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Class-I-Restricted Human Cytotoxic T Lymphocytes Directed against Minor Transplantation Antigens and Their Possible Role in Organ Transplantation¹

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Introduction

The products of the extreme polymorphic genetic systems, which are called major histocompatibility complexes (MHC), play important roles in the immune system. A number of immune response (Ir) genes which regulate the level of response to specific antigens have been mapped to the same region. MHC molecules participate in the interaction of T cells with foreign antigens, possibly providing binding sites for foreign structures such as viruses and soluble antigens and thus permitting their successful recognition by T cells. T-cell interaction with antigen is MHC-restricted, which implies that T cells must possess receptors which simultaneously recognize both the self-MHC molecule and the foreign antigen. During the last decade experimental evidence for this phenomenon has been widely reported in mouse and man.

In this chapter, *in vitro* observations are described concerning the recognition of human minor transplantation antigens by cytotoxic T cells in

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the context of self-MHC. The antigens recognized in the systems described below are the male histocompatibility antigen, H-Y, and other minor histocompatibility antigens. In both cases, the class-I molecules of the MHC are involved in the interactions of the effector cytotoxic T cells with their targets. Detailed analyses of these systems using cell lines and clones have shown that human cytotoxic T cells recognize antigens in a manner analogous to that of cytotoxic T cells in the mouse. We have taken advantage of the availability of cells from bone marrow-grafted patients which had been primed to minor histocompatibility antigens *in vivo* in order to study the role of cytotoxic T cells in their recognition. The results of the *in vitro* studies of patient material allowed us to draw some conclusions towards its clinical relevance and hopefully can contribute to the understanding of the role of non-MHC antigens in organ transplantation.

Cell-Mediated Lympholysis

T cells are very heterogeneous in function as well as in expression of surface markers. Only one of the T-cell subsets, namely the T-effector cells will be discussed. This T-cell subset can be further divided into functionally different T-effector cell subpopulations, of which the cytotoxic T lymphocytes (CTLs) will be described below.

In the early models of cell-mediated immunity the effector populations were obtained from experimental animals or humans who had been immunologically challenged *in vivo*. Immunizations with allogeneic materials usually resulted in high levels of cytotoxicity and they can serve as a model for the analysis of transplantation. One of the best characterized effector cells in these *in vivo* allogeneic models is the CTL [19]. The first unequivocal observation of lymphocyte-mediated cytotoxicity was made by *Govaerts* [46] who observed that thoracic duct lymphocytes, harvested from dogs which had rejected their kidney allograft, were capable of lysing donor cells *in vitro*. Subsequent observations have confirmed the generality of that finding, and the lymphocyte-mediated destruction of many different target cells have been reported [89].

In man, similar studies were reported in situations where allogeneic sensitization occurred *in vivo*. For obvious ethical reasons, the need for a homologous *in vitro* system to replace the *in vivo* sensitization step was clear. The availability of an *in vitro* technique for the induction and differentiation of specific cytolytic effector T cells has made a significant contri-

tribution to the understanding of the role of the MHC antigens in the immune response and has provided an *in vitro* model of the homograft reaction. The so-called cell-mediated lympholysis (CML) technique was developed by *Lightbody et al* [70] and consists of two *in vitro* phases: an induction phase in which lympholytic effector cells are induced and an effector phase in which the effector cells lyse chromium-labeled target cells. The generation of effector cells in the first step is necessary in order to obtain measurable cytotoxicity in the second step. In addition, they used lymphocytes transformed into blasts by treatment with phytohemagglutinin as the source of target cells. The CML assay as just described used relatively large numbers of cells but a micromethod was introduced where the cells were incubated in plates [102]. The disadvantage of this system was that at the end of the incubation period the supernatant from each well had to be removed for gamma counting. This disadvantage was abolished by the technical improvement for harvesting the supernatants in the CML assay with a simple cartridge system [51]. Precise details of the method currently used in our laboratory to detect cytotoxic T-cell responses *in vitro* have been given elsewhere [40].

Recognition Structures for Cytotoxic T Lymphocytes

Class-I Molecules

Class-I molecules (H-2K, D and L in the mouse, and HLA-A, B and C in man) are transmembrane glycoproteins with a molecular weight of 44,000 daltons. The major portion of the molecules is on the outside of the cell membrane and is associated with a 12,000-dalton polypeptide called β_2 -microglobulin. The genetic analysis of the HLA complex was facilitated by computer analyses of reaction patterns obtained from large numbers of antisera tested against random leukocyte samples [95]. Recognition of the multiple alleles of the three HLA class-I loci was facilitated by a series of international cooperative workshops. Recently, also biochemical analysis of the gene products of the class-I loci demonstrated a series of 44,000-dalton glycoprotein chains [116], showing the presence of more than three class-I-like structures. Whether these can serve as recognition structures for CTLs is unknown. The class-I molecules can serve as target determinants for CTLs. This is presumed since target determinants recognized in CML are highly associated with the serologically defined HLA specificities [16, 26, 47-49, 69, 80, 113] and, in addition, demonstrated by the use of a

human B lymphoblastoid cell line which lacked the serologically defined HLA-A2 antigen and had lost susceptibility to lysis by HLA-A2-specific CTLs [2]. Whether or not alloantisera and CTLs recognize the same epitopes on class-I molecules is a topic of current investigation and will be discussed briefly in this chapter.

Class-II Molecules

The class-II molecules (H-2 I region products in the mouse and HLA-D region products in man) consist of two separate glycoprotein chains with molecular weights of approximately 34,000 and 28,000 daltons. Both chains are integral membrane proteins and have a large extracellular and a small intracellular region. The products of the class-II genes are mainly responsible for the proliferative responses in mixed lymphocyte culture. They can be identified by serological as well as cellular techniques. At least three distinct human class-II molecules exist: HLA-DR, DC and SB [53].

Recently, analysis of the HLA-D region at the molecular and DNA level became possible and will lead to the further characterization of the class-II molecules [53, 99]. It is possible to detect cytotoxicity towards class-II HLA-D/DR antigens or determinants closely linked to them [1, 27, 55, 76] and to DC antigens [109].

Class-I Variants

In 1965 *Bailey and Kohn* [3] provided the earliest evidence of H-2 gene mutation by the discovery of incompatibilities within inbred strains. In inbred strains of mice, individuals were found that were capable of rejecting skin grafts from mice of the same strain. The generation and detection of these mutants has been reviewed [59]. A rather high rate of mutations is observed for the H-2K allele [60]. Structural differences in the bm series of mutants of the H-2K allele have been shown by biochemical analysis [84]. At the functional level, the differences have been confirmed by skin grafting and functional assays such as CML [4, 78, 79, 120].

Evidence has been presented for the occurrence of analogous mutations in man. In 1976, individual variation within a serologically defined specificity, i.e. HLA-Bw35, could be detected by CTLs [35]. CTLs were generated, between HLA-A, B and C identical siblings, which specifically recognized two different types of HLA-Bw35 antigenic determinants which are still serologically indistinguishable. Subsequently, by the use of CTLs, subtypes (variants) have been described for HLA-A2 [12, 41, 52, 90, 91, 108], HLA-A3 [14], B7 [108], B27 [18], B40 [73], and Bw44 [56]. For some of these

subtypes defined by alloimmune-specific CTLs biochemical analysis showed structural differences thereby indicating a basis for the aberrant reaction patterns obtained with CTLs [13 83 92]

It is obvious that information is needed about the role of these variants in human organ transplantation. If so the use of unrelated donors should take into account the presence of subtypes or variants. Especially since HLA-matched unrelated donors also in bone-marrow grafting are regularly used.

Non HLA Antigens

Evidence for the existence of non-HLA target determinants recognized by in vitro generated CTLs between HLA identical related and unrelated combinations has been presented by several authors [48 67 75 77 96 97 106]. The nature of these non-HLA target determinants recognized by in vitro CTLs could however not be deduced. It is possible that some of these reactions represent variants. It seems likely that CTLs specific for non-HLA antigens will occur following in vivo immunization i.e. as a result of blood transfusion pregnancy graft rejection and graft-versus-host attack and the chance of finding non-HLA antigen-specific CTLs may be increased in those situations. With the use of in vivo generated CTLs a number of reports described membrane differences detected by CML but not detected by conventional HLA typing [27 29 31-33 36 42 54 68 74 75 88 90 106a 112 114 117 118 121 122]. The nature of some of these target determinants recognized by in vivo generated CTLs and the mechanism by which they can be recognized will be discussed below.

MHC Restriction of CTLs

History and Models

Oldstone et al [85] described variation in susceptibility to infection with lymphocytic choriomeningitis virus (LCMV) in congenic mice differing only at H-2 (H-2q mice were more susceptible than H-2k mice). These variations to LCMV (which is apparently a T-cell-mediated immunopathological process) may be due to genetically determined differences in T-cell responsiveness. The original experiment by *Zinkernagel and Doherty* [123] established the fundamental importance of the role of the MHC in T-cell-mediated immunity namely, virus-immune T cells obtained from LCMV-infected mice showed only in vitro cytotoxic activity when assayed on H-

H-2-compatible LCMV-infected fibroblasts target cells from H-2-incompatible donors were not lysed [123].

During the following years numerous studies in rodents have shown that the activation of T lymphocytes by different foreign antigens is restricted by self-MHC molecules. Thus T lymphocytes appear mainly to be able to recognize foreign antigens when they are presented to the T lymphocytes on cells which share some homology of the H-2 region with the animal under study.

The observations with LCMV were soon confirmed and seemed to be also applicable to a series of other viruses: ectromelia (mouse pox), vaccinia, sendai, influenza, herpes simian (SV40), cytomegalo, alpha viruses and also for tumor-associated viruses [for review see ref. 124]. The H-2-restriction phenomenon was not limited to viruses only. Independently and at the same time as Zinkernagel and Doherty [123], Shearer [101] discovered in 1974 that MHC-restricted T-cell-mediated cytotoxicity can be generated against syngeneic lymphoid cells whose surfaces had been chemically modified. MHC restriction of CTLs was also found for non-H-2 alloantigens, i.e. minor histocompatibility antigens [6], including the male specific H-Y antigen [34]. These reports and the many which followed, show that a main function of class-I molecules is to participate in the interaction of CTLs with their targets. MHC restriction is not unique to cytotoxic T cells as discussed extensively elsewhere in this volume.

The underlying mechanism by which cytotoxic T cells recognize self-MHC and foreign antigen can be explained by two distinct models: (a) the altered-self model, and (b) the double-receptor model. In addition several other models sharing some of their features have been proposed. In the altered-self model T cells are equipped with a single receptor which is specific for a neoantigenic determinant formed by a complex of self-MHC and foreign antigen on the target cell, or one receptor for the complex of the unaltered-MHC molecule and antigen. In the two T-cell receptor model T cells have two recognition molecules: one is specific for self-MHC restriction molecule and the other for foreign antigen. This is called the dual recognition hypothesis. The current thoughts about the two models have been recently summarized by Berzofski [5]. The first clear demonstration of the involvement of products of the human MHC in T-cell-mediated cytotoxicity was reported by our own group [36, 37] and concerned the recognition of the male-specific minor histocompatibility antigen, H-Y. Subsequently, HLA-restricted cytotoxic T-cell responses have been described for virally infected target cells like Epstein-Barr virus [94-115], influenza virus

[11-72] measles virus [65] herpes simplex virus [98] human cytomegalovirus [93] mumps virus [66] and human T-cell leukemia lymphoma virus [82] *hapten modified target cells* like dinitrofluorobenzene-conjugated or dinitrophenyl-conjugated target cells [22-23] trinitrophenyl [100] and fluorescein isothiocyanate-conjugated target cells [28] *soluble antigens* like fetal calf serum [81] and purified protein derivative of tuberculin [119]

The role of the class-I molecules and the clinical implication of the anti-viral CTL responses are treated in more detail elsewhere in this volume. The HLA-restricted CTL responses against minor histocompatibility (i.e. minor H) antigens will be reviewed below and are based on our experiments done over the last years on the detection of minor H antigens and their possible implications in human organ transplantation.

Class I Restricted CTLs Directed against the Minor Transplantation Antigen H-Y

The involvement of H-Y (at that time called Y-factor) in homograft rejection had been postulated by *Fichwald and Slimser* [24] in 1955. The term H-Y antigen was introduced by *Billingham and Silvers* [15] since the Y factor is a transplantation antigen determined by a histocompatibility gene comparable in all respects to the antigens responsible for homograft rejection.

In vitro immune response against the human male specific histocompatibility antigen H-Y was detected in a multi-transfused female aplastic anemia patient (i.e. patient 1). She received a bone marrow graft from her HLA-identical brother. There was a temporary take but the graft was rejected after 20 days. Rejection was accompanied by a spontaneous recovery of the patient's bone marrow hemopoietic function.

Investigation by the use of the CML technique showed unambiguously that the post-transplant peripheral blood lymphocytes (PBLs) of this patient (i.e. patient 1, HLA phenotype HLA A2, A2, Bw44, Bw60, Cw3, Cw5, DR4, DRw6) exhibited strong cell-mediated cytotoxic reactions specific for male HLA-A2-positive target cells [36, 37]. The results obtained with the HLA-A2-restricted anti-H-Y CTLs from the patient are shown in table I.

As shown in table I, cells derived from three HLA-A2-positive females (from a randomly selected panel of 45 HLA-A2-positive women) were also lysed (although at a lower level). Since we obtained our HLA-restricted anti-H-Y CTLs from an in vivo sensitized patient, it is possible that small

Table I Pattern of lysis by CTLs from patient 1 against target cells from unrelated individuals

CML	Serological typing for HLA-A2		Sex of HLA-A2-positive target cells	
	positive	negative	male	female
+	76	0	73	3 ^a
-	42	57	0	42

By studying the reactivity patterns of HLA-restricted anti-H-Y CTLs one can be confronted with variants at the level of the specific restricting element (i.e. HLA-A2 molecule) as well as at the level of the subject of restriction—the antigen H-Y. This table excludes the results obtained by investigating HLA-A2-restricted anti-H-Y CTLs against lymphocytes derived from (1) HLA-A2 variants [41–45] and (2) abnormal sex chromosome constitutions [43].

^a The level of lysis is low but significant.

numbers of T-cell clones may have been directed against other minor H antigens. The presence of these minor H antigens in the males may have been masked by the H-Y-specific lysis. On the other hand, we may be dealing with the phenomenon ‘neoantigens can resemble alloantigens’. It has been shown that T cells which show antigen-specific MHC-restricted reactivity can also demonstrate cross-reactivity. Namely, MHC-restricted CTLs can recognize not only modified self but also histoincompatible (i.e. foreign) MHC molecules and/or modified foreign MHC molecules. This observation is often referred to as ‘the self plus foreign (\approx neoantigen) resembles allo’ phenomenon [10, 71].

In our study all 3 women also had, in addition to HLA-A2, the HLA-Bw60 antigen in common. Analysis on the clonal level of the reactivity patterns of the HLA-A2-restricted anti-H-Y CTLs against these HLA-Bw60-positive female donors may provide us with more definitive answers.

It is worth noting that the level of HLA-A2-restricted anti-H-Y lysis seemed to decline with time and the gradual waning seemed to be associated with the patient’s recovery. Nevertheless, it was possible to restimulate the H-Y-specific lysis by an *in vitro* stimulation using lymphocytes from an HLA-A-, B-, C- and D-identical unrelated male donor, instead of the HLA-identical male sibling donor cells. In that way effector cells were formed with the expected specificity for HLA-A2 male target cells. By using this

protocol A2-restricted H-Y-specific CTLs can be obtained up to the present time – 8 years after grafting. Our first observation was confirmed in 2 additional cases (out of a group of 12 female aplastic anemia patients studied) which showed exactly the same phenomenon namely HLA-restricted anti-H-Y immunity [38, 39]. These 2 cases (patients 2 and 3) were female siblings with severe aplastic anemia. They had both been immunized by multiple blood transfusions (and not by bone marrow transplantation) and in addition patient 2 had had three pregnancies. The pattern of lysis obtained with effector cells of patient 2, after restimulation *in vitro* with the lymphocytes from her healthy HLA-identical male sibling showed HLA-A2-restricted H-Y killing comparable to that in our first case reported earlier. Thus it follows that blood transfusions and possibly pregnancies are sufficient in themselves to induce HLA-restricted anti-H-Y cytotoxicity and that bone marrow transplantation is not a necessary prerequisite [38]. *Singal et al* [104] demonstrated the presence of HLA-A2-restricted anti-H-Y CTLs in multiparous females.

In vitro studies with the lymphocytes of our 3rd patient revealed HLA-restricted CML reactivity to the H-Y antigen in which the self-HLA-A2 or HLA-B7 molecule was required [39]. The results obtained with the HLA-A2 and B7 anti-H-Y CTLs from the 3rd patient (HLA phenotype HLA A2, A28, B7, Bw62, Cw3, DR1, DR2) are shown in table II. As shown in table II, cells derived from an HLA-A2-negative, B7-negative female donor were also lysed, although at a much lower level than was observed for HLA-A2- or B7-positive male target cells. Those target cells represent the only discrepancy in the tests with the CTLs from patient 3.

Interestingly, *Pfeffer and Thorsby* [90] described the detection of HLA-A2- and B7-restricted anti-H-Y CTLs after kidney graft rejection. A previously nonsensitized (never transfused or pregnant) female patient acutely rejected a kidney graft from her HLA-identical brother. Shortly after the graft loss, lymphocytes obtained from the patient showed cytotoxic activity against male target cells sharing the HLA-A2 and (or) B7 antigen with the patient. The latter results and the results which we obtained in our 3 patients suggest that H-Y-specific cytotoxic T cells demonstrate preference for certain (i.e. A2 and B7) restricting elements.

To gain insight into the apparent dual specificity of such CTLs (A2 plus H-Y, B7 plus H-Y) at least three basic cellular approaches can be used: monolayer absorption, cold target inhibition and cytotoxic T cell clones. The results of a cold target inhibition experiment are shown in table III. The HLA-A2- and B7-restricted anti-H-Y CTLs were tested against only one

Table II Pattern of lysis by CTLs from patient 3 against target cells from unrelated individuals

CTL	Serological typing for A2 and B7				Sex of HLA A2 and/or B7+ target cells	
	A2+ B7+	A2+ B7-	A2- B7+	A2- B7-	male	female
+	12	32	7	1 ^a	51	0
-	5	11	2	16	0	18

This table excludes the results obtained by investigation of HLA-A2- and B7-restricted anti-H-Y CTLs against lymphocytes derived from (1) HLA A2 variants and (2) abnormal sex chromosome constitutions (see also legend to table I)

^a HLA-A2-negative and B7-negative female donor

Table III Specific inhibition of HLA-A2- and B7-restricted anti-H-Y CTLs by cold target cells

Cold inhibitors added ^a	Lysis of ⁵¹ Cr-labeled target cells %	
	A2+ve ♂	B7+ve ♂
None	55	65
A2-ve B7-ve male	56	66
A2+ve male	9	72
B7+ve male	60	19
A2+ve B7+ve male	10	9
A2+ve female	60	60
B7+ve female	58	63

^a Hot/cold ratio 1/10; the competitive inhibition protocol has been described in detail [40]. Effector/target ratio 5/1.

type of target cell – either A2 or B7. For example HLA-A2-positive male of isotope-labeled target cells and unlabeled target cells of either HLA-A2-positive male cells or HLA-B7-positive male cells were added. Effective reduction in lysis was observed only when cold target inhibitors of the same HLA specificity as the labeled target cells were added; for example labeled

HLA-A2-positive male target cells were inhibited by unlabeled HLA-A2-positive male target cells but not by unlabeled HLA-B7-positive male target cells (table III)

Each CTL subpopulation is restricted to recognizing the foreign antigen (H-Y and other minor H antigens as will be discussed later) in association with only one HLA molecule. Sensitized killer cells which recognized two combined antigens HLA-A2 H-Y and HLA-B7 H-Y could indeed be divided into two populations one directed against HLA-A2 H-Y and the other against HLA-B7 H-Y.

The consequence of these distinct subsets of cytotoxic T cells is that the individual is not dependent on a single allele that can serve as a source of restriction molecule for T cells. Clonal distribution of HLA-restricted anti-H-Y-specific CTLs has been described also by *Pfeffer and Ihorsby* [90] using cold target inhibition tests.

Clinical Relevance of the H-Y Alloantigenic System

In order to determine the possible influence of H-Y incompatibilities on the results of human kidney allograft transplantation, we examined cadaveric renal allograft survival data (collected under the auspices of Eurotransplant) at 2 years after transplantation for the influence of sex and HLA. The retrospective study showed a significant difference between HLA-A2 females receiving HLA-A2 male kidneys and non-HLA-A2 females receiving non-HLA-A2 male kidneys. The H-Y incompatibilities in donor-recipient combinations sharing the HLA-A2 antigen resulted in a 38% graft survival at 2 years in contrast to 58% in the HLA-A2 non-sharing group [38].

To explain these findings and those reported by *Pfeffer and Ihorsby* [90] the following hypotheses can be considered: (1) a peculiar immunogenicity of the HLA-A2-H-Y complex (and HLA-B7-H-Y complex); (2) the involvement of Ir genes in the anti-H-Y response. A similar phenomenon has been suggested in the mouse [103]. At the moment it is difficult to choose between these two hypotheses. *Opelz and Tezasaki* [87] investigated the influence of sex on histocompatibility matching in a large series of kidney transplant recipients. They observed a striking difference in renal allograft survival between the sexes when matching for HLA-A and B antigens. A highly significant correlation was found between HLA matching and graft survival in male patients. Additionally, the latter observation was most striking in male patients with blood groups other than O.

Some other studies have not been able to detect any influence of sex in kidney transplantation [21, 86] but in those studies male to female trans-

plants in HLA-A2 or HLA-B7-compatible combinations were not specifically investigated

In bone marrow transplantation the situation is as follows. Despite the fact that aplastic anemia and leukemia patients are transplanted with bone marrow from an HLA genotypically identical sibling, rejection and graft versus host disease (GVHD) still remain a serious problem in human bone marrow transplantation. The results reported on the effect of sex match between bone marrow donor and recipient are rather conflicting. *Storb et al* [110] reported a study of prognostic factors associated with GVHD and survival in transplanted aplastic anemia patients. They found that, using a multifactorial analysis, the occurrence of GVHD and survival of patients with aplastic anemia was strongly influenced by whether or not donor and recipient were matched for sex. Male patients seemed to have a high incidence of GVHD. However, the same group [111] recently reported that recipients of marrow from donors of the opposite sex have survivals equal to that of recipients of marrow from sex-matched donors. A publication by *Bottin et al* [17], on the behalf of the Advisory Committee of the International Bone Marrow Transplant Registry, suggested that the use of male donors was preferable to female donors because of a higher rate of engraftment, less severe GVHD and a higher 1-year survival rate. Indeed, investigation of their detailed analysis showed that 64% of male recipients of marrow from female donors had GVHD (male → female 31%, female → female 71%, male → male 42%). Additionally, the detailed analysis of the number of 1-year survivors showed that male recipients of bone marrow from female donors had the worst survival rate. In order to obtain a correct insight into the influence of sex combinations on the outcome of bone marrow transplantation, I think the HLA-A2- and B7-positive recipients should be investigated separately. As discussed earlier in this chapter, H-Y associated with HLA-A2 and probably also with HLA-B7 seems to act as a strong immunogen in females. In addition, involvement of the HLA-A2 and B7 molecules in associative recognition of other minor H antigens is very striking and will be described below.

Class-I-Restricted CTLs Directed against Minor H Antigens

Simultaneously with the description of the estimation of the number of major H loci in mice, *Snell* [105] indicated the existence of what he called rare h genes coded for by separate histocompatibility loci. He noticed at that time the difficulty with which these loci could be detected: 'Is there any way that these loci can be discovered? The answer would appear that there

is no simple method of finding them but that by the use of somewhat laborious methods they can eventually be brought to light. Those statements are still true especially for the human minor H loci. *Coumou et al* [20] classified the tissue antigens which account for the phenomenon of graft rejection into two main groups according to the strength with which they lead to graft rejection: (1) major H antigens which cause rapid acute graft rejection and (2) minor H antigens which account for comparatively slow and more chronic graft rejections.

The minor transplantation antigen H-Y coded for by the Y chromosome has already been discussed above. Other minor H antigens which in mice are coded for by as many as 30–100 genetic loci which are spread all over the genome, act more strongly than others in graft rejection and for the majority only a few allelic forms have been described [58]. With special regard to the cellular recognition of minor H antigens, *Bevan* [6–9] extensively analyzed the cytotoxic T-cell responses towards minor H antigens in association with MHC molecules.

Recently considerable information has been obtained about the possible existence of minor H antigens in man. The occurrence of a severe GVHD in a bone marrow-transplanted male acute myeloid leukemia patient prompted us to investigate the *in vitro* cytotoxic activity of the patient's post-transplantation lymphocytes. The patient (HLA phenotype HLA-A2, A2, B27, Bw62, Cw1, Cw4, DR1, DR4) had been transplanted with bone marrow from an HLA-identical female sibling donor and full chimerism was induced. His clinical recovery was complicated by severe chronic GVHD. The initial experiment [42] demonstrated that post-transplant lymphocytes had strong cytotoxic activity, as measured by the CML assay, against his own pre- but *not* post-transplantation lymphocytes.

As expected the patient's post-transplant lymphocytes did not react with the target cells from the bone marrow donor. Thus, the transplanted lymphocytes (i.e. donor cells) differ for a non-HLA factor (which we designated as minor H antigen) from the patient's own pre-transplant lymphocytes. Subsequently, the patient's post-transplant lymphocytes were analyzed more extensively [44]. In order to do so, large quantities of the patient's CTLs were needed. They were obtained using the following protocol. CTLs with specific cytotoxic activity were generated by stimulating the patient's post-transplant lymphocytes with either his own pre-transplant lymphocytes or with the lymphocytes from an HLA-A-, B-, C- and DR-identical unrelated healthy individual. After 6 days of primary *in vitro* sensitization, cytotoxic T-cell lines were grown (by the use of feeder cells

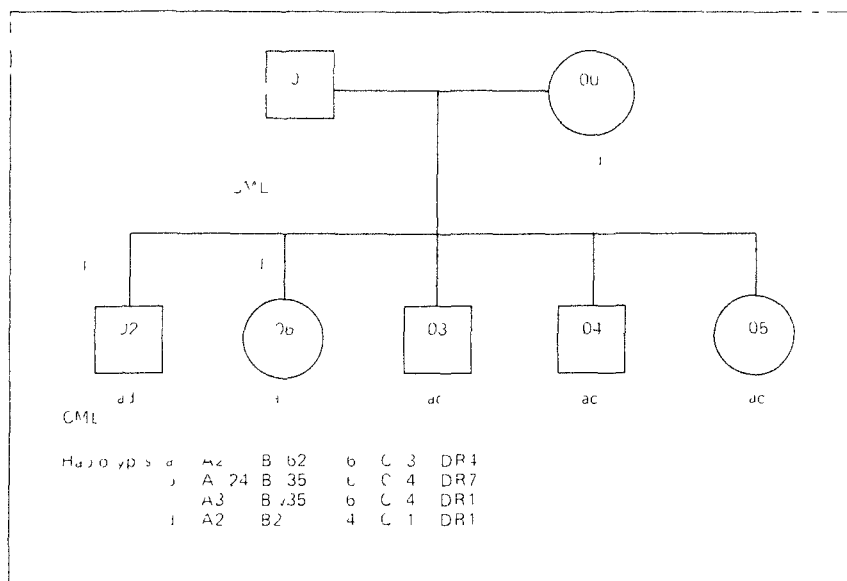


Fig 1 Lysis pattern in patient's family

plus lectin and/or T-cell growth factor) and were tested for specific cytotoxic activity and subsequently frozen in small aliquots. In that way, large amounts of CTLs were harvested, stored and used as typing reagents

The results obtained using as target cells lymphocytes from (1) the patient's own family (2) a comprehensive number of randomly chosen unrelated healthy panel donors, and (3) relatives of the positively lysed panel donors, are as follows

(1) Investigation of patient's family revealed, besides absence of cytotoxicity against the lymphocytes of the bone marrow donor (i.e. D), difference in susceptibility to lysis between *HLA-identical siblings* (J3, J4, J5). The latter observation indicates that we must be dealing with a *non-HLA target determinant* (fig 1)

(2) The results of the panel study are shown in table IV and illustrate that the *non-HLA target determinants* could only be recognized if one of the three different HLA class-I molecules of the patient were present on the target cells. The panel analysis shows that the minor H antigen associated with HLA-A2 has high gene frequency in the random population in contrast to the minor H antigen associated with B27. Nevertheless, we have as

Table II Evidence for one (or more) minor H antigens associated with class I molecules on target cells

CMI	HLA phenotypes			
	A2+ B27- Bw62-	A2- B27+ Bw62-	A2- B27- Bw62+	A2- B27- Bw62-
	91	2	18	0
	4	13	8	55

The incidences of cytotoxicity obtained against cells with or without one of the three restricting molecules is shown. Target cells carrying more than one restricting element are not included. Target cells from established HLA-A2 (and HLA-B27) variants are excluded from this study.

Table I Competitive inhibition experiments

⁵¹ Cr labeled target cells	Lysis %	Cold inhibitors added	Lysis %
1 HLA-A2+ Bw62- B27-	99	a none	
		b HLA-A2+ Bw62- B27-	39
		c HLA-A2- Bw62+ B27-	99
2 HLA-A2+ Bw62- B27-	97	a none	
		b HLA-A2+ Bw62- B27-	26
		c HLA-A2- Bw62- B27+	95
3 HLA-A2- Bw62+ B27-	58	a none	
		b HLA-A2- Bw62+ B27-	19
		c HLA-A2+ Bw62- B27-	55
		d HLA-A2- Bw62- B27+	54

The inhibitory capacity of non labeled target cells carrying only one of the restricting molecules plus the minor H antigen was measured. The ratio labeled/unlabeled target cells was 1/10. The percentage lysis for effector/target ratio of 50/1 is shown. The competitive inhibition protocol has been described in detail [40].

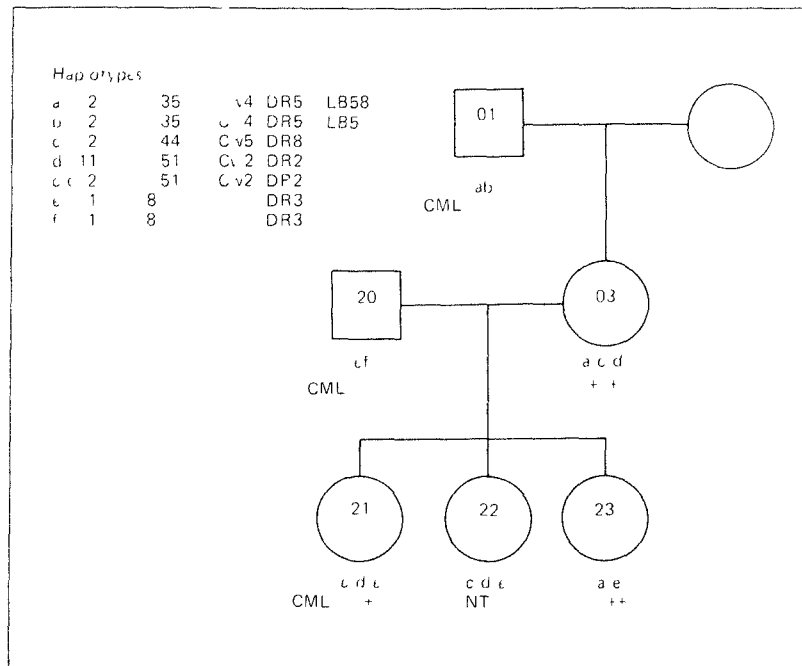


Fig 2 Lysis pattern of HLA-restricted anti-minor H antigen CTLs

yet no data to indicate whether or not the non-HLA antigen which is seen in association with HLA-A2 is the same as those seen in association with HLA-B27 and HLA-Bw62

(3) We investigated the relatives of the positively lysed panel members. The family studies showed a codominant mode of inheritance of the HLA-restricted minor transplantation antigen. In 2 HLA-A/C cross-over families, the target determinant segregated with the HLA-A2 antigen, indicating that if this minor H antigen is coded by chromosome 6, it is located telomeric from HLA-C. An example of the lysis pattern of the HLA-A2-restricted anti-minor H antigen CTLs in 1 HLA-A/C cross-over family is shown in figure 2.

In order to identify the involvement of distinct subsets of CTLs with receptor specificity only for one restricting element, cold target competitor experiments and analyses at the clonal level were carried out. The results of cold target cell inhibition experiments (table V) and T-cell clonal analysis

(table VI) showed unequivocally that we are dealing with three discrete subsets of cytotoxic T cells: one specific for minor H antigen plus HLA-A2, one specific for minor H antigen plus HLA-Bw62 and another one specific for minor H antigen plus HLA-B27.

Inhibition of lysis of the labeled target cells is only observed if cold target cells are added which carry the same class-I-restricting molecule. For example, the percent lysis of target cells 1 is only inhibited by addition of cold inhibitor cells b and not by addition of cold inhibitor cells c which carry another class-I-restricting molecule (i.e. HLA-Bw62).

Distinct subsets of T cells showing receptor specificity exclusively for only one HLA gene product can also be demonstrated by analysis at the clonal level. For example, clone 1 reacts only with HLA-A2+ B27- Bw62- target cells, clone 4 reacts only with HLA-A2- B27+ Bw62- target cells.

In summary, CTLs were harvested from a recipient of bone marrow from his HLA genotypically identical sibling. That recipient was suffering from severe chronic GVHD. These CTLs showed extremely high cytotoxicity *in vitro* against the patient's own pretransplant lymphocytes. Differences in susceptibility to lysis between HLA-identical siblings and positive reactions *in vitro* against target cells which carried the class-I antigens (i.e. HLA-A2, Bw62 and B27) of the patient. These results strongly suggest the presence of one (or more) minor H antigens recognized by MHC-restricted CTLs. At the clonal level it could be demonstrated that distinct cytotoxic T-cell subpopulations demonstrate their specificity for the different class-I-restricting molecules.

Evidence for the existence of human minor H antigens has also been reported by other investigators [25, 106a, 112]. *Lilkins et al.* [25] described the recognition of a human minor alloantigen by lymphocytes derived from a multitransfused aplastic anemia patient. They showed that the lymphocytes from that *in vivo* sensitized patient, restimulated *in vitro* using HLA-mismatched stimulator cells, recognized a non-HLA antigen in an MHC-restricted fashion. Testing of the patient's lymphocytes against a panel of lymphocytes from unrelated individuals indicated that the class-I molecule B7 was involved in the recognition of the minor H antigen. Subsequently, *Tekolf and Shaw* [112] demonstrated that *in vivo* priming by pregnancy seemed to be sufficient to generate cytotoxic T cells against a human minor H antigen. PBLs from a normal female, after secondary *in vitro* stimulation with cells from an HLA-A-, B-, C- and D-matched donor, showed strong cytotoxicity against a minor H antigen in an HLA-restricted fashion. The restricting molecule was again HLA-B7.

Table 11 Clonal analysis of the HLA-A2 Bw62- and B27-restricted anti-minor H antigen CTLs

Clones	E:T ^a ratio	Target cells		
		HLA-A2+ B27- Bw62-	HLA-A2- B27+ Bw62-	HLA-A2- B27- Bw62+
1	8:1	34 ^b	3	2
2	4:1	59	3	4
3	18:1	81	0	1
4	3:1	1	52	1
5	25:1	2	2	24
6	25:1	7	5	13

^a Effector target ratio

^b Percentage lysis

Clinical Relevance of the Minor H Antigen System

The results in bone marrow transplantation are still far from satisfactory. One of the major problems is GVHD which affects approximately 50–60% of the patients grafted with bone marrow from HLA-identical siblings.

H antigens other than HLA certainly exist. Whether or not these non-HLA determinants play a role in the induction of GVHD remains to be proven. Results which might be of importance in the understanding of the biological role of non-HLA transplantation systems and in its involvement in organ transplantation will be discussed below.

In the mouse, the involvement of minor H antigens in GVH reaction has been demonstrated [50, 57, 61–63]. The immunogenetics of GVH reactions to minor H antigens and various properties of cells causing GVHD in mice have lately been summarized by *Korngold and Sprent* [64]. Very little is known about the factors which might influence the development of GVHD following MHC-identical bone marrow transplantation in man. *Sparkes et al* [107] reported on one such factor: a significant correlation was found between compatibility for the blood group system MNSs between donor and recipient and GVHD. Their results suggested that incompatibility for the blood group system MNSs may result in GVHD in man.

The detection of a non HLA minor H antigen as described above prompted us to continue our search for minor H antigens and their role in bone marrow transplantation. The studies were carried out in three stages. In the first stage our goal was the confirmation and extension of our first results i.e. the generation of CTLs with post-transplant lymphocytes of bone marrow-grafted patients and their correlation with the occurrence of GVHD. In the second stage we used the in vitro generated CTLs for the identification of other minor H antigens i.e. increasing the number of anti-minor H cellular-typing reagents. Finally we used those anti-minor H specific CTLs for the retrospective typing of HLA-identical bone marrow donor/recipient pairs.

We studied the post-transplant lymphocytes of 13 HLA-matched bone marrow recipients: 2 with no GVHD, 7 with acute GVHD and 4 with chronic GVHD. The same culture protocol which was successfully used for generation of anti-minor H antigen CTLs [42-44] was applied. Post-transplant lymphocytes from recipients of HLA genotypically identical bone marrow grafts were sensitized in vitro for 6 days with the patient's pre-transplant lymphocytes. The effector cells harvested at day 6 were tested in the CML assay for specific cytotoxic activity. They were expanded also in order to obtain cytotoxic T-cell lines. Thus large amounts of cytotoxic reagents may be obtained, frozen and adequately used after thawing. Table VII shows that with post-transplant lymphocytes from 5 patients, 4 of whom had chronic GVHD, cytotoxicity was observed which was directed against their own pretransplant lymphocytes.

In the second stage we tested the in vitro generated CTLs (derived from 5 patients) for the identification of other minor H antigens. The analysis of the cytotoxic activity using a limited panel of target cells from healthy unrelated individuals identified five different CTL populations directed against different minor H antigens. The *non HLA antigens* (designated minor HA-1, minor HA-2, minor HA-3, minor HA-4 and minor HA-5) and the *restricting* MHC molecules necessary for their associative recognition are shown in table VII. The analysis of the CTL 1 (derived from patient 1) recognizing minor HA-1 in association with class-I molecules HLA-A2, B27 and Bw62 have been discussed earlier in this chapter and was referred to as minor H antigen CTLs. Another minor H antigen (i.e. minor HA-2) was recognized by CTL 2 derived from patient 2. Our search for the restricting molecules by analyzing the cytotoxic activity against a limited number of unrelated target cells has been unsuccessful so far.

Table VII Detection of cell mediated anti-non-HLA activity after bone marrow grafting

Bone marrow recipients	CML		Patient No	HLA typing
	+	-		
No GvHD	0	2	1	A2,A2, B27,Bw62, Cw1 3 DR1 4
Acute GvHD	1	6	2	A1 A11 B8,B40 Cw3, DR2 3
Chronic GvHD	4	0	3	A2 A3, B18,Bw44, Cw7 DR4 6
			4	A1,A2 B7,B8, Cw7 DR2 3
			5	A2 A29 Bw49 Bw44 Cw-, DR5 7

CTLs	MHC-restricting molecules	Non-HLA antigens
1	A2 B27 Bw62	minor HA-1
2	A2 B27 Bw62 A2	minor HA-2
3	A1	minor HA-3
4	A2	minor HA-4
5	A2 Bw44	minor HA-5

The results obtained with anti-minor HA-3 CTL 3 from patient 3 indicate that they possibly recognize not only an HLA-A2-restricted non-HLA antigenic determinant but also a non-self-HLA class-I alloantigen (i.e. HLA-B8). Thus, HLA-A2 (i.e. self) plus minor HA-3 (i.e. X) equals foreign HLA (i.e. B8). This might be an example of cross-reactivity earlier described in mouse [10]. Yet, analysis on the clonal level has to be carried out to justify our observation. Recently, cross-reactivity of HLA-restricted Epstein-Barr virus-specific CTLs for allo-MHC determinants has been described [30]. Analysis of the reactions of CTL 4 obtained from patient 4 identified other minor H antigen (i.e. minor HA-4) recognized with the HLA-A2 molecule as restricting element. The HLA-A2- and HLA-Bw44-restricted CTLs derived from patient 5 recognized a minor H antigen designated minor HA-5. As discussed earlier in this chapter, the minor antigen H-Y is preferentially recognized in conjunction with the HLA-A2 and B7 molecule. The recognition of different minor H antigens (i.e. HA-1, HA-3, HA-4 and HA-5 as shown in table VII) demonstrate again the preference for the HLA-A2 class-I molecule for the associative recognition of minor H antigens.

In our final third stage, the large scale expansion of these minor H-specific CTLs enabled us to store enough minor H reagents in order to

Table VIII Cellular typing of human minor H antigens with MHC-restricted anti-minor H-specific CTLs generated during GVHD

Donor recipients pairs	Minor H	
	compatibilities ^a	incompatibilities ^b
Recipients without GVHD	7	0
Recipients with acute GVHD	4	1
Recipients with chronic GVHD	3	7

The donor recipient pairs are selected for the presence of the restricting class I molecules for required associative recognition of the minor H antigens

^a No incompatibilities found between donor and recipient for minor HA-1 to HA-5

^b Incompatibilities found between donor and recipient for HA-1 to HA-5

provide us with typing reagents. Those minor H cytotoxic reagents were used to type retrospectively a series of HLA-identical bone marrow donor recipient pairs: recipients without GVHD, recipients with acute GVHD and recipients with chronic GVHD. The results of this analysis are shown in table VIII. Also shown are the incompatibilities between HLA-identical bone marrow donors and recipients for minor H antigens found so far mainly in the group of patients suffering from chronic GVHD.

Perspectives

Since donors and recipients are routinely selected for identity at the major H system, rejection and GVHD may be caused by disparity for the products of minor H systems. The effect of weak transplantation antigens on allograft survival may hopefully become predictable when CTL-typing reagents at least for the strongest non-MHC transplantation antigens become available. At present no alloimmune sera are available, for the typing of the non-HLA antigens, which are relevant in the pathogenesis of GVHD. Consequently, when more than one bone marrow donor is available (as well in related as in unrelated situations), CTL typing may mitigate at least one of the obstacles for successful bone marrow transplantation. Our present goals are: (1) the generation of anti-minor H CTLs with post-

transplant lymphocytes from *all* available bone marrow-grafted patients (2) the performance of extensive immunogenetic analyses (using the results obtained with those anti-minor H cytotoxic reagents) which will hopefully provide us with information about the average number of human genetic minor H loci and their alleles and (3) the investigation of the role of the various minor H antigens on the outcome of kidney as well as bone marrow transplantation

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