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Human minor histocompatibility antigens

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For over five decades minor histocompatibility (mH) antigens have continued to fascinate immunologists, mainly because of their minor but distinct role in transplantation but no less because of their as yet unknown nature In the 1970s it became evident that mH antigens were recognized in a MHC restricted fashion, an important feature which was poorly understood at that time Through the innovative work on the MHC class I crystals, significant insight into the nature of mH antigens as MHC-bound peptides has recently been obtained Thanks to extensive murine and human studies, more knowledge, though still the tip of the iceberg, has been gathered with regard to the role of mH antigen specific cytotoxic T cell (CTL) and helper T (Th) cell responses in bone marrow transplantation

In this review, we have restricted ourselves to the discussion of two main aspects of human mH antigen specific CTLs, the first being their long debated mechanistic involvement in the pathogenesis of graft versus host disease (GvHD) after HLA-identical bone marrow transplantation (BMT), the second being the molecular nature of the cell surface epitopes seen by these MHC class I restricted mH antigen specific CTLs

History and definition of mH antigens

At the beginning of this century, Little and Tyzzer[1] observed that the rejection of tumour grafts by inbred strains of mice was regulated by a family of 'ndependently segregating genes The identification of single loci responsible for tumour graft rejection became possible in 1940 when Snell outlined a procedure for the production of so-called congenic-resistant mouse strains Mice of these congenic strains rejected tumour grafts from inbred partners as a result of a genetic disparity for one short donor-strain derived chromosomal segment, thus said to contain a 'histocompatibility' (H), locus [2] One of the 13 H loci originally identified by Snell was the H-2 locus, which was later named the major histocompatibility complex (MHC) This name resulted from the observation that the speed with which tumour graft rejection was induced by disparity at these histocompatibility loci varied greatly, and that the H-2 locus was amongst the strongest (major) ones All other histocompatibility loci found to induce tumour graft rejection were grouped under the complementary name of minor histocompatibility (mH) loci Bailey continued the search for mH loci by using skin instead of tumour graft rejection as a more sensitive histogenic measure for mH incompatibility

In addition to *in vivo* tumour and skin graft rejection, histoincompabitility at these mH loci was found to induce T cell responses which could be analyzed *in vitro* Bevan[3] and Simpson[4] were the first to describe cytotoxic T cell (CTL) responses measurable in a cell lympholysis (CML) assay after *in vivo* priming across multiple or single mH disparities These donor strain reactive CTLs were found to recognize the immunizing mH antigen in a MHC class I restricted fashion and provided a powerful tool for detailed *in vitro* analysis of mH antigens Over 40 mH loci have been defined using Snell's and Bailey's congenic mouse strains, all sharing the common features of inducing skin or tumour graft rejection and of MHC restricted *in vitro* CTL responses (reviewed in[5])

In man, the existence of mH loci became evident with the discovery of the human analogue of the murine MHC the human leucocyte antigens (HLA) by Dausset, Payne and Van Rood [6] Kidney and bone marrow grafting between individuals with genetic identity at the HLA genes was still observed to result in graft rejection or graft-versus-host disease (GvHD) (reviewed in [7]) Unlike the situation in the mouse, the single loci responsible for these immune responses could not be isolated via histogenetic methods Instead, human mH loci were identified with T cell populations obtained after *in vivo* grafting across multiple mH incompatibilities By generating MHC class I restricted CTL lines from such individuals, the *in vitro* definition of single human mH gene products commenced [7] Thus far, the efforts of several investigators have led to the identification of a small number of human mH antigens.

Over the last few years the understanding of the nature of mH gene products has rapidly increased With these insights, the notion grew that the name of *minor histocompatibility antigens*, first introduced 44 years ago to describe transplantation phenomena, might raise false suggestions with respect to their nature First, the term *minor* implies relatively 'not important' In the clinical setting of organ and bone marrow transplantation between HLA-identical, mH antigen mismatched individuals, these antigens are capable of inducing vigorous immune responses leading to graft rejection or graft-versus-host disease. Secondly, the name *minor histocompatibility* suggests we are dealing with a group of related loci forming the counterpart of the complex of major histocompatibility genes However, whereas the MHC gene products form a homogeneous group with respect to function, so far no

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information is available on a potential common function of mH proteins Thirdly, in contrast to the term major histocompatibility antigen, which refers to an immunogenic transmembrane glycoprotein, it is indicated that no molecule exists to which the term minor histocompatibility *antigen* could refer With the forthcoming molecular identification of the epitopes seen by mH antigen specific T cells, and their intracellular sources, more specific and functionally appropriate names will undoubtedly manifest themselves in the coming years

Human mH antigens

Genetics and polymorphism

Human mH antigens have been defined by means of MHC restricted T cells to be obtained from individuals primed *in vivo* with HLA compatible cells Both MHC class II restricted proliferative and MHC class I restricted cytotoxic T cell clones have been used to define mH antigens Description of CTL populations observed after priming after pregnancy, blood transfusion, kidney grafting, and especially after HLA-identical bone marrow grafting (reviewed in [7]), has been very common Alas, the elaborate task of characterization of the epitopes recognized, in terms of population frequencies, segregation patterns and allelism, has only been undertaken for six mH antigens (i e W-1[8] and HA-1 to HA 5[9]) of the small number of mH antigenic CTL epitopes reported so far (Table 1)

As yet no data are available on the total number of loci encoding T cell defined human mH antigens The incidence of T cell mediated immune reactions in 45% of recipients of HLA genotypically identical bone marrow[10] suggests that incompatibility for at least one non-HLA locus is very common This high probability of mH disparity, even between siblings, might theoretically result from a high number of alleles per locus (discussed below) and/or a high number of mH loci in the human genome

The locations of human mH genes which encode CTL defined products are not known, with the exception of the gene encoding the male-specific antigen H-Y Simpson *et al* pinned the H-Y encoding gene on the long arm of the Y chromosome,[11] thereby separating it from the testis determining gene, TDF De Gast used polymorphic blood genetic makers with known gene localization to localize the non-MHC loci responsible for GvHD after related HLA-identical BMT Disparity for the markers rhesus, MNSs and acid phosphatase, located on chromosome 1, 4 and 5 respectively, correlated with increased chronic GVHD (see [7]) Although a direct parallel between this observation and the location of CTL-defined mH loci is not possible, these data would be compatible with the concept that human mH loci, as in the mouse, are located on several chromosomes

The population frequency of mH alleles represents a functionally relevant parameter which could be studied in man Extensive analysis of the mH antigens W-1,[8] and HA 1, HA-2, HA-3, HA-4, and HA 5[9] has indicated phenotype frequencies of 80, 69, 95, 88, 16 and 7%, respectively, within a panel of unrelated individuals all expressing the required MHC class I restriction molecule The contrasting very low or very high frequencies of these human mH phenotypes is compatible with the indicated frequencies of murine alleles [12] The question of how many alleles exist for each locus remains to be settled The fact that *in vivo* priming is required to define any given mH antigen forms an intrinsic complication in the study of potential allelic products, since priming in any HLA-identical transplantation setting is uni-directional Van Els *et al* made the observation that none of a panel of 100 HLA-A2 expressing individuals ever coexpressed the three HLA-A2 restricted mH antigens HA-1, HA-4 and HA-5 However, the numbers did not suffice to prove or disprove whether HA-1, HA-4 and HA-5 might represent allelic products of the same locus [9]

Whereas the mH loci of congenic mice were defined as independently segregating units, the CTL-based definition of human mH antigens does not require this Where analyzed, mH phenotypes were found to be inherited and not to spontaneously arise or disappear within families [7 8] However, the MHC class I restricted recognition of mH antigens provided a structural limitation confounding thorough segregation analysis in families Both Zier *et al* [13] and our own group[14] showed that this frustrating prerequisite could be circumvented by inserting the missing MHC molecule by membrane fusion or gene transfection into cells of family members naturally lacking the restriction molecule Using this technique to generate complete HLA-A2 expressing families, we showed that the segregation patterns of the mH antigens HA-1, HA 2, HA-4 and HA-5 are compatible with them being products of single genes inherited in a Mendelian fashion (Schreuder *et al*, in press)

T cell response to mH antigens

While MHC antigens can be recognized by B and T lymphocytes, responses to mH antigens appear predominantly T cell mediated Though some reports exist on putative mH antigen specific antibodies, mainly towards the male-specific antigen H-Y, these antisera have proved to be weak or nonspecific The only solid exception is the H-3 encoded beta 2 microglobulin (β2m) molecule which induces a strong and specific antibody response [20] The inability to measure strong B cell responses to mH antigens and the apparent exception of the β2m molecule can nowadays be explained by the knowledge that mH antigenic T cell epitopes, with the exception of β2m, are most probably small peptides as will be discussed later

T cell responses to mH antigens in man and mouse are characterized by three main features First, *in vitro* detection of mH antigen specific T cells in general requires an *in vivo* priming phase followed by an *in vitro* boosting phase Secondly, T cells defining mH antigens are MHC restricted An exception to this rule is the murine mH antigen Mls (now known to be a retroviral product[21]) which is seen in the context of any I-A and I-E class II molecule A third common and characteristic feature, so far only proven to regulate T cell responses to murine mH antigens, is the phenomenon of immunodominance

In vivo priming

In vitro coculture of unprimed peripheral blood leukocytes (PBL) or spleen cells of mH antigen disparate donors, does not result in measurable proliferation in mixed lymphocyte reactions (MLR), cytotoxicity in CML assays or lymphokine production A low frequency of mH antigen specific CTL precursors is commonly thought to underlie this phenomenon [22] Another explanation might be that the antigen presenting cells (APC) present *in vivo* are better equipped to present mH antigens than the spleen cells and peripheral blood leucocytes used *in vitro* In fact, in those exceptional cases where investigators did observe primary *in vitro* responses to mH antigens, unusual sources of stimulator cells such as leukaemic cells,[23] epidermal cells[24] or bone marrow cells[25] were employed To fully understand the role of distinct APC in the induction of mH CTL responses, the processing route and requirements of mH antigens should be known, as will be discussed later on CTL responses to viral antigens show a

Table 1 Human mH antigens defined by MHC class I restricted CTL clones

Defining CTL population(s)						mH antigen			
Code	#[a]	Disparity	Priming	Clonal[c]	HLA-restriction	Code	Frequency[b]	Segregation	Ref
—	—	HLA-id sibling	BMT/ host → graft	−	B7	W1	110/141	Mendelian single gene	8
A10 4	1[d]	HLA-id sibling	BMT/ host → graft	−	B7/B27	—	7/9	nt	15
NH-5 2	4[e]	HLA-id sibling	kidney graft	−	B35	hmH A-1	6/6	nt	16
NH-5 4	2	HLA-id sibling	kidney graft	−	B38	hmH A-2	3/4	nt	16
1R35	2	HLA-id sibling	BMT/ host → graft	+	A2 1	H Y	100/100 (m)	Y-chrom	17
5W4	1	?	blood transfusions	+	B7	H Y	60/60 (m)	Y chrom	17
R26	3	HLA-id sibling	BMT/ host → graft	−	B60	H-Y	45/45 (m)	Y-chrom	
A42	2	?	blood transfusions	−	A1	H-Y	32/32 (m)	Y chrom	18
3HA15	4	HLA id sibling	BMT/ graft → host	+	A2 1	HA-1	69/100	Mendelian single gene	7,9
5H17	2	HLA-id sibling	BMT/ graft → host	+	A2 1	HA-2	95/100	Mendelian single gene	7,9
5HO11	3	HLA-id sibling	BMT/ graft → host	+	A1	HA 3	88/100	Mendelian single gene	7,9
5G30	1	HLA-id sibling	BMT/ graft → host	−	A2 1	HA-4	16/100	Mendelian single gene	7,9
5W27	2	HLA-id sibling	BMT/ graft → host	−	A2 1	HA-5	7/100	nt	7,9
cl21	2	HLA-id sibling	BMT/ graft → host	−	B7	HA-6	15/16	nt	19
cl6	1	HLA id sibling	BMT/ graft → host	−	B7	HA-7	13/15	nt	19

[a] number of CTL clones with identical panel recognition,
[b] (number of positive ind /total number of unrelated ind tested, all restriction antigen+),
[c] monoclonality confirmed by TCR-rearrangement patterns,
[d] three more clones were described with B7 and/or B27 restricted activity on a panel of nine,
[e] one more B35 restricted clone analyzed on a panel of four is described

strong analogy to CTL responses to mH antigens and, besides other common features, also in general require an *in vivo* priming phase However, recently it was shown that by employing very potent APC, such as purified dendritic cells, primary viral antigen specific CTLs could be induced *in vitro* (*De Bruijn et al*, in press) We are currently applying this approach to generate a primary anti mH-antigen T cell response *in vitro* However, in doing so, one should be aware that, in several models, *in vitro* priming gave rise to CTLs recognizing epitopes distinct from those induced by *in vivo* priming

MHC restriction

CTLs and Th cells defining mH antigens were observed to be restricted by MHC class I and class II molecules respectively The phenomenon of MHC restriction involves a T cell receptor (TCR) molecule on the CTL which interacts with a tri-molecular complex composed of a MHC class I heavy chain, a noncovalently bound β2m light chain and antigen in the form of a short, strongly bound peptide Recent experiments have indicated that distinct MHC class I alleles contain distinct sets of peptides [26] This observation suggests that MHC restriction involves not only recognition of both MHC and MHC-bound specific peptide, but that the MHC molecule may also be involved at the level of the generation of the specific (mH) peptide

In mice, MHC class I molecules of all haplotypes (H-$2^{b\,d\,k\,s}$) are shown to present (some) mH antigens to CTLs A predominant restriction by the H-2^b haplotype is observed [5] This may not be surprising since the majority of murine mH antigens have been defined using H-2^b mice The phenomenon that some mouse strains of a given H-2 haplotype are so-called 'nonresponders', i e they do not reject skin or develop CTLs when confronted with an immunogenic mH antigen, is not well understood This 'Ir-gene effect' has so far not been observed in man The Ir-gene effect has been investigated most thoroughly by Simpson *et al* for the CTL response to the H Y antigen [27] In man, H Y specific CTLs have been described restricted by several MHC class I molecules ([17,18] and unpublished observations) Of the CTL clones which have been used to define autosomal human mH antigens, many use either HLA-A2[7,9] or HLA-B7[8,15] as restriction molecules However, at this point in time it cannot be excluded that the high frequency of the HLA-A2 allele in the population or a bias of the investigators are responsible for this apparent preferential MHC restriction

Immunodominance

Immunization of mice against multiple mH antigens results in the generation of CTLs specific for only a limited number of so-called 'immunodominant' mH antigens [5] For example, most of the 40 BALB B mH antigens are capable individually

of inducing CTL responses when present as single alloantigens, but do not induce measurable CTL responses when C57BL/6 mice are immunized simultaneously with all BALB B mH antigens [28] Immunodominant mH antigens have been described in several models involving multiple disparities, and a ranking of more or less dominant antigens has been suggested (reviewed in [5]) Since dominating and dominated antigens must be present on the same immunizing cells, immunodominance is thought to be regulated by antigen competition at the level of the antigen presenting cells Though the exact mechanism remains to be elucidated, antigen competition would be very well compatible with the recent view that mH antigen specific CTLs see peptides bound to MHC class I molecules In humans, the number of identified mH antigens is too low to conclude whether immunodominance in general governs the T cell response to multiple mH antigens Assuming that, as in the mouse, the human genome has an abundancy of mH loci, some recent results by our group can at least be called suggestive From the PBL of three individuals, each transplanted across a multiple (and probably distinct) mH barrier, CTL clones reactive to the same antigen termed HA-1 were obtained [9]

mH antigens and bone marrow transplantation

Graft versus host disease (GvHD) still forms a major barrier to successful bone marrow grafting between HLA-identical as well as HLA-matched donor–recipient pairs It is beyond the scope of this review to elaborate on the pathological manifestations of acute and chronic GvHD and the progress made by clinicians in minimizing its complications However, two GvHD risk factors relevant to understand the impact of mH antigens in BMT are discussed below

The incidence of GvHD increases with increasing genetic differences between BM donor and recipient Typical incidence values are (1) <1% for autologous grafting and monozygotic twins, (2) 36% for HLA genotypically identical siblings, (3) 40% for siblings sharing one genotypically identical and one phenotypically identical haplotype of chromosome six, and (4) 50–79% for A, B, DR matched unrelated MLC-negative donors [29] The contribution of mH antigens is illustrated by the difference between groups 1 and 2, which is due to disparities within half of the genome, for which siblings are expected to differ Likewise, donor–recipient incompatibility in group 4 compared to group 2, comprizes additional disparities for mH antigens (total genome), plus MHC linked loci on chromosome 6 Within each group, GvHD occurs more often when female BM is grafted into male recipients, illustrating the effect of an additional incompatibility for the male specific mH antigen H-Y [10]

Mature donor T cells have been shown to be required for the development of GvHD, since pretreatment of bone marrow using T cell specific antibodies or by means of physical separation almost completely prevents clinical GvHD The benefits of using T cell depleted BM in terms of graft-survival are reduced by the associated increased risks of graft failure, infection and recurrence of the original disease [30] Thus, the effects of T cell depletion and histoincompatibility stress the requirement for mature donor T lymphocytes reactive to the histocompatibility antigens of the patient for GvHD to occur

Effector mechanisms in GvHD

mH antigen specific CTLs

The first series of studies performed in mice to identify the T cell subset responsible for the induction of anti-minor GvHD uniformly implicated Lyt2 (CD8) expressing CTL By using BM inocula completely depleted of L3T4 (CD4) T cells, direct evidence was provided that CTLs on their own were sufficient to cause lethal GvHD in the B10 BR into CBA multiple mH disparate model [31] The notion that CTLs might not be required nor sufficient for GvHD was first aroused by Hamilton[32] who noticed the presence of CTLs in the spleens of BM grafted mice lacking signs of GvHD Extensive studies subsequently performed by Korngold and Sprent and Hamilton, employing additional mH disparate strains of mice, revealed that depending on the genetic background (including H-2) and the mH disparity, either the Lyt2 or the L3T4 or both T cell types could cause GvHD (reviewed in [33]) Later, an analogous series of observations was made in man The first reports by Tsoi and our own group revealed the presence of CTLs in the blood of patients undergoing GvHD, which displayed host-antigen specific MHC class I restricted lysis *in vitro* In these initial reports, the presence of host-reactive CTLs in the blood was found to correlate with the occurrence of acute [34] and chronic[7] GvHD, respectively However, in an extensive recent study this correlation was not confirmed [35] Although patients with chronic GvHD tended to develop higher and more persistent levels of anti-host CTL activity than those without GvHD, this finding was not statistically significant In fact, the *in vitro* protocol used in which lymphocytes at several dates after BMT were boosted with pre BMT PBL *in vitro*, revealed host-antigen specific CTLs in all 16 patients in at least one point in time

In a very recent study by our group, the role of anti-minor CTLs was further elucidated by quantifying the frequency of CTL precursors (CTLp) in PBL of BM recipients using a limiting dilution (LD) assay (de Bueger *et al*, in press) HLA identical BMT was found to induce high frequencies of mH antigen specific CTLs detectable in the blood during the early phase (25–100 days) of reconstitution However, the number of CTLp against host antigens showed the same development in time in all patients (i e high 25–100 days, falling in time to become undetectable after 400 days), irrespective of their GvHD status Therefore, the frequency of recipient-reactive CTLp in PBL was found not to predict the incidence of GvHD The same conclusion was recently drawn by Perrault in a murine GvHD model [36] Using a LD assay, frequencies of CTLp against host antigens were measured in 12 strains of mice after grafting with mH mismatched BM Spleen cells of all mice were found to contain high frequencies of host-reactive CTLs shortly after BMT, whether they developed moderate acute GvHD or not

It is tempting to conclude from these murine and human studies that anti-minor CTLs are neither necessary nor sufficient for the development of GvHD However, this hypothesis is contradicted by a large body of evidence suggesting that CD8+ T cells do play a role in inducing GvHD across mH barriers Notably, GvHD in the same mouse strain combinations as analyzed by Perrault[36] had previously been shown to be caused by the CD8+ T cell subset [33] Hence, these recent LD studies do not negate the role of mH antigen reactive CTLs in GvHD, but rather question the clinical value of *in vitro* analysis of post-BMT CTLs obtained from the peripheral blood Further evidence in man favouring a role of CD8+ T cells in GvHD was provided by a recent study showing that the incidence of GvHD was reduced (without an increase in the recurrence of leukaemia) when HLA-identical

BM depleted of the CD8 subset of mature T cells was used [37] *In vitro* data in support of a role of CTLs in GvHD in man were provided by Kaminsky *et al* [22] The frequency of recipient-reactive CTLp present in donor PBL before BMT was found to predict the severity of GvHD after BMT However, it must be stressed that the donor-recipient pairs in this study were unrelated, HLA-matched, MLC nonreactive pairs of individuals Apparently, the CTLp asssay is not sufficiently sensitive to measure significant values before BMT between HLA-genetically identical individuals (de Bueger *et al*, in press), whereas the presence of additional genetic differences in the case of HLA-matched donor-recipient pairs enables *in vitro* measurement of unprimed mH antigen specific CTL responses [22] These additional genetic differences may lie in HLA subtypes not detected by typing or HLA linked loci, as well as in an increased number of incompatible mH loci

Additional effector models

The seemingly contradictory results on the putative correlation between mH antigen reactive CTLs in PBL and GvHD after BMT may be explained by (1) measurement of the 'wrong' CTL population, or (2) the existence of factors, in addition to mH antigen specific CTLs, which codetermine the outcome of HLA identical BMT

Under the first heading above, the blood may not be the right site to monitor those CTLs potentially relevant in GvHD The observed disappearance of anti-host CTLp in time in PBL could well be attributed to an initial peripheral expansion followed by redistribution and migration out of the blood into the target tissues Whereas this lymphocyte trafficking was suggested in rodent models,[38] to date no evidence supports a significant lymphocyte homing to the GvHD affected tissues in man [39] Furthermore, the anti host CTL population quantified *in vitro* may comprise CTL clones reactive to all distinct host mH antigens However, some host mH antigens appear to be more 'dominant' in the induction of GvHD than others (see mH antigen specificity of effector cells) In addition, CTLs reactive to tissue-specific mH antigens may strongly contribute to the local GvHD phenomena, whereas the usage of Epstein Barr virus transformed B lymphoblastoid cell lines (EBV BLCL) or PBL as stimulator cells allows expansion and detection of only those host-reactive CTLs which recognize mH antigens on lymphoid cell types The potential relevance of target tissue specific mH antigens in GvHD is supported by the finding that *in vitro* responses of donor T cells to host epidermal cells, but not to host lymphoid cells, are predictive for the occurrence of GvHD after HLA-identical BMT [40] Also, CTLs specific for the skin specific murine mH antigen EPA-1 inflict GvHD-like tissue destruction when injected *in vivo* [41]

The contribution of factors other than mH specific CTLs must be considered too It is very possible that, though no significant correlation between the presence of CTLs and GvHD is measured, these anti host CTLs do indeed contribute to the induction and/or effector phases of GvHD In particular, if CTLs are required but are dependent for their activity on interaction with Th cells and soluble factors, they would not by themselves be sufficient to account for the development of GvHD The last few years evidence has accumulated that, in addition to CTLs, host antigen specific CD4+ Th cells could be relevant in GvHD Van Els *et al* demonstrated that significant Th cell activity *in vitro* tended to correlate with clinical acute GvHD [42] Mouse strains have been identified in which the L3T4+ subset alone induced GvHD, whereas the additional presence of Lyt2+ cells in the donor inoculum intensified the GvHD reactions [33] Genetic analysis of loci encoded within the murine H-3 and H-4 regions has revealed the existence of separate loci encoding Th cell and CTL mH epitopes Disparity for both Th and CTL epitopes was required to induce a CTL response *in vivo*,[43] indicating the relevance of Th-CTL cell collaboration in the anti H-3 and anti H-4 immune response Most recently, an LD assay was developed to measure the frequency of pre-transplant donor Th cell precursors against host mH antigens Preliminary results on 16 donors of HLA-genotypically identical BM indicated that a high frequency of helper T cells might be predictive of subsequent severe acute GvHD [44]

Since GvHD does not occur in the complete absence of histocompatibility differences, it can be anticipated that somewhere in the cascade of cellular events leading to GvHD, antigen specific T cells must be involved in at least one phase However, this does not exclude an important contribution of antigen nonspecific factors such as lymphokines in the development of GvHD Convincing data were obtained on the role of TNFα in murine and human GvHD Antibodies to TNFα could completely prevent lethal GvHD induced in mH disparate mice [45] In 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 culture medium [46] 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* [47]

Effector cells in the skin

Seeking to define the effector cell type responsible for the local pathogenesis in the target organs of GvHD, particular attention has been paid to the skin This is because the epithelium of the skin is the first and most commonly affected tissue in acute GvHD and because the infectious complications which often accompany chronic GvHD predominantly are caused by lesions in the skin The majority of histopathological studies of murine and human GvHD-affected skin after grafting with unmanipulated, mH disparate BM, describe a moderate, but certainly not extensive, mononuclear cell infiltrate containing increased numbers of CD8 expressing cells The common observation of mononuclear cells in close apposition to necrotizing basal keratinocytes[48] has strongly suggested that CTL mediated lysis of keratinocytes might occur However, one recent report denies the role of CD8+ cells in the pathology of human cutaneous GvHD No significant difference was observed between the absolute numbers or ratios of lymphocyte subsets present in GvHD-affected compared to unaffected skin in 21 patients after HLA-identical BMT [39] Another group of investigators described cell types other than CD8+ CTL in contact with degenerating epithelial cells in the skin of mice suffering from anti-minor GvHD [49 50] In the first of two consecutive reports a skin infiltrating cell population, termed large granular lymphocytes, was described to express a natural killer (NK) cell marker, but none of the T cell markers CD4, CD8 or CD3 [49] In the second report, however, studying the same B10 BR into CBA minor disparate model, these skin infiltrating cells were claimed to have a distinct phenotype (i e NK-marker, CD3 and CD8 negative but CD4 positive) [50] Some of the differences on the lymphocyte subsets proposed as effector cells in these histoimmunochemical studies may relate to the usage of distinct and more or less well defined antibodies Others may reflect the usage of distinct mH disparate combinations of mice as experimental GvHD models The usage of bone marrow grafts selectively depleted of either the CD4 or the CD8 T cell subset allowed Piguet *et al* [51] to directly show that T cells of the phenotype capable of inducing GvHD in

particular mouse strains were indeed always observed in increased numbers in necrotizing skin.

Recent work by our group has revealed that some mH antigen specific CTL clones (i.e. αH-Y, HA-3, HA-4, HA-6 and HA-7) lysed skin epithelial cells in a MHC class I restricted, mH antigen specific fashion.[52,53] These *in vitro* data suggest that at least some mH antigen specific CTL clones (though obtained from PBL and not from the skin itself) have the potential to serve a direct effector function in the local GvHD pathogenesis via CTL-mediated lysis of epithelial target cells. Furthermore, our preliminary results indicate that some, though not all, mH antigen specific CD4+ Th cell clones can be induced to proliferate upon coculture with intact layers of MHC class II+ and ICAM-1+ keratinocytes (de Bueger *et al.*, in press).

Summarizing, both cytotoxic and proliferative T cells might be responsible for the pathogenesis of GvHD. It has been established in murine models that the phenotype of the effector cells involved locally and systematically is influenced by the genetic background and the mH barrier involved. Most importantly, the mere detection of host reactive T cells in lymphatic organs by means of *in vitro* assays does not ensure their relevance as effectors in GvHD.

mH antigenic specificity of effector cells

To identify the nature of mH antigens relevant for the development of GvHD after multiple mH disparate BMT, most investigators in man and mouse have performed *in vitro* analysis of host-reactive T cell lines obtained from spleen, peripheral blood or skin from individuals suffering from GvHD. Only a few investigators have attempted to 'identify' the nature of recipient-specific, MHC restricted mH antigens. In man, we determined the reaction patterns towards a panel of 100 target cells of 12 out of 160 CTL clones obtained from five MHC class I restricted, recipient specific CTL lines.[7,9] Five distinct patterns of panel recognition were found and interpreted to define distinct mH antigens. However, since long-term *in vitro* culturing of the CTL lines preceeded limiting dilution and not all clones per patient were analyzed, no conclusion could be drawn on the relative importance of these five mH antigens in triggering CTLs in fully mH antigen disparate BMT. In the mouse, Wettstein *et al.*[28] used a transplantation barrier consisting of more than 40 known BALB.C mH loci, and analyzed the specificity of generated CTL lines on target cells expressing single (B6.C series of congenic strains) or several (CBX series of recombinant inbred (RI) strains) of the immunizing BALB.C mH loci. From the detection of H-2K^b restricted CTLs with only four distinct specificities, other than the known BALB.C antigens, two main conclusions were drawn. First, mH antigens inducing CTL responses after BMT can *differ* from those defined by skin graft rejection. Secondly, the repertoire of CTLs triggered by multiple mH disparate BMT, which mimicks best the situation encountered in man, can be reactive to only a *limited number* of mH antigens.

However, as discussed above, host-reactive T cells detectable *in vitro* may be present in GvHD-free animals and patients.[32,35] Thus, the approach used in these studies does not *a priori* guarantee that the T cell clones studied recognize 'GvHD relevant' mH antigens. This aspect was addressed in a recent study by Miconnet *et al.*[47] Seven mH antigen specific T cell clones were generated from spleen cells of GvHD mice, expanded and analyzed for lymphokine production, mH antigenic specificity and GvHD-inducing potential upon reinjection. Three CD4+ T cell clones specific for the mH antigens termed Ag-I and Ag-II, each producing high levels of TNFα *in vitro*, induced vigorous GvHD reactions *in vivo*. Two other CD4+ T cell clones (reactive with a mH antigen termed III) and a CD8+ cytolytic T cell clone (specific for Ag IV) did not. However, in trying to interpret these results the investigators were confronted with the complication of having two unknown variables, i.e. (1) the specific mH antigens recognized and (2) the phenotype, lymphokine production, and proliferative and/or cytotoxic characteristics of the T cells responsible for GvH reactions *in vivo*. One could conclude that those mH antigen reactive T cells which produce TNFα are relevant in GvHD; however, one could also argue that T cells specific for the mH antigens I and II, but not for III and IV are relevant in GvHD.

In conclusion, despite numerous efforts, little information is currently available on the number and molecular nature of the mH antigens governing the GvHD responses induced in multiple mH antigen disparate BMT. Striving for controllable GvHD in HLA-identical bone marrow transplantation, it would be desirable if a limited number of dominant antigens were involved, as preliminary data in the mouse have indicated.

Nature of mH antigens

Recently, the general view of the nature of mH antigens has drastically changed. It is now clear that in contrast to an MHC antigen which refers to an immunogenic glycoprotein, an mH antigen does not refer to a single molecular entity. Instead, one should make a distinction between the T cell epitopes seen by mH antigen specific CTLs on one hand, and the intracellular gene products giving rise to them on the other hand. The huge steps made in the elucidation of the 'MHC class I restricted processing pathway'[54] have led to the suggestion that mH antigen specific CTLs recognize peptides, derived from intracellular proteins, which are processed and presented by MHC class I molecules.

mH peptides

Identification

Two main lines of investigation have led to the definition of the limited number of mH peptides which are known to date. The first strategy involves the enormous task of sequencing stretches of genomic DNA known to include the genes encoding the mH T cell epitope. Subsequent cellular testing of synthetic peptides generated according to the deduced protein sequences has resulted in the definition of peptides recognized by mH antigen specific T cell clones.[55] Using this approach CTL clones defining Mta, a maternally transmitted mitochondrial murine mH antigen, were found to recognize synthetic peptides corresponding to a polymorphic part of mitochondrial protein ND-1.[56] Hydrophobic peptides of 17–26 amino acids in length were efficiently recognized; peptides of 12 amino acids were moderately well recognized, whereas shorter stretches did not sensitize target cells for recognition by anti-Mta CTL at all. The H-2^d restricted epitopes on three 'tumour negative' (tum−) variants P91, P35 and P198, generated by chemical mutagenesis of P815 cells, could be mimicked by hydrophobic peptides of 13, 11 and 11 amino acids, respectively (reviewed in [56]). These peptides each differed at one position from their allelic counterparts, which were not recognized by CTLs.

The second approach to identify mH peptides was developed by Rammensee and coworkers,[26,57] and has since then been applied by several investigators for purification of

MHC class I and class II bound peptides. The biochemical procedure involves the extraction of acid soluble low molecular weight material from cells expressing the T cell epitope of interest. Extraction is done of either the complete pool of acid soluble peptides present in a cell or, by including an extra affinity chromatography step, of only those peptides naturally bound to MHC.[58] Subsequent peptide separation on a reverse-phase column by means of HPLC yields fractions which each can be tested for containing T cell epitopes. Using this approach Rötschke *et al.* showed that the H-Y, H-2D^b restricted epitope and the H-4^b, H-2K^b T cell epitope were present in the low MW fractions (<5000 kD) of acid extracts of spleen cells, provided these expressed H-Y and H-2D^b or H-4^b and H-2K^b, respectively. Although the peptidic nature of the H-Y and H-4^b T cell epitopes was indicated by susceptibility to protease treatment and by size estimation based on HPLC elution profiles, the amino acid sequences of the cell surface epitopes of these classical mH antigens are not available yet.[57] Other investigators and our group too have demonstrated the presence of the HLA-B35 restricted hm-2[59] and the HLA-A2 restricted mH epitope HA-2 (de Bueger *et al.*, in press) in the low MW fraction of antigen expressing acid-eluted EBV-BLCL.

In summary, hydrophobic synthetic peptides of 9–26 amino acids long are recognized by MHC class I restricted CTL specific for a number of murine mH antigens. The naturally occuring epitopes, seen by murine and human mH antigen specific CTLs can be isolated from the restricting MHC molecules by acid elution. However, thus far the amino acid sequences of these peptides have not been determined.

Processing and presentation

Little is known thus far on the molecular mechanisms involved in the intracellular generation of MHC class I restricted mH peptides. Those few data available confirm that mH peptides presumably are products of the recently defined MHC class I pathway, analogous to viral and self-peptides.

MHC allele specificity The results by Falk *et al.*[26] very strongly suggest that indeed the H-4^b and H-Y protein fragments mentioned above are selected and presented by MHC class I molecules. They showed that the H-4^b and H-Y peptides detected in whole cell extracts from H-2^b mice were not present in similar extracts from male and H-4^b expressing H-2^d mice, indicating a strong MHC allele dependent effect in the generation of these two mH epitopes. So far, two examples are available where mH peptides have been *directly* eluted from purified MHC class I molecules. In one, the H-2K^d restricted tum− mH peptide P198.3 was eluted from purified H-2K^d molecules.[60] Although the sequence of this natural tumour peptide could not be identified directly by Edman degradation, a synthetic peptide was identified with identical HPLC behaviour as the natural peptide, and the sequence of this synthetic mH peptide was compatible with the binding profile described for H-2K^d bound self-peptides.[58] In the second example the T cell epitope of the human mH antigen HA-2 could be eluted from the purified HLA-A2.1 (de Bueger *et al.*, in press). The HA-2 active HPLC fraction contained merely nonapeptides expressing a Leu at position 2 and 9, therewith confirming the motif previously described for HLA-A2.1 bound self-peptides.[58] However, all our attempts to mimick the natural HA-2 peptide by (mixtures of) synthetic peptides, generated according to the measured profile, were thus far unsuccessful. Therefore, at this stage it cannot be excluded that the natural HA-2 T cell epitope, demonstrated to be a peptide generated and presented by HLA.A2.1, does not follow the binding restrictions known for abundant self-peptides.

Competition An argument in favour of mH peptides following the same intracellular route as viral peptides was provided by Kuzushima.[61] It was shown that viral infection reduced a cell's potential to present mH peptides to class I restricted anti-minor CTLs. Furthermore, the drug brefeldin A, which selectively blocks transport of newly synthesized proteins from the ER, inhibited anti-virus and anti-minor target cell lysis in a similar fashion.

Transport into ER The mutant cell lines RMA-s and 721.174/T2, with a suspected defect in the transport of cytosolic peptides into the class I secretory pathway,[54] have been analyzed for their ability to generate and present mH peptides. In one report RMA-s failed to present mH peptides to the appropriate MHC class I restricted CTLs.[62] However, this finding was contradicted by a study in which mH antigen specific CTL clones did recognize RMA-s cells.[63] The recent observation that RMA-s cells, though ineffectively, do present endogenous antigens, may account for this observation.[64] Because of its 'leaky' phenotype these results with RMA's do not provide clear insight into the processing requirements of mH peptides. More straightforward results were obtained with the human T2 cell line. In contrast to its parental cell line T1, it was found to be unable to present the human mH peptide HA-2 to HLA-A2.1 restricted CTLs. Likewise, T2 failed to process and present antigenic peptides from influenza following viral infection. Yet upon transfection of T2 with the rat ABC transporter cDNAs TAP-1 and TAP-2, the capacity to process and present both the mH antigen HA-2 and the influenza specific peptide for CTL recognition were restored (Momburg *et al.*, in press). These results indicate that peptide import into the ER is required for enabling this mH peptide to bind class I molecules. Moreover, since the signal recognition particle-mediated pathway[54] is unaffected in T2 cells, the failure to present HA-2 indicates this mH peptide may not represent a signal peptide.

mH proteins

The use of transfection models has revealed that proteins in all cellular compartments and of viral, foreign as well as self-origin can in principle give rise to class I associated CTL epitopes. Rammensee *et al.*[65] elegantly illustrated the degeneracy of intracellular localization in the β-galactosidase model by generating transfectants producing either a glycosylated transmembrane, a cytosolic or a secreted form of the protein. All three types of transfectants presented identical peptides to H-2L^d restricted CTL clones. The indicated irrelevance of glycosylation, intracellular destination and presence of signal sequences of proteins for the generation of MHC class I restricted peptides had previously been demonstrated using the influenza virus model.[66] In addition, it has become evident that proteins do not have to be newly synthesized nor do they need to be of endogenous origin. Native ovalbumin, when introduced directly into the cytoplasm, was shown to be recognized by H-2^b restricted, OVA-specific CTLs.[67]

Therefore, of the two criteria used to initially describe murine mH antigens, one, the recognition by MHC class I restricted CTLs, appears to be met by many intracellular proteins. Which intracellular proteins would in principle have the potential to meet the second classical biological criterion of inducing skin graft rejection remains to be determined.

Classical mH proteins

Of the classical mH gene products, defined by skin-graft

rejection between H-2 identical congenic strains of mice, only very few have been formally defined. The 12 kD protein β2m was found to be encoded in the H-3 region on chromosome 12.[19] This protein was classified as a mH antigen since it induces H-2 restricted CTL responses after *in vivo* priming, even though its potential to induce skin graft rejection is unclear. However, β2m as a secreted 12 kD cell surface molecule may not be representative of mH proteins. The three known murine alleles of β2m are recognized as integral subunits of the MHC class I cell surface complex and do not require processing or degradation for recognition by β2m specific CTLs.[68,69] It has been proposed that its behaviour as a mH antigen reflects differential binding of (mH) peptides by the distinct β2m alleles,[69] although this remains to be proven.

A second identified mH protein is the mitochondrial transmembrane protein ND-1. This protein exists in four allelic forms differing by single amino acids in the hydrophobic N-terminal part of the protein, and seems to give rise to epitopes recognized by Mta-specific CTLs.[56] The function of this ND-1 protein remains elusive; it is suggestive that no functional differences could be detected between carriers of the four distinct allelic forms of the protein.

The proteins giving rise to tum− peptides have not been identified. Given the fact that the sequences of the peptides recognized by tum− specific CTLs were identified (see *mH peptides*), the identity of their proteins was expected to be found within protein databases. However, this was not the case. Boon and coworkers have advanced two explanations for this inability to find the protein source of these peptides. Either tum− peptides originate from as yet unidentified cellular proteins or, alternatively, are directly encoded by small pieces of promotorless DNA, so-called 'peptons', and thus would not result from proteolysis of translated functional proteins. This new and provoking concept so far has not been supported by experimental evidence.[70]

In summary, the ubiquitous glycoprotein β2m, involved in cell surface transport and stabilization of MHC class I molecules, and the mitochondrial transmembrane protein ND-1 of unknown function, constitute the only two proteins identified as giving rise to cell surface epitopes recognized by mH antigen specific CTLs.

'New' mH proteins

Viral (regulated) proteins A concept formulated in 1966 was that mH proteins might be encoded or regulated by retroviral genes.[71] This notion was substantiated by Wettstein[72] who described close linkage between ectotropic and xenotropic retroviral sequences and B6 mH loci. However, it could not be excluded that the observed linkage could result from random insertion of retroviral as well as mH loci in the genome. In an attempt to provide direct evidence, Colombo[73] inserted Moloney murine leukemia viral (Mulv) genes into BALB/c, C57B/6 and 129 strain embryos to generate so-called 'Mov co-isogenic' strains. Skin grafts from these Mov strains of mice were rejected by their co-isogenic partners, differing only for the inserted viral gene. The induced H-2^b restricted CTLs were shown to recognize a Mulv-derived product since Mulv- and Rauscher-virus infected background strain target cells were lysed. With these results, Colombo was the first to provide direct evidence that retroviral sequences, when introduced into the genome can result in cell surface epitopes inducing skin graft rejection and class I restricted CTL responses.

In man, virtually nothing is known about retroviral insertions in the genome. At this point the hypothesis that germline inserted (retro-) viral genes encode or regulate (some) mH proteins remains open.

Self-proteins A second general hypothesis held by many investigators in the field of mH antigens is that polymorphic (parts of) intracellular 'self' proteins could represent mH transplantation barriers. The murine mH protein β2m represents such a polymorphic self-protein. However, as discussed above, β2m may be an exception since it is recognized as a whole cell surface protein instead of as a processed peptide. To test the hypothesis that a polymorphic self-protein could act as a transplantation barrier, Speiser *et al.*[74] made use of the known polymorphism of the murine myxovirus resistance nuclear protein MX. Indeed, skin grafts from MX protein-expressing BALB.c mice were rejected by MX-negative BALB.c congenic partners. Furthermore, injection of MX-expressing spleen cells into MX-negative BALB.c congenic mice resulted in MX antigen specific CTL after *in vitro* boosting. Notably, cell surface expression of the MX protein in MX+ mice was shown to depend on stimulation with IFNγ.

Thus, the creation of new mH gene products has indicated that viral as well as self-proteins can fit the description of mH transplantation proteins. Proteins of (retro)viral, foreign or self-origin located in either ER, cytosol or any other organelle can give rise to peptides immunogenic to class I restricted CTL and can represent transplantation barriers. The research of the coming years will have to prove how many and which of these peptides are brought to the cell surface and which actually do represent barriers in transplantation.

Immune surveillance in healthy individuals

Shaping the T cell repertoire

In the thymus, immature bone marrow derived thymocytes undergo T cell receptor (TCR) gene rearrangements, maturation and subsequent selection. This selection process must ensure that self-reactive, potentially autoaggressive, T cells do not reach the periphery (negative selection). At the same time it must ensure that functionally relevant T cells, i.e. self MHC restricted T cells specific for foreign antigens, are allowed to populate the peripheral lymphoid organs (positive selection) (reviewed in [75]). The now established view that mature T cells recognize peptides presented in association with MHC class I and class II molecules has led to the notion that the interaction with peptide/MHC complexes might in a similar fashion regulate the development of maturing thymocytes.

According to the current model, all self-peptides including self mH peptides, produced and presented in sufficient numbers by self MHC molecules, will induce negative selection of the specific T cells. The repertoire of self MHC restricted T cells which is left after negative selection to respond to pathogenic foreign antigens is therefore dependent on the composition of the set of self-peptides present. There are several examples where modification of the TCR repertoire as a result of expression of a certain mH or self-peptide could be demonstrated either by a skewed Vβ usage of the remaining T cells,[76] or by functional deficits in the immune response to foreign antigen.[77] Mice expressing certain alleles of the murine mH antigen Mls are found to lack subsets of T cells using particular TCR genes. However, Mls is not considered a classical mH antigen since Mls-reactive T cells are found at very high precursor frequencies and are restricted by more than one MHC class II allele. For most classical mH antigens it is not known whether they induce T cell subsets which express only particular Vβ genes. Preliminary results from

our laboratory have indicated that distinct CTL clones specific for H-Y use distinct Vβ genes, even when a H-Y peptide in the context of the same MHC class I molecule was seen (Kaminsky *et al*, unpublished observations) By contrast, several CTL clones derived from different individuals, all specific for the mH antigen HA-1, all seemed to use an identical Vβ, but distinct Vα and J segments (pers comm) However, even if T cell responses to classical mH peptides generally involve the predominant usage of certain TCR genes, one would not expect that negative selection of these T cells would change the peripheral TCR repertoire in mH expressing individuals to a measurable extent because of the low precursor frequencies of mH antigen reactive T cell populations in unprimed individuals Moreover, not all 'blind spots' in the TCR repertoire, created as a result of expression of self mH antigens, functionally abolish the ability of the individual to mount a T cell response to foreign antigens That the immune system can indeed cope with such blind spots was illustrated by an experiment of Rammensee *et al* Whereas the anti H-Y response in Mls-1^a negative mice was mainly mediated by Vβ6+ T cells, this response was not abrogated in MlS-1^a positive mice (which lack peripheral Vβ6+ T cells) but, instead, was mediated by T cells carrying distinct TCRs [78]

In contrast to the potentially negative effect of inducing blind spots, expression of self mH antigens may contribute in a positive fashion to the shaping of the T cell repertoire The presence of a distinct set of polymorphic mH peptides in each individual might allow a distinct fraction of self MHC restricted T cells to mature and contribute to the diversity of the TCR repertoire within the species Diversity of the TCR repertoire in its turn would positively influence the survival of the species since a broader range of pathogenic antigens can be recognized

Tumour surveillance

In a healthy individual all T cells reactive to self mH peptides presented by self MHC molecules will be deleted in the thymus (see above) or will be tolerized by means of a peripheral mechanism However, potentially autoreactive T cells specific for peptides derived from polymorphic self-proteins which are, under physiological conditions, not generated, are allowed to mature and can be found in peripheral blood [79] Therefore, it is highly likely that mature antigen specific T cells will be present to recognize mH peptides which become newly generated or overexpressed in the course of life For example, changes in the intracellular protein content as a result of induced transcription of onco genes in a developing tumour cell, might result in new mH peptides presented by the cell's MHC molecules The efficacy of this presently theoretical form of immune surveillance is unclear

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