Els Goulmy

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Minor Histocompatibility Antigens in Man and their Role in Transplantation

INTRODUCTION

The definition of the word 'minor' according to the Concise Oxford Dictionary[1] is: 'comparatively unimportant (for example: minor poet) or in case of operation: presenting no danger to patient's life'. Consequently, in our discipline, the 'majors' (histocompatibility antigens) must account for the greatest obstacle in human organ exchange; a barrier which in all probability was already playing a role in ca 300 AD when two sibling doctors, Saint Cosmas and Saint Damian, carried out a total leg replacement. Nowadays even with the use of a new potent immunosuppressive drug, Cyclosporine A, the renal transplant recipient still benefits from a well matched organ donor.[2]

The fact that minor histocompatibility (minor H) antigens are 'allied' to the major H antigens probably raises one's interest in them. This is based on the assumption that major H molecules serve as salvers for foreign antigens, such as foreign minor H antigens, thus triggering an immunological T cell response.[3]

This chapter will deal with the recognition of human minor H antigens, the genetics of these antigens at the population level as well as in families, and finally their possible impact on the outcome of bone marrow transplantation will be discussed. This review does not pretend to cover all information regarding the influence of non-MHC antigens on the outcome of organ transplantation, but rather reflects a summary of our own cellular immunological *in vitro* studies in relation to bone marrow transplantation in man in particular.

INTRODUCTION TO AND RATIONALE OF THE STUDY

It is now 49 years since the first human bone marrow transplantation was carried out.[4] In the 1950s, the first series of clinical applications of bone marrow grafting was reported.[5,6] Thanks to Mortimer Bortin, a compendium on the results of the

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first 203 human bone marrow transplants performed as therapeutical treatment of severe aplastic anaemia, leukaemia and immune deficiency disease became available in 1970 [7] Although, the graft versus-host (GvH) reaction historically dates from early this century,[8] the high number of deaths in the latter report, was not attributable to GvH disease

Notwithstanding Ceppellini's statement in 1967 'there is little doubt however that the motivation of Nature in selecting for a genetic polymorphism of this complexity, was not an *a priori* hostibility against transplantation surgeons',[9] it is clear that in an artificial situation, such as organ transplantation, the major H antigens function as a major transplantation barrier and thus play an important role in the survival of transplants and patients Consequently, improved success in bone marrow transplantation was reported when matching for the HLA antigens was taken into account [10] Between 1975 and the present day, the long-term results of allogeneic bone marrow transplantation have greatly improved due to the use of HLA matched siblings as marrow donors, advanced pretransplant chemoradiotherapy, the use of potent immunosuppressive drugs as prophylaxis, better antibiotics and isolation procedures

Despite the promising advances, the overall results achieved so far are still not completely satisfactory Graft versus host Disease (GvHD) affects (despite the selection of HLA identical siblings as bone marrow donors) approximately 20–70 percent of the patients depending on their age [11][12] The aetiology of GvHD presumes that immunocompetent donor T cells are reacting against the host tissues Thus, when endeavouring to prevent GvHD development, donation of marrow depleted from mature T cells has become a frequently used regimen Unfortunately, this treatment also has its drawbacks Graft failure or rejection as well as recurrence of the original disease have been reported as major complications following T lymphocyte depleted bone marrow transplant [13][14] The severe, and in some cases fatal complications following these marrow transplants justify the search for at least one of the obstacles to successful bone marrow transplantation

In human transplantation, donors and recipients are routinely screened for identification of the major H system, therefore GvHD and rejection may be caused by the disparity of the products of the so called 'minor' H systems, i e histocompatibility antigens other than those coded for by the MHC

Skin grafting experiments in the mouse demonstrated the presence of a large number of histocompatibility antigens coded for by multiple loci scattered all over the genome They show distinguishable patterns in eliciting allogeneic reaction, skin grafting over a multiple minor H barrier demonstrates a graft rejection time comparable to those that differ only at H 2 [15][17]

Prior to the detection of the possible involvement of human minor H antigens in the development of GvHD after HLA genotypically identical bone marrow grafting,[18] convincing results were reported in the mouse demonstrating that, using congenic strains of mice, incompatibility for non H 2 antigens alone can lead to a high rate of GvH mortality [19][21] Moreover, the T cells causing lethal GvHD across minor H barriers appear to be H 2 restricted Thus T cells responsible for induction of GvHD to minor H antigens do not respond *in vivo* to the same minor H antigens presented on H 2 different cells [22][23]

CELLULAR IMMUNOLOGICAL *IN VITRO* STUDIES
OF HUMAN BONE MARROW TRANSPLANTATION

Figure 3-1 functions as a guideline according to which I will treat the major effects resulting from bone barrow transplantation, illustrated with, as far as possible, the results of our *in vitro* studies. First, the studies dealing with graft failure/rejection will be discussed. Second, the complication of GvHD will be examined, exemplified with *in vitro* studies reflecting the graft-versus-host attack. Finally, I would like to touch briefly on the possible 'graft-versus-leukaemia' (GvL) effect.
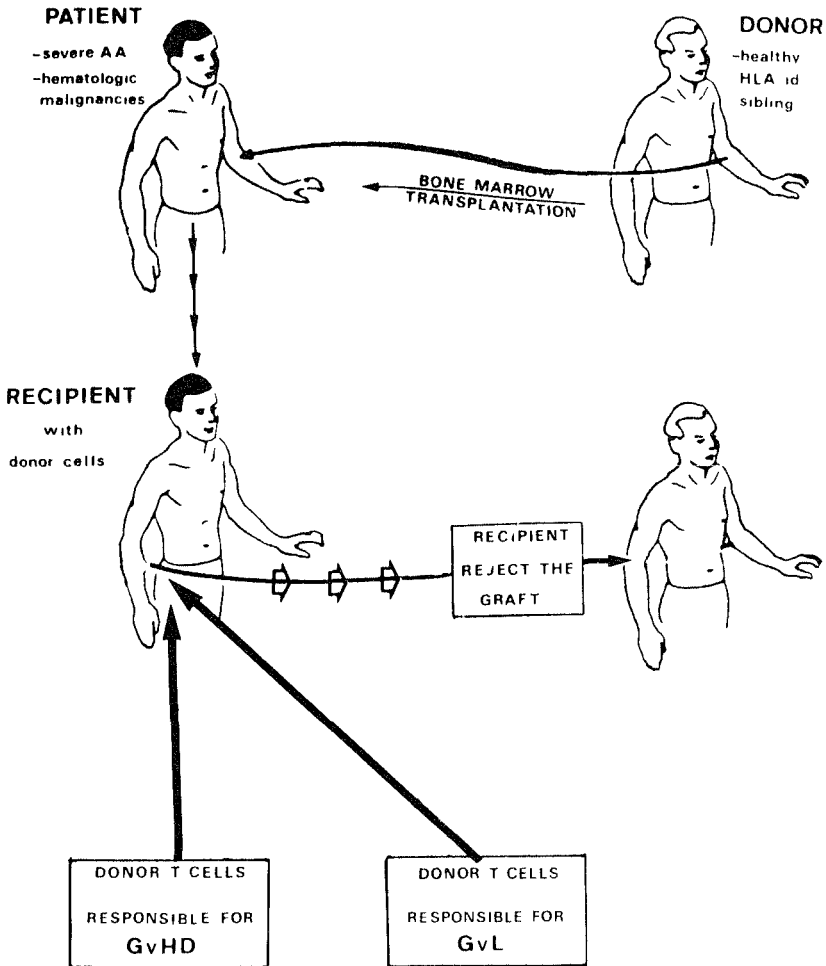

Figure 3-1. Cellular immunological *in vitro* studies of human bone marrow transplantation. AA = aplastic anaemia, GvHD = graft-versus host disease, GvL = graft-versus leukaemia

The Male Specific Antigen H-Y

Cellular Recognition

Our involvement in human minor H antigens started in 1975, virtually by coincidence. On one hand we were interested in mutually cytotoxic T cell (CTl) reactivities between HLA identical siblings *in vitro*, and after *in vivo* sensitization [24,25] On the other hand, a clinical so called 'no take' observation after bone marrow grafting, donated by a male sibling donor to his HLA genotypically identical sister, was reported to us. Fortunately, this clinical event led us not only to the first demonstration in man of the participation of the HLA molecules in the interaction of T cells with foreign antigen, but also brought us into the mysterious world of minor transplantation antigens of which the male specific antigen H Y is the far easiest and also the most extensively minor H antigen studied [25,26]

The first report of H Y as a transplantation antigen is an untitled communication by Eichwald and Silmser in 1955. These authors observed that within two inbred strains of mice, most of the male to female skin grafts were rejected, whereas transplants made in other sex combinations nearly always succeeded [27] The term H Y antigen was introduced by Billingham and Silvers[28] because the male specific antigen can function as a classical transplantation antigen responsible for homograft rejection. An important step in the recognition of the H-Y antigen *in vitro*, using H Y immune spleen cells obtained from *in vivo* immunized female mice, was reported by Gordon et al [29] *In vitro* cell mediated cytotoxic responses to the male specific antigen H Y were found to be H 2 restricted, i e CTl s recognize foreign antigens, such as H Y, only when they are presented to the CTl s on cells which share some homology of the H 2 region as expressed on the effector CTl s. Similarly, the recognition of other minor H antigens by CTl s is also MHC restricted [30]

Results in View of Clinical Cases

Returning to our clinical case, *in vitro* analysis of the posttransplant peripheral blood lymphocytes (PBl s) of the female patient (HLA phenotype HLA A2, A2, B44, B60, Cw3, Cw5, DR4, DRw6) showed unambiguously strong CTl responses specific for *male* HLA *A2* positive target cells [25,26] Whether the H Y specific CTl s actually mediated the allograft rejection, we do not know. It must be remarked, however, that most probably the female patient, who was suffering from severe aplastic anaemia, had been sensitized to the H Y antigen prior to transplantation through multiple (mainly male) blood transfusions and pregnancies. This assumption is based on our subsequent observations. As shown in Table 3 1, PBl s derived from four additional cases showed, after *in vitro* restimulation with HLA identical male cells exactly the same phenomenon, namely HLA restricted (A1, A2 and/or B7) anti H Y cytotoxic T cell activity. In one patient (i e Case 5, Table 3 1), the H Y specific HLA B7 restricted cytotoxicity was detected shortly after an acutely rejected kidney donated by an HLA identical male sibling (unpublished observation) In circumstances similar to ours, other investigators also described the presence of HLA restricted H Y directed cytotoxicity [31,32]

Table 3-1

MHC Restricted Cytotoxic T Cell Responses Against the Minor H Antigen H Y

Patient/Disease	CTLs Derived From	HLA Typing CTLs
1 AA*	♀ post bm grafting	HLA (A2), A2, B44, B60
2 AA	♀ multi transfused	HLA (A2), A3, B60, B62
3 AA	♀ multi transfused	HLA (A2), A28, (B7) B62
4 AA	♀ multi transfused	HLA (A1), A2, B8, B61
5 ——	♀ post renal transplant	HLA A2 A2 (B7), B13

◯ MHC restricting antigens
*Aplastic anaemia

Although in our first case we formally could not prove that the H Y specific CTLs actually mediated the rejection of the male allograft, very recently we have been confronted with a case with a fatal outcome, in which anti H Y CTLs were most probably mainly responsible for graft rejection It concerned a multitransfused female patient suffering from myelodysplasia after treatment for Hodgkin's disease *In vitro* analysis demonstrated the presence of HLA-A1 restricted anti H Y CTLs (Table 3 1, Case 4) Since the father appeared to be the only HLA compatible related donor, he was the obvious choice (despite, unfortunately, the presence of patient's pretransplant anti H Y CTLs) Notwithstanding intensive pretransplant immunosuppressive treatment and the donation of T cell depleted marrow there was no recovery of the patient's bone marrow haematopoietic function (Voogt et al , ms in preparation) With hindsight, the choice of an unrelated HLA identical (or compatible) female bone marrow donor might eventually have led to a less dramatic result The consideration of choosing in favour of an unrelated — or even a partially HLA mismatched donor — will be treated in more detail elsewhere in this chapter

Interestingly, it is not only very recent reports,[33][34] but reports since 1977,[35] which have pointed towards sex mis-match as one of the risk factors associated with GvHD or rejection According to the most recent data collected worldwide and evaluated by the Advisory Committee of the International Bone Marrow Registry [33][34] male to female, and female-to male transplants have a high chance of rejection respectively GvHD in acute myelogenous leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic myelogenous leukaemia (CML) and severe aplastic anaemia (SAA) With special regard to marrow treatment in the latter disease, I think it might be helpful to a certain extent to be aware of the apparently fairly high incidence *in vitro* of CTLs directed against the male specific antigen H-Y A report on the presence of anti H Y cytotoxicity in an untransfused 11 year old female patient with SAA, further strengthens this point [36]

The clinical relevance of the H Y alloantigen on the results of human kidney allograft transplantation has also been determined A 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, 2 years after transplantation

Male allografts from HLA A2 positive donors in HLA-A2 positive females survived for a significantly shorter time than non-A2 male kidneys in non-A2 female recipients [37]

Perspectives

To elaborate on the assumption that minor H antigens such as H-Y do indeed play a role in the success of graft exchange between HLA identical siblings in general and in T cell depleted marrow in particular, we investigated the expression of this minor H antigen on haematopoietic progenitor cells (HPC). For this purpose, an *in vitro* cellular cytotoxicity assay with bone marrow cells as target cells has been developed [38] In brief, *in vitro* sensitized HLA-A2 specific CTLs were incubated with bone marrow mononuclear cells derived from, for example, HLA-A2 positive or HLA-A2 negative donors Complete inhibition of growth was observed in the myeloid, erythroid and multipotential HPC from the HLA-A2 positive individual,[38] whereas normal outgrowth of HPC cultures was observed in the bone marrow cells from HLA-A2 negative donors

Naturally, the next step was to investigate whether or not the *H-Y antigen* is expressed on human HPC The results of these experiments show that indeed the H-Y antigen is expressed on HPC [39] These observations support the notion that the minor H antigen H-Y is by and large likely to play a role in the failure of (stable) marrow engraftment, particularly in the graft failure of Case 4 as discussed in the previous section

Little is known about the nature and exact function of the histocompatibility antigen H-Y Recent examination of sex-reversed humans by combined analyses of different sets of Y-DNA probes and H-Y specific CTLs revealed that the gene for H Y maps to the long arm or centromeric region of the human Y chromosome [40] Hopefully, we will reach the state in which we can study which peptide(s) (obtained either by cleavage of the H-Y gene product or chemically synthesized) forms the immunogenic complex with the MHC class I (HLA-A1, -A2 or -B7) molecules as recognized by the CTLs Thereupon, one could think of making antibodies against such peptide(s) which could block the specific cytotoxic activity as well as neutralize the intact H-Y protein so that the individual is protected from unwanted H-Y directed cytotoxicity

The interaction of the H Y antigenic peptide(s) with its 'salver' is also interesting In the light of our findings the H-Y antigen preferentially binds to the HLA-A2 molecule Therefore analysis of the epitopes on the HLA-A2 molecule required for cellular recognition of the H-Y antigen has been carried out These studies led to the observations that alloimmune HLA-A2 specific CTLs[41 42] as well as HLA-A2 restricted H-Y specific CTLs[43] can distinguish between several, yet serologically identical, HLA-A2 molecules Lately, additional information has become available on the more precise location of the amino acids on the HLA A2 molecule involved in the recognition by CTLs Combined investigations (resulting from a collaborative effort) of the HLA A2 'variant' molecules at the molecular and at the functional level, demonstrated that amino acid changes at position 43 and in the residues 145–157 (i e cellular defined subtypes HLA-A2 2 and HLA A2 3) lead to the loss epitope(s) necessary for associative recognition of the H-Y antigen by HLA-A2 restricted CTLs [43 44]

Interestingly, a single amino acid change from phenyalanine to tyrosine at position 9 in the heavy chain of the HLA A2 molecule (i e cellular defined subtype HLA A2 4) does not affect the recognition of H-Y by HLA A2 restricted CTLs [43] Recently, the crystal structure of the purified HLA A2 molecule provided us with information on the differential functions of the amino acids in the different domains of the HLA-A2 molecule [3] According to the latter authors, residue 9 is one of the positions located in the binding site region for processed antigen and thus might be involved in the binding of the H-Y peptide(s)

This assumption is extremely interesting because of the following observation three different HLA-A2 molecules i e 1) *M7* HLA-A2 2, amino acid changes at positions *43,95,156* (ref 45), 2) *AM* HLA-A2 2Y, amino acid changes at positions *9,43,95,156* (ref 46) and 3) *Cla* HLA-A2 4, amino acid changes at position *9* (ref 44) have been analysed with the HLA-A2 restricted H-Y specific CTLs *Absence* of recognition was *only* observed when male donor M7 was tested as target cells [47] The sole difference between M7 and AM is an additional amino acid change at position *9* in AM which is absent in M7 Despite the limited information which is available so far, these results further strengthen the postulated interaction of position 9 in the α_1 domain of HLA-A2 molecule with antigen The amino acid change at position 9 from Phe to Tyr is identical between Cla and AM but is the difference between AM and M7 These data suggest that tyrosine is a candidate for the binding of H-Y into the groove of the top of the HLA-A2 molecule

The 'Other' Minor Histocompatibility Antigens

Polymorphic Blood Genetic Markers

Before discussing the method of detection, identification and genetics of our minor HA antigenic system and its possible influence on the development of GvHD, the involvement of other putative 'minor' non-HLA histocompatibility antigens in GvHD should be mentioned Sparkes et al [48] reported on a significant correlation between compatibility for the blood group system MNSs betwen donor and recipient and GvHD, incompatibility for this system may result in GvHD Similarly, De Gast et al [49] studied the association between 22 polymorphic blood genetic markers and GvHD Three (i e rhesus, MNSs bloodgroup and acid phosphatase) of the 22 markers appeared to be involved in GvHD Mismatching for all three markers showed an additive effect and significant correlation with GvHD

The Minor Histocompatibility Antigens 'HA', the origin of HA-1

Again, a clinical case opened our eyes to the first demonstration in man of possible involvement of minor H antigens (other than H-Y) in the development of GvHD

The second part of the section on cellular immunological *in vitro* studies of human bone marrow transplantation will deal with GvHD (see Fig 3-1) The occurrence of a severe GvHD in a bone marrow-transplanted male AML patient prompted us to investigate the *in vitro* cytotoxic activity of the patient's posttransplantation lymphocytes The patient had been transplanted with bone marrow from an

HLA identical female sibling donor. His clinical recovery, however, was complicated by severe chronic GvHD. The initial experiment demonstrated that the posttransplant lymphocytes had strong cytotoxic activity against the patient's own *pre*transplant lymphocytes but not against the lymphocytes of his HLA identical donor.[18] This observation in itself supports the notion that whatever the target determinant recognized by the latter CTLs, the HLA genotypically identical donor and recipient differed for it.

From additional analysis of the patient's posttransplant CTL activities, it became apparent that the antigen (which we designated minor H antigen HA 1) was not only present on the patient's own pretransplant cells, but could also be detected on lymphocytes from two out of three haplo identical siblings as well as on the lymphocytes of the parents, and also on lymphocytes from a large number of unrelated healthy individuals. The antigen HA 1 could be recognized by the patient's posttransplant CTL only if one of the patient's HLA class I antigens was present on the target cells.[50] Consequently, HA 1 is recognized in an MHC restricted fashion, an event comparable to the recognition of H Y and similar to that described in the mouse (see p 30).

Since strong anti minor H antigen cytotoxic activity was observed in a patient suffering from severe GvHD after HLA identical bone marrow transplantation, it was reasonable to assume that there might exist a correlation between both *in vivo* and *in vitro* observations. Based on this concordance, we felt it was justified to continue our search for non HLA antigens and their possible role on the outcome of bone marrow transplantation. For this purpose we used the simple, yet laborious, method as outlined in the following section.

Methodology

The basic idea of generating anti host CTLs with specific cytotoxic activity for non HLA antigens is based on the assumption that posttransplant (i e donor) cells when sensitized against the patient's own pretransplant cells, are directed against host specific target structures, such as minor H antigens, which are absent from the donor cells. This supposition is plausible since our studies have been carried out with material obtained from HLA genotypically identical bone marrow donor and recipient combinations.

The protocol, which we commonly use, enables not only the generation of anti host CTLs posttransplantation of bone marrow, but also the establishment and expansion of minor H antigen specific CTL lines. The success of this was due to a crucial, in fact logical, culturing policy and an implicit confidence in succeeding. Peripheral blood lymphocytes (PBLs) taken from the patients shortly after bone marrow transplantation look disastrous. So, the trick is to prime the 6 day old responder/stimulator cell cultures with specific stimulator cells and highly purified interleukin 2 (HP IL 2) for 3–4 days (see Fig 3 2). This restimulation is just sufficient for the few surviving cells to recover and to start proliferating. The HP IL 2 is preferable to recombinant IL 2, because it contains small amounts of extra possible growth factors. Thereafter, the effector cells are regularly fed with, most preferably, the original stimulator cells (or if not available, lymphocytes from HLA identical unrelated healthy individuals) and T cell growth factor (TCGf). Since each individual
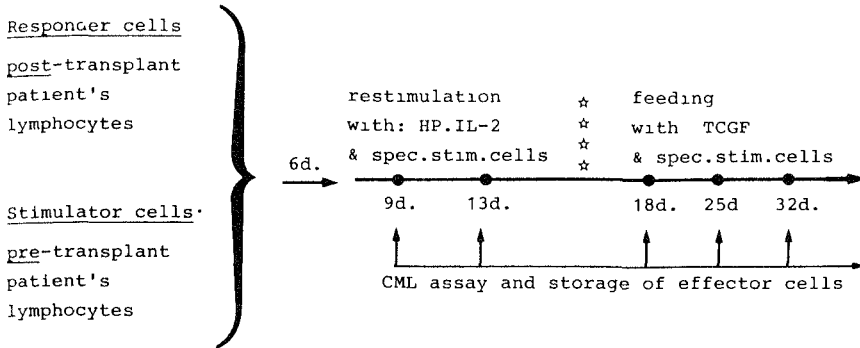
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Responder cells  ⎫
                 ⎪
post-transplant  ⎪
                 ⎪   restimulation       ☆   feeding
patient's        ⎪   with: HP.IL-2       ☆   with   TCGF
                 ⎪                        ☆
lymphocytes      ⎬   & spec.stim.cells    ☆   & spec.stim.cells
              6d.⎪ ──────────────────────────────────────────────────►
                 ⎪        9d.   13d.           18d.  25d.   32d.
Stimulator cells·⎪        ●      ●              ●     ●      ●
                 ⎪
pre-transplant   ⎪        ↓      ↑              ↑     ↑      ↑
patient's        ⎪        CML assay and storage of effector cells
                 ⎪
lymphocytes      ⎭
```

Figure 3-2. Flow chart for generation of anti-host CTLs CML = cell mediated lympholysis HP IL-2 (highly purified inter leukin-2) and TCGF (T cell growth factor) are both commercially available from Biotest HP IL-2 is used in the first restimulation, thereafter TCGF is added for growth promotion Recommended cell numbers at day 0 minimum of 2 10^6 responder cells and 2 10^6 stimulator cells in 2 ml of culture medium

cell-line has its own growth kinetics, the optimal effector cell yield and cytolytic activities can only be achieved by paying attention to each effector cell combination individually Once reasonable growth is obtained (in general between 12 and 20 days of culture), the effector cells are first tested for specific cytotoxic activity i e patient's pretransplant lymphocytes Subsequently, further expansion of the CTLs is provided by the alternate addition of feeder cells and TCGF Specific cytotoxic T cell activity is measured by the use of the cell-mediated lympholysis (CML) assay previously described in detail [51]

Anti-host Cytotoxic T Cell Activities

Next, we aimed at both confirmation and extension of our first results regarding the possible impact of polymorphic genetic systems other than HLA on the development of GvHD in man For this purpose, we investigated posttransplant lymphocytes from a series ($n = 28$) of recipients of HLA identical bone marrow grafts for the presence of anti-host CTL activity Posttransplant lymphocytes from 17 out of 21 patients suffering from GvHD demonstrated CTL activity (Table 3-2), which was directed against patients' own pretransplant lymphocytes Host directed CTL could so far be demonstrated in all (except one) patients suffering from chronic GvHD Furthermore, in five out of eight patients with acute GvHD, anti host CTL activity was also observed One of the latter CTL populations (designated as 'anti HA 3' CTLs) has been analysed extensively and will be discussed in the following sections The remaining four anti host CTL populations generated with posttransplant PBLs from patients with acute GvHD and those generated in the two patients without GvHD are currently under investigation We do not know yet whether the cytotoxic effector cell populations and the target structures they are directed at are different in the acute

Table 3-2

Anti host Cytotoxic T Cell Activity after HLA Identical Bone
Marrow Grafting

Bone Marrow Recipients	Anti host Cytotoxic Activity	
	Yes	No
No GvHD	2	5
Acute GvHD	5	3
Chronic GvHD	12	1

χ $-8\ 62$
p $0\ 00134$

patients or those suffering from chronic GvHD At the moment we cannot also provide
information on the specific reactivity of the populations observed in patients withou
any clinical signs of GvHD, except for one donor/recipient combination in the
'no GvHD' group where an HA-3 incompatibility was observed (see subsequen
sections where the inverse correlation of the HA 3 antigenic determinant with the
occurrence of GvHD will be discussed)

At present, we are also attempting to gain insight into the kinetics of the *in vitro*
anti host responses, because to date our search for anti host CTl activity has been
limited to one posttransplant (±40 days) bleeding date The appearance and eventua
decline of both proliferative and cytotoxic anti host T cell activities have been
systematically analysed The results so far show that host-directed T cells can be
detected from 25 days to 25 months after bone marrow transplantation, the latter
long lasting anti host CTLs was observed in a patient suffering from chronic GvHD
(van Els et al , manuscript in preparation)

The results of the anti-host CTl activities posttransplantation of bone marrow can
be summarized as follows first, posttransplant anti host CTl activity can be generated
in patients suffering from chronic GvHD As far as analysed, these activities are
extremely high and long lasting Second, in five out of eight patients with acute GvHD
anti host cytotoxic responses were found, the latter patterns however are rather variable
in appearance and activity Third, anti host T cell activities can be present in some
patients without GvHD and thus do not uniquely appear in patients with GvHD
suggesting that anti host CTL are not the sole mediators of GvHD These preliminary
findings in man are in line with results reported in mice [52] Finally, that host directed
T cell activities in patients without any clinical signs of GvHD and also in patients
suffering from acute GvHD only are observed, touches upon an interesting issue,
namely it may lead us to the identification of the populations responsible for eliciting
the graft versus leukaemia (Gvl) reaction This will be discussed later

Genetics of the HA Antigenic System

Similar to the initial anti host specific CTLs 'HA 1' (as discussed previously), we
next endeavoured to uncover the specificity of the target structures recognized by
some of the anti host CTLs (see Table 3 2) It is worth noting that such CTLs can
be derived from either male or female patients suffering from different haematologic

Table 3-3
MHC Restricted Cytotoxic T Cell Responses Against Minor Histocompatibility Antigens

CTLs Derived From	HLA Typing CTLs	Minor Histocompatibility Antigens
1 AML ♂ post bm grafting	HLA (A2), A2, (B27), B(62)	HA 1
2 AA ♀ post bm grafting	HLA A1, (A2), B7, B8	HA 2
3 AML ♂ post bm grafting	HLA (A1), A11, (B8), Bw60	HA 3
4 CML ♀ post bm grafting	HLA (A2), A3, B18, B44	HA 4
5 AML ♀ post bm grafting	HLA (A2), A29, (B44), B49	HA 5

( ) MHC restricting antigens

malignancies prior to bone marrow transplant (Table 3-3) Five (including HA 1) out of the 28 anti-host CTL populations (see Table 3 2) underwent comprehensive analyses at the population level as well as in families Comparable to HA 1, anti host CTLs derived from the second, third, fourth and fifth patient were found to be directed against minor H antigens, designated HA-2,3,4,5 respectively, requiring self HLA class I antigens for their recognition These conclusions are based on the reaction patterns exhibited by CTLs HA-1 to HA-5 against a panel of unrelated healthy individuals as shown in Table 3 4 The common denominator of HA 1,2,4 and 5 specific CTLs is the preferential use of the apparently popular MHC class I restriction molecule HLA-A2 Notwithstanding, likewise the HA-1 specific CTLs, the HA-3 and HA-5 specific CTLs showed lysis of target cells which carried another self MHC class I molecule (HLA A1, B8 and B44 respectively) We have, as yet, no data to indicate whether or not the minor H antigens which are seen in association with HLA A2 are distinct from those recognized by HLA-A1 or -B8 (in the case of HA 3) or HLA-B44 (in the case of HA 5) directed CTLs Table 3 4 shows the frequency distribution of the four HLA A2 restricted CTLs in a reasonable number of healthy unrelated HLA-A2 positive individuals It is clear that the minor H antigens HA-1,2,4 and 5 are found at a high frequency in the random population The same applies to the minor H antigen HA-3 (data not shown)

The presumption that the anti HA-1 and HA 2 CTLs are in fact recognizing one determinant and that the anti-HA-4 and HA 5 CTLs are recognizing a second determinant, allelic to HA-1 and HA-2, is untenable as will be clear from the inspection of Table 3-4 Moreover, one feels inclined to conclude that the anti HA 1 and HA 2 CTLs (r value = 0 44) but especially the anti HA-4 and anti HA 5 CTLs (r value = 0 87) might recognize identical minors Despite their apparent identity, there are a number of cases where the CTLs type the same target cells differently Cold target inhibition experiments, performed with the effector cell-lines, confirmed this at least with regard to the incapability of HA 1 or HA-4 cold target cells to inhibit HA 2 specific cytotoxic activity (Fig 3 3) Cold target inhibition studies for HA 4 versus HA 5 as well as analyses at the clonal level are presently undertaken to prove or disprove their identity At this stage we conclude that the antigen HA 1 is different from HA 2, and that both are different from the antigens HA 4 and HA-5, it is highly probable that HA 4 and HA 5 are identical

Table 3-4

Minor Histocompatibility Antigens CTL Typing in HLA A2+ Individuals

n	HA 1	HA 2	HA 4	HA 5
70	+	+	+	+
1	+	+	+	−
3	+	+	−	+
21	+	+	−	−
3	+	−	+	+
1	−	+	+	+
1	−	+	+	−
1	−	+	−	+
11	−	+	−	−
6	−	−	+	+
3	−	−	−	−
%	81	90	68	69

	+/+	+/−	−/+	−/−	r
HA 1/HA 2	95	3	14	9	0 44
HA 4/HA 5	80	2	4	35	0 87
HA 1/HA 4	74	24	8	15	0 32
HA 1/HA-5	76	22	8	15	0 34
HA 2/HA 4	73	36	9	3	0 02
HA 2/HA 5	75	34	9	3	−0 01

$r - \chi^2/\bar{n}$, all χ were calculated with Yates' correction No incidences of cytotoxicity have been obtained against HLA A2 negative ($n = 70$) target cells The genotype frequencies of HA 1, 2 4 and 5 within the HLA A2 positive population is 56 percent, 69 percent 43 percent and 45 percent respectively

In order to gain insight into the genetics of the HA antigenic system, we investigated the relatives of the bone marrow donor/recipient combinations (between whom we generated the several HA specific CTLs, see Table 3-2 and 3-3), with HA-1 to HA-5 specific cytotoxic T cell reagents The pedigree of one of these five families is given in Fig 3-4 The reactivity patterns in these patients' families (and also in other randomly chosen families, Goulmy et al , ms in preparation) showed clearly segregation of the HA antigens They also demonstrate that HLA identical siblings can differ for the HA antigens For example in the family of patient HA-4 (Fig 3-4), healthy siblings 09 typed differently from her HLA identical siblings 04, 06 and 10 These latter siblings typed identically for HA-1,2,4 and 5 among each other, likewise siblings 05 and 11 Of interest is that the HA-2 antigenic determinant which is present on the maternal cells (00) is apparently lost in the third generation (i e 82, Fig 3 4) Finally, as exemplified in Fig 3-4 and observed in all but one (i e HA-3) donor/recipient combinations which led to the creation of the HA specific CTLs, the donors and recipients differ for more than one minor HA antigen Consequently, CTLs generated from posttransplant lymphocytes from patient 4 leading to the cytotoxic T cell population designated as HA-4 specific CTL clones (Fig 3 4) must also be present directed against HA-1 and HA-5 The panel analysis (Table 3-4) performed with the bulk effector cell populations HA 1,2,4 and 5 is consistent
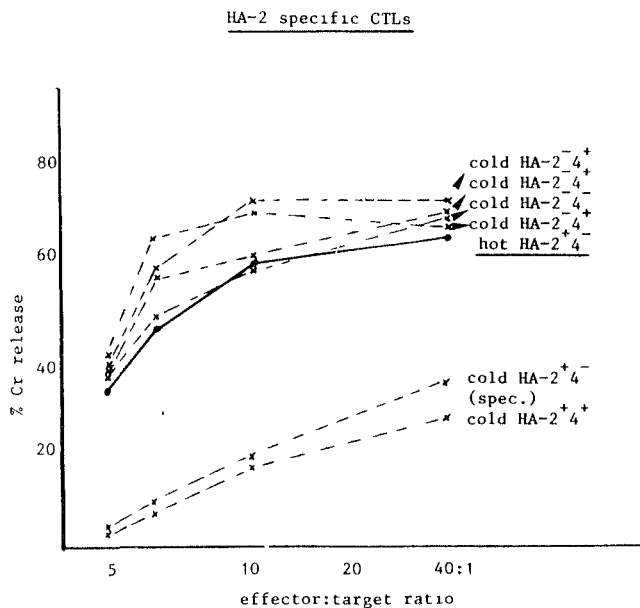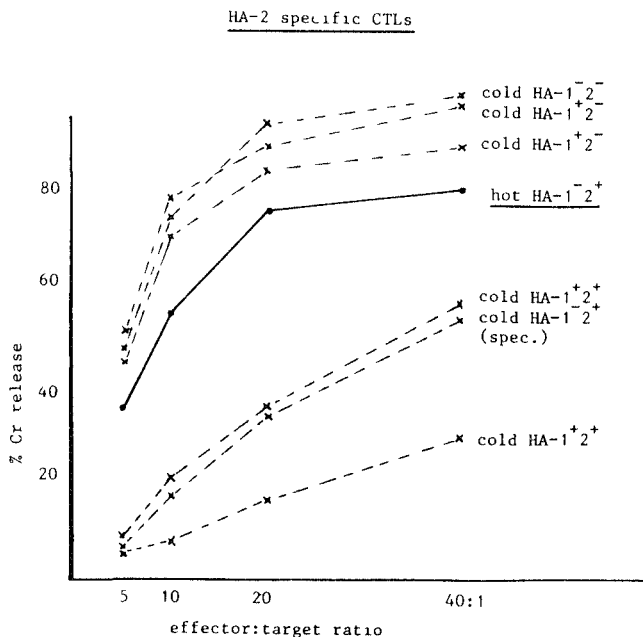
HA-2 specific CTLs

HA-2 specific CTLs

Figure 3-3.   Cytotoxic T cell activity with specificity for the HA-2 antigen; competitive inhibition experiments Hot : cold target cell ratio is 1 : 10.
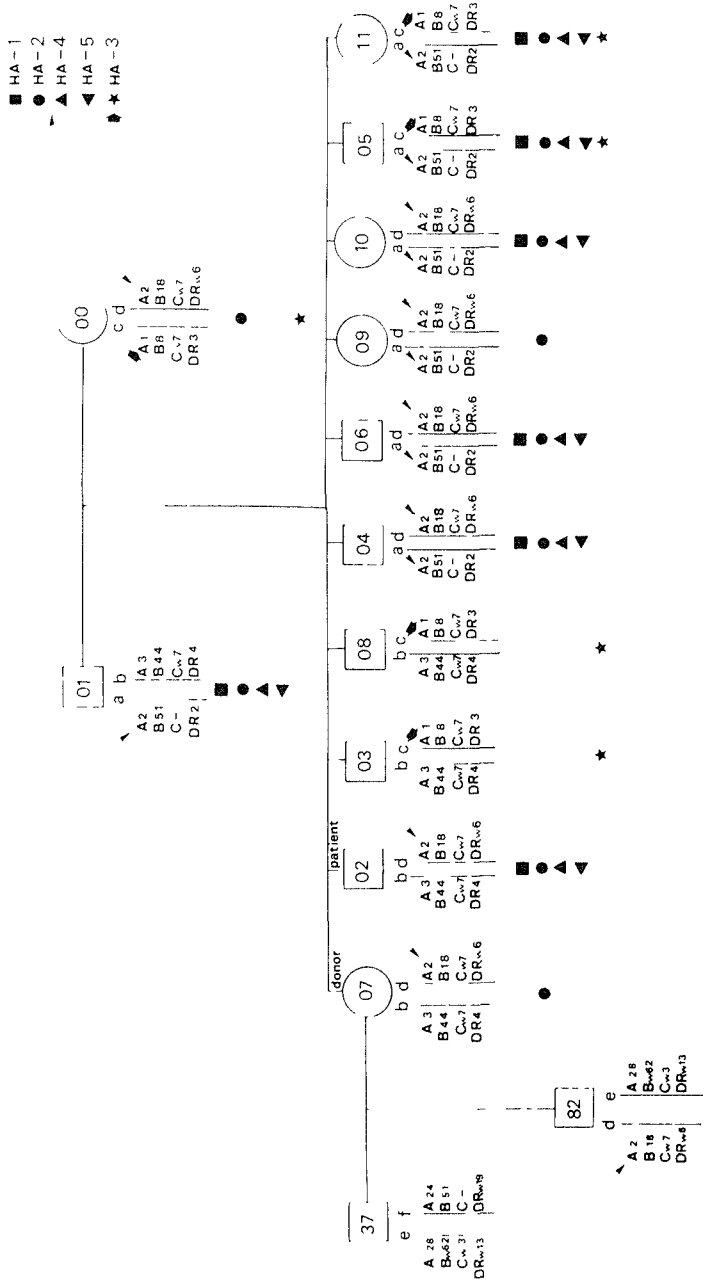
Figure 3-4. Pedigree of family members of patient HA-4. ➤ indicates the presence of the HLA-A2 antigen; ▲ indicates the presence of the HLA-A1 antigen. The presence of the HA antigens is indicated with their specific symbols as specified in the figure.

with the latter notion, i e the HA-1 specificity is present in the HA-4 effector cell bulk population

Evaluating the analyses in families, in the population, and the cold target inhibition studies carried out so far, the propositions for the HA antigenic system are as follows the HA 1 antigenic specificity is included in the HA-2 'specific' CTLs but is different from the HA-2 antigenic determinant and, both are different from HA-4 and HA-5, the latter two are most probably alike A pronouncement upon the possible location of the genes coding for the HA-1,2,4 and 5 products is, at this point of investigation, not justified Current examination of a large number of families will hopefully enable us to answer the question of whether or not they are linked to HLA

Results in Relation to the Clinical Situation

Based on the observation that host-directed CTLs specific for minor H antigens can be generated posttransplantation of bone marrow in patients suffering from GvHD, a retrospective analysis has been performed to study the relationship between HA incompatibilities and the occurrence of GvHD in a series ($n = 87$) of HLA identical donor/recipient combinations The five well defined minor H antigen specific CTLs (HA 1 to HA 5) were used as cellular typing reagents Table 3-5 demonstrates that incompatibilities for HA-1,2,4 and/or 5 between bone marrow donor and recipient occurred preferentially in the group of patients suffering from GvHD In some ($n = 4$) HLA A2 positive pairs, HA-1,2,4 or 5 was absent in the recipient, but present in the donor These 'reverse' incompatibilities can play a role in GvHD, and will always influence possible graft rejection

Concentrating on the HLA-A1 restricted minor H antigen HA-3, our studies to date do not imply a correlation of the HA-3 antigen with the occurrence of GvHD Despite the fact that the HA-3 specific CTLs were originally generated in a patient suffering from acute GvHD, the retrospective typing analysis in HLA A1 positive pairs (Table 3 5) demonstrated HA-3 incompatibility between donor and recipient in patients without any clinical signs of GvHD after transplant ($n = 3$) Moreover, the three patients suffering from acute and/or chronic GvHD which typed nonidentical

Table 3-5

Cellular Typing for Human Minor H Antigens with MHC Restricted Minor H Antigen Specific CTLs (a retrospective study)

Donor/Recipient Pairs	HLA A2 Positive Pairs Typing for HA 1,2,4 and 5		HLA A1 Positive Pairs Typing for HA 3	
	Identical	Non identical	Identical	Non identical
Recipients without GvHD	14	2(2)	7	3
Recipients with acute GvHD	20	8(2)	8	1(1)
Recipients with chronic GvHD	10	6	6	2(2)

The numbers in parentheses indicate the number of bone marrow donor/recipient pairs in which the HA incompatibility was present on the donor cells By subtracting these numbers, the minor H antigen incompatibilities HA 1 2,4 and 5 and GvHD are significantly associated (p 0 042 if calculated as groups without, acute and chronic separately)
Donor/recipient pairs used for generating the HA 1 to HA 5 specific CTLs are not included

with their donor for HA 3, were mismatched in the 'reverse' direction (i e HA 3 present in the donor, but absent in the recipient)

The number of bone marrow donor/recipient pairs investigated to date exceeds the number presented earlier [53] It must be remarked that this analysis showed a significant association between the HA 1,2,4 and 5 incompatibilities and the occurrence of GvHD Now that we have investigated almost twice the number of donor/recipient pairs, the latter correlation hardly reaches a significant level (Table 3 5, $p = 0\ 04$) One of the possible explanations for these observations is that in at least four of the ten HA 1,2,4 and five identical donor/recipient combinations in the group of patients suffering from chronic GvHD, we generated anti host CTL populations which could recognize minor H antigens other than HA 1,2,4 or 5 Moreover, assuming that HA 4 is indeed identical to HA 5 and that HA 3 incompatibility does not play a role in the development of GvHD, then we must realize that we used only three minor H antigen typing reagents which obviously is not sufficient for a large number of donor/recipient combinations

The dichotomy among the minor H antigens regarding their postulated different roles in clinical bone marrow transplantation may possibly be explained by their differential tissue distribution To date, experiments carried out at the HPC level (see previous discussion on the expression of the H Y antigen on HPC) support the notion of differential expression of HA 3 versus the HA 1,2,4 and 5 antigenic determinants (Voogt et al , ms in preparation) These studies may bring us closer to understanding the different impacts of the HA antigens on the development of GvHD and host versus graft disease (HvGD)

Perspectives

At present our studies are extended to a posttransplant follow up time of 2 years As mentioned earlier, the cytotoxic and proliferative variation of activity and the phenotypic markers of the anti host T cell lines of a series of patients are presently under study (van Els et al , manuscript in preparation) Hopefully, these studies will provide us with information about the average number and the individual immunogenicity of the minor H antigens, so that the most immunodominant HAs can be mapped according to their impact in GvHD or rejection

The HA specific CTL populations and CTL clones can be expanded on a large scale, frozen and, when preferred, used directly after thawing as typing reagents providing in principle, the clinician with results within 4 hours Prospective donor typing for the most common and 'strongest' minor H antigens may be helpful in avoiding at least one of the obstacles to successful bone marrow transplantation This might be especially true in such cases as when patients lack an HLA identical sibling, where more than one potential unrelated donor is available In this connection, it would be helpful if collaborative studies could be undertaken aimed at exchanging and comparing information and material between interested parties

Recognition of human minor H antigens has been reported by several investigators Elkins et al [54] and Zier et al [55] described the recognition of a human minor alloantigen by lymphocytes derived from a multitransfused aplastic anaemia patient They showed that the lymphocytes from this 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, and in families, indicated that the class I molecule B7 was involved in the recognition of the minor H antigen Tekolf and Shaw[56] 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 Also, after irreversible rejection of an HLA identical sibling kidney allograft, CTLs directed against minor H antigens could be demonstrated *in vitro*[57] An elegant study was performed by Sondel et al [58] who attempted to generate effector cells specific for leukaemic blasts Despite the fact that indeed the effector cells recognized the leukaemic target cells the non leukaemic lymphocytes from both parents of the patient were also lysed, suggesting the recognition of a minor H antigen by these CTLs

However, a different approach for generating anti minor H antigen specific responses has been forwarded by Tekolf and Shaw[59] Their data indicated that minor H antigen specific CTLs can be generated *in vitro*, without prior *in vivo* priming, by using a limiting dilution system through which suppressor cells are diluted out of the culture system Finally, Irle et al [60] described anti minor H antigen responses posttransplantation of bone marrow, in essence this was an experimental set up comparable to ours The posttransplant CTL clones were directed against a minor H antigen whose recognition was HLA B7 restricted It is of note that HLA B7, along with HLA A2, appears to function well as a restricting antigen for minor H antigens This can be concluded from these studies and also from the preference of the H Y antigen for HLA A2 and B7 as noted earlier Moreover, among our new series of patients who are presently under investigation, HLA B7 in addition to A2 turned out to be their favourite restricting molecules

The future use of more unrelated marrow donors makes it obligatory to know how to distinguish between pernicious and harmless minor H and major H antigens With regard to the former, one could image that for example by mismatching for HLA A2 and selecting the serologically cross reactive HLA A28 antigen, no generation of HLA A2 restricted anti minor H antigen responses would be induced Moreover, with regard to the major histocompatibility antigens, identification of the functionally important molecules could lead practically to more potential donors and would hopefully diminish the chance of graft failure or GvHD Disparities in the class II region[61-63] might be of particular interest because of their role in activation of the immune response although at least in a related situation, there is no consensus on the correlation between DP incompatibility and the occurrence of GvHD [64]

FUTURE INVESTIGATIONS AND THEORETICAL CONSIDERATIONS

Which Cells Cause GvHD to Minor H Antigens?

In the mouse, a variety of studies have been carried out to explore the identity and function of cells responsible for GvH reaction After the initiating experiments of Boak and Wilson[65] who showed that allogeneic lymphoid cell populations devoid of donor T cells do not induce GvH disease, and those of Korngold and Sprent[70]

who showed that by removing mature T cells from the marrow, lethal GvHD across minor H barriers could be prevented, the question of which donor T cells populations are involved in the induction of GvHD was largely surveyed A preliminary report on the characterization of the cells involved in H-2 restricted GvH reactions showed that both the Lyt 1^+2 and Lyt 1 2^+ populations were involved [66] Recently, more precise information became available showing that Lyt 2^+ T cells as well as L3T4$^+$ cells cause GvHD to minor H antigen differences [67] Moreover, the T cell subsets initiating the GvHD may differ for each strain combination, a mixture of the Lyt 2^+ and L3T4$^+$ T cell subsets, however, results in a severe form of GvHD [68] The complexity of the immunological reactions initiating GvHD due to minor H antigen differences becomes even greater when we have to take into account Parkman's findings that possibly different cellular mechanisms act in acute versus chronic GvHD [69] Parkman characterized, by photypic and functional analysis at the clonal level, the cells involved in acute GvHD and found that they were different from the clones established during the chronic phase of the disease [69]

In man, much less is known about the characteristics of the cells responsible for initiating GvHD across non-MHC barriers During the last few years, several investigators aimed at unmasking at least some of the minor H antigen specific cells playing a functional role in the course of the events after transplantation of bone marrow Irle and colleagues[70] studied the change, during *in vitro* culturing, of specific cytolytic activity patterns in a series of minor H antigen specific clones obtained from a marrow transplant patient after *in vivo* priming Tilkin et al [71] described a proliferative as well as cytotoxic mainly CD4 positive T cell line, isolated after transplantation of bone marrow, recognizing a minor H antigen in the context of self MHC class II The peculiarity of the latter T cell line, which was of donor origin, is that it showed autoreactivity and consequently might be of importance in the reconstitution of the marrow and recovery of the immune system after transplantation

As mentioned in earlier sections, we recently gathered information upon the composition and the function of the minor H antigen specific effector cells at different times after transplantation of bone marrow

Expression of Minor H Antigens in the Skin

One of the affected organs during GvHD after bone marrow grafting is the skin The assumption that donor T cells are responsible for the epidermal lesions is generally accepted in mouse and man In the mouse, Piguet et al [72] investigated the epidermal lesions of GvHD elicited by minor loci, whole MHC differences or a MHC class I or II difference alone They showed that donor T lymphocytes L3T4$^+$ Ly2 as well as L3T4 Ly 2$^+$ can elicit the epidermal lesions in the various donor/recipient combinations Similarly, epidermal damage resulting from a marrow exchange over a minor H antigen difference alone was reported earlier [73 74] These experimental animal data lead to the search for the target structure(s) causing the damage of the epidermis The antigens functioning as sites of attack could be for instance tissue restricted histocompatibility antigens such as the epidermal alloantigen Epa 1 [75] In subsequent studies, Steinmuller et al demonstrated that by immune lymphocyte

transfer tests i e injection of anti Epa 1 CTL clones, GvH lesions developed in H 2k, Epa 1 positive hosts [76]

In man, Guyotat et al [77] carried out histological and immunopathological studies on serial skin biopsies from patients after bone marrow transplantation Dermal and epidermal infiltration by CD8 + cells correlated with the severity of GvHD The primary sites of attack in early GvH in the skin are the rete ridge keratinocytes [78] The antigenic target structures involved could be tissue specific antigens (expressed on epithelial but not on lymphoid cells) like in the studies of Tsoi et al [79] On the other hand, minor H antigen specific proliferative responses were also described Reinsmoen et al [80] took skin biopsies at the site of the GvHD lesions, cultured them and observed secondary proliferative responses in the presence of the patient's pretransplant cells With regard to our own studies, we are presently exploring the expression of the minor H antigens HA 1 to HA 5 on keratinocytes with the HA specific cytotoxic typing reagents Two most elegant approaches for detecting minor H antigen differences *prior* to transplant were reported by Vogelsang et al [81] and Bagot et al [82] using a skin explant model and a mixed epidermal cell lymphocyte reaction respectively Both test systems appeared to have predictive value, which obviously is very useful for donor selection The minor H antigens inducing the activities in the latter systems remained, to my knowledge, unidentified

The Desired Side-effect of GvHD

We posed ourselves the question whether the posttransplant anti host CTL activity that runs through this chapter like a continuous thread might also be of advantage to the bone marrow transplant recipients? Do the patients benefit from anti host CTL activity? The hypothesis that posttransplantation of bone marrow anti host CTL activity may have a beneficial effect is based on the assumption of the postulated anti leukaemic potential as a 'desired' side effect of the post bone marrow transplant complication GvH [83] [85] The current thoughts about GvL following bone marrow transplantation has been recently summarized by Butturini et al [86]

In the third part of Fig 3-1 showing donor T cells responsible for GvL, only preliminary results from our laboratory are available The information from the initial experiments can be summarized as follows first, some HA specific CTL seemed to react with leukaemic cells, second, the HA antigen expression, as tested by quantitative cold target inhibition experiments, can vary which might be depending on the differentiation state of the leukaemic cells As mentioned earlier the host directed T cell populations generated in patients suffering from acute GvHD (work in progress) and in patients without any clinical signs of GvHD are of course of prime importance for the determination of their role in the GvL reaction Regarding the few *in vitro* immune studies in man to date on the mechanisms preventing leukaemia relapse after bone marrow transplantation, one detailed clinical and laboratory study by Sondell et al deserves special notice [87] These authors described the recurrence of the disease despite the engraftment of donor marrow and the (secondary) capability of the donor cells to destroy the leukaemic cells Hopefully, such studies will facilitate the search for the exact balance between GvH and GvL, yielding a higher efficacy for clinical bone marrow transplantation

What are Minor H Antigens?

The answer to this question can be either very short or extremely long, becau
nobody knows exactly A variety of proposals about the nature of the minor H antige
suggest that they may be quite different from one another and quite different fro
the functions of the antigens of the MHC complex,[88] they are probably membrar
bound and present on several tissues, it is unlikely that they are primarily concerne
with transplantation *per se*, they may be a diverse group of molecules participatir
in various cellular housekeeping functions and their antigenicity may come vei
incidentally, perhaps as a result of their expression in the plasma membrane,[89] o
minors are frankly mysterious entities, they fail to induce an antibody response, the
are naturally processed fragments of polymorphic nucleoproteins that associate wit
MHC products [90] None of these utterances can yet be proved false As little as w
know now from the studies on human minor H antigens, it is clear from our dat
that even the minor H antigens which we detected by CTLs can be quite differer
from one another This is supported by the observation that the HA 3 antigen
determinant most probably differs from HA 1,2,4 and 5 in its role in the developmer
of GvHD and by its expression on HPC In general, no antibody responses again
minor H antigens are observed except for H Y [91] The absence of these antibodic
falls in the same category as the 'MHC restricted plus X' antibodies Although th
number of antigen specific MHC restricted antibodies reported does not reac
overwhelming levels, the independent observations recently summarized,[?] strongl
support the notion that they exist One of the previous proposals on minor H antigei
furnishes food for reflection 'such mysterious entities' could well be processe
antigenic peptides from viruses In the framework of the multifactorial aetiology o
GvHD,[93] it is of interest to examine our HA specific cellular reagents for possibl
reactivity against virus infected target cells Finally, findings in the mouse by Colomb
et al [94] add an extra dimension to the creative thoughts on the nature of minor F
antigens These authors reported on the expression of new non H 2 histocompatibilit
antigens (as defined by skin graft rejection and by CTL s) through germ line insertio
of a gene from retroviral origin

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