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Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy

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Summary. Bone marrow transplantation (BMT) is the present treatment for hematological malignancies Two major drawbacks of allogeneic BMT are graft-versus-host disease (GVHD) and leukemia relapse The use of HLA-matched siblings as marrow donors results in the best transplant outcome Nonetheless, the results of clinical BMT reveal that the selection of MHC-identical donors' bone marrow (BM) is no guarantee for avoiding GVHD or ensuring disease-free survival even when donor and recipient are closely related It is believed that non-MHC-encoded so-called minor histocompatibility antigens (mHag) are involved in both graft-versus-host and graft-versus-leukemia activities The recent new insights into the chemical nature of mHag not only reveal their physiological function but, more importantly, provide insights into their role in BMT Together with the information on the human mHag genetics and tissue distribution gathered in the past, we may now apply this knowledge to the benefit of human BMT Directly relevant is the utility of mHag molecular typing for diagnostics in BM donor selection Most promising is the use of mHag-specific cytotoxic T cells for adoptive immunotherapy of leukemia

Introduction

Minor histocompatibility antigens (mHag) are products of genetic loci responsible for graft rejection As the MHC encoded major H systems, the minor H antigens have important biological functions besides their role in organ and bone marrow transplantation (BMT) Their latter characteristic, however, was first disclosed Both types of transplantation antigens were described by Snell (1) and dissevered from one another on the basis of their respective power in murine tumor graft rejection models (2) 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 showed distinguishable patterns in eliciting allogeneic reaction, skin grafting over a multiple minor H barrier demonstrated a graft rejection time comparable to those that differed only at H-2 (2–4)

In human transplantation, donors and recipients are routinely screened for identification of the major H system, therefore, graft-versus-host disease (GVHD) and rejection may be

Table 1. MHC class I restriction of H-Y-specific cytotoxic and proliferative T-cell responses

UPN[a]	PBLs derived[b]	CD8 CTL	CD4 CTL & Th
1	Post BMT	HLA A2 H Y	HLA A2 H Y
			HLA B60 H Y
2	After multiple transfusions	HLA A2 H Y	
3	After multiple transfusions	HLA A2 H Y	
		HLA B7 H Y	
4	After multiple transfusions	HLA A1 H Y	
5	Post renal transplant	HLA B7 H Y	

[a]unique patient number all patients are female

[b]peripheral blood lymphocytes

caused by the disparity of the products of the minor H systems, i e histocompatibility antigens other than those coded for by the MHC

The description by Zinkernagel & Doherty (5) of the classical immunological phenomenon of the MHC-restricted recognition of viral antigens by T cells appeared also to apply to the recognition of non-MHC alloantigens In the seventies, murine (6, 7) and human (8, 9) mHag were defined *in vitro* by MHC-restricted T cells

In man, mHag studies have predominantly been performed in the HLA-identical BMT setting The efforts of several investigators have led to the identification of a relatively small number of mHag Both cytotoxic T cells (CTLs) and T-helper (Th) cells recognizing mHag in a classical MHC-restricted fashion were described MHC molecules serve as templates (10) for peptides derived from intracellularly processed proteins (11, 12) This knowledge was essential for the prediction that mHag are naturally processed fragments of intracellular proteins that associate with MHC molecules (13, 14) Indeed, this supposition was recently verified both for murine (15) and human mHag (16)

This review summarizes our current knowledge of the impact of mHag on the outcome of BMT and discusses the putative clinical applicabilities now that biochemical identification of mHag is possible

The male-specific mHag H-Y – cellular recognition

The involvement of H-Y (at that time called Y-factor) in homograft rejection had been postulated by Eichwald & Silmser (17) in 1955 The term H-Y antigen was introduced by Billingham & Silvers (18) 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 She received, after anti-thymocyte globulin (ATG) pretreatment, a bone marrow (BM) graft, donated by her HLA-genotypically identical male sibling *In vitro* analysis of the post-transplant peripheral blood lymphocytes (PBLs) 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 (8, 9) Whether the H-Y-specific CTLs 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 anemia, had been sensitized to the H-Y antigen prior to transplantation through multiple blood transfusions and pregnancies Interestingly, the anti-H-Y response in the latter patient appeared broader than the HLA-A2-restricted CD8 CTL clones We isolated two CD4 cytotoxic and proliferative H-Y-specific clones one restricted via HLA-A2 and the other one recognized an H-Y T-cell epitope in association with HLA-B60 (*Table 1*) (19)

Although in our first case we could not formally prove that the H-Y-specific CTLs actually mediated the rejection of the male BM allograft, some years ago we were confronted with a case with a fatal outcome in which anti-H-Y CTLs were most probably mainly responsible for BM graft failure It concerned a multi-transfused female patient suffering from myelodysplasia after treatment for Hodgkin's disease *In vitro* analysis prior to BMT demonstrated the presence of HLA-A1-restricted anti-H-Y CTLs (*Table 1* UPN 4) Since the father appeared to be the only HLA-compatible related donor, he was the obvious choice (despite the presence of the patient's pretransplant anti-H-Y CTLs) Notwithstanding intensive pretransplant immunosuppressive treatment, there was no recovery of the BM hematopoietic function (20) In view of the latter case, expression of mHag on hematopoietic stem cells (HPCs) might be relevant in presensitized patients receiving an mHag-positive T-cell-depleted marrow graft For that purpose, the expression of the male-specific antigen H-Y was studied for its expression on HPCs It became clear that, indeed, H-Y is expressed on CFU-GEMM, CFU-GM and BFU-E (21) The assumption that H-Y sensitization can readily occur following blood transfusion and organ transplantation is based on our subsequent observations As shown in *Table 1*, PBLs derived from three 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 CTL activity In one patient (*Table 1* UPN 5), the H-Y-specific HLA-B7-restricted cytotoxicity was detected shortly after a kidney donated by an HLA-identical male sibling acutely rejected (unpublished observation) In circumstances similar to ours, other investigators have also

Table 2. **Identification of human mHag**

Restriction molecule	mHag	peptide (amino acids)	Chromosomal location	origin funct on
HLA B7	H Y	SPSVDKARAEL (11AA)	Y	SMCY presently unknown
HLA A2 1	H Y	FIDSYICQV (9 AA)	Y	SMCY
HLA A2 1	HA 2	YIGEVLVSV (9 AA)	?	non filamentous class I myosin[a] involved in cell locomotion and organelle transport

[a]postulated origin based on homology of 7 out of 9 AA

described the presence of HLA-restricted H-Y-directed cytotoxicity (22–24)

To elaborate on the function of the antigen-presenting molecule as well as on the antigen recognized, *in vitro* studies were carried out with HLA-A2 "variant" molecules and abnormal chromosomal sex patterns, respectively The analysis of the epitopes on the HLA-A2 molecule required for cellular recognition of the H-Y antigen led to the observations that alloimmune HLA-A2-specific CTLs (25, 26) as well as HLA-A2-restricted H-Y-specific CTLs (27) can distinguish between different HLA-A2 molecules Combined investigations (resulting from a collaborative effort) of the HLA-A2-subtype molecules at the functional level demonstrated that amino acid changes at position 43 and in the residues 145-157 (i e cellularly defined subtypes HLA-A2 2 and HLA-A2 3) lead to the loss of epitope(s) necessary for associative recognition of the H-Y antigen by HLA-A2-restricted CTLs (27, 28) Interestingly, a single amino acid change from phenylalanine to tyrosine at position 9 in the heavy chain of the HLA-A2 molecule (i e cellularly defined subtype HLA-A2 4) did not affect the recognition of H-Y by HLA-A2-restricted CTLs (27) These analyses, carried out well before the crystal structure of HLA-A2 became available, led us to postulate crucial MHC/peptide-binding sites as well as to distinguish harmful from irrelevant amino acid changes in the HLA-A2 molecule The identification of the HLA-A2-binding H-Y peptide (see below) together with the availability of the HI A-A2 crystal structure ensure that the postulated MHC/peptide-binding sites can now be verified

The function and the chromosomal location of the histocompatibility antigen H-Y were also sought We studied lymphocytes from individuals with a discrepancy between the karyotype and phenotypic sex Besides a clear positive reaction with the cells of an XY female, the H-Y-specific CTLs showed no reactivity when analyzed against XX males (29) 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 (30), thereby separating the H-Y gene from the testis-determining factor (TDF) locus In additional studies, it could be shown that a loss of spermatogenesis did not correlate with absence of the mHag H-Y CTL recognition, thereby separating the azoospermia factor (AZF) locus from the locus coding for the mHag H-Y (31) Extensive deletion-mapping studies using specific DNA markers revealed that the H-Y antigen, as determined by our HLA restricted H Y-specific CTL clones, maps to a portion of deletion interval 6 on the long arm of the human Y chromosome (32, 33)

The male-specific mHag H-Y – biochemical identification

Being among the H-Y "searchers" since 1976, we were challenged to identify the human mHag H-Y The mHag-specific T-cell clones have been used for the biochemical identification of the H-Y peptides The biochemical isolation procedure, i e affinity chromatography combined with microcapillary reversed-phase high-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (34), was successfully used for the identification of the mHag peptides The H-Y antigen presented by the HLA-B7 molecule was the first one described (35) (*Table 2*) The HLA-B7-restricted H-Y T-cell epitope was identified as an 11-residue peptide derived from the human homologue of the selected mouse cDNA on the Y (Smcy) gene (see below) encoded on the Y chromosome (35)

The genetic mapping of the mouse Y chromosome has suggested between two and five distinct loci encoding H-Y antigens (36) However, a murine H-Y epitope restricted by H-2K^k has also been shown to be derived from the murine Smcy protein (37) The demonstration that two H-Y epitopes from either mouse or human are derived from the same protein makes SMCY the prime target in searching for other H-Y epitopes Therefore, we set out to identify the H-Y T-cell epitope presented by the HLA-A2 molecule Indeed, the H-Y peptide recognized by our HLA-A2-restricted T-cell clones also originates from the SMCY protein (*Table 2*) (38) Two HLA-A2-restricted H-Y-specific T-cell clones were used in this study

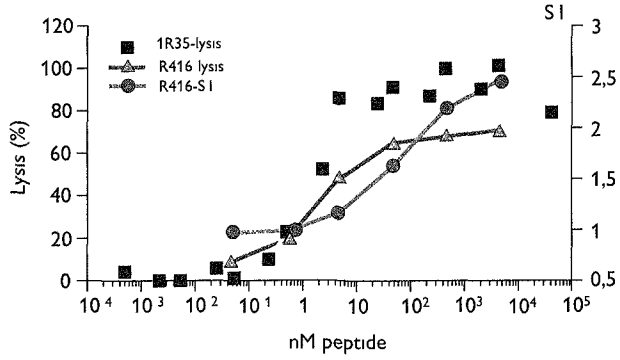

Fig. 1. Cytotoxic and proliferative HLA-A2 H-Y peptide-specific responses. For both responses, 10,000 responder cells and 50,000 female stimulator/target cells pulsed with various amounts of H Y peptide are used Effector/target ratios are 1 1 1 and 1 8 1 for IR35 and R416, respectively
S I Stimulation Index (measurement of proliferation)

(*Table* 1) a CD8 CTL clone (designated as IR35) and a CD4 cytotoxic and proliferative T-cell clone (designated as R416) (19), IR35 and R416 were derived from the same individual (*Table* 1 UPN1) Both clones recognize the 9-residue peptide FIDSYICQV (*Fig* 1) with significant cytolytic and proliferative responses Interestingly, post-translation modification of this H-Y epitope significantly altered the recognition, especially of the CD4 H-Y T-cell clone The latter clone clearly preferred the cysteinylated form of the H-Y peptide, whereas the CD8 H-Y T-cell clone recognized both peptide forms equally well (38)

The importance of the SMCY protein as a major source of H-Y determinants was recently further underlined Preliminary results from a collaborative study (Roosnek et al manuscript in preparation) showed HLA-A2-restricted H-Y reactivity against one dominant H-Y epitope 15 male-specific CTL clones isolated from 3 individuals recognized the same HPLC-purified peptide fraction The latter clones all reacted with the FIDSYICQV synthetic peptide, earlier identified as the HLA-A2-restricted H-Y T-cell epitopes derived from SMCY (*Table* 2) (38) Simmons et al (39) observed that HLA-B27-presented peptides that are produced and recognized in B27-transgenic rats are not encoded by Smcy, even though the gene seems to be necessary for their generation Hence, there is evidence for a trans-mediated effect of Smcy in giving rise to these peptides Interestingly, additional murine studies demonstrated that H-Y peptides could be products of genes (other than Smcy) on the Y chromosome (40)

The Smcy was earlier reported by Agulnik et al as a new mouse Y chromosome gene having its human homologue SMCY mapping to the same Yq deletion interval as the mHag H-Y-controlling locus (41) The latter authors demonstrated the evolutionary conservation of the Smcy gene by the isolation of Smcy homologous from human and horse genomic fragments (41) In view of the latter notion, we investigated whether the mHag are evolutionarily conserved between human and non-human primates Indeed, human HA-1, HA-2 and H-Y peptides can be recognized on the cell surface of non-human primate cells transfected with human class I genes by our human HA-1-, HA-2- and H-Y-specific class I-restricted CTL clones Furthermore, the mH peptides could be eluted from HLA-A2 1 molecules expressed on the transfected non-human primate cells This implies that the human mH peptides have been conserved for at least 35 million years (42) Indeed, concurrent with our latter study, Kent-First et al (43) demonstrated the expression of the SMCY gene in early primate development Moreover, the SMCY gene was shown to be widely expressed (41, 43) This is in concordance with our previous mHag H-Y tissue distribution studies, wherein we demonstrated ubiquitous expression of the human mHag H-Y (44) The precise function of the SMCY gene is still not known It is expressed very early in embryogenesis (41) SMCY is homologous to SMCX (located on the X chromosome) at the amino acid level at 84 4% (43) SMCY (and SMCX) proteins share significant sequence homology to retinoblastoma-binding protein suggesting that the SMCY gene may code for a transcription factor (43)

***Table* 3. No influence of an H-Y mismatch on GVHD.** Results of H-Y typing according to the GVHD status in HLA-A1 and HLA-A2 donor/recipient pairs

Donor/recipient pairs	HLA A1		HLA A2	
	GVHD		GVHD	
	yes	no	yes	no
male/male	5	9	16	11
female/female	9	7	17	12
male/female	8	1	18	12
female/male	6	5	18	10
not tested	0	0	2	1
total pairs	28	22	71	46

The male-specific mHag H-Y – clinical relevance

It has been suggested that GVHD is more frequent in male recipients of marrow from female donors (45) This effect was seen primarily with female donors who had been pregnant or had received a transfusion (45) Indeed, the above-described H-Y responses (*Table* 1) were preceded by thorough *in vivo* sensitization events However, a mismatch for H-Y between donor

Table 4. **Characteristics of HLA class I-restricted mHag**

	H Y	HA 1	HA 2	HA 3	HA 4	HA 5	References
Restriction molecules	A1 A2						
	B7 B60	A2	A2	A1	A2	A2	9 19 20 85 87
Mendelian segregat on		yes	yes	yes	yes	yes	86
Phenotype frequency %	50	69	95	88	16	7	85
Tissue distr bution	broad[a]	limited[b]	limited	broad	broad	limited	44
TCR usage	variable	skewed	variable	not tested	not tested	not tested	88

[a]expression on hematopo etic and non hematopoietic cell lineages

[b]expression on hematopo etic cell lineages

and recipient, with the H-Y present in the male recipient and not in the female donor, did not lead to an increase in GVHD in our recent study (46) *Table* 3 summarizes the results of typing for H-Y according to the GVHD status in 50 HLA-A1- and 117 HLA-A2-matched donor-recipient pairs Neither in the HLA-A1 nor in the HLA-A2 pairs is there a significantly increased frequency of GVHD in the sex mismatch (i e female donor/male recipient) combination

The absence of an H-Y effect was observed earlier by Ramsay et al (47) Also, in zero-mismatched living donor renal transplants, no H-Y effect could be demonstrated (48) Immunodominance amongst the mHag as well as absence of synergistic effects between CTLs and Th cells in mounting an efficient mHag immune response (as discussed below) may explain these apparently controversial reports

The non-Y-linked mHag – mHag-specific T-cell subsets and GVHD

Besides the Y-linked mHag, one can assume that, as in the mouse, the human genome has an abundance of mH loci encoding proteins that generate mH peptides that are either processed via the MHC class I pathway or presented in the context of MHC class II Both mHag-specific class I-restricted CTLs and class II-restricted Th cells are probably mediating GVHD in HLA-matched BM transplants In the mouse, a variety of studies has been carried out to explore the identity and function of cells responsible for GVH reactions After the initial experiments of Boak & Wilson (49), who showed that allogeneic lymphoid cell populations devoid of donor T cells do not induce GVHD, and those of Korngold & Sprent (50), 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-cell populations are involved in the induction of GVHD was largely surveyed in the murine model The T-cell subsets initiating GVHD can differ for each strain combination (51, 52) It has also been reported that the T cells involved in acute GVHD were found to be different from the clones established during the chronic phase of the disease (53) Although CD8 T cells are often reported to be involved in murine GVHD models (50, 54), in some strain combinations CD4 T cells can also mediate GVHD (55, 56) Both T-cell subsets have the potential to cause GVHD (57, 58) In man, the presence of a reduced number of CD4 cells in the donor marrow inoculum appeared to be compatible with slow but sustained engraftment and a low incidence of serious acute GVHD (59) CD8 T-cell depletion in HLA-identical sibling transplants reduces the incidence of GVHD (60, 61) On the other hand, in *vitro* observed mHag-specific CTL responses did not necessarily correlate with the development of human GVHD either on the bulk or on the CTL precursor frequency level (62, 63) The same phenomenon was previously noticed in a murine GVHD model (64) and confirmed on the CTL precursor level as well (65)

Several reports have demonstated the presence of anti-host mHag-specific CTLs in patients suffering from GVHD after HLA-genotypically identical BMT (62, 66–72) Also, class II-restricted anti-host CTLs with a CD4 phenotype were observed in a patient suffering from severe GVHD after allogeneic BMT (24) In addition to CTLs, in *vitro* studies reporting on host directed Th cells have been described in patients having GVHD (67, 73–75) Van Els et al (76) reported on the long-term kinetics of Th cells in response to host mHag in 16 patients and demonstrated that significant Th-cell activity in *vitro* correlates with clinical acute GVHD These anti-host Th cells carry the CD4 phenotype and recognize mHag in the context of HLA-DR and -DP (77) Post-transplant host-directed Th-cell responses measured at the Th-cell precursor level correlate with GVHD (78) Prior to HLA-identical BMT, putative mHag-specific Th-cell precursor frequencies can be measured (79, 80) In addition to anti-host-reactive CD4 T cells, IL-2-secreting CD8 T cells are also detected prior to HLA-identical sibling BMT (81)

Table 5. Characteristics of human mHag

	References
• MHC restricted recognition by T cells	
presentation via various class I	85 90–96
and class II molecules	24 74 77 97–99
• Variable phenotype frequencies	85 90–99
• Mendelian segregation	86 90 92–98
• Tissue distribution	
limited and ubiquitous	44 71 96 100

The non-Y-linked mHag – cellular recognition

Our first non-Y-linked mHag cellularly identified on the clonal level originated from a male acute myelogenous leukemia (AML) patient transplanted with BM from an HLA-identical female sibling donor His clinical recovery, however, was complicated by severe acute and chronic GVHD The initial experiment demonstrated that the post-transplant lymphocytes had strong cytotoxic activity against the patient's own pretransplant lymphocytes but not against the lymphocytes of his HLA-identical donor (66) This observation in itself supported 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 post-transplant CTL activities, it became apparent that the antigen (which we designated mHag HA-1) was not only present on the patient's own pretransplant cells, but could also be detected on lymphocytes from 2 out of 3 haplo-identical siblings, as well as on the lymphocytes of the parents and on the lymphocytes from a large number of unrelated healthy individuals The antigen HA-1 could be recognized by the patient's post-transplant CTLs only if one of the patient's HLA class I antigens was present on the target cells (82) Consequently, HA-1 is recognized in an MHC-restricted fashion, an event comparable to the recognition of H-Y With respect to our earlier studies on the impact of sex mismatch in BMT, the in vitro observed CTL response in this female/male donor-recipient combination appeared not to be directed against H-Y

Next, we aimed at both confirmation and extension of the latter results regarding the possible impact of polymorphic genetic systems other than HLA on the development of GVHD in man For this purpose, we investigated post-transplant lymphocytes from a series (N=34) of recipients of HLA-identical BM grafts for the presence of anti-host CTL activity Post-transplant lymphocytes from 21 out of 25 patients suffering from GVHD demonstrated CTL activity which was directed against the patient's own pretransplant lymphocytes (83) Host-directed CTLs could be demonstrated in 6 out of 9 patients suffering from acute GVHD grade 2 or more Furthermore, in 15 out of 16 patients with chronic GVHD, anti-host CTL activity was also observed It is worth noting that such CTLs can be derived from either male or female patients suffering from different hematologic malignancies prior to BMT Similar to the initial anti-host-specific CTLs HA-1 (as discussed above), we next endeavored to uncover the specificity of the target structures recognized by some of the anti host CTLs (*Table* 4) Five (including HA-1) out of 21 anti-host CTL populations 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 mHag-designated HA-2,-3,-4 and -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 N=100 unrelated healthy individuals

The common denominator of HA-1-, 2-,-4- and -5- specific CTLs is the preferential use of the MHC class I restriction molecule HLA-A2 (*Table* 4) Whether this reflects the relatively high phenotype frequency of HLA-A2 1 (i e 49% in the Caucasian population) or suggests that HLA-A2 1 is optimally equipped to serve as the template for peptide presentation is unclear According to the latter proposition, it is of interest to note that allelic differences exist in the interaction of MHC class I molecules with transporters associated with antigen processing (84) Among other HLA alleles, HLA-A2 shows that a high affinity for TAP is required for translocation of cytosolic peptides, such as minor H peptides (84a, 84b) In addition, however, it is possible that TAP supports correct folding and loading of a subset of MHC class I molecules (84)

Table 4 also shows the results of the phenotype frequency analyses carried out for mHag HA-1 to HA-5 These studies revealed that some mHag, i e HA-1, HA-2 and HA-3, appeared frequently (69–95%), while others, i e HA-4 and -5, occurred with lesser (7–16%) frequencies in the healthy population (85) An analysis of their genetic traits demonstrated a Mendelian mode of inheritance (*Table* 4) (86) These four antigens can each be considered as the product of a gene with one allele expressing the detected specificity, and one or more alleles not expressing it Although our family data did not provide sufficient information concerning linkage between the different mH loci themselves and HLA, all our tests were compatible with the hypothesis that these loci are independent of each other and independent of HLA (86) The CTL clones listed in *Table* 4 were also used to analyze functional expression (i e

Table 6. **Specificity analysis of mHag-specific CTL clones**

UPN[a]	sex do/rec	CTL lines	no of clones analyzed	mHag specificities
1	female/male	HA 1	7	HA 1
			9	unknown
4	female/female	HA 4	8	HA 4
			10	HA 1
			11	unknown
5	male/female	HA 5	4	HA 5
			11	HA 1
			16	unknown

[a] patients who suffered from severe GVHD

read-out is cell-mediated-lympholysis) of the mHag on various tissues and cells Differential expression was observed some, i e H-Y, HA-3 and HA-4, are ubiquitously expressed, whereas the expression of other mHag, i e HA 1 and HA 2, is limited to cells of the hematopoietic lineage only (44) The additional information on the TCR usage for recognizing the MHC/HA-1 mHag ligand (88) will be touched upon in more detail later in this paper

In circumstances similar to ours, several other investigators also described the cellular identification of more (yet a relatively small number) non-Y-linked mHag specificities (for an overview see (89)) The characteristics, as presented for H-Y and HA-1 to HA-5 (*Table 4*), are representative for other human mHag identified so far (24, 44, 71, 74, 77, 85, 86, 90–100) *Table 5* summarizes the general features presently known for human mHag a) recognition by T cells in association with various MHC class I and MHC class II molecules, b) occurrence with variable phenotype frequencies in the random (though HLA-restricted) population, c) segregation in a Mendelian fashion, and d) either limited or ubiquitous cell and tissue expression It is important to note that these conclusions are drawn from the outcome of functional *in vitro* cellular assays It is almost superfluous to state that confirmative studies on the molecular level need to be carried out

The non-Y-linked mHag – biochemical identification

Proteins of (retro) viral, foreign or self-origin located in ER, cytosol or any other organelle can give rise to peptides immunogenic to class I-restricted CTLs and can represent transplantation barriers (101–105) With respect to the non-Y linked classical mHag, the mouse maternally transmitted antigen (Mta) was the first one identified at the molecular level (106) This mitochondrial H antigen is a peptide derived from the amino terminus of the ND1 protein (15)

Four alleles have been detected at one locus, each different by a single amino acid (106) The first human non-Y-linked mHag biochemically identified was HA-2 (16) The HLA-A2-bound HA-2 peptide most probably originates from an as yet unidentified member of the non-filament-forming class I myosin family, a large family of proteins that are involved in cell locomotion and organelle transport (*Table 2*) At present, we are investigating whether, indeed, a class I myosin gene is the source of the HA-2 peptide Identification of the HA-2 gene will provide the basis for its differential expression in the population (*Table 4*) Its allelic polymorphism can be a result of presentation of homologous but non-identical peptides, a failure to present a peptide because it has lost its MHC-anchor residue or polymorphism in the class I antigen-processing system The amino acid sequence of the HA-1 mHag has just been elucidated as well (J M M Den Haan et al manuscript in preparation)

The non-Y-linked mHag – clinical relevance

The putative influence of known mHag disparities between HLA-identical BM donors and recipients on the development of GVHD has been retrospectively analyzed Elkins et al (107) analyzed 67 pairs for incompatibility for mHag W1 in relation to GVHD No influence of W1 on GVHD could be demonstrated because the number of W1 mismatches was too low (i e there was a high phenotypic frequency) The study by Behar et al (108) dealt with allelic differences between donor and recipient for the polymorphic adhesion molecule CD31 CD31 mismatches between BM donor and recipient are associated with an increased risk of severe GVHD grade 3 or 4 (P=0 004) The platelet-endothelial-cell adhesion molecule 1 (CD31) has a broad expression, and it is constitutively expressed on vascular endothelial cells, BM stem cells, platelets and leukocytes (108) Interestingly, anti-CD31 monoclonal antibodies seemed to differentially recognize the allelic forms No CD31-specific T-cell responses were reported, which separates this transplantation antigen from the classical ones described in man and rodents earlier In a subsequent study comprising a large series of BM donor/recipient pairs, the postulated correlation between CD31 matches and occurrence of severe GVHD could not be confirmed (109)

We analyzed the influence of mHag HA-1, -2, 4 and -5 mismatches between HLA-identical BM donor/recipient pairs (i e BM donor mHag-negative and BM recipient mHag-positive) on the occurrence of acute GVHD grade 2 or more The results in adult patients can be summarized as follows a mismatch for HA-1 and/or HA-2, -4, -5 was significantly associ-

Table 7.
A. HA-1 effect analyzed on GVHD

	Adults and Children GVHD		Adults GVHD	
	no	yes	no	yes
HA 1 #	2	11	0	10
HA 1 =	50	52	28	43
Odds ratio	5 4		∞	
95% confidence interval	1 0 56		1 3 ∞	
P value (2 sided)	0 05		0 02	

B. No subdominant H-Y effect on GVHD analyzed in 102 HA-1-matched patients

	GVHD	
	no	yes
H Y #	37	39
H Y =	13	13
Odds ratio	0 95	
95% confidence interval	0 35 2 55	
P value (2 sided)	1 000	

ated with GVHD (P=0 006) The main effect of the significant association with the development of GVHD appeared to be caused by an HA-1 mismatch, since a single HA-1 mismatch between donor and recipient reached a P value of 0 02 (*Table 7A*) (46) It is clear that these studies need confirmation in larger groups of patients

Immunodominance of mHag

In 1966, Graff, Hildemann & Snell, using a panel of congenic-resistant mice differing at multiple mH loci, concluded from their skin allograft studies as follows *"The strengths of the barriers imposed by the non H-2 histocompatibility loci were quite variable, the median survival times for the various loci ranging from 15 to > 300 days"* (110) Subsequent series of murine skin-grafting responses, *in vivo* priming experiments and GVHD models clearly showed that the immune responses were dominated by a small number of mHag Hereby the phenomenon of immunodominance of murine mHag was clearly established (111–116) Later, the immunodominance was also verified on the mHag peptide level Bulk CTL responses generated across multiple mH barriers appeared to be directed against only a few mH peptides (117–120) Whether or not a single mHag disparity can cause GVHD, an experimental condition which will never occur in man, is not yet clear (121,122)

The fact that a significant number of BM transplants between HLA-identical siblings (with optimal immunosuppression) do not lead to GVHD suggests a hierarchy in mHag immunogenicity (123) Two sets of our data are indicative for mHag immunodominance Firstly, CTL clones reactive to the same mHag HA-1 were obtained from peripheral blood lymphocytes of 3 individuals each transplanted across a multiple and probably distinct mH barrier (*Table 6*) (85) Secondly, in the study mentioned earlier of 148 BM HLA-identical donor/recipient pairs, investigating the influence of mHag HA-1 to HA-5 mismatching on the development of GVHD, a mismatch of only HA-1 was significantly associated with GVHD in adult patients (46)

The hierarchy in mHag immunodominance also implies the existence of subdominant mHag, as exemplified for murine mHag previously (113) We observed the absence of an H-Y mismatch effect (discussed above under the heading *The male-specific mHag H-Y – clinical relevance*) (*Table 3*) In view of the existence of subdominant mHag, we analyzed our mHag disparities and human GVHD data by omission of the dominant HA-1 antigen (*Table 7*)

No H-Y effect could be demonstrated in 102 HA-1-matched BM donor/recipient pairs (*Table 7B*) It is of interest to note that Wettstein (124) reported on the immunodominant behavior of an autosomal murine mHag H-3 over the H-Y antigen in the generation of CTLs

How to become a "wicked" minor

We now know that mHag are naturally processed proteins of peptidic nature Any protein, whether it is cellular- or membrane-associated, can give rise to mH peptides To become a "wicked" minor, a condition *sine qua non* is that the minor protein source must possess some degree of polymorphism The immunogenicity of a potentially large number of mHag is restricted by various factors Some of the possible factors underlying the mHag immunodominance, illustrated by as yet very little information on human major minors, will be discussed below

The synergistic effects of MHC class I mHag-specific CTLs and MHC class II mHag-specific Th cells promoting an effective mH immune response

In the murine model, an early report of an effective H-Y response brought about by H-Y-specific CTLs and Th cells was published by Von Boehmer & Haas (125) Genetic analysis of loci encoded with the murine H-3 and H-4 regions has revealed that the existence of separate loci encoding Th-cell and CTL mH epitopes was required to induce a CTL response *in vivo*,

Table 8. Synergistic effects of mHag-specific CTLs/Th cells on GVHD

Anti host T cell activities	GVHD status	
	no	acute > 2
CTL and Th	2	10
CTL alone	2	2
Th alone	0	1
no CTL no Th	3	0

indicating the relevance of Th-CTL cell collaboration in the anti-H3 and anti-H4 immune response (126, 127) With regard to the murine mH-H3 complex, recent genetic linkage studies demonstrated that the CTL and Th epitope are encoded by distinct genes, the H3a (encoding the CTL epitope) and the H3b (encoding the Th epitope) map approximately 12 cM apart on the mouse chromosome 2 (128) Nonetheless, CTL and Th epitopes can also be encoded by the same gene From a melanoma patient, CD4 T cells isolated from tumor-infiltrating lymphocytes recognized an immunodominant epitope coded for by a gene which also encodes class I CD8 T-cell epitopes (129) As discussed above (under the heading *mHag-specific T-cell subsets and GVHD*), it is likely that both CTL and Th-cell subsets play a role in the development of human GVHD We analyzed 20 patients to determine whether anti-host CTL and Th-cell responses occurred simultaneously at different times post-HLA-identical sibling BMT *Table* 8 shows anti-host CTL and Th-cell responses in 10 out of 13 patients with severe GVHD On the contrary, in the "no GVHD" group of patients, both CTL and Th-cell responses were detectable in only 2 out of 7 patients analyzed These preliminary results support the notion that CTL and Th mHag epitopes collaborate in the anti-host GVHD immune responses in man as well Naturally, identification of the CTL and Th mHag involved in these responses needs to be determined

T-cell repertoire dependency

Immunodominance may also depend on the available TCR repertoire A single murine class I allo peptide appeared dominant in Vβ8-positive but not in Vβ8-negative mouse strains, indicating that the dominant peptide recognition was dependent upon Vβ8-positive T cells (130) We observed by analyzing TCR usage of 12 clones derived from 3 individuals (*Table* 6 UPN 1, 4, 5) that the TcRβ chains all used the TCRβV6S9 gene segment and showed remarkable similarities within the N-D-N regions (*Table* 4) (88)

Peptide affinity

One of the mechanisms of immunodominance also resides at the level of peptide/MHC-binding properties The affinity of MHC class I-peptide binding is crucial for the outcome of an immune response, even in the situation of subdominant epitopes (131) Murine mHag T-cell responses appeared to be influenced by differential binding of the minor peptide to class I molecules (132) Using an equilibrium-binding assay to measure relative affinities, the mHag HA-2 and the H-Y peptide are classified among the highest affinity naturally processed peptides that have been identified to date The concentration of the HA-2 peptide as competitor peptide that resulted in 50% inhibition of the iodinated peptide binding (IC50) was 6 7 nM, and the IC50 value for H-Y was 16 nM (*Table* 9), the IC50 values for other published peptides vary from 11–214 nM for HLA-A2 (133, 134)

Table 9 also illustrates the half-maximal lysis values of the human mHag peptides HA-2 and H-Y The synthetic peptide concentrations required to reconstitute 50% specific CTL recognition are low compared to the values of the T-cell epitopes reported earlier (135) This reflects high affinity of the peptide for MHC or high affinity of the T-cell receptor

Production of cytokines

Antigen presentation by professional antigen-presenting cells (APCs) accounts for the primary initiation process of GVH pathogenesis Cytokines do play a significant role in both acute and chronic GVHD (see (136) for a comprehensive review) In a murine model, IL-1α has been postulated as a critical effector molecule in mHag-directed GVHD (137) Antibodies to TNFα could completely prevent lethal GVHD induced in mH-disparate mice (138) Also, the GVHD-inducing potential of some mH antigen-specific T-cell clones has been shown to correlate with the levels of TNFα clones produced in *vitro* (139) T-cell-derived lymphokines (IL-3, IL-4, and CSF) are produced *in vivo* and in *vitro* in response to mHag The properties of these produced activities are similar to those that responded to irradiated syngeneic cells, but there was a difference in the time course of the lymphokine production between GVH mHag-disparate mice and the syngeneic transplant mice (140)

In man, by means of a GVHD-predictive assay, the in *vitro* GVH reactivity to host skin tissue was found to correlate with the levels of TNFα and INFγ secreted into the supernatant of HLA-matched patient/donor mixed lymphocyte cultures (141)

Tissue distribution

Presentation of immunogenic MHC/mH peptide complexes by professional APCs is essential for induction of anti-host cellular immune responses In this regard, it is worthwhile mentioning that the human mHag HA-1, which is shown to be significantly associated with GVHD (as discussed earlier), is clearly

expressed on the APCs, i e dendritic cells (DCs) and Langerhans cells (LCs) (142) The latter BM-derived APCs are most potent in inducing alloreactive T-cell responses (143, 144) The conditioning regime prior to BMT will eliminate most of the recipient's hematopoietic cells, yet residual recipient cells including DCs can be present Host LCs can persist for a long time after BMT (145)

Human mHag new concepts for marrow transplantation and adoptive immunotherapy

The putative clinical potentiality of mHag is presently based upon *in vitro* results of functional and clinically related studies performed in the past Bearing this restricted information in mind, three areas of clinical application are worthwhile mentioning (*Table 10*)

The utility of diagnostics in BM donor selection is self-evident Several mHag are now biochemically identified We are currently identifying the mH genes which will provide us with the mHag allelic counterparts In the near future, molecular typing for mHag loci can be performed Depending on how major the immunodominant minors turn out to be in the HLA-matched unrelated donor/recipient combinations, one may consider overruling a minor-major mismatch The speculative proposal of the use of immunodominant mHag as GVHD prophylaxis is based upon putative immunomodulation of the GVHD response with mHag peptide analogues Designing mHag peptide analogues which function as MHC or T-cell receptor antagonists might interfere with the harmful anti-host mHag-directed T-cell reactivities post-HLA-identical BMT The presence of human mHag peptides in non-human primates (42) could serve as a translational model MHC peptide antagonists will compete for MHC binding Inhibition of secondary mixed lymphocyte reaction and prevention of murine GVHD across mH barriers by high-affinity class II binding peptides were recently demonstrated (146, 147) TCR peptide antagonists competing by their structural similarities for TCR engagements are probably more efficient (148) Whether or not a single TCR antagonist can cause significant inhibition of mH-directed T-cell responses is questionable A major obstacle is the involvement of the various MHC molecules together with their respective mH allopeptides, *not* taking into account the possible subdominant mHag responses popping up Nonetheless, it is worthwhile analyzing, once the major mH protein sources are available, whether at least the harmful mH anti-host responses can be eliminated Two studies reporting on adequate inhibition of a CTL and a Th-cell response against HIV and influenza hemagglutinin, respectively, with TCR antagonist peptides (149, 150) are encouraging

***Table 9.* mHag HA-2 and H-Y peptide affinities**

	IC50 (nM)	Half maximal lysis[a]
A2 HA 2	67	40 pM
A2 H Y	16	3 pM
A2 H X[b]	540	not tested
B7 H Y	34	10 pM
B7 H X	140	100 nM
Reference values	11–214 for HLA A2	10 pM to 50 nM

Reconstitution of the HA 2 and H Y epitopes with synthetic peptides indication of the synthetic peptide concentration to achieve 50% lysis with the mHag specific CTL clones

[b] A homologue of SMCY is SMCX (see under the heading *The male specific mHag H Y–biochemical identification)* The amino acid sequence of the H Y peptide of SMCY differs only at two amino acid positions from SMCX

Induction of tolerance using mHag with broad tissue distribution

Achieving tolerance prior to transplant in mHag-negative BM donors to prevent GVHD and in mHag-negative BM recipients to prevent rejection would decrease the necessity for the use of pharmacological immunosuppression

Acquired tolerance for mHag after MHC-identical BMT does occur and has been reported in mouse and man (151–154) A common denominator in two of the latter reports (one murine study (152) and one human study (154)) was the involvement of ubiquitously expressed mHag Induction of tolerance for mHag in immune mature adults prior to BMT requires comprehensive analysis A nice example of induction of transplantation tolerance for mHag was recently demonstrated by Davies et al (155) Life-long tolerance for multiple murine mHag was achieved as a result of suppression via linked recognition

Adoptive immunotherapy of leukemia

Last but not least, immunotherapy for leukemia using CTLs specific for mHag peptide for the treatment of refractory, residual or relapsed leukemia is most promising The mHag with restricted tissue distribution (e g HA-1 and HA-2) are the candidates for adoptive immunotherapy of leukemia This proposal is supported by three sets of important clinical results First, adoptive immunotherapy of buffycoat mononuclear cells and IFNα induced cytogenetic remissions in relapsed CML patients after allogeneic BMT (156–159) However, this donor

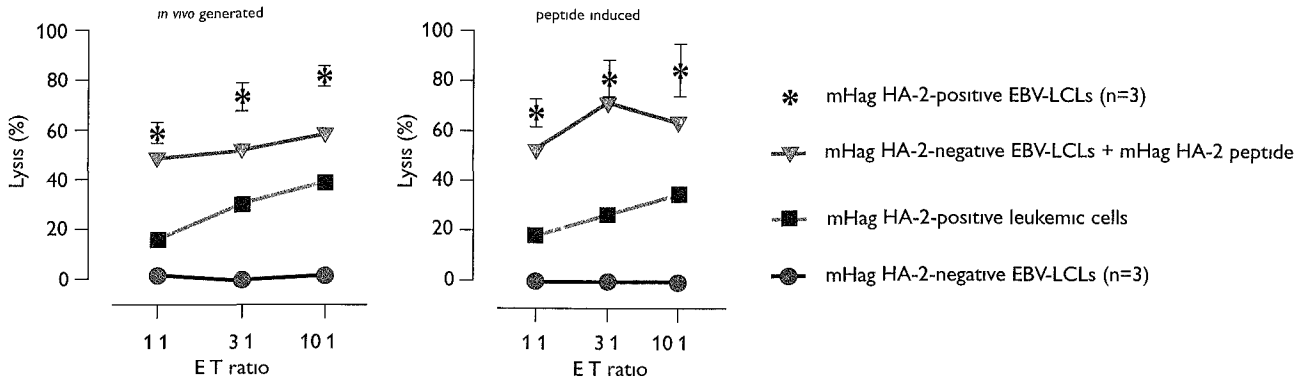

Fig. 2. Generation of mHag peptide-specific CTLs. Peripheral blood lymphocytes were pulsed with the HA-2 synthetic peptide and used as stimulator cells for autologous T cells The T cell line obtained was cloned under limiting dilution conditions 0 3 cell/well

leucocyte therapy is associated with a significant occurrence of marrow aplasia and GVHD (160) In addition, donor leucocyte infusions for relapsed ALL and AML patients are far less effective (160, 161) Second, adoptive immunotherapy with donor Epstein-Barr virus (EBV)-specific CTLs eradicated EBV-associated post-transplant lymphoproliferative disease without causing GVHD (162) Third, adoptive transfer of cytomegalovirus-specific T-cell clones were effective in restoring immunity (163)

The advantage of using mHag-specific CTLs as adoptive immunotherapy of leukemia lies in their restricted and specific target cell damage Thus, we will take advantage of three of the known characteristics of human mHag (*Table* 5), i e 1) MHC-restricted recognition by T cells, 2) variable phenotype frequencies, i e mHag polymorphism, and 3) restricted tissue distribution Moreover, since mHag are clearly expressed on circulating leukemic cells and clonogenic leukemic precursor cells of both myeloid and lymphoid origin (164, 165), both types of leukemias can be targeted We will generate mHag peptide CTLs *ex vivo* from mHag-negative BM donors for mHag-positive patients Our preliminary results are promising We prepared peptide-specific CTL clones from an HLA-A2-positive mHag HA-2-negative healthy blood donor by pulsing autologous APCs with HA-2 synthetic peptide Proliferating clones were expanded and tested for specific cytotoxic activity against mHag HA-2-positive and mHag HA-2-negative EBV-LCLs and HA-2-negative EBV-LCLs loaded with the HA-2 peptide and against mHag HA-2-positive leukemic cells The results of one mHag peptide-induced CTL clone are shown in *Fig* 2 The results are compared with those obtained with our pre-existing (*in vivo* induced) mHag HA-2-specific CTL clone assayed against the same target cells

Upon transfusion (either pre-BMT as part of the conditioning regimen or post-BMT as adjuvant therapy), the mHag peptide-specific CTLs will eliminate the mHag-positive patient's leukemic cells and, if of the patient's origin, also the patient's hematopoietic cells but will spare the patient's non-hematopoietic cells If necessary, subsequent mHag negative donor BMT will restore the patient's hematopoietic system A universal option would be to generate "prefab" mHag peptide-specific CTLs by using mHag-negative healthy blood donors with frequent HLA-homozygous haplotypes Patients who are HA-1- or HA-2-positive (and their BM donors HA-1- or HA-2-negative) and who match the HLA typing of the CTL donor can be treated with these "ready to be used" allo HA-1 or HA-2 peptide-specific CTLs Transduction of these CTLs with a suicide gene makes elimination of the CTLs possible in case adverse effects occur Future research should also focus on the possible need for mHag Th epitopes for optimal therapeutic efficacy

Table 10. Human minor histocompatibility antigens: new concepts for marrow transplantation and adoptive immunotherapy

Immunodominant mHag
• BM donor selection
• GVHD prophylaxis/treatment
mHag with broad tissue distribution
• induction of tolerance
mHag with restricted tissue distribution
• adoptive immunotherapy of leukemia

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