A genetic analysis of human minor histocompatibility antigens demonstrates Mendelian segregation independent of HLA

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Abstract. An analysis of the genetic traits of human minor histocompatibility (mH) antigens is, unlike with inbred mice, rather complicated. Moreover, the fact that mH antigens are recognized in the context of MHC molecules creates an additional complication for reliable segregation analysis. To gain insight into the mode of inheritance of the mH antigens, we relied upon a series of HLA-A2-restricted cytotoxic T-cell (CTL) clones specific for four mH antigens. To perform segregation analysis independent of HLA-A2, we transfected HLA-A2-negative cells with the *HLA-A2* gene: this results in the cell surface expression of the HLA-A2 gene product and, if present, mH antigen recognition. The mode of inheritance of the HLA-A2-restricted mH antigens HA-1, -2, -4, and -5 was analyzed in 25 families whose members either naturally expressed HLA-A2 or were experimentally rendered HLA-A2-positive. Analysis of distribution of the mH antigens in the parent population among the mating types, together with their inheritance patterns in the families, demonstrated that the four mH antigens behaved as Mendelian traits, whereby each can be considered a product of a gene with two alleles, one expressing and one not expressing the detected specificity. We also showed that the loci encoding the HA-1 and HA-2 antigens are not closely linked to HLA (lod scores $Z(0 = 0.05) < -4.0$). Some indication was obtained that the HA-4- and HA-5-encoding loci may be loosely linked to HLA. While we are aware of the limited results of this nonetheless comprehensive study, we feel the similarity in immunogenetic traits between human and mouse mH antigens is at least striking.

Introduction

An argument that leucocyte groups do exist was found in family studies which showed that leucocyte iso-antigens could be inherited (Van Rood 1962). Since the mode of inheritance of human minor histocompatibility (mH) antigens is so far unknown, their existence is apparently questionable. The classical definition of mH loci is, however, also based on their immunogenicity in skin transplant experiments in mice (Snell 1948). The mH antigenic immunogenicity in humans is reflected by the occurrence of graft-vs-host-disease (GVHD) as well as the rejection of grafts in HLA genotypically identical bone marrow donor/recipient combinations (Thomas et al. 1975; Deeg and Storb 1984).

Until recently, mH antigen identification was approached solely by in vitro cellular means. In that way, a limited number of T helper (Th) cells and cytotoxic T lymphocyte (CTL) mH antigenic determinants was identified (see review Goulmy 1988). Characteristic for mH antigens is that they are presented by MHC class I and II molecules. By using selective depleted T-cell subsets, we showed that the mH antigen Th cell responses are mediated by CD4 + ve class II (HLA-DR or -DP) restricted T cells; the CTL responses have the CD8 phenotype and recognize the mH antigens in the context of class I (HLA-A or -B) molecules (Goulmy 1988; Van Els et al. 1990).

No information exists on the origin of human mH antigens. The fact that they are presented by MHC class I (and also MHC class II) molecules favors the idea that they are naturally processed fragments of intracellular proteins which associate with MHC products. Recently, mH antigen identification was approached by biochemical purification techniques. Rötzschke and colleagues were the first to isolate naturally occurring peptides that represented classical mouse mH antigens (Rötzschke et al. 1990). Recently, following

similar immuno and biochemical procedures, we and others have isolated human mH antigenic peptides (Sekimata et al 1992, De Bueger et al 1993). Despite the latter advances, the exact amino acid sequence and identity of the protein from which the classical murine or human mH antigens originate remain to be determined.

We recently also obtained information on the polymorphism of the human mH antigens. We used a series of distinct CTL clones specific for five HLA class I-restricted mH antigens to analyze the phenotype frequencies of mH antigens HA-1, -2, -4, and -5 in the healthy population. These immunogenetic studies revealed that some appeared with high frequencies (HA-2 95%), whereas other mH antigens occurred with low frequencies (HA-5 7%, Van Els et al 1992). Using the same CTL clones as genetic probes, we analyzed the mode of inheritance of four HLA-A2-restricted mH antigens in twenty-five randomly chosen families. Segregation of human mH antigens was proposed earlier (Goulmy et al 1982b, 1988, Zier et al 1983). Here we report that mH antigens can be considered as independent dominant Mendelian traits. Moreover, lod score analyses were carried out on the present family material to analyze the linkage between the mH antigen loci themselves and between the loci encoding these mH antigens and HLA.

Materials and methods

mH antigen HA-1, -2, -4, and -5 specific CTL clones Five cytotoxic T cell lines (CTLs), designated HA 1 through -5, were isolated from peripheral blood lymphocytes (PBL) obtained from five patients after *in vivo* sensitization by a bone marrow allograft from their HLA genotypically identical siblings (Goulmy 1988). The CTL lines were suspended at 1.5 cells/ml in a feeder cell mixture and plated at 0.2 ml/well (i.e., 0.3 cells/well) of 96-well round bottom microtiter plates. Clones with antihost cytotoxic activity were selected and large scale expansion (30 to 60-fold) of these clones for extensive panel typing (N = 100) was performed. Four distinct clusters of mH antigen-specific clones, restricted by HLA-A2 (HA-1, -2, -4, and -5), were identified in this way (Van Els et al 1992).

Cell mediated lympholysis (CML) assay Cell-mediated lympholysis was measured *in vitro* by using a standard chromium release assay (Goulmy 1982a).

Class I gene transfection We used electroporation (Potter et al 1984) to introduce cloned HLA genes into the Epstein-Barr Virus (EBV) transformed B-cell lines. We transfected the HLA A2 gene cloned in the pHEBO vector (Sugden et al 1985, Shimizu et al 1986) into HLA-A2 negative cells. Fluorescence activated cell sorter (FACS) analyses using the class I and HLA A2 specific monoclonal antibodies were carried out and demonstrated the surface expression of the HLA-A2 gene product on all transfected cells. Subsequently, the transfected cell lines were subjected to mH typing (Goulmy et al 1991).

Families Twenty five families, previously HLA typed for various reasons, were available. Four of these were kindly provided by

J. Dausset. In fourteen families both parents were HLA A2 positive. From nine of these families lymphocytes from the HLA A2 negative members (N = 14) were rendered A2 positive by electroporation. In addition lymphocytes from all the members of five HLA A2 negative families were rendered A2 positive by electroporation. Those five families were only tested for HA 1 and HA 2.

Test for segregation in families If the genetics of mH antigens are comparable to blood groups they should be inherited in a simple way (Race and Sanger 1954). They should be controlled by sets of allelic genes that follow the Mendelian laws. We tested the hypothesis that each of the HLA A2 restricted mH antigens is inherited as an independent dominant Mendelian character.

The material presented here enabled us to perform the following analyses:

- 1) Using the parents as a random population to compare their respective phenotype (pf) and gene (gf) frequencies, with those previously published (Van Els et al 1992),
- 2) To test whether Mendelian traits are randomly distributed among the mating types of the parents,
- 3) To test whether Mendelian traits are inherited by the progeny in a predictive fashion. However, as pointed out by Smith (1956), a slight over representation of individuals expressing the dominant trait may influence the χ^2 unfavorably. We followed the method suggested by Smith (1956), which compares the distribution of recessive offspring within and among the families tested, with the expected distribution. The χ^2 was calculated as $\chi^2 = (\text{observed} - \text{expected})^2 / \text{variance}$.

Recessive children can be a result of $+\times-$ mating if the parental genes were $+-\times--$ or from $a+\times+$ mating if the parental genes were $+-\times+-$.

The test can then be divided into two parts: 1) matings with at least one recessive child should result in a reasonable number of recessive children in all, 2) The distribution of matings resulting in recessive children can be checked against those without any recessive children.

The gf of HA-1, HA-4, and HA-5 were published previously as 0.44322, 0.08348, and 0.03564, respectively, whereas the pf of HA-2 was estimated as 95% (see Table 1). The mH antigen phenotypes are designated HA-1, HA-2, and so on, and the non-expressing phenotype ha-1, ha-2, and so on. The frequency of the allele encoding the expressed mH antigen can be called p and of the nonexpressed allele q, where $p+q=1$. The pf and gf are shown in Table 1.

When the number of observations is very close to the expected number, χ^2 values are low and consequently, p values are high, i.e., >0.05 , indicating a good fit to the expectations.

Lod score analyses for linkage of each of the mH antigens with HLA was performed in double backcross families using the formula of Morton (1956):

$$Z(\theta) = \log 2^{s-1} + \log [\theta^s (1-\theta)^{s-r} + \theta^{s-r} (1-\theta)^s],$$

where θ = the recombination fraction (theta),

s = the total number of children,

and r = the number of children having one type.

Table 1. Phenotype (pf) and gene (gf) frequencies of the HLA-A2 restricted mH antigens (Van Els et al 1992)

Spec	Phenotype frequency	gf p = expressing	gf q = non expressing
HA-1	69%	0.44	0.56
HA-2	95%	0.77	0.23
HA-4	17%	0.09	0.91
HA-5	8%	0.04	0.96

Table 2. Antigen expression in families**A** Expression of HA-1 and HA-2 antigens

Fam Name	No children	HA-1		Children		HA-2		Children	
		P1	P2	pos	neg	P1 [†]	P2 [†]	pos	neg
Nach	3	+	+	3	0	+	+	3	0
Nijs*	3	+	+	3	0	+	+	3	0
Hart	4	+	+	2	2	+	+	1	3
Dirw ⁺	4	+	+	4	0	+	+	4	0
Pool ⁺	4	+	+	3	1	+	+	4	0
Otte ⁺	5	+	+	5	0	+	+	5	0
Creg	6	+	+	6	0	+	+	6	0
Hoge	6	+	+	6	0	+	+	6	0
Kop	8	+	+	8	0	+	+	8	0
deRy*	9	+	+	9	0	+	+	9	0
vdBr ⁺	10	+	+	7	3	+	—	10	0
Wild*	3	+	—	1	2	+	+	3	0
WdV1	3	+	—	3	0	+	+	3	0
WdV2	4	+	—	1	3	+	+	4	0
Goek*	4	+	—	3	1	+	+	4	0
Back ⁺	5	+	—	5	0	+	+	5	0
Dyeu	5	+	—	3	2	+	+	5	0
Viss	5	+	—	4	1	+	+	5	0
Koud*	5	+	—	5	0	+	+	5	0
Gys*	10	+	—	8	2	+	+	10	0
Rooy	13	+	—	13	0	+	+	5	8
Pasc	4	—	—	0	4	+	+	4	0
Capt*	4	—	—	0	4	+	+	4	0
Aang*	5	—	—	0	5	+	+	5	0
Robr*	7	—	—	0	7	+	—	7	0

B Expression of HA-4 and HA-5 antigens

Fam Name	No children	HA-4		Children		HA-5		Children	
		P1	P2	pos	neg	P1	P2	pos	neg
Nijs*	3	+	—	2	1	—	—	0	3
Nach	3	+	—	3	0	—	—	0	3
Dyeu	5	+	—	3	2	+	—	2	3
Robr*	7	+	—	6	0	—	—	0	6
Kop	8	+	—	3	5	—	—	0	8
Gys*	10	+	—	1	7	—	—	0	8
WdV1	3	—	—	0	3	—	—	0	3
Hart	4	—	—	0	4	+	—	2	2
WdV2	4	—	—	0	4	+	—	1	3
Koud*	5	—	—	0	4	—	—	0	4
Creg	6	—	—	0	6	—	—	0	6
Hoge	6	—	—	0	6	—	—	0	6
deRy*	9	—	—	0	9	+	—	2	7
Rooy	13	—	—	0	13	—	—	0	13
Pasc	4	nt	nt	nt	nt	—	—	0	4
Viss	5	nt	nt	nt	nt	—	—	0	5

* Families with at least two *HLA-A2*-positive haplotypes, cells from A2-negative family members were rendered A2-positive by electroporation

+ *HLA-A2*-negative families, rendered *HLA A2*-positive by electroporation

† P1, P2 parent 1, parent 2

Table 3. Segregation of HA-1

A Number of ha-1 children in families with at least one ha-1 child						
Mating type	Fam size	No fams	No of ha-1 children			
			obs	exp	variance	X ²
+ × +	4	2	3	2 926	0 840	0 074 (n s)
	10	1	<u>3</u>	<u>2 649</u>	<u>1 592</u>	
			6	5 575	2 432	
+ × −	3	1	2	1 714	0 490	3 956 (p = 0 047)
	4	2	4	4 266	1 564	
	5	2	3	5 162	2 164	
	10	1	<u>2</u>	<u>5 005</u>	<u>2 478</u>	
			11	16 147	6 696	
B Number of families with at least one ha-1 child vs all families						
Mating type	Fam size	No fams	No of fams with ha-1 children			
			obs	exp	variance	X ²
+ × +	3	2	0	0 595	0 418	0 675 (n s)
	4	3	2	1 058	0 685	
	5	1	0	0 393	0 239	
	6	2	0	0 847	0 489	
	8	1	0	0 464	0 249	
	9	1	0	0 477	0 249	
	10	<u>1</u>	<u>1</u>	<u>0 486</u>	<u>0 250</u>	
		11	3	4 320	2 579	
+ × −	3	2	1	1 256	0 467	0 258
	4	2	2	1 347	0 880	
	5	4	2	2 782	0 849	
	10	1	1	0 718	0 203	
	13	<u>1</u>	<u>0</u>	<u>0 717</u>	<u>0 202</u>	
		10	6	6 820	2 601	
Total X ² (a+b)					4 d f	4 963 (n s)

Results and discussion

mH antigen HA-1. In Table 2A the distribution of HA-1 in the 25 families is shown.

1) The HA-1 phenotype distribution among the 50 parents was compared with the expected distribution as shown below:

Phen	Genotype	N exp	N obs
HA-1	++ = $p^2 = 0.44^2$	= 0 194	
	+ - = $2pq = 2 \times 0.44 \times 0.56$	= 0 493	
		0 687	34 3
ha-1	-- = $q^2 = 0.56^2$	= 0 314	15 7
		50 0	50 0
$0.49 = X^2$			

We can conclude that the HA-1 distribution in the parent population is not significantly different from the expected values ($X^2 = 0.49$, $p > 0.05$).

2) The distribution of mating types over the families was compared with the expected distribution, which can be calculated as follows:

Mating types	exp	obs
HA-1 × HA-1 = 0.686^2	= 0 470	11 75
HA-1 × ha-1 = $2 \times 0.686 \times 0.313$	= 0 432	10 80
ha-1 × ha-1 = 0.313^2	= 0 097	2 45
	1 000	25 00
		25 1 087 = X^2

We can conclude that the distribution of the mating types of the 25 families is according to expectation ($X^2 = 1.087$, $p > 0.05$).

3) Using the methods described by Smith (1956) we tested first the distribution of recessive children from + × + matings. As can be seen in Table 2A, 11 families had this mating type, three of which had ha-1 offspring. The expected number of ha-1 children and its variance were calculated for each family size observed. The combined results allowed for comparison with the observed number of ha-1 children and was according to expectation ($X^2 = 0.0742$, Table 3A). If HA-1 is a Mendelian trait the number of families that have at least one ha-1 child can be estimated using the HA-1 gf, $q = .44$. Although the number of families with ha-1

Table 4. HA-2 Phenotype distribution

A In parent population			
	obs	exp	X ²
HA-2	48	47.36	0.04
ha-2	<u>2</u>	<u>2.64</u>	<u>0.01</u>
	50	50.00	0.05 (n s)

B Among mating types			
Mating	obs	exp	X ²
+ × +	23	22.56	0.008
+ × -	2	2.37	0.057
- × -	<u>0</u>	<u>0.06</u>	<u>0.060</u>
	25	24.99	0.125 (n s)

C Segregation of HA-2 comparing				
1) Number of ha-2 children in families with at least one ha-2 child (methods analogous to those given in Table 3)				
Mating type	ha-2 children			
	obs	exp	variance	X ²
+ × +	11	3.520	1.435	38.98
+ × -	0	-	-	-

2) Number of families with at least one ha-2 child vs all families				
Mating type	Fam. with ha-2 child			
	obs	exp	variance	X ²
+ × +	2	2.387	2.138	0.07
+ × -	0	0.745	0.466	<u>1.19</u>
Total X ² (c 1+2)			3 d f	40.24

children was small, it was according to expectation ($X^2 = 0.675$, Table 3B). Ten families showed the + × - mating type. The number of ha-1 children in these families was slightly different from the expected number ($X^2 = 3.956$, $p = 0.047$), but the fact that they were observed in six of ten families was according to expectation ($X^2 = 0.258$, Table 3). The total X^2 (a+b) indicates that the HA-1 segregation agrees satisfactorily with our hypothesis of a genetic trait that follows Mendelian laws (Table 3). The same methods as described above were used to analyze the segregation of HA-2, HA-4, and HA-5.

mH antigen HA-2. As can be seen in Table 2A, only two parents had the ha-2 phenotype. However, this was according to our expectations as shown in Table 4A. The observation of only two families with the + × - mating type was not significantly different from that expected (Table 4B).

The number of ha-2 children detected in the two + × + families was much larger than expected

($X^2 = 38.98$), but this may be due to having had so few families. However, the fact that only two such families were found among all the families was according to expectation (see Table 4c). The very high frequency of HA-2 greatly restricts the number of analyzable families.

mH antigen HA-4. Six of 28 parents were HA-4 positive, and they all belonged to + × - matings (Table 2B). Both observations were according to expectation (Table 5A, B). Since the + × + mating type was not represented, we could only test the number of recessive children in the + × - matings. Their number, as well as the number of families in which they were observed, fulfilled the criteria for Mendelian segregation (Table 5C).

mH antigen HA-5. The HA-5 specificity is even less frequent than of HA-4 (gf.p = 0.04). The chance of finding HA-5-positive individuals is low and that of + × + matings is extremely low. However, the four

Table 5. HA-4 phenotype distribution

A In parent population				
	obs	exp	X ²	
HA-4	6	4.81	0.29	
ha-4	<u>22</u>	<u>23.19</u>	<u>0.06</u>	
	28	28.00	0.35	
B Among mating types				
Mating	obs	exp	X ²	
+ × +	0	0.42	0.42	
+ × -	6	3.92	1.13	
- × -	<u>8</u>	<u>9.66</u>	<u>0.27</u>	
	14	14.00	1.82	
C Segregation of HA-4 comparing				
1) Number of ha-4 children in families with at least one ha-4 child				
Mating type	ha-4 children			
	obs	exp	variance	X ²
+ × +	0	-	-	-
+ × -	15	12.327	5.462	1.308
2) Number of families with at least one ha-4 child vs all families				
Mating type	Fam. with ha-4 child			
	obs	exp	variance	X ²
+ × +	0	-	-	-
+ × -	4	5.16	0.645	<u>2.086</u>
Total X ² (c 1+2)			2 d f	3.394 (n s)

observed HA-5-positive parents giving rise to four + × - matings all agreed with the expected values. Thus, HA-5 behaves as a Mendelian trait (Table 6).

mH antigens and HLA. We have shown that each of the four mH antigens can be considered a product of a locus with two alleles. Are these loci 1) independent of HLA and 2) independent of each other?

For each of the mH antigens a number of double back-cross families were analyzed for linkage with HLA. In families with the + × + HA mating types, only the HA-negative children were counted. The results are shown in Table 7. HA-1 and HA-2 did not appear to be closely linked to HLA, since their lod scores are below -2.0 at recombination fractions 0 = 0.05, and 0.10. HA-4 showed a positive lod score which was highest at 0 = 0.1. However, this score was not sufficiently high to ensure linkage between both loci. Weak but insignificant positive linkage between HLA and HA-5 was found. In one family an HLA-A:B recombinant hap-

pened to be informative for HA-5. If HA-5 is linked to HLA its location is most likely centromeric to HLA-A.

Linkage studies between the mH antigens themselves revealed hardly any informative double back-cross families. This is due to the very high frequency of HA-2 and the very low frequencies of HA-4 and HA-5. Two families were informative for HA-1:HA-5 linkage and one family for HA-2:HA-5, both resulting in weak negative lodscores (Table 7). There were no informative families for the other combinations.

Although these data are really very few and insignificant, they at least do not contradict the above observation that HA-1 and HA-2 loci are definitely not linked to HLA, whereas HA-5 may be linked. The linkage between HA-1 and HA-2 could not be established with only one informative family.

To summarize, all four HLA-A2-restricted mH antigens behaved as Mendelian traits in tests for their phenotype distribution and segregation. Each can be considered a product of a gene with two alleles, one

Table 6. HA 5 phenotype distribution

A In parent population				
	obs	exp	X ²	
HA 5	4	2.51	0.88	
ha 5	<u>28</u>	<u>29.49</u>	<u>0.07</u>	
	32	32.00	0.95	
B Among mating types				
Mating	obs	exp	X ²	
+ × +	0	0.1	0.10	
+ × -	4	2.3	1.26	
- × -	<u>12</u>	<u>13.6</u>	<u>0.19</u>	
	16	16.0	1.55	
C Segregation of HA 5 comparing				
1) Number of ha 5 children in families with at least one ha 5 child				
Mating type	ha 5 children			
	obs	exp	variance	X ²
+ × +	0	-	-	-
+ × -	15	11.356	4.861	2.73
2) Number of families with at least one ha 5 child vs all families				
Mating type	Fam with ha-6 child			
	obs	exp	variance	X ²
+ × +	0	-	-	-
+ × -	4	3.765	0.213	<u>0.26</u>
Total X ² (c 1+2)			2 d f	2.99 (n s)

Table 7. Lod scores for linkage between HLA and HA 1, HA-2, HA-4, and HA 5

	$\theta = 0.05$	0.10	0.20	0.30
<i>HLA HA 1</i> (counts)				
(2 1, 4 0, 3 2, 6 4, 2 0, 1 1, 3 0, 3 0)	-4.815	-2.357	-0.519	0.010
<i>HLA HA 2</i> (3 0, 1 2, 5 3, 5 3)	-4.000	-2.211	-0.772	-0.232
<i>HLA HA-4</i> (4 1, 4 0, 0 3, 4 2, 8 0)	1.905	2.275	2.037	1.344
<i>HLA A HA 5</i> (1 4, 0 4, 0 4, 6 3)	-0.187	0.597	1.101	0.207
<i>HLA BDR HA 5</i> (1 4, 0 4, 0 4, 7 2)	1.181	1.549	1.490	0.975
<i>HA 1 HA 5</i> (4 1, 1 1)	-0.907	-0.466	-0.070	0.019
<i>HA 1 HA 2</i> (2 0)	0.258	0.215	0.134	0.064
<i>HA 2 HA 5</i> (2 1)	-0.721	-0.444	-0.194	-0.076

expressing and one not expressing the detected specificity. Due to the high frequency of HA-2 ($pf = 95\%$, $gf = 0.77$), only two families were detected with HA-2-negative children. The distribution of negative children in these two families was not according to Mendelian segregation, which might be due to the small number of

observations. But all other tests were in favor of the hypothesis that HA-2 is also encoded by an independent gene.

We concluded that the loci encoding HA-1 and HA-2 are not linked to the *HLA* region. Our family data did not provide sufficient information concerning linkage

between the other mH-encoding loci and HLA or between the mH loci themselves

The mode of inheritance of the respective HA antigens has considerable implications for bone marrow transplantation. Since the HA antigens are not tightly linked to HLA and also probably not to each other, the selection of HLA-identical family members does not implicate matching for the mH antigens. However, among family members the chance of being HLA and HA-identical is higher than among HLA-identical unrelated individuals (Martin 1991). It is clear that the differences in frequency distribution of the mH antigens is also important. The chance of matching two unrelated HLA-identical individuals for mH antigens with very high (HA-2) or very low frequencies (HA-5) will be higher than for those minors with an equal allelic distribution, such as HA-1. However, our observations only concern HLA-A2-restricted mH antigens, and many more as yet unknown antigens may play a role in GVHD. Although multiple mH antigen disparities between HLA-matched individuals may exist, T-cell responses against immunodominant mH antigens will prevail, as was clearly demonstrated earlier in mouse models (Wettstein and Bailey 1982) and more recently supposed by our own studies (Van Els et al 1992).

Our contribution concerning the mode of inheritance of minor histocompatibility antigens and their distribution in the population may help to elucidate further causes of GVHD in the future.

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