiercy J-M, Morel C, Freidel AC, Zwahlen F, Gebuhrer L, Betuel H, Jeannet M, Mach B: Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. Proc Natl Acad Sci USA 88:7121, 1991.
2. Tiercy J-M, Grundschober C, Jeannet M, Mach B: A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes. In Hui KMB (ed): Handbook of HLA Typing Techniques. Boca Raton, FL, CRC, 1993, p 117.
3. Rufer N, Tiercy J-M, Speiser D, Helg C, Gratwohl A, Chapuis B, Jeannet M, Roosnek E: Characterization of pretransplant CTL activity in "perfectly matched" unrelated bone marrow donor/recipient combinations. Bone Marrow Transplant 11(Suppl 1):20, 1993.
4. Santamaria P, Reinsmoen NL, Lindstrom AL, Boyce-Jacino MT, Barbosa JJ, Faras AJ, McGlave PB, Rich SS: Frequent HLA class I and DP sequence mismatches in serologically (HLA-A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA-identical unrelated bone marrow transplant pairs. Blood 83:280, 1994.
5. Rufer N, Breur-Vriesendorp BS, Tiercy J-M, Slavcev AS, Lardy NM, Francis P, Kressig R, Speiser DE, Helg C, Chapuis B, Gratwohl A, Jeannet M, Roosnek E: High-resolution histocompatibility testing of a group of sixteen B44-positive, ABDR serologically matched unrelated donor–recipient pairs: analysis of serologically undisclosed incompatibilities by cellular techniques, isoelectrofocusing, and HLA oligotyping. Hum Immunol 38:235, 1993.
6. Kaminski E, Hows J, Man S, Brookes P, Mackinnon S, Hughes T, Avakian O, Goldman JM, Batchelor JR: Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantation. Transplantation 48:608, 1989.
7. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY: Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N Engl J Med 323:1818, 1990.
8. Roosnek E, Hogendijk S, Zawadynski S, Speiser D, Tiercy J-M, Helg C, Chapuis B, Gratwohl A, Gmür J, Seger R, Jeannet M: The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. Transplantation 56:691, 1993.
9. Breuning MH, Breur BS, Engelsma MY, Goulmy E, Ivanyi P: Activation of cytotoxic T lymphocytes in HLA-A, -B and -C-identical responder–stimulator pairs. II. New subtypes of HLA-Bw35. Tissue Antigens 24:90, 1984.
10. Theiler G, Pando M, Delfino JM, Takiguchi M, Satz ML: Isolation and characterization of two new functional subtypes of HLA-B35. Tissue Antigens 41:143, 1993.
11. Chertkoff LP, Herrera M, Fainboim L, Satz ML: Complete nucleotide sequence of a genomic clone encoding HLA-B35 isolated from a Caucasian individual of Hispanic origin: identification of a new variant of HLA-B35. Hum Immunol 31:153, 1991.
12. Lederer E, Nossner E, Wank R, Schendel DJ: Analysis of HLA-B35 variants and B35 haplotypes by isoelectric focusing and Southern blot analysis. Immunogenetics 30: 63, 1989.
13. Zemmour J, Little AM, Schendel DJ, Parham P: The HLA-A,B "negative" mutant cell line C1R expresses a novel HLA-B35 allele, which also has a point mutation in the translation initiation codon. J Immunol 148:1941, 1992.
14. Ooba T, Hayashi H, Karaki S, Tanabe M, Kano K, Takiguchi M: The structure of HLA-B35 suggests that it

is derived form HLA-Bw58 by two genetic mechanisms. Immunogenetics 30:76, 1989.

15. Roosnek E, Lanzavecchia A: Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. J Exp Med 170: 297, 1989.
16. Tiercy J-M, Djavad N, Rufer N, Speiser D, Jeannet M, Roosnek E: Oligotyping of HLA-A2, -A3, and -B44 subtypes: detection of subtype incompatibilities between patients and their serologically matched unrelated bone marrow donors. Hum Immunol 1994 (in press).
17. Kernan NA, Bartsch G, Ash R, Beatty P, Champlin R, Filipovich A, Gajewski J, Hansen JA, Henslee-Downey J, McCullough J, McGlave P, Perkins HA, Phillips GL, Sanders J, Stoncek D, Thomas ED, Blume KG: Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med 328:593, 1993.
18. Beatty PG, Clift RA, Michelson EM, et al.: Marrow transplantation from related donors other than HLA-identical siblings. N Engl J Med 313:765, 1985.
19. Ash R, Casper JT, Chitambar CR, Hansen R, Bunin N, Truitt RL, Lawton C, Murray K, Hunter J, Baxter-Lowe LA, Gottschall JL, Oldham K, Anderson T, Camitta B, Menitove J: Successful allogeneic transplantation of T-cell-depleted bone marrow from closely HLA-matched unrelated donors. N Engl J Med 322:485, 1990.
20. Neefjes JJ, Breur-Vriesendorp BS, van Seventer GA, Ivanyi P, Ploegh HL: An improved biochemical method for the analysis of HLA-class I antigens: definition of new HLA-class I subtypes. Hum Immunol 16:169, 1986.
21. Yang SY: Population analysis of class I HLA antigens by one-dimensional isoelectrofocusing gel analysis electrophoresis: workshop summary report. In Dupont B (ed): Immunobiology of HLA. New York, Springer-Verlag, 1989, p 309.
22. Steinle A, Reinhardt C, Nössner E, Uchanska-Ziegler B, Ziegler A, Schendel DJ: Microheterogeneity in HLA-B35 alleles influences peptide-dependent allorecognition by cytotoxic T cells but not binding of a peptide-restricted monoclonal antibody. Hum Immunol 38:261, 1993.
23. Hows J, Bradley BA, Gore S, Downie T, Howard M, Gluckman E: Prospective evaluation of unrelated donor bone marrow transplantation. Bone Marrow Transplant 12:371, 1993.
24. Beatty PG, Anasetti C, Hansen JA, et al.: Marrow transplantation from unrelated donors for treatment of hematologic malignancies: effect of mismatching for one HLA locus. Blood 81:249, 1993.
25. Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE, Thomas ED, Hansen JA: Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. Hum Immunol 29:79, 1990.
26. Anasetti C, Amos D, Beatty PG, et al.: Effect of HLA compatibility on engraftment of bone marrow transplants in patients with leukemia or lymphoma. N Engl J Med 320:197, 1989.
27. Tiercy J-M, Roosnek E, Speiser D, Cros P, Allibert P, Mach B, Jeannet M: Replacement of HLA class II serology by the HLA-DR microtitre plate oligotyping assay: a one-year experience in unrelated bone marrow donor selection. Br J Haematol 85:417, 1993.

Chapter 4

Sequencing of a new HLA-B41 subtype (B*4102) that occurs with a high frequency in the Caucasoid population

Immunogenetics (1995) 41: 333

SEQUENCE REGISTER

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Sequencing of a new HLA-B41 subtype (*B*4102*) that occurs with a high frequency in the Caucasoid population

Received: 23 November 1994 / Revised: 6 December 1994

Incompatibilities detected by the cytotoxic T-lymphocyte precursor frequency test [(CTLpf) (Rufer et al. 1993)] in serologically matched unrelated bone marrow donor/recipient pairs are usually caused by HLA subtypes (Bodmer et al. 1994) that escape detection by our routine tissue typing procedures. Here we present the case of a patient for whom three of six serologically HLA-identical unrelated donors were incompatible by CTLpf analysis. By testing patient-specific clones on a panel of serologically typed individuals, the incompatibility was mapped to the B41 antigen. We report here the complete nucleotide and deduced amino-acid sequence of a novel B41 subtype, now referred to as *B*4102*, from one of these donors.

cDNA polymerase chain reaction (PCR) cloning from donor SBD4 (HLA A1,2; B8,41; DRB1*0301,1303; DRB3*0101), selection and sequencing of the HLA-B41-specific M13 clones were done as previously described (Gauchat-Feiss et al. 1994). The full-length sequence of *B*4102* is shown in Figure 1. *B*4102* differs from *B*4101* by 6 nucleotide substitutions in exon 3 (α2 domain): TTGG ⇒ CCTC at positions 354–357, G ⇒ C at position 363, and T ⇒ C at position 369. These correspond to the following amino acid changes : Trp ⇒ Leu at codon 95 and Arg ⇒ Ser at codon 97. Two neutral substitutions occured at codons 94 and 99, respectively (Fig. 2). In order to discriminate the two *B41* alleles, we set up a simple oligotyping procedure, based on exon 3 group-specific PCR, followed by hybridization with probes W95 [(5'-CTCTGCCAAGTGTG) (B*4101)] and S97 [(5'-CGTACATGCTCTGG) (B*4102)]. Oligotyping of the patient and four of his donors showed that two donors shared the *B*4101* allele with the patient, whereas the remaining two donors were *B*4102*, thereby confirming the CTLpf results. A further analysis of a panel of 11 B41-positive Caucasoid individuals showed that *B*4102* is as frequent as the *B*4101* allele (7/16).

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The nucleotide sequence data reported in this paper have been submitted to the EMBL database and have been assigned the accession number X81363. The name *B*4102* was officially assigned by the WHO Nomenclature Committee in November 1994. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature Report (Bodmer et al. 1994), names will be assigned to new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature Report

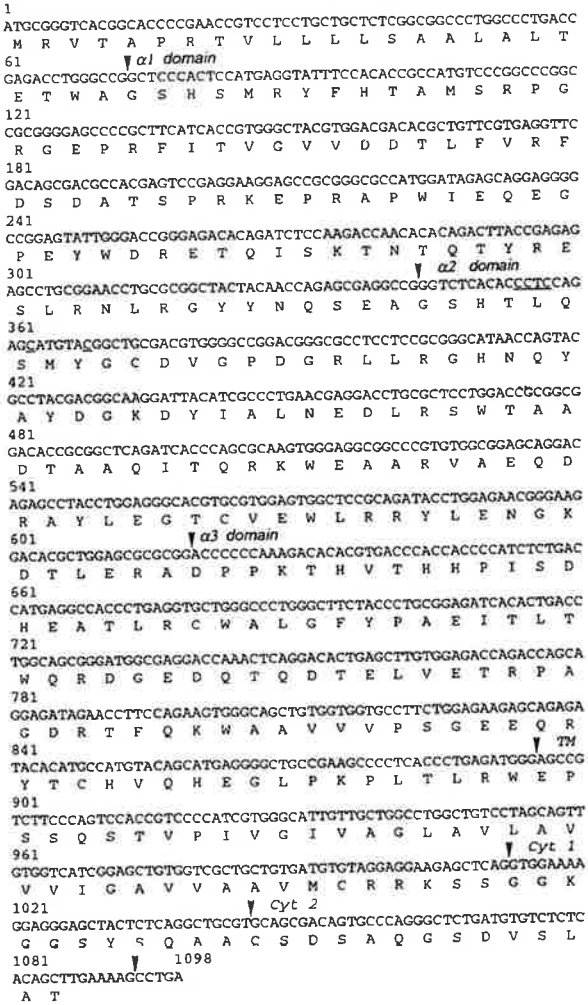

Fig. 1 Complete nucleotide (1–1098) and deduced amino acid sequence of the *B*4102* allele. Nucleotides differing from *B*4101* are *underlined*. *Arrowheads* mark exon boundaries

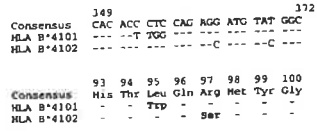

Fig. 2 Comparison of the *B*4101* and *B*4102* nucleotide and amino acid sequences from codons 93–100 of the α2-domain

References

Bodmer, J. G., Marsh, S. G. E., Albert, E. D., Bodmer, W. F., Dupont, B., Erlich, H. A., Mach, B., Parham, P., Sasazuki, T., Schreuder, G. M., Strominger, J., Svejgaard, A., and Terasaki, P. I. Nomenclature for factors of the HLA system 1994. *Tissue Antigens 44:* 1–18, 1994

Gauchat-Feiss, D., Breur-Vriesendorp, B. S., Rufer, N., Jeannet, M., Roosnek, E., and Tiercy, J.-M. Sequencing of a novel functional HLA-B44 subtype differing in two residues in the alpha 2 domain. *Tissue Antigens 44:* 261–264, 1994

Rufer, N., Breur-Vriensendorp, B. S., Tiercy, J.-M., Slavcev, A. S., Lardy, N. M., Francis, P., Kressig, R., Speiser, D. S., Helg, C., Chapuis, B., Gratwohl, A., Jeannet, M., and Roosnek, E. High resolution histocompatibility testing of a group of sixteen B44-positive, ABDR serologically matched unrelated donor-recipient pairs. *Hum Immunol 38:* 235–239, 1993

Chapter 5

High resolution HLA matching associated with decreased mortality after unrelated bone marrow transplantation

High resolution HLA matching associated with decreased mortality after unrelated bone marrow transplantation

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Blood 1996 (in press)

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5.1 ABSTRACT

As compared with related HLA identical sibling donors, bone marrow transplantation (BMT) with phenotypically HLA ABDR-'compatible' unrelated donors is associated with increased mortality. This may be due to hidden HLA incompatibilities not detected by conventional typing. We have analyzed 44 unrelated patient-donor pairs who were matched for HLA-A, -B, and -DR by routine tissue typing. Our comprehensive HLA typing approach consisted of serology, cytotoxic T cell precursor (CTLp) tests, T cell cloning, oligotyping and DNA sequencing. By these techniques we identified numerous HLA allele mismatches not detected by the previously applied routine typing. 24 patient-donor pairs were highly matched and had a low CTLp frequency, while the remaining 20 pairs were allele mismatched for HLA-A,-B,-C,-DR,-DQ antigens, and/or had a positive CTLp test. Patient and donor age, diagnosis, and treatment did not differ significantly between the 'matched' and 'mismatched' transplants. The probability for severe acute graft-versus-host disease grades III-IV was 21% in the 'matched' and 47% in the 'mismatched' patients (p=0.0464). Transplant related mortality was 21% and 57% (p=0.0072) and actuarial patient survival rates at three years 61% and 13% (p=0.0005). We conclude that both HLA class I and class II allele mismatches between unrelated phenotypically ABDR-'compatible' patient-donor pairs are frequent and associated with increased incidence of posttransplant complications.

5.2 INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is an accepted treatment for numerous hematological disorders.[1-3] The success rate of BMT has steadily increased in recent years, but graft-versus-host disease (GvHD) and other immune dysfunctions are still major causes of posttransplant mortality.[4,5] The degree of HLA compatibility between patient and donor significantly influences the incidence and severity of such complications.[6-11]

Only about 35% of patients have a genotypically HLA identical sibling donor. For many of the remaining patients it is now possible to find an HLA ABDR-'compatible' unrelated donor among approximately 2.6 million volunteers so far registered. It is important to note, however, that such donors will only be phenotypically (but not genotypically) HLA matched. Moreover, there is distinct evidence that fine HLA specificities frequently differ between conventionally typed HLA ABDR-'compatible' unrelated individuals,[11-16] a relevant finding for graft outcome.[17] Because standard HLA typing techniques do not discriminate between many of the existing (and in part as yet not characterized) polymorphic HLA alleles, an unrelated donor might be phenotypically ABDR-'compatible' but nevertheless incompatible at the DNA sequence level for one or more HLA alleles.

Compared with HLA genotypically identical related BMT, unrelated grafting is associated with a higher mortality rate.[7-10] We addressed the hypothesis that this may be due to HLA allele mismatches undetected by routine typing methods.[12]

5.3 PATIENTS AND METHODS

Patient population. All 34 consecutive transplants performed in the years 1990-1994 in patients from Switzerland were included in this study; no patients were excluded. In addition, all patients (5) from Helsinki and all patients (5) from Hamburg for whom sufficient blood cells had been cryopreserved to allow cytotoxic T lymphocyte precursor (CTLp) and HLA subtype analysis were included.

Diagnoses, stage of disease at the time of transplantation, age of patients and donors, CMV serology, preconditioning, and GvHD prophylaxis are shown in Table 1. 40% of the donors were female (55% in the 'matched', 22% in the 'mismatched' group), 48% of the patients were female (48%,47%), and 45% of the transplants were sex mismatched (61%,32%). There was no statistically

significant difference between 'matched' and 'mismatched' grafts with respect to diagnosis, age, sex matching, CMV serology, preconditioning, and GvHD prophylaxis.

HLA typing and patient-donor matching. According to our selection policy, no unrelated donors other than those who were HLA ABDR 'compatible' defined by HLA-A and -B serology (including splits) and generic oligotyping for HLA DR1-14 were accepted for bone marrow donation. Blood samples of the 44 patients and 167 unrelated donors recruited through the unrelated bone marrow donor registries were analyzed. Each patient was transplanted with the donor who had the highest achievable HLA-compatibility. HLA-A and -B was typed by serology. The subtypes of HLA-A2, A3, B35, and B44 were determined as described.[16,18] HLA-C SSO-oligotyping was done using an HLA-C specific PCR with primers 5CIn1-61 and 3BCIn3-12[19] followed by hybridization with 26 SSO probes including 19 probes form ref.[20] implemented with 7 probes testing codons Q35, A73, R91, H129, E155, K173, and S178. Hybridization patterns obtained with these 26 probes allowed the discrimination of 23 HLA-C alleles. HLA-DRB DNA typing was performed by the DR microtiter plate oligotyping assay,[21] and additional DRB1 subtypes, DQB1, and DPB1 alleles were determined by hybridization with sequence specific oligonucleotide (SSO) probes after polymerase chain reaction (PCR) amplification.[14,22] The CTLp test was conducted in GvH direction as described by Kaminski et al.[23] The test was considered positive when the precursor frequency was three or more per million PBMC. Subsequently, we generated CTL clones from the positive wells of the CTLp test which confirmed it's positive result and allowed the characterization of the mismatched HLA antigens using panel cells expressing various HLA phenotypes. Finally, the HLA alleles encoding the incompatible antigens recognized by the CTL clones were analyzed by DNA sequencing[24] and/or oligotyping as described above. No HLA-A or -B allele differences could be detected in 24/25 unrelated control pairs with a negative CTLp test. Therefore, a negative CTLp test was predictive for HLA-A and -B compatibility at the allele

(sequence) level[25] making it non-essential to molecularly type all HLA-A and -B alleles of the CTLp negative patient-donor pairs. In parallel to the CTLp test performed in GvH direction, all incompatibilities in GvH direction (donor homozygous / patient heterozygous) but not in rejection direction (patient homozygous / donor heterozygous) were considered as mismatches.

Bone marrow transplant procedure. Patients were transplanted in the five different centers (University Hospitals of Basel, Geneva, Hamburg, Helsinki, and Zürich). Preconditioning was as indicated in Table 1. Preconditioning of the 6 non-leukemic patients was fractionated total body irradiation (3/6), total lymphoid irradiation (1/6), cyclophosphamid (4/6), busulphan (2/6), and VP16 (3/6). GvHD prophylaxis (Table 1) was comparable in the matched and mismatched groups: in vivo T cell depletion, cyclosporin A (CsA) and methotrexate (MTX) were applied in 28% of the patients (29% in the 'matched', 26% in the 'mismatched' group); in vivo T cell depletion and CsA in 7% (4%,11%); CsA and MTX in 61% (63%,58%); and CsA alone in 4% (4%,5%). In vivo T cell depletion employed the monoclonal antibody Campath-1G (day -4 to +5; 10 mg i.v./day). GvHD was classified according to the criteria described by Glucksberg et al.[26]

Statistics. All patients were followed throughout the entire study period (January 1990 to May 1995) and for at least 6 months after BMT or until death. The mean ± SD follow-up of surviving patients was 866 ± 529 and 926 ± 526 days for the 'matched' and 'mismatched' group, respectively. The Kaplan-Meier method and the logrank-test were employed to calculate and compare the probabilities of GvHD, TRM and survival.[27] The remaining parameters were assessed using the Chi-square test. Not significant (n.s.) is indicated where $p>0.05$.

Table 1. Patient characteristics and treatment

	all patients	HLA 'matched'	p	HLA 'mismatched'
(number of patients)	(44)	(24)		(20)
age				
patient age (years; mean±SD)	24.3±12.7	23.4±11.7	n.s.	25.3±13.6
donor age (years; mean±SD)	38.6±8.5	38.1±6.4	n.s.	39.0±10.1
diagnosis				
acute myeloid leukemia (n)	13	8		5
acute lymphoid leukemia (n)	6	2		4
chronic myeloid leukemia (n)	19	12		7
myelodysplastic syndrome (n)	4	2		2
Wiskott-Aldrich syndrome (n)	1	-		1
Histiocytosis (n)	1	-		1
BMT in advanced disease *				
relapse (n)	4	1		3
accelerated phase (n)	3	1		2
blast crisis (n)	1	1		-
CMV serology (donor/patient)				
negative / negative (%)	41	45	n.s.	35
negative / positive (%)	5	-	n.s.	12
positive / negative (%)	30	30	n.s.	29
positive / positive (%)	24	25	n.s.	24
preconditioning				
fract. total body irradiation (%)	86	87	n.s.	83
total lymphoid irradiation (%)	9	9	n.s.	8
cyclophosphamid (%)	93	93	n.s.	92
GvHD prophylaxis				
in vivo T depletion (%)	35	33	n.s.	37
CsA (%)	100	100	n.s.	100
MTX (%)	89	92	n.s.	84

* 'Relapse' refers to patients with acute leukemia in relapse; 'accelerated phase'/'blast crisis' refers to patients with advanced chronic myeloid leukemia. The mean ± SD time from diagnosis to transplantation was 603 ± 267 and 680 ± 494 days for the 'matched' and 'mismatched' group, respectively. (n), number of patients.

5.4 RESULTS

HLA incompatibilities not identified by routine HLA typing were detected using high resolution HLA matching techniques. While 24 (55%) of the patient-donor pairs were HLA matched for A, B, C, DRB1, B3, B5, DQB1 alleles (Table 2a), mismatches were found in 20 (45%) pairs (Table 2b; HLA mismatches in bold characters). In nine patient-donor pairs there were mismatches for DRB1/B3/B5 and/or DQB1 alleles. Three of them were mismatched for two alleles, either DRB1/DQB1, DRB1/B5, or DQB1/C (UPN 376, 420, 326). The remaining eleven patient-donor pairs were matched for class II antigens but mismatched for HLA-A, -B, -C and/or had positive cytotoxic T cell precursor tests (Table 2b). The subsequent cellular and molecular analysis confirmed that the CTL activity was in most cases directed against mismatched HLA class I antigens. The CTLp test was positive in all cases of HLA-A, -B, or -C mismatches with the exception of two patient-donor pairs with HLA-C incompatibilities (UPN 298, 145). Furthermore, there were three CTLp positive patient-donor pairs (UPN 285, 390, 440) without detectable HLA mismatches but with CTL clones specific to particular HLA class I antigens. We could not identify structural differences of these antigens indicating that the CTLs reacted against mismatched 'minor' transplantation antigens. In summary, we identified 20 patient-donor pairs with HLA mismatches and/or a positive CTLp test. Full compatibility for HLA-A, -B, -C, -DRB,-DQB and a negative CTLp test was found in 24 patient-donor pairs. In the following we will refer to the former 20 as the 'mismatched' transplants and the latter 24 as the 'matched' transplants.

HLA-DPB1 oligotyping revealed that only eight (18%) patient-donor pairs were DPB1 matched (23% in the 'matched' and 13% in the 'mismatched' group). Because of this high mismatch frequency, DPB1 compatibility was not taken into account for allocation to the 'matched' and 'mismatched' group.

High degree acute GvHD grade III-IV was observed in 21% of the 'matched' and 47% of the 'mismatched' transplants (p=0.0464, Table 3). In contrast, there was no difference in moderate to severe acute GvHD grade II-IV. The mean grade of

skin GvHD was also similar in the two groups, but the mean grade of liver and gut GvHD was significantly higher in patients receiving 'mismatched' grafts. The incidence of limited and extensive chronic GvHD was comparable in the two groups with a relatively low overall incidence (17%) of extensive chronic GvHD.

Infectious diseases were only slightly but not significantly more frequent in the mismatched as compared to the matched group (Table 3). Bacterial sepsis, CMV pneumonia, and toxoplasmosis were observed in both groups, whereas the two cases of aspergillosis occurred in the matched group.

Major causes of death were GvHD, infection, and leukemia relapse (Table 3). Graft failure was observed in two patients, both with CML transplanted more than one year (14 and 18 month) after diagnosis, one in the first chronic phase and the other in blast crisis. Three of the four patients with acute leukemia transplanted in relapse died from a further relapse diagnosed between 90 and 861 days after BMT. In contrast, the four patients transplanted in advanced CML died from acute GvHD grade IV (2x), interstitial pneumonitis and graft failure.

The transplant related mortality (TRM) was significantly more frequent in 'mismatched' than 'matched' grafts (Figure 1; p=0.0072). Similarly, the survival probability, according to Kaplan-Meier analysis, was significantly better for the 'matched' than the 'mismatched' transplants (Figure 2; p=0.0005).

In view of the known poor prognosis associated with transplantation in advanced disease stage, the comparison of 'matched' and 'mismatched' transplants was repeated after exclusion of the eight patients with advanced disease. The results were comparable to those obtained without exclusion of high risk patients, with acute GvHD III-IV being 23% and 54% (p=0.041), TRM 14% and 70% (p=0.0003) and three year survival probability 67% and 21% (p=0.0002) in 'matched' (n=21) and 'mismatched' (n=15) grafts, respectively.

The complete typing of all six classical HLA loci allowed the evaluation of HLA class I mismatches in class II matched BMT and vice versa. Therefore, we performed an additional analysis after splitting the 'mismatched' transplants in

two groups, i.e. HLA class I and HLA class II mismatched grafts and compared them to the 'matched' group defined above. No significant correlation was observed between matching and severe acute GvHD grades III-IV (Table 4). TRM was significantly higher in the HLA class II mismatched transplants. Finally, patient survival correlated significantly with both class I and class II mismatches.

Table 2a. Histocompatibility of the 'matched' patient-donor pairs

UPN		A	B	Cw	DRB1	DRB3,5	DQB	DPB	CTLp	aGvH
383	p	2	22,62(15)	1,3	0401		0302	0401	neg	II
	d	2	22,62(15)	1,3	0401		0302	0401	Alive	
349	p	3,26	7,49(21)	0702	0405,1501	DRB5*0101	0302,0602	02011,0401	neg	I
	d	3,26	7,49(21)	0702	0405,1501	DRB5*0101	0302,0602	02011,0401	Alive	
373	p	2,28	44(12),44(12)	0501	1301,0401	DRB3*0101	0301,0603	0401	neg	III
	d	2,28	44(12),44(12)	0501	1301,0401	DRB3*0101	0301,0603	0401	Dead	
538	p	2,29(19)	18,4403	7	0701,1101	DRB3*02	nd	0201,0401	neg	III
	d	2,29(19)	18,4403	0701,1601	0701,1101	DRB3*02	nd	0201,0401	Alive	
269	p	1,3	7,4403	0702,1601	1501,07	DRB5*0101	0201	0401,1101	neg	II
	d	1,3	7,4403	0702,1601	1501,07	DRB5*0101	0201	0401,1101	Dead	
360	p	1,31(19)	8,51(5)	0501,0701	0301,1301	DRB3*0101,02	0201,0603	0101,0201	neg	-
	d	1,31(19)	8,51(5)	0501,0701	0301,1301	DRB3*0101,02	0201,0603	0101,0201	Alive	
259	p	2,29(19)	4403,51(5)	nd	07,1101	DRB3*02	0201,0301	**1101,1401**	neg	II
	d	2,29(19)	4403,51(5)	1203,1601	07,1101	DRB3*02	0201,0301	**0401**	Alive	
388	p	2,28	8,13	6,7	0301,0701	DRB3*0101	0201	**0101**,0401	t.f.	II
	d	2,28	8,13	6,7	0301,0701	DRB3*0101	0201	0401,**1701**	Dead	
138	p	3,32(19)	3501,22	0304,04	0101,1401	DRB3*02	0501,0503	**0402**	neg	II
	d	3,32(19)	3501,22	0304,04	0101,1401	DRB3*02	0501,0503	**0401**	Alive	
165	p	1,2	8,44(12)	0701,08	0301,0801	DRB3*0101	0201,04	0401,**0101**	neg	II
	d	1,2	8,44(12)	0701,08	0301,0801	DRB3*0101	0201,04	0401	Alive	
286	p	2	44(12),51(5)	0501	0401,1301	DRB3*0101	0301,0603	**02011**,0401	neg	-
	d	2	44(12),51(5)	0501	0401,1301	DRB3*0101	0301,0603	**0101**,0401	Alive	
242	p	2,3	7,35	04,0702	0101,1501	DRB5*0101	0501,0602	0401,**0501**	neg	II
	d	2,3	7,35	04,0702	0101,1501	DRB5*0101	0501,0602	0401	Alive	
278	p	1,3	7,60(40)	0304,0702	1501,1302	DRB3*0301	0602,0604	**0201,0301**	neg	III
	d	1,3	7,60(40)	0304,0702	1501,1302	DRB3*0301	0602,0604	**0401**	Alive	
366	p	2,25(10)	37,62(15)	0303,06	0401,1001		0305,0501	**0402,0901**	neg	-
	d	2,25(10)	37,62(15)	0303,06	0401,1001		0305,0501	**0401**	Dead	
382	p	1,2	8,44(12)	0501,0701	0301,1301	DRB3*0101	0201,0603	**0101**,0401	neg	-
	d	1,2	8,44(12)	0501,0701	0301,1301	DRB3*0101	0201,0603	0401,**0402**	Alive	
353	p	3,32(19)	7	0702	1501	DRB5*0101	0602	**0201,0501**	neg	II
	d	3,32(19)	7	0702	1501	DRB5*0101	0602	**0401**	Dead	
503	p	1,2	8,35	4,7	0101,0301	DRB3*0101	0501,0201	0401,**1501**	neg	III
	d	1,2	8,35	4,7	0101,0301	DRB3*0101	0501,0201	0401,**1101**	Dead	
418	p	0201	3501,62(15)	3	1301,0802	DRB3*02	nd	**0301**,0401	neg	II
	d	0201	3501,62(15)	3	1301,0802	DRB3*02	nd	0401	Alive	
384	p	24(9),26(10)	55(22),62(15)	0303	16,1101	DRB3*02,5*02	0301,0502	**0901**,0401	neg	IV
	d	24(9),26(10)	55(22),62(15)	0303,**01**	16,1101	DRB3*02,5*02	0301,0502	**02012**,0401	Dead	
222	p	2,32(19)	27,61(40)	02	1101	DRB3*02	0301	**0401,0201**	neg	II
	d	2,32(19)	27,61(40)	01,02	1101,**0103**	DRB3*02	0301	**0402**	Alive	
414	p	2	27,35	4	0101		nd	**0401**	neg	II
	d	2	27,35	4,**1**	0101		nd	**0101,1301**	Alive	
531	p	2,11	52(5),38(16)	nd	1104,1301	DRB3*0101,02	nd	0201,**0202**	neg	I
	d	2,11	52(5),38(16)	nd	1104,1301	DRB3*0101,02	nd	0201,**0301**	Dead	
307	p	26(10),29(19)	3501,38(16)	0401,1203	0101,1301	DRB3*0101	0501,0603	nd	neg	I
	d	26(10),29(19)	3501,38(16)	0401,1203	0101,1301	DRB3*0101	0501,0603	nd	Alive	
512	p	1	8	7	1301,0301	DRB3*0101	nd	nd	neg	II
	d	1	8	7	1301,0301	DRB3*0101	nd	nd	Alive	

Table 2b. Histocompatibility of the 'mismatched' patient-donor pairs

UPN		A	B	Cw	DRB1	DRB3,5	DQB	DPB	CTLp	aGvH
376	p	2,11	35,57(17)	04,06	0401,**0103**		0501,**0301**	nd	neg	III
	d	2,11	35,57(17)	04,06	0401,**0101**		0501,**0302**	nd	Dead	
347	p	3	7	0702	07,**0404**		0201,0302	**0301,0401**	neg	-
	d	3	7	0702	07,**0402**		0201,0302	**0501,02011**	Dead	
433	p	1,24(9)	27,39(16)	2	0101,**1101**	DRB3*0202	0301	**0401**,0201	pos	IV
	d	1,24(9)	27,39(16)	2	0101,**1102**	DRB3*0202	0301	**0301**,0201	Dead	
387	p	1,3	50(21),57(17)	06	07,**1104**	DRB3*02	0301,0303	nd	neg	IV
	d	1,3	50(21),57(17)	04,**06**	07,**1102**	DRB3*02	0301,0303	nd	Dead	
420	p	1,0201	2702	2	0101,**1601**	DRB5***0201**	nd	**0401,0402**	t.f.	II
	d	1,0201	2702,**2705**	2,**1**	0101,**1501**	DRB5***0101**	nd	**0301**	Alive	
266	p	23(9),24(9)	4403,60(40)	0304,04	07,1301	DRB3***0101**	0201,0603	0402,**0301**	neg	I
	d	23(9),24(9)	4403,60(40)	0304,04	07,1301	DRB3***02**	0201,0603	0402,**0201**	Dead	
364	p	11,26(10)	35,60(40)	0304,04	0101,0402		0501,**0302**	**02011**,0401	neg	III
	d	11,26(10)	35,60(40)	0304,04	0101,0402		0501,**0305**	0401,**0501**	Dead	
330	p	2,3	8,22	1,7	1501,0301	DRB3*0101,5*0101	**0602**,0201	**0402,0401**	neg	II
	d	2,3	8,22	1,7	1501,0301	DRB3*0101,5*0101	**0601**,0201	**0101,0601**	Dead	
326	p	24(9),31(19)	7,62(15)	**0303,15**	1501,0401	DRB5*0101	0602,**0301**	0401,**0501**	t.f.	IV
	d	24(9),31(19)	7,62(15)	**0702**	1501,0401	DRB5*0101	0602,**0302**	0401	Dead	
331	p	**0201**,0301	18,3501	04,0701	0101		0501	0401,0402	pos	II
	d	**0206**,0301	18,3501	04,0701	0101		0501	0401,0402	Alive	
172	p	**0201**,3	4402,40	0304,0501	0101,1302	DRB3*0301	0501,0604	0401,**0301**	pos	III
	d	**0213**,3	4402,40	0304,0501	0101,1302	DRB3*0301	0501,0604	0401,**0201**	Dead	
180	p	0201,**0205**	4402,60(40)	0501,0304	1501,0404	DRB5*0101	0302,0602	0401,0402	pos	-
	d	0201,**0201**	4402,60(40)	0501,0304	1501,0404	DRB5*0101	0302,0602	0401,0402	Dead	
285	p	0201	60(40)	0304	1302	DRB3*0301	0604	nd	pos	III
	d	0201	60(40)	0304	1302	DRB3*0301	0604	nd	Dead	
390	p	2,0301	7,1401	0702,0802	0401,1501	DRB5*0101	0302,0602	**0401,0601**	pos	I
	d	2,0301	7,1401	0702,0802	0401,1501	DRB5*0101	0302,0602	**02011,0402**	Dead	
440	p	1,25(10)	8,3501	nd	0101,1401	DRB3*0201	0501	**0401**	pos	II
	d	1,25(10)	8,3501	nd	0101,1401	DRB3*0201	0501	**0402,0201**	Dead	
298	p	3,23(9)	4403,27	**01**,04	0301,07	DRB3*0101	0201	**0201,2301**	neg	II
	d	3,23(9)	4403,27	**02**,04	0301,07	DRB3*0101	0201	**0401**	Alive	
273	p	24(9),3	27,7	**0304**,0702	0401,1501	DRB5*0101	0302,0602	0401,**1401**	pos	II
	d	24(9),3	27,7	**02**,0702	0401,1501	DRB5*0101	0302,0602	0401,**0402**	Dead	
145	p	3,32(19)	7,39(16)	**0501**,0702	1501,1301	DRB3*02,5*0101	0602,0603	**0201,0402**	neg	-
	d	3,32(19)	7,39(16)	**0304**,0702	1501,1301	DRB3*02,5*0101	0602,0603	**1001,0401**	Alive	
352	p	0301,24(9)	18,3501	04,**0701**	0301,1501	DRB3*02,5*0101	0201,0602	nd	pos	II
	d	0301,24(9)	18,3501	04,**0501**	0301,1501	DRB3*02,5*0101	0201,0602	nd	Dead	
528	p	0201,32(19)	4402,14	**5**,8	0102,1104	DRB3*02	nd	nd	pos	IV
	d	0201,32(19)	4402,14	**7**,8	0102,1104	DRB3*02	nd	nd	Dead	

The precise HLA matching was determined as described in 'Patients and Methods'. Patient-donor pairs matched for all the HLA-A, -B, -C, -DRB1/B3/B5, and -DQB1 alleles, and with a negative CTLp test, were assigned to the 'matched' group (Table 2a). In contrast, the 'mismatched' group (Table 2b) consisted of patient-donor pairs with **mismatches (in bold characters)** at the HLA-A, -B, -C, -DRB1/B3/B5, and/or -DQB1 locus, and/or with a positive CTLp test. Incompatibilities in rejection direction only (patient homozygous, donor heterozygous) were not considered as mismatches (UPN 384, 222, 414). In three patient-donor pairs we could not identify structural differenes of the HLA class I antigens recognized by the CTL clones (UPN 285: A*0201; UPN 390: B*1401; UPN 440: B*3501). In one of them (UPN 285) the CTL clones were found to be reactive against a male specific 'minor' transplantation antigen in association with HLA-A*0201. d, donor; nd, not done; p, patient; t.f., technical failure; UPN, unique patient number.

Table 3. Clinical results

(number of patients)	all patients (44)	HLA 'matched' (24)	p	HLA 'mismatched' (20)
acute GvHD (%)				
grades II-IV (%)	73	71	n.s.	75
grades III-IV (%)	30	21	0.0464	47
skin GvHD (grade; mean±SD)	1.8 ± 1.1	1.8 ± 1.1	n.s.	1.6 ± 1.1
liver GvHD (grade; mean±SD)	1.1 ± 1.5	0.7 ± 1.2	0.0469	1.6 ± 1.6
gut GvHD (grade; mean±SD)	1.0 ± 1.3	0.6 ± 1.0	0.0340	1.4 ± 1.5
chronic GvHD (%)	55	55	n.s.	56
limited (%)	38	35	n.s.	44
extensive (%)	17	20	n.s.	12
infectious diseases: total (n)	23	11	n.s.	12
bacterial sepsis (n)	4	2	n.s.	2
CMV pneumonia (n)	7	3	n.s.	4
toxoplasmosis (n)	2	1	n.s.	1
aspergillosis (n)	2	2	n.s.	-
other, not specified (n)	8	3	n.s.	5
causes of death @				
GvHD	10	4	n.s.	6
infection #	15	5	<0.05	10
leukemia relapse	4	1	n.s.	3
graft failure	2	2	n.s.	-

@ The numbers of patients with the causes considered to be (co-)responsible for death are shown (1 or 2 causes per patient). # Infections include CMV pneumonia (5), aspergillosis (2), bacterial sepsis (3), toxoplasmosis (1), and not specified (4). The numbers of patients that could be evaluated for acute GvHD were 23/19 and for chronic GvHD 20/9 for the 'matched' / 'mismatched' groups, respectively. HLA matching, GvHD, TRM, and survival did not differ significantly between the five participating transplantation centers (data not shown). (n), number of patients.

Table 4. Clinical results of HLA class I and class II allele mismatched transplants

	HLA 'matched'	HLA class I mismatched		HLA class II mismatched	
(number of patients)	(24)	(11)	p	(7)	p
severe acute GvHD III-IV (%)	21	38	n.s.	43	n.s.
transplant related mortality (%)	21	45	n.s.	64	0.038
survival (%)	61	18	0.011	14	0.002

The results of the 24 HLA 'matched' patients are the same as shown in Table 3 and Figures 1 & 2. The 20 'mismatched' patients therein have been divided in two groups: the 11 patients mismatched for HLA class I and the 7 patients mismatched for HLA class II. The p values (logrank-test) compare these two groups with the 24 'matched' patients. Percentages indicate probabilities determined by the Kaplan-Meier method.[27] Two patients (UPN 326 and 433) were not considered for this analysis because of combined HLA class I (HLA-C and CTLp positive) and class II (DQB1 and DRB1) mismatches.

Figure 1

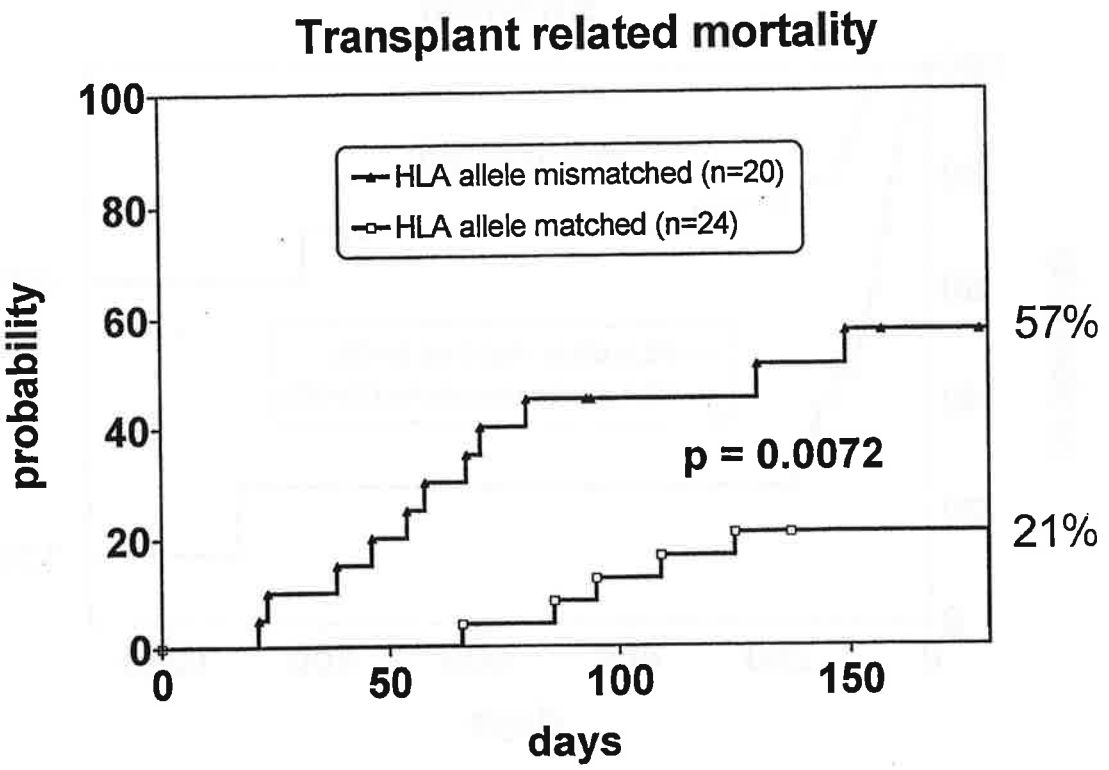

Figure 1.

Probability of transplant related mortality. Comparison between patients with fully HLA matched and patients with HLA mismatched bone marrow grafts.

Figure 2

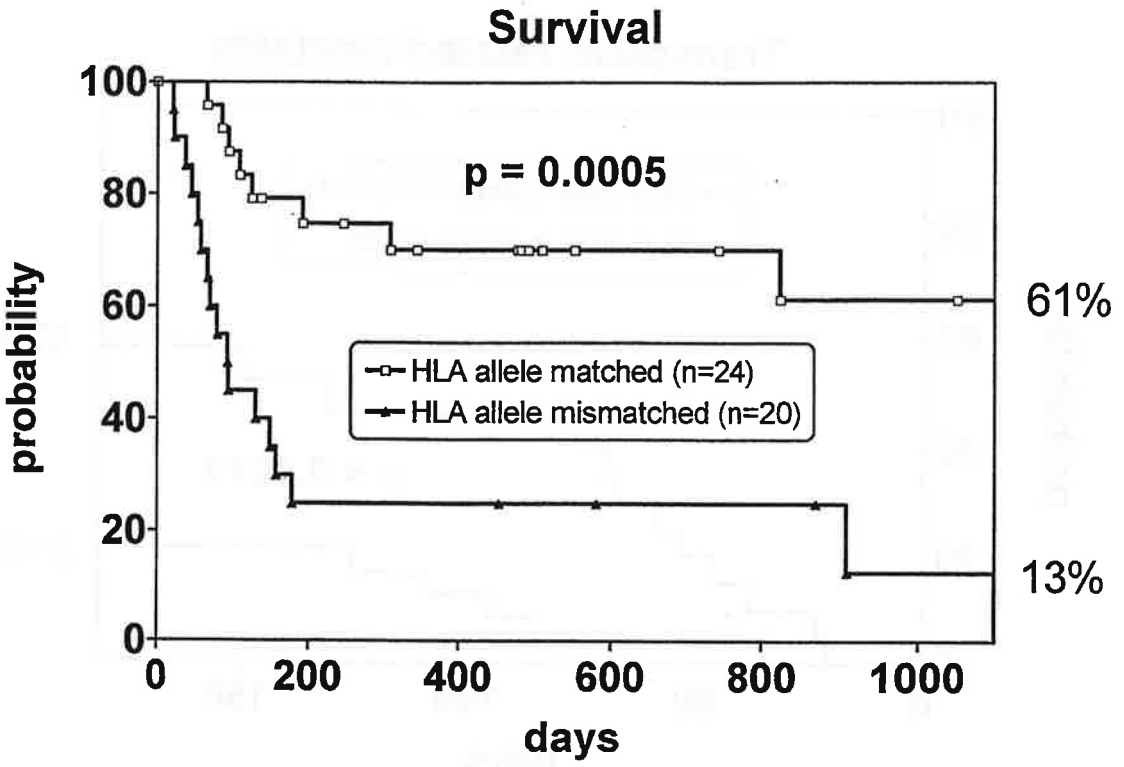

Figure 2.

Survival probability of patients transplanted with HLA allele matched and mismatched donors.

5.5 DISCUSSION

In related BMT, the degree of HLA compatibility correlates with the incidence of posttransplant complications.[3,6] Unfortunately, the situation in unrelated BMT is less clear.[9-11] Routine HLA typing techniques are sufficiently precise for the matching of siblings but are not suitable to detect minor polymorphic differences of human transplantation antigens. Therefore, unrelated patient-donor pairs are often mismatched for antigens not discriminated by conventional typing. The introduction of oligotyping for HLA class II[14] has been a considerable progress and has recently allowed to demonstrate a significant correlation between HLA-DRB1 allele matching and GvHD.[28,29] However, the clinical significance of high resolution HLA class I plus class II matching has not yet been demonstrated. This lack of comprehensive HLA matching studies is due to the low resolution of the routine HLA class I typing methods. Although DNA sequencing of donor and patient HLA genes allows the precise evaluation of individual HLA matching,[12] further technological progress is required before this approach can be applied routinely. Our strategy consisting of a combination of serology, cellular techniques, and oligotyping allowed identification of most HLA allele incompatibilities, while DNA sequencing was only necessary in the few remaining cases of previously not characterized alleles.[16,25]

The finding that patient survival was significantly better in the 'matched' group indicates that HLA allele incompatibilities only detected by high resolution typing and missed by conventional techniques are clinically relevant. There were no major differences between the 'matched' and the 'mismatched' group with respect to demographics, diagnosis, risk factors and treatment modalities, apart from an imbalance concerning the eight patients transplanted in relapse, accelerated phase or blast crisis. However, after exclusion of these patients, the differences in TRM and patient survival remained statistically significant.

Moderate to severe GvHD grades II-IV was frequently observed and did not correlate with the degree of HLA matching. However, in the HLA 'mismatched' group the grades of acute liver and gut GvHD were significantly higher and severe acute GvHD III-IV occurred more frequently. Therefore, the 'matched' and 'mismatched' groups differed in the severity but not the incidence of GvHD. Interestingly, the overall incidence of severe acute GvHD grades III-IV was rather low when compared with findings in other studies.[9,10]

No major differences in the incidence of infectious diseases were observed between the two groups. However, the incidence of fatal infections was higher in the 'mismatched' vs. the 'matched' group (10 vs. 5; Table 3). Therefore, the two groups differed in the severity rather than the incidence of infections. In contrast to the relatively low overall incidence of severe GvHD, the overall incidence of lethal infections was relatively high in our patients. This is in disagreement with some earlier observations showing a correlation between the rates of severe acute GvHD and infection.[30] In our study, only 4 of the 15 patients with death associated infections suffered from severe acute GvHD III-IV. A possible explanation is that in vivo T cell depletion and strong immunosuppressive therapy efficiently prevented severe acute GvHD but favoured the development of severe infections. It is desirable to improve our means to predict GvHD in order to adapt GvHD prophylaxis individually for each patient. One step towards this goal is the precise characterization of HLA matching. Other GvHD influencing factors such as 'minor' transplantation antigens,[31] bacterial infections,[32] irradiation, or pretransplant immune reactivity must also be considered. New transplantation protocols perhaps including the transfusion of large numbers of donor hemopoietic stem cells[33] may further reduce the incidence and/or the severity of GvHD.

The frequency of HLA-C incompatibilities was relatively low confirming the reported high linkage disequilibrium between the HLA-C and the HLA-B locus. The clinical results in the five patients with isolated HLA-C mismatches were as follows: severe acute aGvHD 1/5, TRM 2/5, and survival 2/5. Thus, our study did not allow to draw firm conclusions on a possible prognostic value of isolated HLA-C incompatibilities.[12]

In agreement with previous reports showing that HLA-DP antigens are very frequently mismatched,[12,13,15] only 18% of the patient-donor pairs in our study were matched for HLA-DP. This is due to the considerable DPB1 polymorphism and the low linkage disequilibrium between DPB1 and DR/DQ loci. For the majority of patients it remains impossible to find an unrelated HLA matched donor with HLA-DP compatibility, making it difficult to evaluate the clinical significance of HLA-DP matching.[12,13,15]

By making the criteria for compatibility more severe, the chance of finding a matched donor decreases. To compensate for this, we analyzed up to 25 donors for those patients who had difficulties in finding a highly matched donor. We found that approximately 10-15 unrelated donors per patient can be analyzed within a reasonable time.[34] Nevertheless, the patient's disease stage and treatment policy have to be taken into account for correct decisions on search and matching strategy. In urgent situations, such as aplastic anaemia, it is not possible to prospectively perform extended time consuming (cellular) investigations. In non-urgent cases however, there is usually sufficient time (i.e. about three months) for the allocation of an optimal donor, if the donor search is started early after diagnosis. During 1994, our search and matching strategy has allowed us to find a highly HLA compatible (HLA-A, -B, -DRB1/B3/B5, -DQB1 subtype matched and CTLp negative) donor for 35% of our patients searching for an unrelated donor.

We conclude that HLA allele mismatches are associated with increased posttransplant mortality. Although the patient groups were small and heterogeneous, the differences in clinical outcome were statistically significant. Nevertheless, our findings should be confirmed in larger patient populations. HLA typing on DNA sequence level will allow to evaluate the clinical importance of mismatches of individual HLA alleles. In the future, this will eventually lead to the characterization of clinically acceptable mismatches.[10,11] Until then, avoiding incompatible donors by the selection of the most compatible available donor using high resolution HLA typing may further improve the prognosis of patients undergoing unrelated BMT.

ACKNOWLEDGMENTS

We are indebted to F. Berthet, C. Bosshard, M. Bujan, F. Dumont-Girard, V. Elamly, D. Gauchat-Feiss, J. Gmür, C. Gonet, C. Goumaz, M. Kern, B. Kervaire, M. Leutenegger, B. Mermillod, A. Morell, A. Morrison, A. Moser, A. Nadolny, B. Orth, M. Ossola, P. Roux-Chabbey, U. Rüdt, G. Salvisberg, V. Sarkissian, U. Schanz, P. Schneider, Y. Schreiber, R. Seger, G. Silvano, B. Speck, S. Stadelmann, S. Stöckli, M. Stockschläder, L. Volin, I. Wenger, and E. Widmer for their support. We also acknowledge the staff of the national registries for volunteer bone marrow donors, and the physicians, nurses, ward clerks, secretaries and technicians of the donor, collection, and transplant centers for their efforts and cooperation.

Abbreviations: BMT, bone marrow transplantation; CsA, cyclosporin A; CTLp, cytotoxic T lymphocyte precursor; d, donor; GvHD, graft-versus-host disease; PBMC, peripheral blood mononuclear cells; MTX, methotrexate; p, patient; PCR/SSO, polymerase chain reaction/sequence specific oligotyping; t.f., technical failure; TRM, transplant related mortality; UPN, unique patient number.

5.6 REFERENCES

1. Thomas ED: Karnofsky memorial lecture: marrow transplantation for malignant diseases. J Clin Oncol 1:517, 1983
2. O'Reilly RJ: Allogeneic bone marrow transplantation: current status and future directions. Blood 62:941, 1983
3. Martin PJ, Hansen JA, Storb R, Thomas ED: Human marrow transplantation. An immunological perspective. Adv Immunol 40:379, 1987
4. Ferrara JLM, Deeg HJ: Graft-versus-host disease. N Engl J Med 324:667, 1991
5. Vogelsang GB, Hess AD: Graft-versus-host disease: New directions for a persistent problem. Blood 84:2061, 1994
6. Kumar L, Goldman JM: Bone marrow transplantation for patients lacking an HLA-identical sibling. Curr Opin Hematol 1:234, 1993
7. Hows JM, Yin JL, Marsh J, Swirsky D, Jones L, Apperley J, James D, Smithers S, Batchelor J, Goldman JM, Gordon-Smith E: Histocompatible unrelated volunteer donors compared with HLA nonidentical family donors in marrow transplantation for aplastic anemia and leukemia. Blood 68:1322, 1986
8. McGlave P, Scott E, Ramsay N, Arthur D, Blazar B, McCullough J, Kersey J: Unrelated donor bone marrow transplantation therapy for chronic myelogenous leukemia. Blood 70:877, 1987
9. Kernan NA, Bartsch G, Ash RC, Beatty PG, Champlin R, Filipovich A, Gajewski J, Hansen JA, Henslee-Downey J, McGullough J, McGlave P, Perkins HA, Phillips GL, Sanders J, Stroncek D, Thomas ED, Blume KG: Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med 328:593, 1993
10. Beatty PG, Anasetti C, Hansen JA, Longton GM, Sanders JE, Martin PJ, Mickelson EM, Choo SY, Petersdorf EW, Pepe MS, Appelbaum FR, Bearman SI, Buckner CD, Clift RA, Petersen FB, Singer J, Stewart PS, Storb R, Sullivan KM, Tesler MC, Witherspoon RP, Thomas ED: Marrow transplantation from unrelated donors for treatment of hematologic malignancies: Effect of mismatching for one HLA locus. Blood 81:249, 1993
11. Van Rood JJ: Immunogenetics. Curr Opin Hematol 1:429, 1994
12. Santamaria P, Reinsmoen NL, Lindstrom AL, Boyce-Jacino MT, Barbosa JJ, Faras AJ, McGlave PB, Rich SS: Frequent HLA class I and DP sequence mismatches in serologically (HLA-A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA-identical unrelated bone marrow transplant pairs. Blood 83:280, 1994
13. Pawelec G, Ehninger G, Schmidt H, Wernet P: HLA-DP matching and graft-versus-host disease in allogeneic bone marrow transplantation. Transplantation 42:558, 1986

14. Tiercy J-M, Morel C, Freidel AC, Zwahlen F, Gebuhrer L, Betuel H, Jeannet M, Mach B: Selection of unrelated donors for bone marrow transplantation is improved by HLA class II genotyping with oligonucleotide hybridization. Proc Natl Acad Sci USA 88:7121, 1991
15. Petersdorf EW, Smith AG, Mickelson EM, Longton GM, Anasetti C, Choo SY, Martin PJ, Hansen JA: The role of HLA-DPB1 disparity in the development of acute graft-versus-host disease following unrelated donor marrow transplantation. Blood 81:1923, 1993
16. Tiercy J-M, Djavad N, Rufer N, Speiser D, Jeannet M, Roosnek E: Oligotyping of HLA-A2, A3-, and B44-subtypes: Detection of subtype-incompatibilities between patients and their serologically matched unrelated bone marrow donors. Human Immunol 41:207, 1994
17. Fleischhauer K, Kernan NA, O'Reilly J, Dupont B, Yang SY: Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N Engl J Med 323:1818, 1990
18. Gauchat-Feiss D, Rufer N, Speiser D, Jeannet M, Roosnek E, Tiercy J-M: Heterogeneity of HLA-B35: oligotyping and direct sequencing for B35 subtypes reveals a high mismatching rate in B35 serologically compatible kidney and bone marrow donor/recipient pairs. Transplantation 60:869, 1995
19. Cereb N, Maye P, Lee S, Kong Y, Yang SY: Locus-specific amplification of HLA class I genes from genomic DNA: locus-specific sequences in the first and third introns of HLA-A, -B, and -C alleles. Tissue Antigens 45:1, 1995
20. Kennedy LJ, Poulton KV, Dyer PA, Ollier WER, Thomson W: Definition of HLA-C alleles using sequence-specific oligonucleotide probes (PCR-SSOP). Tissue Antigens 46:187, 1995
21. Cros P, Allibert P, Mandrand B, Tiercy J-M, Mach B: Oligonucleotide genotyping of HLA polymorphism on microtitre plates. Lancet 340:870, 1992
22. Tiercy J-M, Grundschober C, Jeannet M, Mach B: A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes, in Bidwell J, Hui K: (eds): Handbook for HLA Tissue Typing Laboratories, , CRC Press Inc, 1993, p 117
23. Kaminski E, Sharrock C, Hows J, Ritter M, Arthur C, McKinnon S, Batchelor JR: Frequency analysis of cytotoxic T lymphocyte precursors: Possible relevance to HLA-matched unrelated donor bone marrow transplantation. Bone Marrow Transpl 3:149, 1988
24. Gauchat-Feiss D, Breur-Vriesendorp BS, Rufer N, Jeannet M, Roosnek E, Tiercy J-M: Sequencing of a novel functional HLA-B44 subtype differing in two residues in the alpha 2 domain. Tissue Antigens 44:261, 1994

25. Rufer N, Tiercy JM, Breur-Vriesendorp B, Gauchat-Feiss D, Shi X, Slavcev A, Lardy N, Speiser D, Gratwohl A, Chapuis B, Jeannet M, Roosnek E: Histoincompatibilities in ABDR-matched unrelated donor recipient combinations. Bone Marrow Transpl 16:641, 1995

26. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, Lerner KG, Thomas ED: Clinical manifestations of graft-versus-host disease in human recipients of marrow from HLA-matched sibling donors. Transplantation 18:295, 1974

27. Kaplan EL, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457, 1958

28. Petersdorf EW, Longton GM, Anasetti C, Martin PJ, Mickelson EM, Smith AG, Hansen JA: The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor bone marrow transplantation. Blood 86:1606, 1995

29. Nademanee A, Schmidt GM, Parker P, Dagis AC, Stein A, Snyder DS, O'Donnell M, Smith EP, Stepan DE, Molina A, Wong KK, Margolin K, Somlo G, Littrell B, Woo D, Sniecinski I, Niland JC, Forman SJ: The outcome of matched unrelated donor bone marrow transplantation in patients with hematologic malignancies using molecular typing for donor selection and graft-versus-host disease prophylaxis regimen of Cyclosporine, Methotrexate and Prednisone. Blood 86:1228, 1995

30. Storb R, Deeg HJ, Whitehead J, Appelbaum FR, Beatty P, Bensinger W, Buckner CD, Clift R, Doney K, Farewell V, Hansen J, Hill R, Lum L, Martin P, McGuffin R, Sanders J, Stewart P, Sullivan K, Witherspoon RP, Yee G, Thomas ED: Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. N Engl J Med 314:729, 1986

31. Goulmy E, Gratema JW, Blokland E, Zwaan FE, Van Rood JJ: A minor transplantation antigen detected by MHC-restricted cytotoxic T lymphocytes during graft-versus-host disease. Nature 302:159, 1983

32. Heidt PJ, Vossen JM: Experimental and clinical gnotobiotics: Influence of the microflora on graft-versus-host disease after allogeneic bone marrow transplantation. J Med 23:161, 1992

33. Reisner Y, Martelli MF: Bone marrow transplantation across HLA barriers by increasing the number of transplanted cells. Immunol Today 16:437, 1995

34. Speiser DE, Tiercy J-M, Rufer N, Chapuis B, Morell A, Kern M, Gmür J, Gratwohl A, Roosnek E, Jeannet M: Relation between the resolution of HLA-typing and the chance of finding an unrelated bone marrow donor. Bone Marrow Transpl 13:805, 1994

Chapter 6

Recognition of major and minor histoincompatibilities after transplantation with marrow from HLA closely matched donors

Recognition of major and minor histoincompatibilities after transplantation with marrow from HLA closely matched donors

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6.1 ABSTRACT

To determine the respective role of major and minor histoincompatibilities in transplantations with marrow from donors other than HLA-identical siblings, we characterized the antigens recognized during graft versus host disease (GvHD) in five patients transplanted with marrow from donors who were histoincompatible for different types of HLA antigens. Patient 1 was mismatched for one "ABDR-antigen" (HLA-A2 versus A3). Two patients were mismatched for antigens that would usually not be taken into account by standard selection procedures. Patient 2 was mismatched for a "HLA-A subtype" (A*0213 versus A*0201), while patient 3 was mismatched for HLA-C (HLA-Cw5 versus HLA-Cw7). All three mismatches were detected by a pretransplant cytotoxic precursor (CTLp) test. Of the 2 other combinations that were CTLp-negative, patient 4 was ABCDRDQ matched at the molecular level, but mismatched for DP (DP*1101 vs. DP*0201), while patient 5 was matched for all 6 loci.

Analysis of the specificity of the cytolytic T cell clones isolated post transplantation, showed that the incompatibilities detected by the pretransplant CTLp-assay, were the targets recognized during GvHD. Independent of whether the incompatibility consisted of a "full" mismatch, a "subtype" mismatch or an HLA-C mismatch, all clones recognized the incompatible HLA molecule. Only in the two CTLp-negative combinations was the anti-recipient CTL-activity directed against minor histocompatibility antigens (mHag).

These data show that also the incompatibilities not considered by routine tissue typing techniques are relevant mismatches recognized after transplantation. Furthermore, they indicate that major histocompatiblity mismatches represent immunodominant epitopes that constrain the allogeneic response towards mHags and that therefore, mHag incompatibilities might be less important in donor/recipient pairs already mismatched for a major histocompatibility antigen.

6.2 INTRODUCTION

Allogeneic bone marrow transplantation is an important therapy for patients with hematological disorders or inborn errors of the immune system.[1-3] Success rates depend heavily on the histocompatibility between donor and recipient and, when available, an HLA-identical sibling will always be the donor of choice. With the establishment of international registries,[1,3] a substantial effort has been made to provide alternative donors for the patients who lack such an optimal donor. Unfortunately, such phenotypically matched donors appear to be less compatible than HLA-identical siblings which is reflected by an increased incidence of post-transplant complications such as Graft-versus-Host Disease (GvHD) or graft-rejections. [1,4-6] Several factors could account for the differences in success rates of HLA-matched sibling- and of matched unrelated donor transplantations.

Firstly, matching of unrelated donors is usually done on basis of HLA ABDR-compatibility only,[1] and although these criteria were sufficient to guarantee full compatibility between two related individuals, they do not so when donor and recipient are unrelated. Even when fully matched for HLA-A, -B and -DR, an unrelated donor might still be mismatched for HLA-C, -DQ and/or -DP antigens. In addition, the resolution of routine typing methods is too low to make out the extremely high polymorphism of the human MHC. Therefore, ABDR-matched donors might still be incompatible for variants of these antigens that are not discriminated by routine laboratory techniques.

A second reason for the increased frequency of post-transplant complications in unrelated marrow transplantation could be that donor and recipient are mismatched for a higher number of minor transplantation antigens (mHag) than related combinations.[7] These antigens which are peptides derived from polymorphic intracellular proteins presented to T lymphocytes in a MHC-restricted fashion,[8-12] can form important transplantation barriers. Obviously, these antigens will play a role in the onset of GvHD only when the recipient expresses a mHag that is not present in the donor. This situation will occur more frequently when donor and recipient are unrelated and do not share 50 percent of their genetic information. Moreover, because a substantial part of unrelated donors are recruited from international registries, donor and recipient might express distinct mHags that are characteristic for the geographical region from which they originate.

The relative risk of being mismatched for HLA-C/DQ/DP antigens, HLA-A/B subtype variants or mHags is unknown. For instance, it is not clear whether additional mismatches such as a difference in sex would still increase the risk of post transplant complications once the donor is not entirely HLA matched. In fact, it is only with the recent development of high resolution compatibility testing, that the extent of HLA mismatches in unrelated donor/recipient combinations has become evident[13] and that such analyses have become possible. The scarce data available suggest that at least some of these incompatibilities normally not typed for, might contribute to GvHD: HLA-C as well as HLA class I subtype variants are recognized by cytotoxic T lymphocytes (CTL) before transplantation.[13,14] Moreover, recognition of an HLA-B subtype during GvHD[15] or during marrow rejection[16] has been reported. In this paper, we have studied to what extent HLA incompatibilities that are usually not taken into account during routine selection procedures, participate in the onset of GvHD. Our data show that the incompatibilities detected by pre-transplant CTL precursor (CTLp)-assays that often remain undisclosed by routine selection procedures,[13] form the targets recognized during GvHD. These results demonstrate the value of high resolution class I typing techniques such as CTLp-tests or molecular class I typing for the selection of unrelated bone marrow donors.

6.3 PATIENTS, MATERIALS AND METHODS :

Selection and HLA typing of donor-recipient pairs: This study comprises 5 patients transplanted between 1990 and 1994. Except for Pat 1, who was transplanted with the bone marrow from his brother, all other patients were transplanted with marrow from an unrelated bone-marrow donor identified through the international unrelated bone marrow registries and further selected on basis of HLA matching by serotyping for HLA-A, B and by oligotyping for HLA-DR 1-14.[17] In addition the following high resolution techniques were applied: High resolution DR/DQ-typing[18] discriminating the vast majority of the HLA-DR and-DQ genes and HLA class I oligotyping for HLA-A2,-A3 and -B44,[19] was performed as described previously. HLA-C oligotyping was performed essentially according to Kennedy et al.,[20] HLA-DP typing was performed using the Inno-Lipa reverse dot blot kit (Endotell). CTLp analysis was performed as described before,[21] by incubation of irradiated (2500 rad) recipient mononuclear cells (5.10^4) with graded numbers (5.10^4-$1.25.10^3$) of donor cells in 96 well, 200 ml round bottom plates (Costar, Cambridge, USA). On day 3 and 6, 25 ml of fresh medium with 100 U/ml rIL-2 (Roche, Nutley, USA) was added to all wells. After 10 days of culture, the wells were tested against ^{51}Cr-labeled phytohemeagglutinin (PHA) stimulated recipient T cells blasts.

Typing of mHags HA-1, HA-2 and HA-4: Expression of the mHags was determined by measuring the lysis of ^{51}Cr-labeled PHA-blasts by the respective mHags-specific T cell clones.[22,23]

Detection and cloning of recipient specific T cells before and after transplantation: All procedures have been described before.[14,24] In brief, peripheral blood samples of the donor and of the patient before and after transplantation were collected, separated by Ficoll-Paque and cryopreserved. Cytotoxic T cell clones were established from the positive wells of the pre- (donor against patient) and post-transplant (patient against patient before transplantation) CTLp assays and specificity towards recipient cells was tested using autologous donor cells and third party cells that do not express HLA antigens in common as negative controls. Further specificity testing was done against a panel of cells expressing well characterized HLA-A, -B, and Cw antigens.

6.4 RESULTS

Histoincompatibilities in five closely matched donor/recipient pairs: To determine to which extent mismatched major- or minor compatibility antigens would provoke an allogeneic response after bone marrow transplantation, we studied five patients who received marrow from donors who were histoincompatible for different types of HLA antigens. The patients were transplanted for either chronic myeloid leukemia (first chronic phase) or for acute myeloid or lymphoblastic leukemia in first remission (Table 1) and suffered from GvHD grade ≥ 2 that started within the first weeks after transplantation. Patient 3 was followed 3 months after transplantation after she was reinfused with donor buffycoat cells in attempt to eradicate the recurring disease. Donor and recipient were typed by serology and high resolution molecular typing techniques (for details see materials and methods). Furthermore, pretransplant donor anti-recipient cytotoxic activity was measured by CTLp frequency analysis. This combination of techniques documented the following MHC mismatches (Table 2) : Patient 1, who received bone marrow from his brother, was mismatched for one "ABDR-antigen" (HLA A2 versus A3). This mismatch was clearly at the origin of the positive CTLp-test as all patient specific T cell clones isolated recognized the HLA A*0301 molecule. Of the other four patients, who received marrow from unrelated donors considered to be "ABDR-matched" by routine typing methods, patient 4 and 5 were ABCDRDQ matched at the molecular level. Patient 2, expressing the common HLA-A2 variant HLA-A*0201 was class I subtype mismatched with his donor expressing HLA-A*0213. This serologically indistinguishable difference was efficiently detected by the CTLp test. Patient 3 was mismatched for HLA-C (Cw5 vs. Cw7) which resulted in a vigorous pretransplant CTL activity. No cytotoxic activity was found against patient 4 and 5 who were class I matched with their respective donors.

HLA-serotype-, HLA-subtype- as well as HLA-C-mismatches are the immunodominant epitopes recognized during GvHD: To study which incompatibilities would serve as the target recognized during GvHD, we analyzed the specificity of patient specific CD8-positive T cell clones that were isolated between one and nine months after transplantation. Table 3 shows that the same incompatibilities detected by the CTLp test before transplantation were recognized after transplantation. Independently of whether the incompatibility

consisted of a "full" HLA-A mismatch (Pat 1: A*0201 versus A*0301), a "subtype" mismatch (Pat 2: A*0213 versus A*0201), or an HLA-C mismatch (Pat 3: HLA-Cw5 versus HLA-Cw7), all clones isolated during GvHD recognized the incompatible HLA molecule. Moreover, no activity against mHags could be detected in any of these three patients transplanted with an HLA-incompatible marrow while such activity was readily detected in the patients who had received a graft from a donor matched for HLA-ABCDRDQ at the molecular level (Pat 4 and 5). The fact that in these latter patients the anti-mHag clones occurred at high frequencies, stresses the significance of the absence of anti-mHag activity in the patients grafted with an HLA-incompatible marrow, suggesting that major histocompatiblity mismatches represent immunodominant epitopes that constrain the allogeneic response towards mHags.

Characterization of the alloantigens recognized in two HLA-ABCDRDQ-matched unrelated donor/recipient combinations. To identify the antigens potentially being recognized in the last two patients, we oligotyped for HLA-DPB1 and typed for three (HA-1, HA-2 and HA-4) of the well characterized HLA-A*0201 restricted mHags using the respective mHag-specific T cell clones.[22,23] Patient 4 was DP-mismatched (DP*1101 vs. DP*0201) and incompatible[&] for HA-1, a mHag known to be involved in GvHD.[25] Patient 5 was compatible for HLA-DP and the three mHags tested for, but was sex mismatched which could result in recognition of H-Y by the female donor. In both patients, high frequencies of recipient specific CTL's were found (data not shown). To resolve which incompatibilities were recognized, we established 65 independent T cell clones that were cytotoxic for the patient cells without recognizing the donor, and tested them for the capacity to lyse panel cells expressing well defined HLA antigens (Table 4). In patient 4, only HLA-A*0201-positive target cells were recognized, and part of the cytotoxic activity could be identified as anti-HA-1. In patient 5, a number of panel cells expressing either HLA-A*0201, HLA-B*4402 or HLA-Cw5 in common with the patient were recognized. Figure 1 shows a study of the specificity of the CTL clones in two informative families. Apart from anti-H-Y, five other specificities, independently segregating from HLA as well as from each other, could be assigned. In the first family, the restricting element is inherited from the mother,

[&] for the sake of simplicity we use the term incompatible for a mHag only when this incompatibility is relevant for the induction of graft-versus-host disease, that is in the particular case when the patient expresses the mHag while the donor does not.

who herself is negative for the respective mHags, while the HLA-A*0201-negative father provides the genes (including H-Y) encoding for the mHags. Of the siblings, one male who had inherited the restricting element HLA-A*0201 of the haplotype C was recognized by the anti-H-Y clones but not by the anti-"minor-I" and anti-"minor-II" clones. The second male expressed H-Y and the "minor-II" in the context of the HLA-A*0201 of the haplotype D and the female sibling expressed both mHags restricted by the HLA-A*0201 of the haplotype D. This typical Mendelian segregation could also be observed for the mHags restricted by HLA-B*4402 and HLA-Cw5 respectively in the family of patient 5 (family 2). While the patient expressed the "minor-III" inherited from his father and the minors-IV and V inherited from his mother, the cells of his sister that did express the two relevant restriction elements were only recognized by the anti-"minor-III" clones.

The analysis of the frequency of the respective mHags found in patient 5 showed that with the exception of "minor-III", the mHags were expressed by 30 to 60 % of the Caucasian individuals tested (Table 4). As a consequence, these mHags will be mismatched frequently and might therefore form significant transplantation barriers in unrelated as well as in related donor/recipient combinations.

Table 1. Patients: Characteristics, conditioning and occurrence of GvHD

	Age	Sex (R/D)	Diag	Conditioning	TCD[1]	GvHD[2] a/c
1	34	M/M	CML	TLI/TBI/CTX/MP	-	II /limited
2	17	M/M	ALL	TBI/CTX/VP16/CyA	-	III /limited
3	36	F/M	AML	TBI/CTX/CyA	+	III
4	23	M/M	CML	TBI/CTX/CyA	+	III
5	18	M/F	AML	TBI/CTX/CyA	+	III

Abbreviations: ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; TLI, total lymphoid irradiation including the spleen; TBI, fractionated total body irradiation; CTX, cyclophosphamide; MP = methylprednisolone; VP16, etoposide; CyA, cyclosporin A.
[1]T cell depletion, Campath-1G in vivo; [2]GvHD : acute/chronic

Table 2. HLA incompatibilities and analysis of pre-transplant donor anti-recipient CTL-activity

	ABDR[1] matched	HLA class I incompatibilities[2] (D ↔ R)	HLA class II incompatibilities (D ↔ R)	# CTLp / 10^6 cells	antigen recognized by CTLs[3]
1	no	A*0201↔ A*0301	=	11	A*0301
2	yes	A*0213 ↔ A*0201	DP*0201 ↔ DP*0301	6	A*0201
3	yes	Cw5 ↔ Cw7	DP*0301 ↔ DP*0202	20	Cw7
4	yes	=	DP*1101 ↔ DP*0201	0	n.a.
5	yes	=	=	0	n.a.

[1]as defined by serology for HLA A/B and generic oligotyping for DR.
[2]Complete typing: Pat. 1:
HLA-A*0201,0301;-B5,49;-C7,15;-DRB1*2,1101;-DRB3*02;-DQw3,-DP*0401,0201.
Pat. 2:
HLA-A*0201,3;-B*4402,40;-Cw3,w5;-DRB1*0101,1302;-DRB3*0301;-DQ*0501,0604;-DP*0401,0301.
Pat. 3: HLA-A*0301,24;-B18,*3501;-Cw4,w7;-DRB1*1501,0301;-DRB3*02;-DQ*0201,0602;-DP*0201,0202.
Pat. 4: HLA-A*0201,29;-B18,*4403;-Cw7,*1601;-DRB1*0701,1101;-DRB3*02;-DP*0201,0401.
Pat. 5: HLA-A*0201,28;-B*4402,4402;-Cw5,w5;-DRB1*0401,1301;-DRB3*0101;-DQ*0301,0603;-DP*0401.
[3]Analysis of the T cell specificity by cloning of recipient specific CTL's, n.a., not applicable

Table 3: Analysis of the specificity of the T cell clones established before transplantation or during Graft-versus-Host Disease

	N° of T cell clones		Antigen recognized before BMT	Antigen recognized during GvHD
	pre-BMT	post-BMT		
1	5	5	A*0301	A*0301
2	1	2	A*0201	A*0201
3	4	9	Cw7	Cw7
4	n.a	7	n.a.	minor antigens
5	n.a	58	n.a.	minor antigens

BMT, bone marrow transplantation, n.a = not applicable

Table 4. Restriction elements and frequencies[1] of HA-1 and 5 novel mHags.

minor antigens	A*0201-pos. cells	B*4402-pos. cells	Cw5-pos. cells	Frequencies of the mHag
HA-1[2]	**38 /76**	0 /31	0 /16	50%
minor-I[3]	**46/118**	0/61	0/30	39%
minor-II[3]	**16/27**	0/11	0/9	59%
minor-III[3]	0/103	**3/59**	0/29	5%
minor-IV[3]	0/96	**19/50**	0/24	38%
minor-V[3]	0/106	0/53	**16/30**	54%
H-Y[3]	**67/99**	0/59	0/29	n.a[4]

[1] Frequencies in a panel comprised of individuals from a local panel and from a panel from which the bone marrow donor originated. Results are expressed as # targets recognized/ # targets tested
[2] Isolated from patient 4
[3] Isolated from patient 5
[4] not applicable (male cells exclusively)

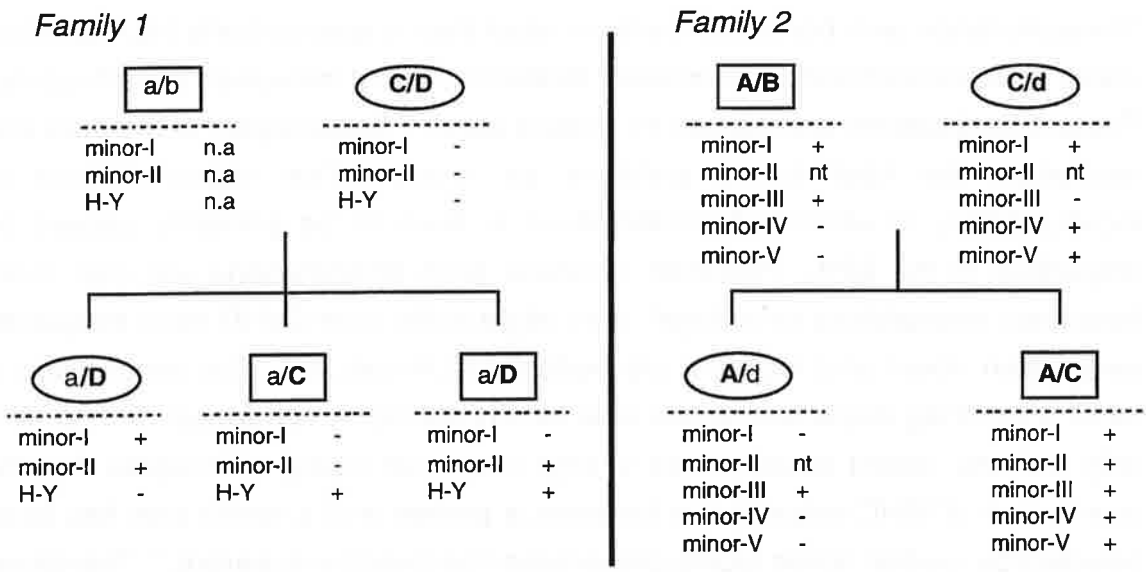


Fig. 1. HLA independent segregation of minor I-V: Results are expressed as presence (+) or absence of (–) of the mHag as shown by lysis of the target by the respective mHag specific T cell clone.
Haplotypes (haplotypes with one or more of the relevant restricting element in capitals):
Family 1: a= A10/B7 ; b= A3/B41; C= A*0201 /B50; D= A*0201/B7
Family 2: a= A28/B4402/Cw5; B= A*0201/B39; C= A*0201/B*4402/Cw5; d= A24/B55
n.a., not applicable (cells do not express one of the restricting elements), n.t., not tested ▭ male cells; ⬭ female cells

6.5 DISCUSSION

Transplantation with bone marrow from other than a genotypically HLA identical donor is associated with an increased incidence of post transplant complications. These complications are initiated by mature donor T lymphocytes in the graft that recognize the host tissue antigens as foreign. The higher degree of incompatibility in unrelated combinations is likely to be primarily caused by disparities in the MHC. However, because such combinations are also more frequently mismatched for mHags[7], they might suffer from GvHD more frequently even when donor and recipient are perfectly HLA-matched. The contribution of MHC and mHag disparities to the total incompatibility is not known. In fact, it is only with the recent development of high resolution typing techniques that the true extent of MHC-mismatches between a patient and a donor that has been selected by routine tissue typing procedures has become apparent.[13] Therefore, clinical data on the importance of such mismatches are still rare and a final conclusion can only be made when large numbers of extremely well typed combinations have been studied. Still less is known about the importance of mHag-mismatches in unrelated donor/recipient pairs. To assess the increased risk of being mismatched for a mHag when donor and recipient are unrelated, one has to bear in mind that these incompatibilities are of different nature than MHC-incompatibilities. Because mHags are self peptides that are derived from endogenously synthesized proteins, they form transplantation barriers only when two conditions are fulfilled. Firstly, the patient's variant of the mHag must be absent in the donor. This will obviously occur more frequently in a donor who is unrelated than in a donor who is born from parents of whom at least one is known to pass the patient's variant to it's progeny. This risk factor can be calculated with population genetics using a biallelic codominant model, and will vary between 1 and 1.8 depending on the frequency of the genes encoding for the mHag.[7,26] Secondly, the peptides derived from the polymorphic variants should also have the right sequence to be presented by the MHC. Because this condition will not always be met, the risk factor decreases (1-1.4) but will still remain significantly higher than in related combinations. However, whether this higher degree of mHag incompatibility contributes significantly to the post transplant complications will depend on the extent that mHag incompatibilities are recognized in patients transplanted with an unrelated donor who will be seldomly compatible for all MHC loci.

To study this question, we have identified the targets recognized during GvHD in patients transplanted with bone marrow from donors who were incompatible for different types of MHC molecules. It should be noted that in unrelated donor/recipient pairs, this reflects by far the most common situation to date. Apart from mismatches that have to be accepted because a more compatible donor is not available,[1,4,5,27] a significant part of the donors selected by standard laboratory procedures will be mismatched for antigens that these techniques do not discriminate. Indeed, the donors recruited for the patients 2, 3 and 4 described in this report, would have been considered as perfectly matched by the vast majority of tissue typing laboratories, in spite of the presence of one HLA-A, one HLA-C and three HLA-DP mismatches. Such mismatches are only detected by methods that include high resolution DNA typing techniques and/or CTLp assays. The results of our study argue strongly in favor of the use of such high resolution compatibility testing by showing that all three class I incompatibilities detected by the CTLp test before transplantation of which neither the HLA-A2 subtype- nor the HLA-C mismatch would have been noticed by routine typing, were the dominant targets recognized during GvHD. The presence of such mismatches will increase the severity of post transplant complications and therefore, when a search has identified more than one ABDR-matched donor, such tests will be extremely useful in donor selection and ultimately lead to better survival rates.[21,28-31]

Unfortunately, a donor fully compatible for all six HLA loci will not be found for many patients. Even if HLA-DP would not be taken into account, a donor matched for HLA-A,-B,-C,-DR and -DQ will only be found for approximately 35% of the patients.[17,31] At present, very little is known about the relative importance of the respective HLA loci. As a whole, mismatched MHC antigens elicit more vigorous reactions than mHags do,[26,32,33] but whether this also holds for HLA-C which is expressed at much lower densities than the other class I molecules[34] has to be verified. At present however, any MHC mismatch is still to be considered as a serious risk factor and in such a situation, one might be reluctant to transplant a patient when for instance, donor and recipient are sex mismatched as well. Interestingly, our data show that the contribution of mHags to GvHD might be minimal when recipient and donor are mismatched for a MHC class I antigen. This finding is not so unexpected in view of the well documented immunodominant role of "strong" antigens that can prevent "weaker" antigens from being recognized. In mice several mHags are immunodominant over the

male antigen and no H-Y specific CTL responses are generated when these dominant mHags are mismatched.[35] In addition, the response to one antigen is suppressed when a cell co-expresses an other antigen to which the animal has been primed previously.[36,37] Because MHC-incompatibilities comprise numerous epitopes[38] that already induce vigorous responses without priming, it is likely that some of these epitopes will be dominant and prevent the immune response to mHags that are considered to be relatively weak transplantation antigens.[26,33]
While no mHag-specific CTLs were found in the patients mismatched for a class I antigen, the anti-recipient response in the patients transplanted with a class I matched unrelated donor was very reminiscent of the ones described in HLA-identical related combinations.[39,40] In patient 4, all CTLs recognized mHags and part of the response was directed to HA-1, one of the dominant HLA-A*0201 restricted mHags involved in GvHD.[25] In patient 5, matched for HA-1, HA-2 and HA-4, the response was directed to H-Y and to five other non-sex linked mHags restricted by HLA-A*0201, HLA-B*4402 and Cw5 respectively. Although these alleles are among the most frequent ones in humans, none of these 5 mHags have been described in patients transplanted with an HLA identical sibling. Therefore, one might argue that a significant number of mHags will be mismatched only when donor and recipient originate from distinct geographical areas with different frequencies of mHag in the population. However, we found no indication that this would already hold for a donor recruited from a registry in an other European country. All 6 mHags analyzed occurred with similar frequencies in the local population as well as in the population from which the donor was recruited. Because the frequency of 4 of 5 of the novel mHags was such that also the majority of HLA-identical siblings must be mismatched for at least one of these mHags, we think that the fact that none of these mHags have been described before is again a consequence of immunodominance. It is very likely that the majority of mHags will only generate an immune response in the absence of more dominant mHags and that therefore some of the mHags recognized in patient 5 will only be found in the rare combinations that are compatible for HA-1, HA-2 and HA-4.
In conclusion, our data illustrate two immunological phenomena that are relevant for marrow transplantation with unrelated donors. Firstly, they confirm that T cells react to serologically silent class I mismatches as vigorously as to incompatibilities that are discerned by the routine techniques used for donor selection. Apparently, this does not only hold for the HLA-A and HLA-B subtype

variants[15,16,19] but also, as we show in this report for HLA-C incompatibilities. Because a pre-transplant CTLp assay detects serotype-, as well as subtype- and HLA-C incompatibilities,[13,14,19] this explains why this assay predicts post-transplant complications in unrelated donor/recipient combinations matched by routine typing.[21,28,30,31] Secondly, it shows that, because only a limited number of immunodominant epitopes are recognized during an immune response, other incompatibilities might be simply ignored. In patients transplanted with an HLA-identical sibling, this mechanism restricts the CTL response to a few mHags such as H-Y[41] or, in HLA-*A0201-positive patients to HA-1.[22,25] Here we show that the same mHags are recognized in patients transplanted with an unrelated class I matched donor. In mismatched combinations however, the response to the incompatible MHC molecule will dominate, and mHag-incompatibilities do not seem to have an additional effect. This does explain why the effect of gender mismatches that are clear risk factors in transplantations with HLA-identical siblings,[42-44] is much less apparent in unrelated donor/recipient combinations.[13,45] Because in most of these studies the donors have been matched by routine HLA-typing, a significant number must be mismatched[13] which will restrain the response to a mHag such as H-Y to the fully compatible combinations only.

Our study has been focused on the role of MHC class I incompatibilities after transplantation. In the 5 patients studied, the only incompatible class II locus was DP, an antigen of which the relevance is not clear yet.[46,47] Because in the class I matched, DP-incompatible patient 4, a vigorous anti-mHag response still occurred, it is tempting to speculate that DP-incompatibilities may be dominated by the response to mHags and that therefore, the role of DP-mismatches in bone marrow transplantations with unrelated donors is indeed less significant.[46]

Acknowledgments: We thank Christophe Grundschober for HLA-C oligotyping, Dr. Laurent Excoffier for helpful discussion and Florence Dumont-Girard for critical reading of the manuscript.

6.6 REFERENCES

1. Kernan NA, Bartsch G, Ash R, Beatty P, Champlin R, Filipovich A, Gajewski J, Hansen JA, Henslee-Downey J, McCullough J, McGlave P, Perkins HA, Phillips GL, Sanders J, Stoncek D, Thomas ED, Blume KG: Analysis of 462 transplantations from unrelated donors facilitated by the national marrow donor program. N Engl J Med 328:593, 1993
2. Anasetti C, Howe C, Petersdorf EW, Martin PJ, Hansen JA: Marrow transplants from HLA matched unrelated donors: An NMDP update and the Seattle experience. Bone Marrow Transplant 13:693, 1994
3. Gratwohl A, Hermans J: Bone marrow transplantation activity in Europe 1992: Report from the European Group for Bone Marrow Transplantation (EBMT). Bone Marrow Transplant 13:5, 1994
4. Ash R, Casper JT, Chitambar CR, Hansen R, Bunin N, Truitt RL, Lawton C, Murray K, Hunter J, Baxter-Lowe LA, Gottschall JL, Oldham K, Anderson T, Camitta B, Menitove J: Successful allogeneic transplantation of T-cell-depleted bone marrow from closely HLA-matched unrelated donors. N Engl J Med 322:485, 1990
5. Beatty PG, Anasetti C, Hansen JA, Longton GM, Sanders JE, Martin PJ, Mickelson EM, Choo SY, Petersdorf EW, Pepe MS, et al : Marrow transplantation from unrelated donors for treatment of hematologic malignancies: effect of mismatching for one HLA locus. Blood 81:249, 1993
6. McGlave P: Treatment of CML with unrelated donor marrow transplant. Leuk Lymphoma 11 Suppl 1:209, 1993
7. Martin PJ: Increased disparity for minor histocompatibility antigens as a potential cause of increased GVHD risk in marrow transplantation from unrelated donors compared with related donors. Bone Marrow Transplant 8:217, 1991
8. Goulmy E, Termijtelen A, Bradley BA, Van Rood JJ: Y-antigen killing by T cells of women is restricted by HLA. Nature 266:544, 1977
9. Rotzschke O, Falk K, Wallny HJ, Faath S, Rammensee HG: Characterization of naturally occurring minor histocompatibility peptides including H-4 and H-Y. Science 249:283, 1990
10. de Bueger M, Goulmy E: Human minor histocompatibility antigens. Transpl Immunol 1:28, 1993
11. Den Haan JMM, Sherman NE, Blokland E, Huczko E, Koning F, Drijfhout JW, Skipper J, Shabanowitz J, Hunt DF, Engelhard VH, Goulmy E: Identification of a graft versus host disease-associated human minor histocompatibility antigen. Science 268:1476, 1995
12. Wang W, Meadows LR, Den Haan JMM, Sherman NE, Chen Y, Blokland E, Shabanowitz J, Agulnik AI, Hendrickson RC, Bishop CE, Hunt DF, Goulmy E, Engelhard VH: Human H-Y: A

male-specific histocompatibility antigen derived from the SMCY protein. Science 269:1588, 1995

13. Rufer N, Tiercy J-M, Breur-Vriesendorp B, Gauchat-Feiss D, Shi X, Slavcev A, Lardy N, Speiser D, Gratwohl A, Chapuis B, Jeannet M, Roosnek E: Histoincompatibilities in ABDR-matched unrelated donor recipient combinations. Bone Marrow Transplant 16:641, 1995
14. Rufer N, Breur-Vriesendorp B, Tiercy J-M, Gauchat-Feiss D, Shi X, Slavcev A, Lardy N, Chapuis B, Gratwohl A, Speiser D, Jeannet M, Roosnek E: HLA-B35-subtype mismatches in ABDR serologically matched unrelated donor/recipient pairs. Hum Immunol 41:96, 1994
15. Keever CA, Leong N, Cunningham I, Copelan EA, Avalos BR, Klein J, Kapoor N, Adams PW, Orosz CG, Tutschka PJ, Baxter-Lowe LA: HLA-B44-directed cytotoxic T cells associated with acute graft-versus-host disease following unrelated bone marrow transplantation. Bone Marrow Transplant 14:137, 1994
16. Fleischhauer K, Kernan NA, O'Reilly RJ, Dupont B, Yang SY: Bone marrow-allograft rejection by T lymphocytes recognizing a single amino acid difference in HLA-B44. N Engl J Med 323:1818, 1990
17. Speiser D, Tiercy J-M, Rufer N, Chapuis B, Morell A, Kern M, Gmür J, Gratwohl A, Roosnek E, Jeannet M: Relation between the resolution of HLA-typing and the chance of finding an unrelated bone marrow donor. Bone Marrow Transplant 13:805, 1994
18. Tiercy J-M, Grundschober C, Jeannet M, Mach B: A comprehensive HLA-DRB, -DQB, and -DPB oligotyping procedure by hybridization with sequence-specific oligonucleotide probes, in Hui KMB (ed): Handbook of HLA typing techniques, Boca Raton, CRC Press Inc., 1993, p 117
19. Tiercy J-M, Djavad N, Rufer N, Speiser D, Jeannet M, Roosnek E: Oligotyping of HLA A2, A3, and B44 subtypes: Detection of subtype-incompatibilities between patients and their serologically matched unrelated bone marrow donors. Hum Immunol 41:207, 1994
20. Kennedy LJ, Poulton KV, Dyer PA, Ollier WER, Thomson W: Definition of HLA-C alleles using sequence-specific oligonucleotide probes (PCR-SSOP). Tissue Antigens 46:187, 1995
21. Roosnek E, Hogendijk S, Zawadynski S, Speiser D, Tiercy J-M, Helg C, Chapuis B, Gratwohl A, Gmür J, Seger R, Jeannet M: The frequency of pretransplant donor cytotoxic T cell precursors with anti-host specificity predicts survival of patients transplanted with bone marrow from donors other than HLA-identical siblings. Transplantation 56:691, 1993
22. van Els CA, D Amaro J, Pool J, Blokland E, Bakker A, van Elsen PJ, Van Rood JJ, Goulmy E: Immunogenetics of human minor histocompatibility antigens: their polymorphism and immunodominance. Immunogenetics 35:161, 1992

23. Schreuder GMT, Pool J, Blokland E, Van Els C, Bakker A, Van Rood JJ, Goulmy E: A genetic analysis of human minor histocompatibility antigens demonstrates Mendelian segregation independent of HLA. Immunogenetics 38:98, 1993
24. Rufer N, Breur-Vriesendorp BS, Tiercy J-M, Slavcev AS, Lardy NM, Francis P, Kressig R, Speiser DE, Helg C, Chapuis B, Gratwohl A, Jeannet M, Roosnek E: High-resolution histocompatibility testing of a group of sixteen B44-positive, ABDR serologically matched unrelated donor-recipient pairs: Analysis of serologically undisclosed incompatibilities by cellular techniques, isoelectrofocusing, and HLA oligotyping. Hum Immunol 38:235, 1993
25. Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg JH, Vossen JM, Gratwohl A, Vogelsang GB, van Houwelingen HC, Van Rood JJ: Influence of minor histocompatibility antigen mismatches on the development of graft-versus-host disease after bone marrow transplantation from HLA identical donors. N Engl J Med 1996 (in press)
26. Graff RJ, Bailey DW: The non-H-2 histocompatibility loci and their antigens. Transplant Rev 15:26, 1973
27. Anasetti C, Beatty PG, Storb R, Martin PJ, Mori M, Sanders JE, Thomas ED, Hansen JA: Effect of HLA incompatibility on graft-versus-host disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. Hum Immunol 29:79, 1990
28. Kaminski E, Hows J, Man S, Brookes P, Mackinnon S, Hughes T, Avakian O, Goldman JM, Batchelor JR: Prediction of graft versus host disease by frequency analysis of cytotoxic T cells after unrelated donor bone marrow transplantion. Transplantation 48:608, 1989
29. Petersdorf EW, Longton GM, Anasetti C, Martin PJ, Mickelson EM, Smith AG, Hansen JA: The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. Blood 86:1606, 1995
30. Spencer A, Brookes PA, Kaminski E, Hows JM, Szydlo RM, Van Rhee F, Goldman JM, Batchelor JR: Cytotoxic T lymphocyte precursor frequency analyses in bone marrow transplantation with volunteer unrelated donors: Value in donor selection. Transplantation 59:1302, 1995
31. Speiser DE, Tiercy J-M, Rufer N, Grundschober C, Gratwohl A, Chapuis B, Helg C, Löliger C, Siren M-K, Roosnek E, Jeannet M: High resolution HLA matching associated with decreased mortality after unrelated bone marrow transplantation. 1996 (submitted)
32. Sherman LA, Chattopadhyay S: The molecular basis of allorecognition. Annu Rev Immunol 11:385, 1993
33. Speiser DE, Zurcher T, Ramseier H, Hengartner H, Staeheli P, Haller O, Zinkernagel RM: Nuclear myxovirus-resistance protein Mx is a minor histocompatibility antigen. Proc Natl Acad Sci U S A 87:2021, 1990

34. Neefjes JJ, Ploegh HL: Allele and locus-specific differences in cell surface expression and the association of HLA class I heavy chain with beta 2-microglobulin: differential effects of inhibition of glycosylation on class I subunit association. Eur J Immunol 18:801, 1988

35. Wettstein PJ: Immunodominance in the T cell response to multiple non-H-2 histocompatibility antigens. III. Single histocompatibility antigens dominate the male antigen. J Immunol 137:2073, 1986

36. Schutze MP, Langlade-Demoyen P, Przewlocki G, Leclerc C: Alloantigen pretreatment induces a down-regulation of the in vivo subsequent development of cytotoxic T lymphocytes directed against linked alloantigens . Eur J Immunol 19:1365, 1989

37. Przewlocki G, Leclerc C: Role of major and minor histocompatibility antigens in the suppression of alloreactive cytotoxic responses induced by alloantigen pretreatment. Res Immunol 141:839, 1990

38. Matzinger P, Bevan MJ: Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens? Cell Immunol 29:1, 1977

39. van Els CA, Bakker A, Zwinderman AH, Zwaan FE, Van Rood JJ, Goulmy E: Effector mechanisms in graft-versus-host disease in response to minor histocompatibility antigens. I. Absence of correlation with cytotoxic effector cells. Transplantation 50:62, 1990

40. van Els CA, Bakker A, Zwinderman AH, Zwaan FE, Van Rood JJ, Goulmy E: Effector mechanisms in graft-versus-host disease in response to minor histocompatibility antigens. II. Evidence of a possible involvement of proliferative T cells. Transplantation 50:67, 1990

41. Voogt PJ, Fibbe WE, Marijt WA, Goulmy E, Veenhof WF, Hamilton M, Brand A, Zwann FE, Willemze R, Van Rood JJ, et al : Rejection of bone-marrow graft by recipient-derived cytotoxic T lymphocytes against minor histocompatibility antigens. Lancet 335:131, 1990

42. Eisner MD, August CS: Impact of donor and recipient characteristics on the development of acute and chronic graft-versus-host disease following pediatric bone marrow transplantation. Bone Marrow Transplant 15:663, 1995

43. Gale RP, Bortin MM, van Bekkum DW, Biggs JC, Dicke KA, Gluckman E, Good RA, Hoffmann RG, Kay HE, Kersey JH, et al : Risk factors for acute graft-versus-host disease. Br J Haematol 67:397, 1987

44. Ho WG, Champlin RE, Winston DJ, Feig SA, Gale RP: Bone marrow transplantation in patients with leukaemia previously transfused with blood products from family members. Br J Haematol 67:67, 1987

45. Petersdorf EW, Stanley JF, Martin PJ, Hansen JA: Molecular diversity of the HLA-C locus in unrelated marrow transplantation. Tissue Antigens 44:93, 1994

46. Petersdorf EW, Smith AG, Mickelson EM, Longton GM, Anasetti C, Choo SY, Martin PJ, Hansen JA: The role of HLA-DPB1 disparity in the development of acute graft-versus-host disease following unrelated donor marrow transplantation. Blood 81:1923, 1993
47. Anasetti C, Etzioni R, Petersdorf EW, Martin PJ, Hansen JA: Marrow transplantation from unrelated volonteer donors. Annu Rev Med 46:169, 1995

Chapter 7

Further studies on the histoincompatibilities detected in four patients after bone marrow transplantation

7.1 Do alloreactive T cell responses become "less cross-reactive" after bone marrow transplantation ?

7.1.1 Introduction

Alloreactive cytotoxic T lymphocytes (CTL) play a crucial role in graft-versus-host disease (GvHD) and graft-rejection. It is now widely accepted that these cells react against incompatible MHC-antigens. Because classical serological HLA-typing techniques are unable to identify each HLA-variant precisely, numerous HLA-mismatches are still present even in HLA closely-matched unrelated donor-recipient pairs (1,2) and form important transplantation barriers by inducing GvHD.

Several models have been proposed to explain the high frequency of alloreactive T cells precursors (3-5). The model first presented by Matzinger and Bevan might be the one which correlates the best with the actual knowledge of antigen presentation (4). They propose that the specific recognition of foreign MHC molecules by T lymphocytes could be influenced by self-structures associated with those molecules. Since a single MHC molecule thereby can present many different cellular peptides and different T cell receptors may interact with those MHC + X, the high frequency of alloreactivity is a consequence of the diversity of these multiple "binary complexes" recognized. Many studies have been focused on the role of endogenous peptides in MHC allorecognition (6-12) and at present, it is generally accepted that alloreactive responses involves both the MHC molecule and its associated peptide ligand.

To get inside into the precise nature of alloreactive T cell recognition after bone marrow transplantation, two transplanted patients mismatched either at the HLA class I -A or -C locus have been studied in more detail.

7.1.2 Patients, materiel and methods

HLA matching of donor-recipient pairs. Since these two patients belong to the same group of patients already described in Chapter 6, the HLA typing procedures used for these D/R pairs can be found in "patient, materials and methods" of that chapter (on pp 106). Patient 1 (P1) was mismatched for HLA-A2 vs -A3 with his donor, while Patient 3 (P3) is incompatible with his donor for HLA-Cw and -DP. Complete HLA typing is as following:

pairs	A	B	C	DRB1	DRB3	DQ	DP
P1	0201,**0301**	5,49	7,15	2,1101	02	w3	0401,0201
D1	0201,**0201**	=	=	=	=	=	=
P3	0301,24	18,3501	4,**701**	1501,0301	02	0201,0602	0201,**0202**
D3	=	=	4,**5**	=	=	=	0201,**0301**

Primary CTLp test. Serial dilutions of donor mononuclear cells (5.10^4-$0.125.10^4$) were cultured in 200 µl 96-round bottom plates with constant numbers (1.10^6/ml) of irradiated recipient mononuclear cells as stimulators. On day 3 and 6, 25 µl of fresh medium with 100 U/ml rIL-2 (Roche. Nutley, USA) was added to all wells. After 10 days of culture, the wells were tested against ^{51}Cr-labeled, phytohemeagglutinin-stimulated recipient T cell blasts.

Post-transplant CTLpf test. Limiting numbers of post-transplant patient PBMCs (4.10^4-1.10^4) were cultured in 96 U-bottom plates with constant numbers (1.10^6/ml) of irradiated pre-transplant recipient mononuclear cells. As a positive control, post-transplant patient cells (1.10^4) were cultured with irradiated third-party cells (1.10^6/ml). Addition of IL-2 and the cytotoxic assay were performed as described above.

Cloning of class I specific T cells. Cytotoxic T cell clones were established from the positive wells of the primary and post-transplant CTLpf assays as described in appendix 1. Specificity towards recipient cells was tested using autologous donor cells and third-party cells that do not express HLA antigens in common as

negative controls. Pre- and post-transplant T cell clones were tested against a panel of third party cells expressing well-defined HLA-A,-B subtypes and HLA-Cw antigens in common with the patient in order to determine their fine specificity.

HLA-C typing. Oligotyping for HLA-A3 and -Cw7 was performed as described before (13). This method based on a PCR/SSO-oligotyping protocol is able to discriminate the four HLA-Cw7 and the two HLA-A3 variants.

7.1.3 Results

A) Analysis of the alloreactive T cell responses found in the HLA-Cw7-mismatched donor/recipient pair after bone marrow transplantation.

This patient was followed 3 months after bone marrow transplantation after she was reinfused with donor buffy-coat cells in attempt to eradicate the recurring disease. Severe GvHD developed about 10 days after the beginning of the buffy-coat treatment, leaving the patient pancytopenic, a few days later. All the T cell clones presented in this study were isolated following buffy-coat treatment during GvHD.

The complete analysis of the specificities recognized by the T cell clones before and after BMT is presented in Table 1. As already described in Chapter 6, the same incompatible HLA-Cw7 molecule detected by the CTLpf test and the CTL clones before BMT, was the target recognized after transplantation. However, this recognition of HLA-Cw7 could be divided into two distinct patterns: seven clones isolated before transplantation and at the beginning of the buffy-coat treatment on day 12 lysed all third-party cells expressing the broad, serologically defined HLA-Cw7 antigen, while others, isolated ≥ 12 days after infusion of the buffy-coat lysed only the HLA-Cw7 targets expressing the same HLA-C*0701 alleles as the patient. Apparently, the 12 days period in vivo had selected the more specific alloreactive cells rather than the crossreactive ones.

The two alloreactive cytotoxic patterns followed two sequential steps in time. The crossreactive CTL pattern present before transplantation and quite early after buffy-coat treatment is entirely replaced by the more specific alloreactive pattern after the day 12 of treatment.

Table 1: Cytotoxic T clones loose their cross-reactivity during severe GvHD after buffy-coat treatment

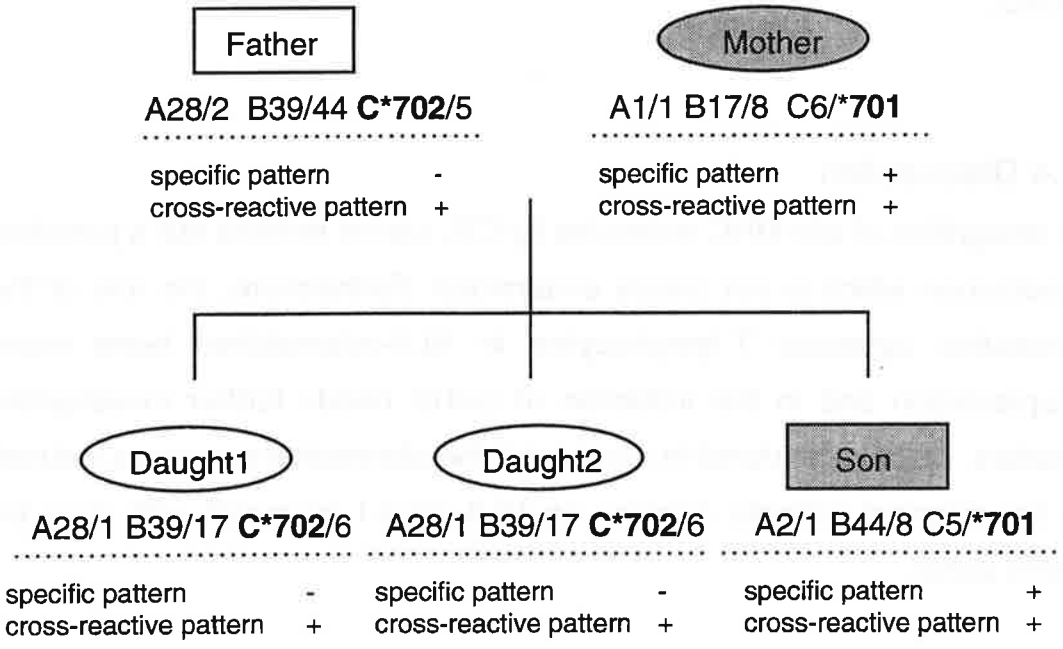
Third-party cells[1]							Pre-BMT[2] (n = 4)	+ 12d[2] (n = 3)	+ 12d[2] (n = 2)	+ 18d[2] (n = 3)
class I	A		B		C		cross-reactive pattern		specific pattern	
Patient	3	24	18	3501	**701**	4	+	+	+	+
Donor	3	24	18	3501	5	4	-	-	-	-
4033	1	28	8	14	**701**	8	+	+	+	+
4099	11	31	35	x	**701**	4	+	nt	+	+
4095	1	2	8	62	**701**	3	+	+	+	+
4018	2	x	7	18	**701**	**702**	+	+	+	+
4079	2	3	7	8	**701**	**702**	+	+	+	+
4070	2	33	14	39	**702**	8	+	+	-	-
4015	2	3	7	44	**702**	x	+	+	-	-
4091	3	32	7	37	**702**	6	+	+	-	-
4075	2	3	7	57	**702**	3	+	+	-	-

[1] 12 T cell clones were tested against nine third-party cells expressing either HLA class I C*701 and/or C*702.
[2] T cell clones were obtained before BMT and 12 or 18 days after the beginning of the buffy-coat treatment.
[3] Results are expressed as + if third-party is recognized and lysed by the CTLs or - when there was no recognition.

The two alloreactive CTL patterns could also be demonstrated in a family study (Figure 1). HLA-C*0701 and -C*0702 alleles were both present and expressed among the different members of this family.

These two cytotoxic alloreactivity patterns correlated exactly with the segregation of the HLA-Cw7 subtypes in the family. The limited, non cross-reactive CTL's recognized only the HLA-C*0701 subtype present in mother's HLA haplotype that was inherited by the son, whereas, the cross-reactive CTL's could also recognize the HLA-C*0702 allele expressed in the father and his two daughters.

Figure 1 : HLA-Cw7 subtype segregation in one family study by anti-Cw7 CTL clone analysis



B) Analysis of the alloreactive T cell responses found in a HLA-A mismatched donor/recipient pair after bone marrow transplantation.

The alloreactive T cell clones already described in Chapter 6, recognized the incompatible HLA-A3 molecule before and after transplantation. However, distinct post-transplant CTL patterns could be identified (Table 2).

The donor cytotoxic T cell clones isolated before and one month after transplantation, recognized all HLA-A3 molecules present on third-party cells. However, two T cell clones isolated two months after transplantation, presented distinct recognition patterns. The third-party cells shown in the first part of Table 2, could be separated into two groups: some (4082 to 4043) were only recognized by clone A, whereas others (TBD to 4003) were only lysed by clone B. Finally, the third-party cells listed in the second part of that table (4030 to 4037), were recognized by both T cell clones (A + B).
Oligotyping for HLA-A3 did not show any difference at the subtype level; all third-party cells expressed the common HLA-A*0301 allele (Table 2). Therefore, it is very unlikely that the two distinct CTL clones recognize different subtypes of HLA-A3.

7.1.4 Discussion

The recognition of allo-MHC molecules by CTL clones remains still a paradoxical phenomenon which is not clearly understood. Furthermore, the role of these alloreactive cytotoxic T lymphocytes in HLA-mismatched bone marrow transplantation and in the induction of GvHD needs further investigations. Therefore, we have analyzed in more detail the alloreactive responses induced in two transplanted patients carrying an HLA-class I mismatch with their bone marrow donor.

These studies demonstrated that the HLA incompatible molecule identified in those patients before BMT, were the target recognized after transplantation during severe GvHD. Secondly, they showed the existence of specific and cross-reactive patterns of alloreactivity. This limited, non cross-reactive pattern was characterized in each case, by different features.

In Patient 1, the difference between the specific and the cross-reactive pattern was based on the discrimination of two HLA-class I subtypes (C-*0701,C-*0702).

Table 2 : Cytotoxic donor T clones lyse three different patterns of HLA-A3 antigens after bone-marrow transplantation during severe GvHD

Third-party cells[1]							Pre-BMT[2]	+ 1 month[2]	+ 2 months[2]	
HLA :	A		B		C		n = 5	n = 2	clone A n = 1	clone B n = 1
Patient	2	**301**	5	49	7	15	+	+	+	+
Donor	2	2	5	49	7	15	-	-	-	-
4082	**301**	32	7	x	7	x	+	+	+	-
SalzP	2	**301**	7	14	7	8	+	+	+	-
LB	2	**301**	7	14	7	8	+	+	+	-
4045	**301**	29	7	38	7	x	+	+	+	-
4043	**301**	10	7	44	7	x	+	+	+	-
TBD	2	**301**	7	14	7	8	+	+	-	+
0091	2	**301**	7	14	7	8	+	+	-	+
4019	**301**	11	7	55	3	x	+	+	-	+
4003	**301**	1	51	55	3	x	+	+	-	+
4030	**301**	x	7	18	x		+	+	+	+
4091	**301**	32	7	37	6	7	+	+	+	+
4011	**301**	23	53	38	4	x	+	+	+	+
4039	**301**	30	7	18	7	x	+	+	+	+
4037	**301**	x	44	35	4	5	+	+	+	+

[1] 16 third-party cells expressing HLA class I-A3 antigen were tested for lysis by pre- and post-transplantation isolated T cell clones.
[2] T cell clones were obtained before BMT and 1 and 2 months after BMT, respectively.

This was however, unlikely for the second patient. Indeed, in that particular case, one plausible explanation for the observation of these two limited and partially complementary T cell responses (clone A + B) might be provided by the recognition or not of a particular peptide presented by HLA-A*0301. This question will be addressed by isolating that particular peptide by acid elution and reverse phase HPLC technology.

It is interesting to speculate that the mechanism of peptide recognition proposed for the clones that can discriminate HLA-A*0301 individuals, is also operative for the clones that discriminate HLA-C*0701 from HLA-C*0702.
Since recent studies have demonstrated a direct role of the associated peptide ligands in allorecognition (15,16), the difference "seen" by the CTL clones between HLA-C*0701 and -C*0702 alleles could be due to the presentation of different set of peptides. If one would accept that recognition of peptides become more important for the clones with higher specificity after BMT, one could argue that the discrimination of the two HLA-Cw7 subtypes is made on basis of the distinct peptides bound by HLA-C*0701 and -C*702, respectively. The specific CTL pattern would only recognize the peptides presented by HLA-C*0701, whereas the less specific pattern would also cross-react with the peptides expressed by HLA-C*0702 allele.

For instance, position 99 (α-2) that differs in C*0701/*0702 (Table 3) is a key position for peptide binding (14) which makes it likely that although the two subtypes differ by 5 amino acids only, the peptides bound will be very different.

In conclusion, the specific T cell clone patterns observed in both patients after bone marrow transplantation might indicate a pivotal role for peptides in the allorecognition of these incompatible MHC molecules. Furthermore, these T cell clones acquire more specific features, since T cell responses found before transplantation are extended and probably less specific.

Table 3 : Polymorphic amino acid residues encoded by HLA-C*701 and C*702 alleles.

	α–1		α–2		α–3
	1	66	99	136	273
consensus	C	K	Y	A	R
C*701	-[1]	N	-	-	S
C*702	G	-	S	X	-

[1]Dashes indicate identity with the consensus sequence.

One could argue that the limited T cell recognition pattern represents very specific and high affinity alloreactive T cell responses and that this particular subset is responsible for the occurrence of GvHD severity. Because these cells will be stimulated, the cross-reactive and less specific T cell response will be diluted progressively and finally be entirely replaced by these restrictive T cell allo-responses.

7.1.5 References

1. Santamaria P, Reinsmoen NL, Lindstrom AL, et al. Frequent HLA class I and DP sequence mismatches in serologically (HLA-A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA-identical unrelated bone marrow transplant pairs. Blood. 1994; 83:280.
2. Rufer N, Tiercy JM, Breur-Vriesendorp BB, et al. Histoincompatibilities in ABDR-matched unrelated donor recipient combinations. Bone Marrow Trasnplant. 1995; 16:641.
3. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. Annu Rev Immunol. 1993; 11:385.
4. Matzinger P, Bevan MJ. Hypothesis: why do so many lymphocytes respond to major histocompatibility antigens. Cell Immunol. 1977; 29:1.

5. Bevan MJ. High determinant density may explain the phenomenon of alloreactivity. Immunol Today. 1984; 5(5):128.
6. Eckels DD, Gorski J, Rothbard J, Lamb JR. Peptide-mediated modulation of T cell allorecognition. Proc Natl Acad Sci USA. 1988; 85:8191.
7. Demotz S, Sette A, Sakaguchi K, Buchner R, et al. Self peptide requirement for class II major histocompatibility complex allorecognition. Proc Natl Acad Sci USA. 1991; 88:8730.
8. Panina-Bordignon P, Corradin G, Roosnek E, Sette A, Lanzavecchia A. Recognition by class II alloreactive T cells of processed determinants from human serum proteins. Science. 1991; 252:1548.
9. Heath WR, Kane KP, Mescher MF, Sherman LA. Peptide-dependent recognition of H-2K^b by alloreactive cytotoxic T lymphocytes. Nature. 1989; 341:749.
10. Heath WR, Kane KP, Mescher MF, Sherman LA. Alloreactive T cells discriminate among a diverse set of endogenous peptides. Proc Natl Acad Sci USA. 1991; 88:5101.
11. Rötzschke O, Falk K, Faath S, Rammensee HG. On the nature of peptides involved in T cell alloreactivity. J Exp Med. 1991; 174:1059.
12. Udaka K, Tsomides TJ, Walden P, Fukusen N, Eisen HN. A ubiquitous protein is the source of naturally occurring peptides that are recognized by a CD8$^+$ T-cell clone. Proc Natl Acad Sci USA. 1993; 90:11272.
13. Tiercy JM, Djavad N, Rufer N, et al. Oligotyping of HLA A2, A3, and B44 subtypes: detection of subtype-incompatibilities between patients and their serologically matched unrelated bone marrow donors. Hum Immunol. 1994; 41:207.
14. Zemmour J, Parham P. Distinctive polymorphism at the HLA-C locus : implications for the expression of HLA-C. J Exp Med. 1992; 176:937.
15. Tallquist MD, Pease LR. Alloreactive 2C T cells recognize a self peptide in the context of the mutant K^{bm3} molecule. J Immunol. 1995; 155:2419.
16. Tomiyama H, Takamiya Y, Hill AB, et al. Isolation of a human allo-peptide presented by HLA-B51 molecules. Int Immunol. 1995; 7:905.

7.2 How are minor histocompatibility antigens characterized ?

7.2.1 Introduction

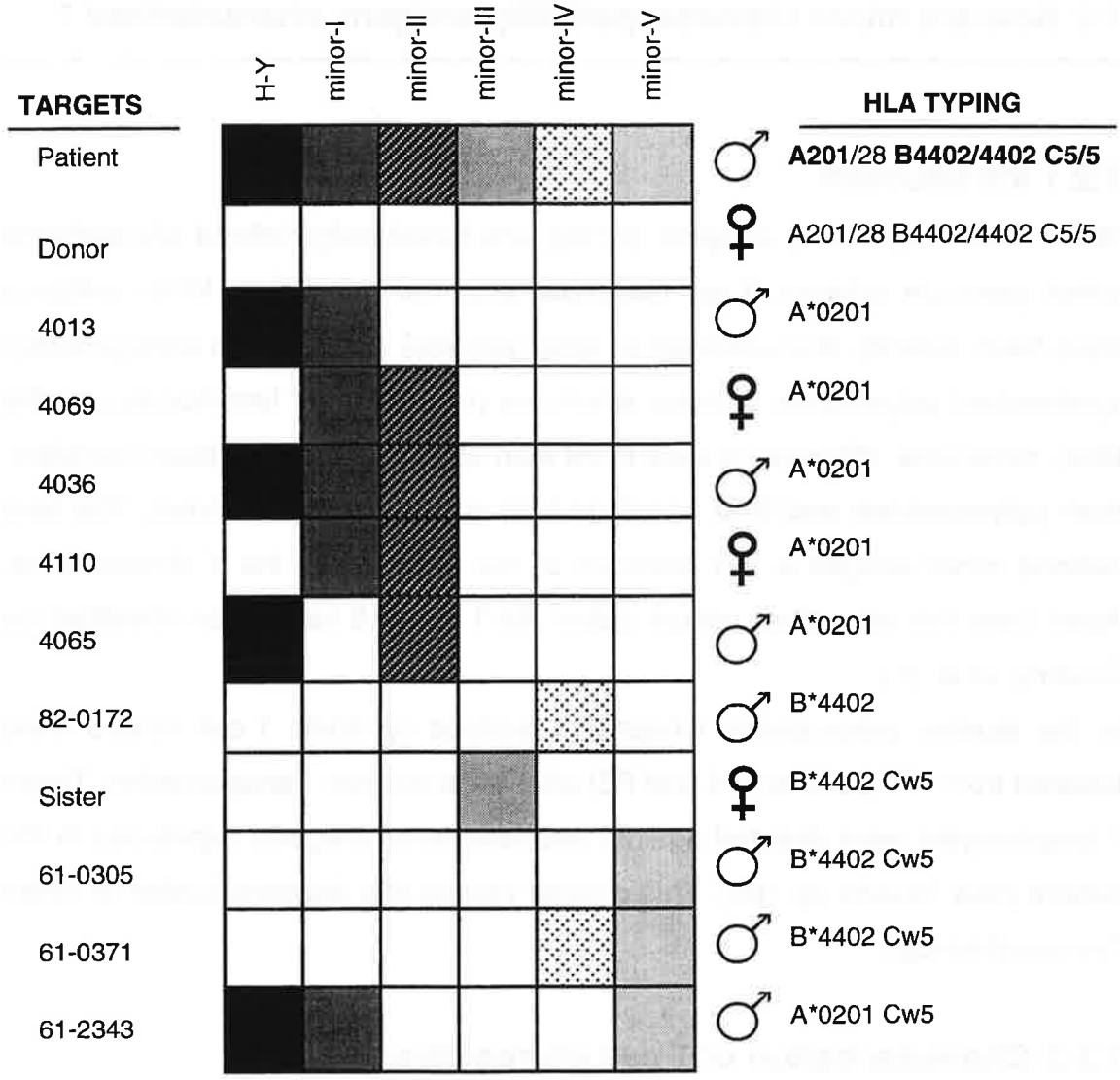
Minor histocompatibility antigens (mHag) are functionally defined allo-antigens which generate cytotoxic T cell responses after transplantation. Minor antigens have been recently characterized as small peptides derived from endogenously synthesized polymorphic proteins which are presented toT lymphocytes by the MHC molecules. Whereas in mice more than 40 minor loci have been identified, their polymorphism and their variety in human is presently unknown. The best defined minor antigen is H-Y encoded on the long arm of the Y chromosome. Apart from this one, other minors called HA-1 to HA-5 have been identified by Goulmy, et al. (1).

In the studies presented in Chapter 6, various cytotoxic T cell clones were isolated from two patients (P4 and P5) after bone marrow transplantation. These T lymphocytes were directed against five new minor antigens expressed in the patient (see Results pp.106). This chapter reports the characterization of these five novel mHags.

7.2.2 Characterization of T cell alloreactive patterns

Since minor antigens are small peptides presented by the MHC molecules, the first step is to characterize the MHC-restriction element recognized. The second step, once the HLA allele presenting the minor antigen has been identified, is to characterize the pattern of reactivity of each cytotoxic T clone. Therefore, the various T clones are tested against third-party cells expressing well-defined HLA-A, -B subtypes and HLA-Cw antigens in common with the patient. A representative example is shown in Figure 1.

Figure 1: Analysis and characterization of five new minor antigen patterns



Black and graded squares represent the recognition of the third-party target cells by the six alloreactive T cell clones (H-Y to minor-V), whereas no recognition of those targets is symbolised by blank squares.

[1] only alleles in common with the patient are depicted.

For instance, the first colomn / black pattern shows that a T cell clone specific for H-Y, when tested against third-party cells, recognizes only some of these cells (4013, 4036, 4065, and 61-2343) in ^{51}Cr-release assays. The HLA-haplotypes carried by those cell lines are then compared to each other. Because, only HLA-A*0201 positive cells are recognized by the clone, this MHC molecule must be the restricting element. Since, only male cells are lysed, the minor antigen recognized by this cytotoxic T cell clone could be identified as the H-Y minor antigen. The five other minor antigens (minor-I to -V) were characterized by using the same approach.

7.2.3 Characterization of minor antigens in family studies

A further confirmation that the cytotoxic activity was directed against minor antigens could be obtained in studies of two informative families (see pp. 106). Such studies allow the identification of these minor antigens as being encoded by genes that segregate independently from HLA molecules and from each other.

7.2.4 Analysis by elution of cellular peptides

It is only recently that isolation of minor antigen peptides from MHC class I and class II molecules has become possible. Such studies were carried on by Rammensee et al. and the methodology is based on acid elution and subsequent reverse phase HPLC separation (2). These reports have demonstrated the crucial role of peptides in the allorecognition by anti-minor T cell clones.

By using these HPLC techniques, we have further characterized some of our minor antigens. Briefly, the peptides were isolated from PHA-blast of either the patient 5's father or mother by acid elution and subsequently separated by reverse phase HPLC. This part of the work has been carried on by E. Wolpert from the Karolinska Institute (Stockolm). T2-Tap1/2 mutant target cells expressing HLA*0201 allele are then incubated with minor antigen specific T cell clones and the various fractions containing the purified cellular peptides. The specific fraction which contains the minor antigen peptide is thereby identified.

For example, we have analysed fractions with anti-minor-I and -H-Y T cell clones. The results are shown on Figure 2 and 3.

Minor antigen pattern I has been shown by family studies to be expressed in both the mother and the father of patient 5. The 40 fractions isolated from the father or from the mother containing the purified peptides were incubated with T2-A*0201 cell lines and anti-minor I T cell clones. Fraction 19 is the only fraction recognized in three separate experiments by the T cell clones. This fraction contains therefore minor-I. Finally, peptide fractions 26-27-29 isolated from the father cells, were recognized by the anti-H-Y minor antigens. Although these results remain preliminary, it is likely that those fractions contain the male specific H-Y minor antigens.

Figure 2: Fraction 19 of patient5's mother and father contains minor-I

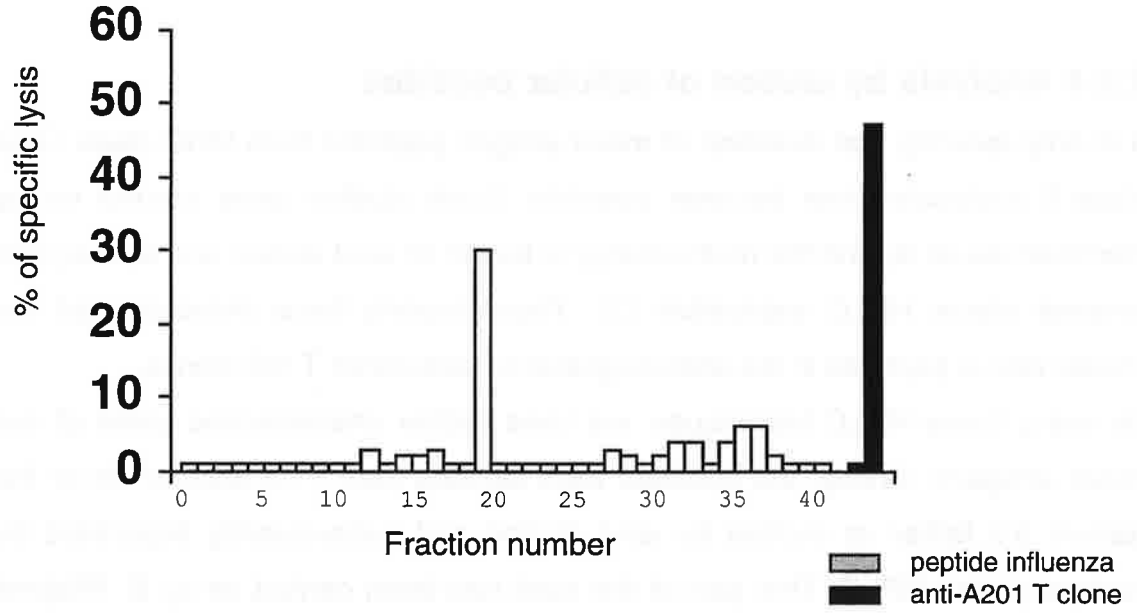
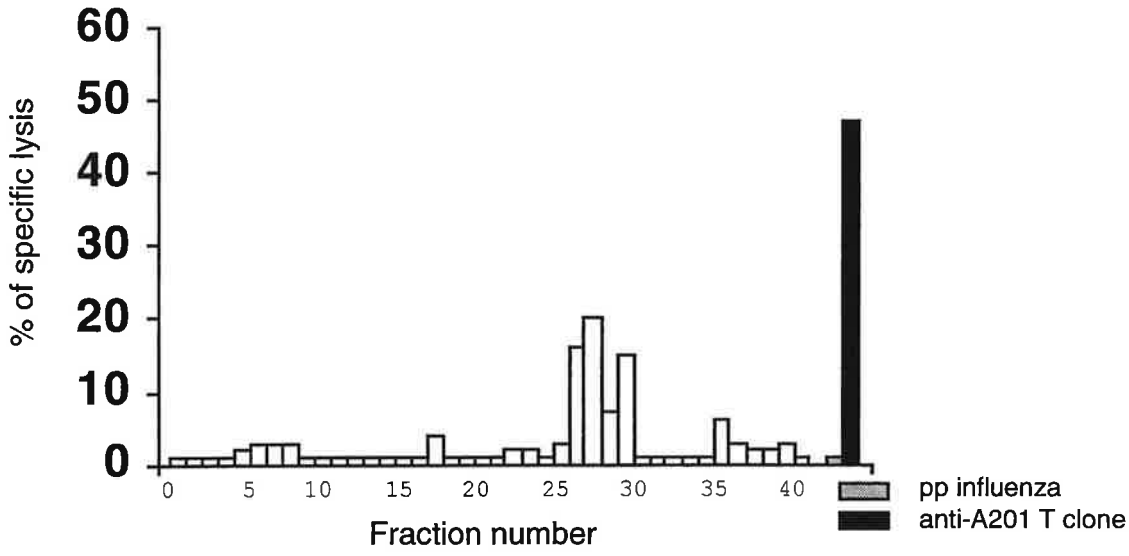


Fig 2 and 3. The influenza peptide is used as a negative control, whereas the expression of HLA-A*0201 at the T2 cell surface is monitored by anti-A201 T clones.

Figure 3: The peptide fraction 26-27-29 (father) contains H-Y minor antigen



In conclusion, the major difficulty in the analysis of human minor histocompatibility antigens lies in the fact that the precise nature of the polymorphic protein containing the minor antigen epitope is usually unknown. Therefore, in most cases, minor antigens are defined by either patterns of recognition by CTL-clones, family segregation studies or peptide HPLC purification. The identification of those polymorphic minor antigens is however possible, but more sophisticated technology is needed in order to isolate and sequence the 8 amino- acid derived from the MHC presented epitope (3).

References:

1. Van Els CACM, D'Amaro JD, Pool J, et al. Immunogenetics of human minor histocompatibility antigens: their polymorphism and immunodominance. Immunogenetics. 1992; 35:161.
2. Rötzschke O, Falk K, Faath S, Rammensee HG. On the nature of peptides involved in T cell alloreactivity. J Exp Med. 1991; 174:1059.
3. Wang W, Meadows LR, den Haan JMM, et al. Human H-Y: a male-specific histocompatibility antigen derived from the SMCY protein. Science. 1995; 269:1588.

Chapter 8

GENERAL CONCLUSION

BMT: a subtle balance between success and failure

For many patients suffering from hematological malignancies, bone marrow transplantation represents the only efficient therapy. Various pre-transplant parameters affect (disease-free) survival and post-transplant complications such as Graft-versus-Host Disease. For instance, the choice of the bone marrow donor clearly influences survival rates (1).

The best choice as a bone marrow donor is an HLA-genotypically identical related individual. Such combinations will always be "perfectly matched" because the errors of the serological techniques used for the identification of an HLA-identical sibling are easily corrected by looking at the four haplotypes in the family. Moreover, due to the fact that those identical siblings inherit the same haplotypes, they will share the same HLA-subtypes and full identity will be guaranteed. Finally, as demonstrated by Martin (2), the disparity of minor antigens might be greater between HLA-identical unrelated individuals than in identical siblings pairs.

However, such an optimal donor is only found for 25 to 30 % of the patients. Therefore bone marrow transplantation with HLA "ABDR-matched" unrelated donors has been developed as an alternative. Unfortunately, such transplantations are hampered by an increased incidence of GvHD and lower survival rates. These complications can be mainly explained by the fact that unrelated "ABDR-matched" combinations are less compatible than identical siblings. The definition of "an HLA ABDR-matched donor" often used in the literature has never been an exact one. In this thesis, this terminology has been used to define a donor compatible at HLA-A, -B and -DR locus by classical serological typing methods and oligotyping for DR 1-14. Although this definition suggests that a donor/recipient pair is "ABDR-identical", other undetected mismatches such as HLA-subtype, -Cw, -DQ and -DP incompatibilities may still

be present in those individuals. One should therefore refer to "ABDR-identical" pair as an HLA *closely* -matched combination only.
Serologically defined antigens can be seen as the top of an iceberg, where many histoincompatibilities will remain "hidden". It is only by the use of extended technologies such as oligotyping, CTLpf assays and class I-subtype specific T cell clones, that further histoincompatibilities can be identified. Because before BMT, frequencies of T cell precursors against minor histocompatibility antigens are usually too low to be detected by CTLpf assays, incompatibilities among those antigens will also remain undetected (Figure 1).

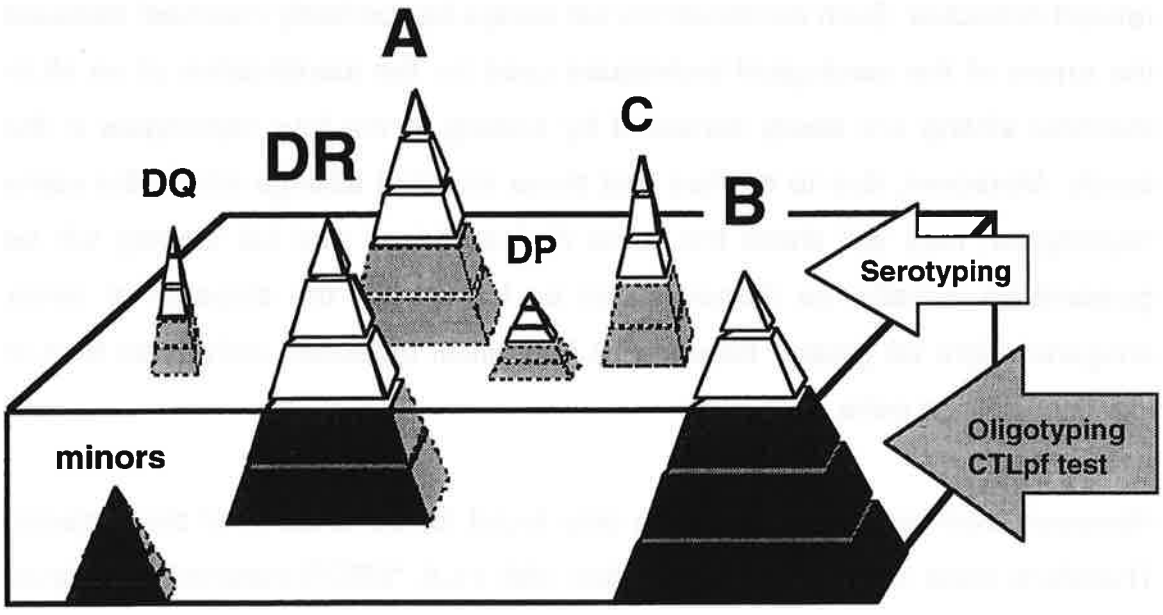


Figure 1: Many histoincompatibilities remain undetected when only classical typing techniques are used.

At present, several studies have analyzed the degree of HLA mismatches in serologically "ABDR-matched" donor/recipient pairs in detail (3,4). Frequent HLA class I and class II mismatches were detected in the different combinations analyzed. For instance, in our own report, 72 % of the "ABDR-identical" D/R pairs

has been shown to be still incompatible for various HLA class I and/or class II subtypes.

The application of such high resolution technology has important consequences for the search of a potential bone marrow donor.
For instance, the identification of a perfectly matched unrelated donor seems to be an impossible challenge, due to the enormous polymorphism at the HLA genes. Indeed, although these sophisticated typing techniques do provide precise tools for HLA-typing, many unrelated potential donors will now be defined as HLA-incompatible. Therefore, the key question has become to determine which mismatch is acceptable and which is not.
Unfortunately, this question has not been studied carefully although many reports have tried to assess the effect of HLA-mismatches on the outcome of a bone marrow transplant. This is due to the fact that most of these studies used classical serotyping methods only. Therefore, many HLA-incompatibilities must have remained undetected so that the fact that no clear correlation on GvHD incidence was found between "ABDR-matched" and HLA serologically mismatched D/R pairs did not come as a surprise (5,6).
Because high resolution typing technique reveals precisely every HLA-incompatible subtype, it is only by using these efficient tools that studies on the relative importance of those variants after bone marrow transplantation could be undertaken.

Several recent studies based on high resolution HLA-typing have now evaluated the impact of the degree of HLA-identity on the clinical outcome (7-9). Our study and others, demonstrated that patients transplanted with highly matched unrelated donors have significantly better survival rates than patients transplanted with less compatible donors emphasizing the crucial role of HLA-incompatibilities in post-transplant complications.

In order to assess precisely the relative importance of each HLA-mismatch on the onset of GvHD, five transplanted patients were analyzed in more detail. These studies showed firstly, that high resolution typing techniques detects *relevant* incompatibilities, since the HLA-mismatches found before transplantation were also the targets recognized after bone marrow transplantation. They confirm that serologically undetected HLA-mismatches can induce a strong cytotoxic T cell response against these hidden incompatibilities after bone marrow transplantation. Secondly, it is only when the donor/recipient pairs are fully HLA-identical, that post-transplant CTL response are directed against minor histoincompatibilities. Finally, five new minor antigens restricted by various HLA-molecules have been identified, suggesting a greater polymorphism among these minor antigens than initially expected.

One of the central issues of this study is immunodominance, an immunological concept already described in mice (10). We report here, that cytotoxic T cell responses against HLA-mismatches are immunodominant and that anti-minor cytotoxic responses occur only in fully HLA-identical D/R pairs. This phenomenon can be explained by the fact that MHC-incompatibilities comprise numerous epitope that already induce vigorous responses without priming, whereas the immune response to miHAs is considered as a relative weak response which induces low T cell frequencies (11).

Therefore, one positive aspect from the view point of the patient is that minor antigen responses might be irrelevant when recipient and donor are already mismatched for a MHC class I molecule. As a consequence, one could imagine that a sex-mismatch in a D/R pair incompatible at the HLA-C locus, should not be considered as an additional, cumulative mismatch and therefore hinder the choice for that potential unrelated donor when no others are available.
The phenomenon of immunodominance could also be observed in cytotoxic anti-minor antigen responses. Indeed, only a few minor antigen patterns were

identified in the patients and two of them were characterized as the dominant H-Y and HA-1 minor antigens already described in the literature (12).

Taken together, the order among the various immunodominant molecules in bone marrow transplantation might be as depicted in Figure 2.

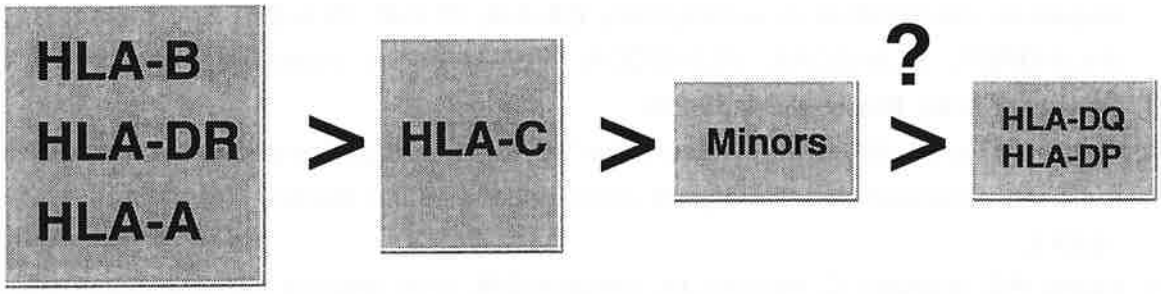


Figure 2: Representation of the relative dominant order of mismatches after bone marrow transplantation.

Taken as a whole, the results presented in this thesis will allow a better understanding of the parameters that influence the outcome of a bone marrow transplantation. It is obvious that the analysis of the relevance of major and minor histo-incompatibilities after bone marrow transplantation and their significance in Graft-versus-Host Disease has only been possible because the resolution of the tissue typing used has been high enough to define the number of HLA mismatches in a combination precisely. Therefore, apart from helping to select the most compatible donor, they might also give insight into which mismatches might be "acceptable" ones.

This will finally lead to a better insight in the role of histocompatibilities in bone marrow transplantation so that an optimal strategy of donor selection and immunosuppression can be chosen.

References

1. Beatty PG, Clift RA, Mickelson EM, Nisperos BB, et al. Marrow transplantation from related donors other than HLA-identical siblings. New Engl J Med. 1985; 313:765.
2. Martin PJ. Increased disparity for minor histocompatibility antigens as a potential cause of increased GvHD risk in marrow transplantation from unrelated donors compared with related donors. Bone Marrow Transplant. 1991; 8:217.
3. Santamaria P, Reinsmoen NL, Lindstrom AL, et al. Frequent HLA class I and DP sequence mismatches in serologically (HLA-A, HLA-B, HLA-DR) and molecularly (HLA-DRB1, HLA-DQA1, HLA-DQB1) HLA-identical unrelated bone marrow transplant pairs. Blood. 1994; 83:280.
4. Rufer N, Tiercy JM, Breur-Vriesendorp BB, et al. Histoincompatibilities in ABDR-matched unrelated donor recipient combinations. Bone Marrow Trasnplant. 1995; 16:641.
5. Beatty PG, Anasetti C, Hansen JA, Longton GM, et al. Marrow transplantation from unrelated donors for treatment of hematologic malignancies: effect of mismatching for one HLA locus. Blood. 1993; 81:249.
6. Anasetti C, Beatty PG, Storb R, Martin PJ, et al. Effect of HLA incompatibility on Graft-versus-Host Disease, relapse, and survival after marrow transplantation for patients with leukemia or lymphoma. Human Immunol. 1990; 29:79.
7. Spencer A, Brookes PA, Kaminski E, et al. Cytotoxic T lymphocyte precursor frequency analyses in bone marrow transplantation with volunteer unrelated donors: Value in donor selection. Transplantation. 1995; 59:1302.
8. Petersdorf EW, Longton GM, Anasetti C, et al. The significance of HLA-DRB1 matching on clinical outcome after HLA-A, B, DR identical unrelated donor marrow transplantation. Blood. 1995; 86:1606.
9. Speiser DE, Tiercy JM, Rufer N, Grundschober C, et al. High resolution HLA matching associated with decreased mortality after unrelated bone marrow transplantation. Blood. 1996; (in press).
10. Sercarz EE, Lehmann PV, Ametani A, et al. Dominance and crypticity of T cell antigenic determinants. Annu Rev Immunol. 1993; 11:729.
11. Sherman LA, Chattopadhyay S. The molecular basis of allorecognition. Annu Rev Immunol. 1993; 11:385.
12. De Bueger M, Goulmy E. Human minor histocompatibility antigens. Transplant Immunol. 1993; 1:28.

Chapter 9

APPENDIX 1

9.1 Cytotoxic T lymphocyte precursor frequency test (CTLpf)

9.1.1 Introduction

The CTLpf assay is based on limiting dilution analysis and allows the estimation of alloreactive cytotoxic T cell precursor frequencies in peripheral blood. In this system, alloreactive T cell precursors are clonally expanded in the presence of stimulatory cells and IL-2. The specific cytolytic activity of developing cytotoxic T cells is then tested in a conventional ^{51}Cr- release assay after 10 days of culture. The T cell precursor frequencies are finally estimated from the extrapolation of the fraction of negative cultures found among the various cell titrations based on the principle of Poisson's equation (Figure 1).

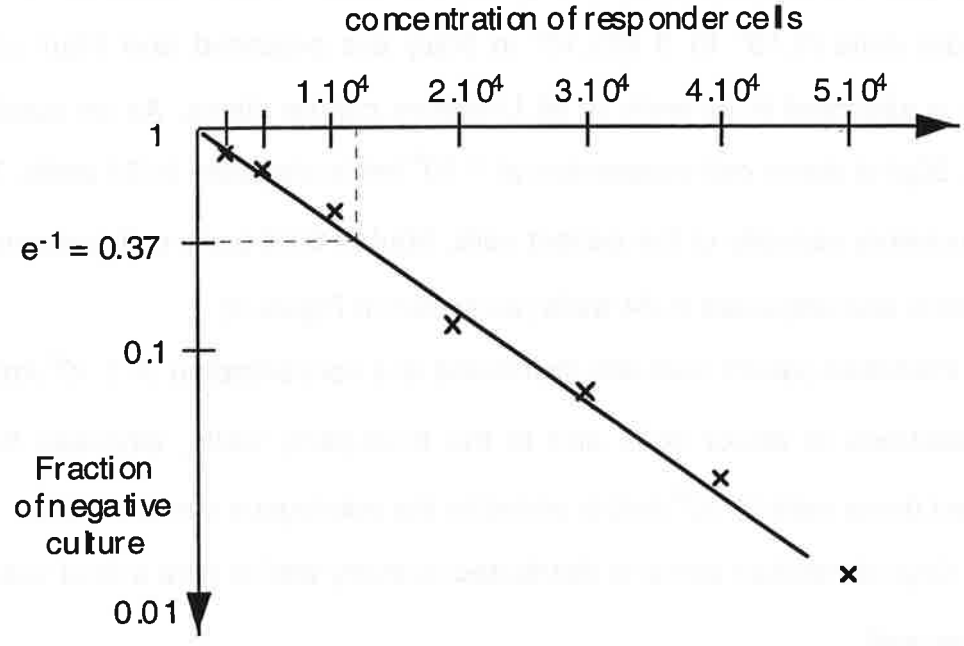


Figure 1: Example of a positive CTLp frequency in an HLA-mismatched D/R pair (a CTLp frequency less than 1:300'000 is considered as negative).

9.1.2 Protocol

This protocol is based on the original protocol as published by Kaminski et al. in Transplantation (1989) 48:608.

Peripheral blood mononuclear cells (PBMCs) are separated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and cryopreserved until required. An HLA-incompatible individual (third-party) is used as a positive control.

Complete Medium: RPMI 1640 (Gibco Life Technologies, Paisley, Scotland) is supplemented by 10% heat-inactivated human serum (Blood Transfusion Center, Bern, Switzerland) , 1% of glutamine (2mM, Gibco), 1% of penicillin-streptomycin (Gibco) and 2-mercapto-ethanol (5.10^{-5} M, Sigma, St Louis, MO).

Day 0: the CTLpf assay is done in Graft-versus-Host Disease direction. Responder cells are *donor* cytotoxic T lymphocyte precursors.

The responder (donor and third-party) and the stimulatory (patient) cells are thawed and resuspended in complete medium. Serial dilutions of donor responder cells (5.10^4 to $0.125.10^4$ in 50µl) are prepared and 50µl of each dilution is aliquoted in 24 wells of 96 U-bottom culture plates. As an autologous control, 50µl of donor cell suspension at 1.10^6 /ml is aliquoted in 24 wells. To test the stimulatory capacity of the patient cells, 50µl of third-party cell suspension at 2.10^5 /ml is also aliquoted in 24 wells (as shown in Figure 2).

50µl of irradiated patient cells are distributed at a concentration of 1.10^6 /ml in the serial dilutions of donor cells and in the third-party wells, whereas 50µl of irradiated donor cells (1.10^6 /ml) is added to the autologous control wells.

Finally, 50µl of medium alone is distributed in every well to give a final volume of 150µl per well.

25µl of recombinant IL-2 (Hoffman-La Roche, Basel, Switzerland) at 100 U/ml is added on **day 3 and 6** to provide CD4+ T cell help.

On day 10, the cultures are assayed for cytotoxicity against ^{51}Cr-labeled PHA (Murex Diagnostics Ltd, Dartford, England) blasts from the patient.

Briefly, 150µl of cell suspension are transferred into new 96U-bottom well plates.

Total chromium release is determined by adding 150µl of medium plus 1% Tween 20 (Sigma, St Louis, MO), whereas spontaneous release is determined by adding 150µl of medium alone.

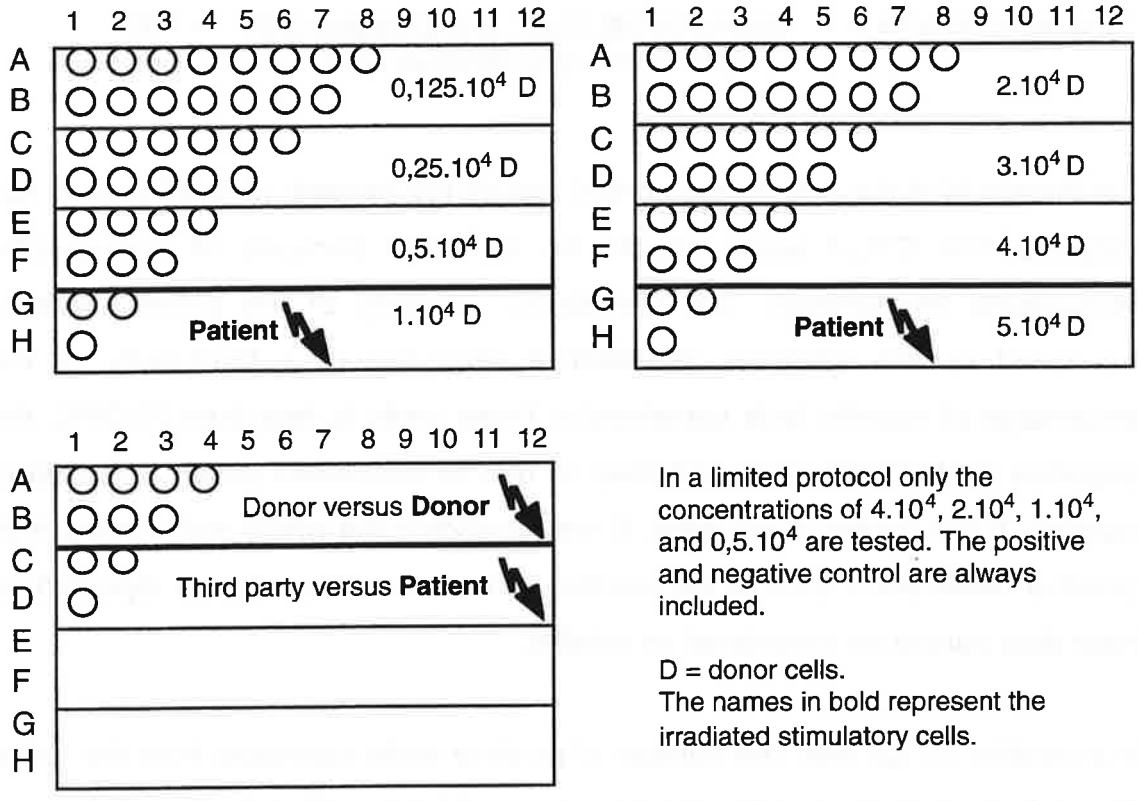


Figure 2: the CTLpf test protocol.

PHA blasts of the patient are incubated at 37^{0}C in 0.1 ml ^{51}Cr (200 µCi, Amersham Rahn, Zurich, Switzerland). After one hour incubation, the target cells are washed 3 times and 50µl of radiolabeled cell suspension at 2.10^5 /ml is

aliquoted in each well. 100µl of supernatants is removed after 4 hours-incubation at 37^0C and tested for ^{51}Cr release using a gamma counter.

1.3 Analysis of the raw data :

Wells are scored as positive if the ^{51}Cr release is greater than the mean plus 3xSD of the ^{51}Cr release of the autologous control.

The percentage of specific lysis is calculated by the following formula :

$$\% \text{ of specific lysis} = \frac{\text{experimental cpm - spontaneous cpm}}{\text{total cpm - spontaneous cpm}} \times 100$$

The threshold of the autologous control should not exceed 15 % of lysis. If this happens, the CTLpf assay should be repeated because of non-specific background interference. The stimulatory capacity of the patient cells is monitored by the cytotoxic alloreactive response of a third-party. If the percentage of specific lysis contained in these wells is less than 20-25%, the response is obviously weak and may be due to decreased patient stimulatory capacity. If this control is too weak, it may invalidate the whole test. Finally, if all "positive" wells are < 20% of the positive control, the CTLpf test is repeated as these data cannot be considered as reliable.

In a positive CTLpf test, the number of positive wells increases from the lower dilution to the higher one following the kinetics of limiting-dilution. (Figure 3).

In a negative CTLpf test, very low % of lysis are found among each dilution (Figure 4).

In a border-line CTLpf test, only very few positive wells are found. The interpretation of such assay remains difficult and the CTLpf test should be repeated (Figure 5).

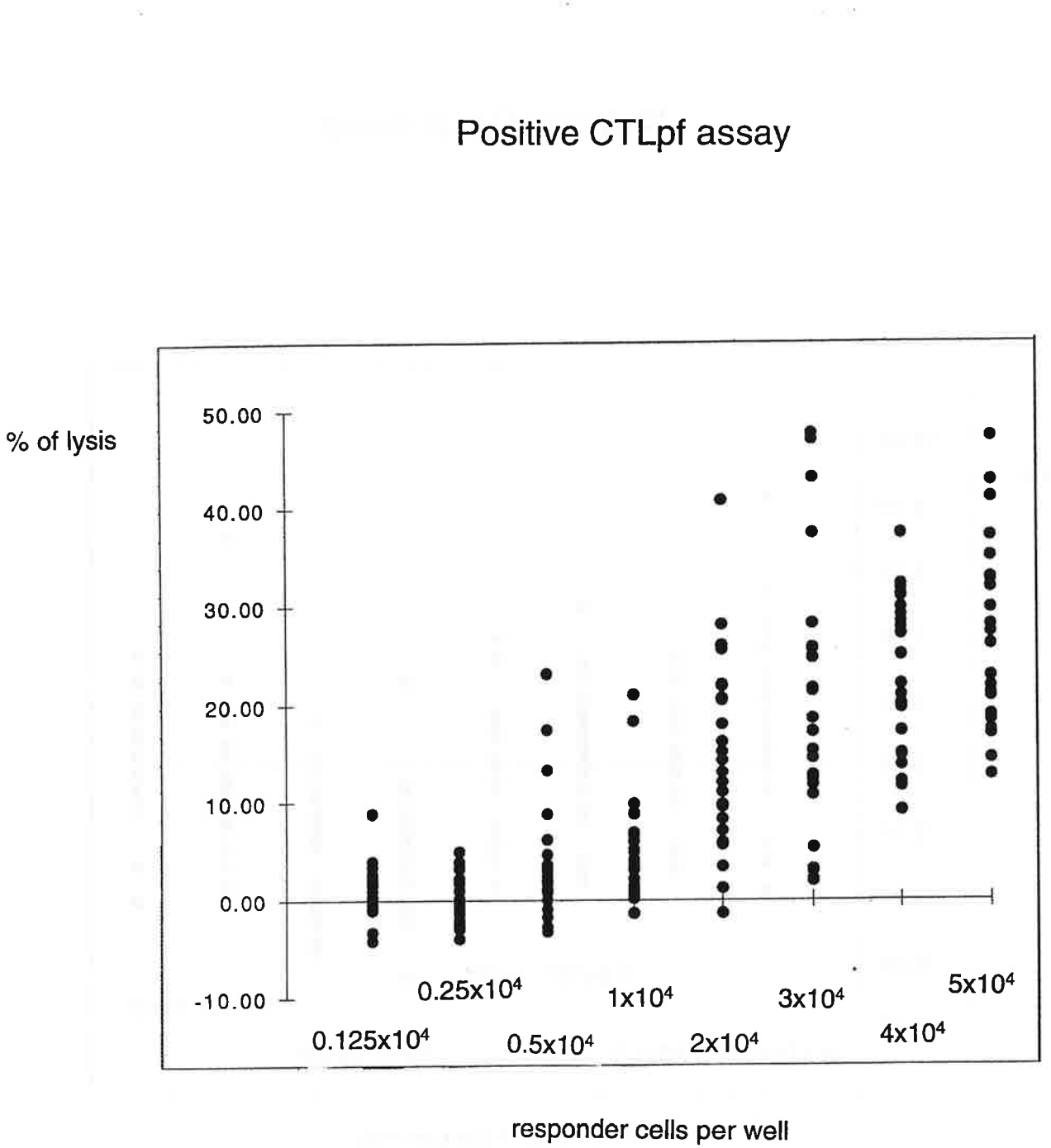


Figure 3. Limiting dilution culture from concentration $0.125x10^4$ to $5x10^4$ of a positive CTLpf assay. Percentage lysis of each well is plotted against the responder cell number per well.

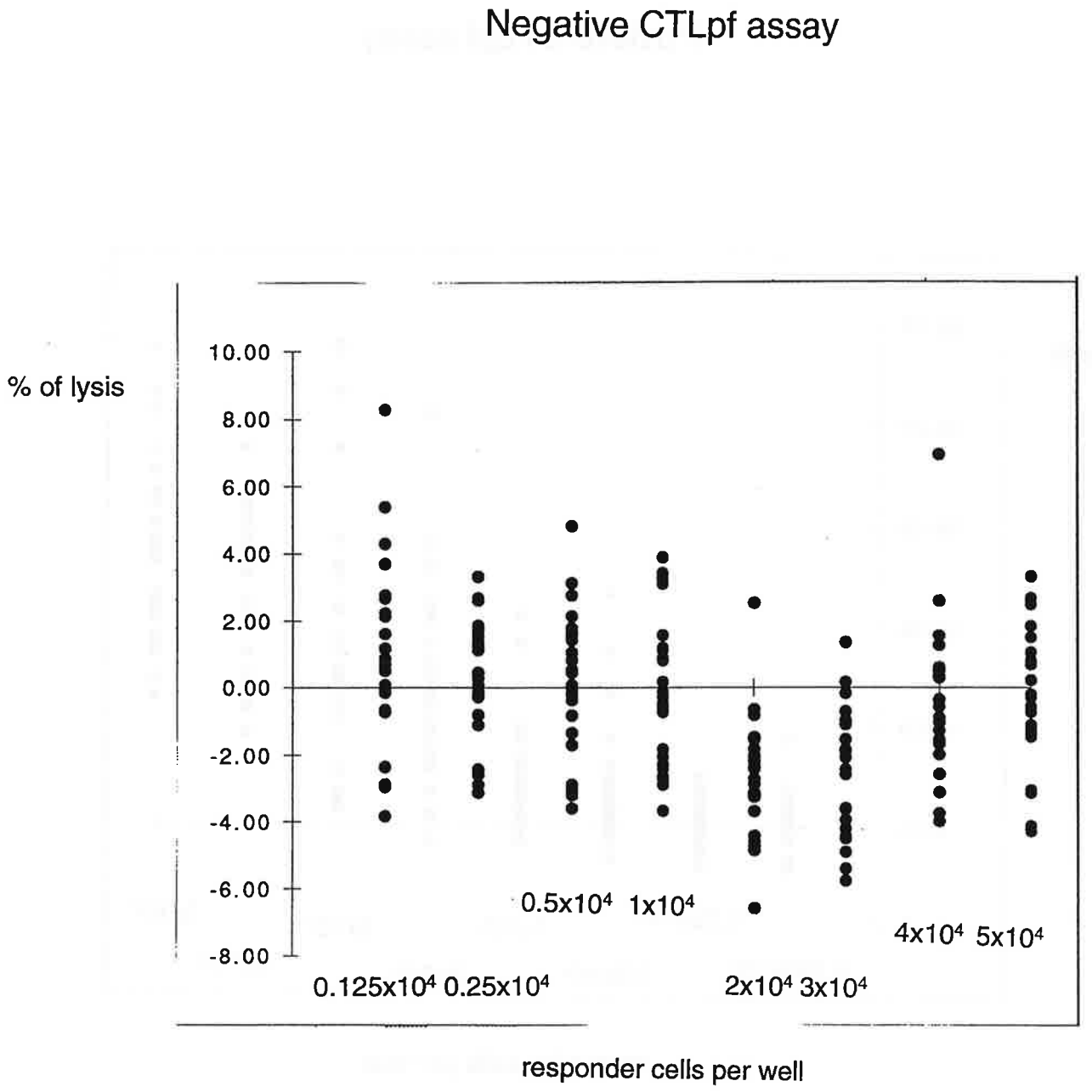


Figure 4. Limiting dilution culture from concentration $0.125x10^4$ to $5x10^4$ of a negative CTLpf assay. Percentage lysis of each well is plotted against the responder cell number per well.

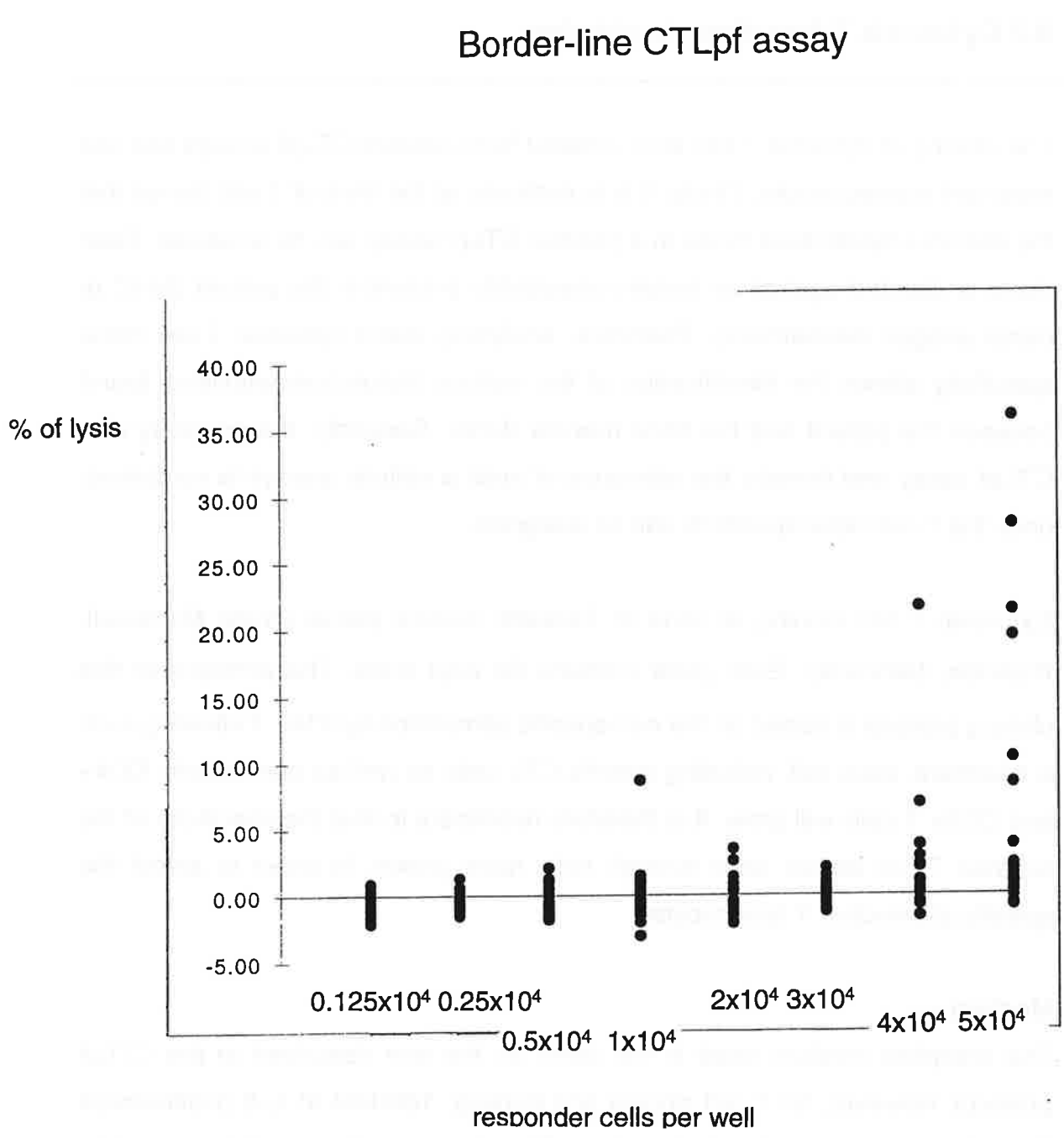


Figure 5. Limiting dilution culture from concentration $0.125x10^4$ to $5x10^4$ of a border-line CTLpf assay. Percentage lysis of each well is plotted against the responder cell number per well.

9.2 Cytotoxic T lymphocyte cloning

The cloning of cytotoxic T cell lines isolated from positive CTLpf assays has two important consequences. Firstly, it is in particular at the level of T cell clones that the histoincompatibilities found in a positive CTLpf assay can be analyzed. Each clone is directed against an histoincompatibility present in the patient (MHC or minor antigen mismatches). Therefore, analyzing donor cytotoxic T cell clone specificity allows the identification of the various histoincompatibilities found between the patient and his bone marrow donor. Secondly, the positivity of a CTLpf assay and thereby the relevance of such a cellular assays is confirmed, once the T cell clone specificity can be assigned.

Cytotoxic T cell cloning is done in Terasaki culture plates (Nunc Microwell, Roskilde, Denmark). Each plate contains 60 20µl wells. The principle of this cloning protocol is based on the non-specific stimulation by PHA. Following such a treatment, each cell, including specific CTL cells as well as non-specific CD4+ and CD8+ T cells will grow. It is therefore necessary to test the specificity of the isolated T cell clones once enough cells have grown, in order to select the specific alloreactive T lymphocytes.

Medium :

The complete medium used is the same as the one described in the CTLpf protocol. However, for T cell cloning and nursing, 100U/ml of IL-2 (Hoffman-La Roche) is supplemented to that medium. The cloning medium contains complete medium supplemented by 100U/ml of IL-2, 1 µg/ml of PHA and $0.4x10^6$/ml of fresh irradiated PBMC feeder cells (2500 rad). Finally, the restimulation medium is almost the same medium as the cloning one, the only difference is the concentration of irradiated feeder cells (1.10^6 /ml).

T cell line cloning :

Cytotoxic T cell lines (from a positive CTLpf test) are counted and resuspended in cloning medium at a final concentration of 25 cells/ml (=0.5 cell / well). In such a dilution (based on Poisson's equation), monoclonal T cells will be obtained. However, since only a few wells will grow on each Terasaki plate (about 5-10/60 positive wells), 10 plates for each line have to be used. 20µl of cell suspension is then aliquoted and the Terasaki plates are incubated at 37°C for about 10 days. At the same time, patient and donor blasts are restimulated with PHA-restimulation medium at a concentration of 10^6 cells/ml in 24 well plates and fed the following days with complete medium plus IL-2 (100U/ml).

At day 10 of culture, the Terasaki plates are screened under a microscope and the confluent wells are transferred into 96U bottom well plates.

Cytotoxic T cell assay:

Patient PHA blasts are labeled in ^{51}Cr as already described in the CTLpf test protocol and are resuspended at a concentration of 5.10^4 /ml. Half of the T cell clone quantity contained in each 96 plates is transferred into new 96U bottom well plates (total volume of 100µl). The spontaneous and the maximum ^{51}Cr release are prepared as already described. 100µl of ^{51}Cr labeled target cells is distributed to all wells. After 4 hours of incubation at 37^0C, 100µl of supernatants is harvested and tested for ^{51}Cr release using a gamma counter. The specific T cell clones are selected from the positive % of specific lysis.

Restimulation and culture of the specific T cell clones :

100µl of restimulation medium is added in each 96 U bottom well containing a specific cytotoxic T cell clone. During the following days, the growing T cell clones are duplicated and fed with complete medium+IL-2. After about 10 days, the T cell clones can be used for further specificity analysis, for FACS analysis or be frozen.

Chapter 10

APPENDIX 2

10. Analysis of the probabilities disparity in GvH direction between a patient and his potential HLA-identical bone marrow donor :

a) found within his family (related donor)

b) from the international registries (unrelated donor).

The following model has been used to study the disparity of minor antigens among two related and two unrelated individuals. Because most of the minor antigens identified in mice have only two alleles, this will probably also be the case for the minors found in human. Therefore, the model used is based on a biallelic model in which any gene encoding for a minor antigen has two alleles: alleles "A" and "a" with gene frequencies p and q and Hardy-Weinberg equilibrium. Since the dangerous situation in bone marrow transplantation is when one minor antigen allele is present in the patient and absent in the donor, the following probabilities of minor disparity among two individuals will be given in the sense of graft-versus-host disparity.

10.1 Probabilities of GvH disparity at a single biallelic locus with two codominant alleles "A" and "a".

In this model described by PJ. Martin, both alleles are codominant and will be processed and presented by the MHC molecules equivalently.

10.1.1 Probabilities of GvH disparity in unrelated transplantation

For any given two loci having codominant alleles "A" and "a" of a frequency p and q = 1- p, the population genotype frequencies of AA, Aa and aa can be calculated from the Hardy-Weinberg equilibrium as p^2, 2pq and q^2.

Table 1: Individual 1(Donor) ----> Individual 2 (Patient)

	PATIENT		
DONOR	AA p^2	Aa 2pq	aa q^2
AA p^2	no disparity	2 p^3q	p^2 q^2
Aa 2pq	no disparity	no disparity	no disparity
aa q^2	p^2 q^2	2 pq^3	no disparity

AA, Aa and aa represent the paternal and maternal genotypes.

A relevant disparity only occurs when the patient expresses an antigen which is not present in the donor. Thereby, GvH disparity occurs when AA individuals serve as donors for Aa or aa patients or when aa individuals serve as donors for AA or Aa patients (Table 1). The probability of such disparity between any two unrelated individuals can be calculated as :

$(p^2 \times 2pq) + (2\,p^2\,q^2) + (q^2 \times 2pq) = 2\,p^3\,q + 2\,p^2\,q^2 + 2\,p\,q^3 =$ **$2\,pq \times (1-pq)$**

10.1.2 Probabilities of GvH disparity in sibling transplantation

To extrapolate the probability of GvH disparity between any two siblings, the probability of such disparity between the progeny of the same parents has to be calculated first. There will be no GvH disparity among the progeny AA x AA, AA x aa, and aa x aa (Table 2).

Table 2: Progeny GvH disparity (Mother x Father)

X	MOTHER		
FATHER	AA p^2	Aa 2pq	aa q^2
AA p^2	no disparity	2 p^3q	no disparity
Aa 2pq	2 p^3q	4 p^2 q^2	2 pq^3
aa q^2	no disparity	2 pq^3	no disparity

However, if the progeny are AA x Aa, Aa x AA, Aa x Aa, Aa x aa or aa x Aa, GvH disparity can occur. Then, the respective probability of the parent's genotype has to be calculated first.

The probability for example of two parents being AA / Aa mating is 2 p^3q . The different children expected from such mating are AA and Aa. GvH disparity occurs when AA serve as a donor for Aa recipient and the probability of such disparity is 0.5 x 0.5 = 0.25. The same probability of disparity can be calculated from the progeny of aa x Aa. Among the progeny of Aa x Aa, GvH disparity occurs when AA individuals serve as donors for Aa recipients (probability = 0.25 x 0.5 = 0.125) or for aa recipients (probability = 0.25 x 0.25 = 0.0625), or when aa individuals serve as donors for Aa recipients (probability = 0.25 x 0.5 = 0.125) or for AA recipients (probability = 0.25 x 0.25 = 0.0625). This gives the overall probability of such disparity = 0.375 (2x0.0625 + 2x0.125) (Table 3).

Table 3: Probability of GvH disparity in each combination

	PATIENT		
DONOR	AA	Aa	aa
AA	0	0.25	0
Aa	0.25	0.375	0.25
aa	0	0.25	0

The probability of GvH disparity between any two related individuals is then calculated as :

$(0.25)x(\ 2\ p^3q)x(2) + (0.25)x(2\ pq^3)x(2) + (0.375)x(4\ p^2q^2) =$

$p^3q + 0.375(4\ p^2q^2) + pq^3 =$ **pq x (1-0.5pq)**

The probability of GvH disparity is less for sibling transplants than for unrelated transplants, when the two equations are compared and plotted on a graph (see Introduction Figure 16a).

However, these probabilities are based on a model which has probably less significance in reality. Indeed, the two alleles giving rise to the presented epitopes by the MHC molecules will probably not share the same binding affinities for these molecules. Therefore, alleles "A" and "a" could not be *codominantly* expressed and an adapted model has to be used.

10.2 Probabilities of GvH disparity at a single biallelic locus in which one allele is always expressed whereas the second one is never expressed:

Such a model represents another extreme situation which is closer to the type of responses one can find in MHC peptides presentation. In that model, one allele (either "A" or "a") is always found processed and presented by the MHC molecule at the cell surface, whereas the second one will never be expressed.

10.2.1 Probabilities of GvH disparity in unrelated transplantation

Again, the population frequencies of the genotypes AA, Aa and aa were calculated from the Hardy-Weinberg equilibrium as p^2, 2pq and q^2. Two situations have to be taken into account here: firstly, when allele "A" is always expressed and "a" is never expressed (Table 4). Secondly, when allele "A" is never expressed and allele "a" is always bound to the MHC molecules (Table 5).

Table 4: Donor ---> Patient when allele "A" is always presented

	PATIENT		
DONOR	AA p^2	A - 2pq	- - q^2
AA p^2	no disparity	no disparity	no disparity
A - 2pq	no disparity	no disparity	no disparity
- - q^2	$p^2 q^2$	$2 pq^3$	no disparity

Table 5: Donor ---> Patient when allele "a" is always presented

DONOR	PATIENT -- p^2	- a 2pq	aa q^2
-- p^2	no disparity	2 p^3q	p^2 q^2
- a 2pq	no disparity	no disparity	no disparity
aa q^2	no disparity	no disparity	no disparity

The "-" sign represents the respective non-expressed allele.

Thereby, GvH disparity occurs when the expressed allele ("A" or "a" respectively) is present in the recipient and not in the donor marrow. The probability of such disparity between any two unrelated individuals can be calculated as :

$(p^2 q^2 + 2pq^3)x\ 1/2 + (p^2 q^2 + 2 p^3q)\ x\ 1/2 =$ **pq x (1- pq)**

10.2.2 Probabilities of GvH disparity in sibling transplantation

The probability of GvH disparity between the progeny of two parents is first calculated. GvH disparity will occur among the progeny (A- x A-) and (A- x --) if allele "A" is always expressed, and among the progeny (-a x -a) and (-a x --) if it is allele "a" that is only present. The respective probabilities of each combination are shown in Table 6, if allele "A" is the one expressed and in Table 7, if allele "a" is always present.

Table 6: Probability of GvH disparity in each combination ("A" present)

DONOR	PATIENT AA p^2	A- 2pq	-- q^2
AA p^2	no disparity	no disparity	no disparity
A- 2pq	no disparity	0.1875	0.25
-- q^2	no disparity	0.25	no disparity

Table 7: Probability of GvH disparity in each combination ("a" present)

DONOR	PATIENT -- p^2	- a 2pq	aa q^2
-- p^2	no disparity	0.25	no disparity
- a 2pq	0.25	0.1875	no disparity
aa q^2	no disparity	no disparity	no disparity

The progeny of (A- x --) will be (A-) and (--). Therefore, the dangerous situation here, occurs when the donor has not inherited the minor antigen allele (--) whereas the patient presents that allele (A-). The probability of such GvH disparity is 0.5 x 0.5 = 0.25. The same probability of disparity can be calculated from the progeny of (-a x --).

Among the progeny of (A- x A-) parents, GvH disparity occurs again when the donor does not possess the minor allele (--) and the patient has either one allele (A-) or two (AA). The probability of such disparity is 0.25x0.25 + 0.25x0.5 = 0.1875. The same probability is found for the progeny of (-a x -a) matings.

The probability of GvH disparity between any two related individuals is calculated as :

$((0.1875)x(4p^2q^2) + (0.25)x(2pq^3)x2) + (0.1875)x(4p^2q^2) + (0.25)x(2p^3q)x2))x1/2$

$= 0.5pq \times (p^2 + 1.5pq + q^2) =$ **$0.5pq (1-05pq)$**

The two extrapolated equations found here represent when compared to those of PJ. Martin's model, the same equations but divided by a factor of two. Thereby, the same curves can be drawn (see Introduction Figure 16b), but the probability of GvH disparity will be decreased by half. However, the relative risk for GvH disparity still remains significantly higher for unrelated combinations than in related individuals.