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The Detection of HLA Antigens in Bloodstains

W. R. Mayr

Institut für Transfusionsmedizin, Klinikum der RWTH, Pauwelsstraße, D-5100 Aachen, Federal Republic of Germany

Summary. After a survey of the genetics of the HLA system, the possibility to type bloodstains for HLA gene products is discussed.

Key words: HLA system, detection in bloodstains – Bloodstains, HLA system

Zusammenfassung. Nach einer Beschreibung der Genetik des HLA Systems wird die Möglichkeit der HLA-Typisierung von Blutspuren diskutiert.

Schlüsselwörter: HLA System, Nachweis in Blutspuren – Blutspuren, HLA-System

Introduction

The HLA complex (for review see Mayr 1986 and 1988) governs gene products with an extremely high polymorphism and, therefore, it is very useful in cases of disputed paternity with a chance of paternity exclusion of 96%. Because of this prodigious polymorphism and because of the high probability to discern between individuals by HLA typing, several attempts have been made to use the HLA antigens for the identification of bloodstains. The aim of this article is not to review all the data which have been obtained when various methods have been applied for the identification of bloodstains, but to mention the main difficulties and the possible sources of errors which can arise when such investigations are performed.

The HLA System

The HLA system encompasses approximately one thousandth (1/1000) of the human genome. It represents the major histocompatibility complex (MHC) of

man and contains several closely linked loci. The chromosomal localization of the HLA system is on the short arm of chromosome 6 between the bands 6p21.31distal (HLA-D region) and 6p21.33distal (HLA-ABC). Within the HLA system, several loci can be distinguished according to the biochemistry and the function of the gene products:

a) the *HLA-ABC* (class I) loci coding for glycosilated polypeptides with a molecular weight of approximately 44,000 daltons. By using molecular genetics, it could be shown that the HLA complex contains around 30 such loci; several of them, however, seem to be pseudogenes which are not expressed. The loci *HLA-A, HLA-B,* and *HLA-C* are class I loci as well as other loci defining alloantigens and possibly differentiation antigens on T-lymphocytes;

b) the *HLA-D* region (class II) loci coding for α and β chains (glycosilated polypeptides with 33,000 and 29,000 daltons, respectively);

c) at least four loci defining proteins of the complement system (these proteins are sometimes called class III gene products): *C2, C4A, C4B,* and *Bf* (Bf = factor B of properdin = C3-proactivator). The loci *C4A* and/or *C4B* can be duplicated;

d) the structural gene for the 21-hydroxylase (the lack of this enzyme causes one form of the congenital adrenal hyperplasia). There are two 21-hydroxylase loci in the HLA system: *21-OHB* and an inactive pseudogene, *21-OHA*.

The alleles of each locus of the HLA system, which are situated on one chromosome, are usually transmitted en bloc. The combination of all these alleles carried by one chromosome is called a haplotype.

The *HLA-ABC* (class I) loci define polypeptides which are post-translationally glycosylated. To this molecule, β2-microglobulin, a non-polymorphic polypeptide coded for by chromosome 15 (localization of the locus: between band 15q21 and 15q22), is bound in a non-covalent form. The heterodimer of the HLA-ABC gene product and β2-microglobulin carries the serologically defined class I antigenic epitopes; for the existence of these sites, the presence of β2-microglobulin is necessary.

The class I gene product consists of an extracellular part (278 aminoacids with the NH_2-terminal end of the molecule), a transmembrane part (28 hydrophobic aminoacids) and an intracellular part (32 aminoacids with the -COOH end of the molecule). The extracellular part comprises three domains (α1, α2, and α3 with 90, 92, and 96 aminoacids, respectively). The antigenic determinants are carried by the α1 and the α2 domains, the α3 domain is relatively constant.

The HLA-ABC gene products are present with quantitative differences on all nucleated cells of the organism. Reticulocytes, as well as some erythrocytes, carry class I antigens; the antigens of the Bennett-Goodspeed system are HLA-ABC factors which can be demonstrated on red blood cells (Bg^a = HLA-B7, Bg^b = HLA-B17, Bg^c = HLA-A28). Soluble HLA-ABC antigens are also present in the serum in an immunologically active form; the concentration of these antigens, however, is very variable.

The loci *HLA-A, B* and *C* show a remarkable multiple allelism: the specificities which are officially recognized at present by the WHO nomenclature committee are listed in Table 1 (Bodmer et al. 1988). From Table 1, it can be seen

that several specificities defined by sera with broad reactivity (supertypic factors) can be split into antigens defined by sera with a narrower specificity (subtypic factors). This phenomenon is due to the fact that the HLA-ABC molecules carry several antigenic determinants; e.g., the gene product of A9 possesses in addition to the A9 epitope an antigenic site specific for A23 or for A24. For the formal genetics of HLA, it is possible to replace the allele governing the supertypic factor by the alleles defining the subtypic specificities.

Population and family studies demonstrate that not all alloantigens of the loci *HLA-A, B,* and *C* can be recognized serologically. The alleles corresponding to the unknown factors are called *AX, BX,* and *CX.*

A strong linkage disequilibrium can be seen between various *HLA-ABC* alleles, i.e., that some *HLA-ABC* haplotypes are found more frequently and others more rarely than expected.

The alloantigens governed by the *HLA-D* region (class II antigens) are heterodimers of two polypeptides which are glycosylated post-translationally in a different way. The α chain possesses two carbohydrates and the β chain only one. Both chains are non-covalently linked and show a similar structure: an extracellular part with two domains (85–95 and 107 aminoacids, respectively, with $-NH_2$), a transmembrane region (23 hydrophobic aminoacids) and an intracellular part (8–15 aminoacids, with -COOH). The alloantigenic epitopes are situated on the outer domain of the extracellular part; the domain being close to the membrane shows nearly no variability and has similarities with the α3 domain of the class I heavy chains.

The *HLA-D* region can be subdivided into at least three subregions, *HLA-DR, HLA-DQ,* and *HLA-DP,* each containing several loci.

The HLA-DR alloantigens are situated on molecules consisting of the gene products of the *DRA* and the *DRB1* locus. On the DRα chain (coded for by *DRA*), no polymorphism has been found, thus showing that the polymorphic alloantigens must reside on the DRβ chain (governed by *DRB1*). The HLA-DR alloantigens are not present on all cells of the organism, they can be detected on B-lymphocytes, activated T cells, monocytes, macrophages, and on some endothelial and epithelial cells. In the HLA-DR system, there is also a remarkable multiple allelism with the factors listed in Table 1. For the moment, not all DR gene products are detectable; the gene coding for the "unknown" specificities is called *DRX.*

The factors which have been named MT2 and MT3 are now designated DRw52 and DRw53. They are probably alloantigens on molecules built up by the gene products of *DRA* and *DRB3* or *DRB4,* respectively, but might be present on other DR molecules. Due to a very strong linkage disequilibrium, DRw52 includes DR3, DR5, DRw6, and DRw8; and DRw53 encompasses DR4, DR7, and DRw9.

In the *HLA-DQ* subregion, there are four loci: *DQA1, DQB1, DQA2,* and *DQB2.* The DQ alloantigens (previously designated MB, DC, or DS) also consist of two chains (coded for by *DQA1* and *DQB1*). They are present on B-lymphocytes, on subpopulations of moncytes and on endothelial cells. The α and β chains of the *DQA1* and *DQB1* genes are polymorphic; it is not yet clear whether the serologically defined alloantigenic epitopes are situated on the α or

Table 1. Complete listing of recognized HLA specificities

A	B	C	D	DR	DQ	DP
A1	B5	Cw1	Dw1	DR1	DQw1	DPw1
A2	B7	Cw2	Dw2	DR2	DQw2	DPw2
A3	B8	Cw3	Dw3	DR3	DQw3	DPw3
A9	B12	Cw4	Dw4	DR4	DQw4	DPw4
A10	B13	Cw5	Dw5	DR5	DQw5 (w1)	DPw5
A11	B14	Cw6	Dw6	DRw6	DQw6 (w1)	DPw6
Aw19	B15	Cw7	Dw7	DR7	DQw7 (w3)	
A23 (9)	B16	Cw8	Dw8	DRw8	DQw8 (w3)	
A24 (9)	B17	Cw9 (w3)	Dw9	DR9	DQw9 (w3)	
A25 (10)	B18	Cw10 (w3)	Dw10	DRw10		
A26 (10)	B21	Cw11	Dw11 (w7)	DRw11 (5)		
A28	Bw22		Dw12	DRw12		
A29 (w19)	B27		Dw13	DRw13 (w6)		
A30 (w19)	B35		Dw14	DRw14 (w6)		
A31 (w19)	B37		Dw15	DRw15 (2)		
A32 (w19)	B38 (16)		Dw16	DRw16 (2)		
Aw33 (w19)	B39 (16)		Dw17 (w7)	DRw17 (3)		
Aw34 (10)	B40		Dw18 (w6)	DRw18 (3)		
Aw36	Bw41		Dw19 (w6)			
Aw43	Bw42		Dw20	DRw52		
Aw66 (10)	B44 (12)		Dw21			
Aw68 (28)	B45 (12)		Dw22	DRw53		
Aw69 (28)	Bw46		Dw23			
Aw74 (w19)	Bw47		Dw24			
	Bw48		Dw25			
	B49 (21)		Dw26			
	Bw50 (21)					
	B51 (5)					
	Bw52 (5)					
	Bw53					
	Bw54 (w22)					
	Bw55 (w22)					
	Bw56 (w22)					
	Bw57 (17)					
	Bw58 (17)					
	Bw59					
	Bw60 (40)					
	Bw61 (40)					
	Bw62 (15)					
	Bw63 (15)					
	Bw64 (14)					
	Bw65 (14)					
	Bw67					

Table 1 (continued)

A	B	C	D	DR	DQ	DP
	Bw70					
	Bw71 (w70)					
	Bw72 (w70)					
	Bw73					
	Bw75 (15)					
	Bw76 (15)					
	Bw77 (15)					
	Bw4					
	Bw6					

The subtypic specificities are characterized by the addition of the corresponding supertypic specificity; e.g., A23 and A24 are subtypic specificities of A9

on the β chain. Until now, nine factors have been recognized. The *DQA2* and *DQB2* loci are also polymorphic and represent with a high probability intact genes and not pseudogenes; for the moment, however, the gene products of these two loci are not known. Between *DRB1* and the locus defining the DQ polymorphism a strong linkage disequilibrium exists: DQw1 is associated with DR1, DR2, DRw6, and DRw10; DQw2 with DR7 and DRw17; DQw3 with DR4 (Dw15 negative), DR5, and DR9; and DQw4 with DR4 (Dw15 positive), DRw8, and DRw18.

Furthermore, a linkage disequilibrium exists between *HLA-DR* and *HLA-ABC* alleles. This linkage disequilibrium within the HLA complex includes of course the *DQ* loci, but also other loci of the MHC, e.g., *C2, C4A, C4B,* or *Bf.*

The *HLA-DP* subregion contains also four loci. The HLA-DP specificities (previously named SB) are built up by the complex of a DPα and a DPβ chain (governed by *DPA1* and *DPB1*). *DPA1* shows no polymorphism; *DPA2* and *DPB2* are probably pseudogenes. For the time being, six HLA-DP specificities are known (DPw1-DPw6). They are routinely defined by a cellular method (PLT = primed lymphocyte typing), but some DP factors may be detectable by serologic techniques.

The HLA-Dw specificities detected by the mixed lymphocyte culture (MLC) can be arranged in another series of specificities; however, it is highly probable that they are not coded for by other loci of the HLA complex but that the reactivity in the MLC is caused by differences in the whole HLA-D region.

Based on the alleles of Table 1 and one X allele in *HLA-A, B,* and *C,* respectively, the numbers of possible HLA-ABC phenotypes reaches 9,700,936 and the number of possible *HLA-ABC* genotypes is higher than 44 millions. With the DR specificities of Table 1 and one X allele, the number of DR phenotypes can be computed to be 106. Because of the extreme linkage disequilibrium between *DR* and DQ alleles, the DQ specificities do not increase this polymorphism. The total number of phenotypes considering the officially recognized HLA-A, B, C, and DR specificities amounts to 1 billion, the number of

possible genotypes is around 10 billions. These figures are obviously too small as the X alleles are heterogeneous and as several alleles can be further subdivided by serologic, cellular, or biochemical techniques (e.g., A2, A9, Aw19, B7, B8, B15, B16, B27, B44, Cw3, DR2, or DR4). The most common HLA phenotype, HLA-A1,3; B7,8; Cw7; DR2,3, has a frequency of less than 0.5%. The HLA system therefore shows a polymorphism which is not reached by any of the other expressed genetic markers of man.

HLA and Bloodstains

The HLA factors are routinely defined by using the microlymphocytotoxicity assay, which is mainly performed in the NIH standard technique. In this test, living lymphocytes (peripheral blood lymphocytes for HLA-ABC typing or purified B-lymphocytes for HLA-D, around 2,000 cells in 1 µl medium) are incubated with specific antisera (1 µl), and in a second step, rabbit serum (5 µl), which serves as complement source, is added. If the cells carry the antigen corresponding to the antibodies in the specific serum, the complement is activated by the antigen-antibody-complexes on the cell surface; the activated complement causes a damage of the cell membrane and the death of the lymphocytes. In the case that the cells do not possess the antigen, they remain living. The discrimination between living and dead cells is performed by the addition of stains, such as eosin or trypan blue which give a coloration of the dead cells. Alternatively, fluorescence techniques can be used, e.g., the addition of fluorescein-diacetate or carboxy-fluorescein-diacetate to mark the living cells or of ethidium bromide or propidium iodide for the coloration of the nuclei of the dead lymphocytes. For HLA-ABC typing, complement-fixation tests on platelets can also be used.

The human sera used for the detection of HLA antigens are usually immune sera; naturally occurring antisera with HLA specificity are extremely rare. The immunization is caused by pregnancies, blood transfusions, and/or organ transplantations. Monoclonal antibodies which are mainly produced by xenogenic immunizations (usually by the immunization of mice with human lymphocytes and the fusion of the murine immune spleen cells with mouse myeloma cells) are also available for some specificities.

Until now, the HLA typing of bloodstains has involved only HLA-ABC factors and was mainly performed by absorption tests (for review see Newall 1985) which evaluated the inhibition of the lymphocytotoxicity of specific HLA antisera after the addition of the bloodstains (or extracts of the bloodstains or of other materials containing HLA-ABC antigens). Besides the difficulties arising from the necessity to use uniform test cells and a standardized pool of rabbit complement, these absorption tests are rendered extremely difficult by the fact that many cross-reactions exist in the HLA system. Such cross-reactions can be due to different reasons (Tanigaki and Tosi 1982):

a) similarities of the structure of epitopes so that all the epitopes are able to react with the antibody. This kind corresponds to the cross-reactivity in the classical sense as described by Landsteiner;

b) existence of identical epitopes on the gene products of several alleles (e.g., anti-HLA-A9 sera react with a common epitope present on the gene products of *HLA-A23* and *A24*);

c) presence of an epitope on the gene product of one locus and strong linkage disequilibrium between the corresponding gene and some alleles of a closely linked locus (such situations are frequent in the HLA-D region, in which, e.g., an anti-DQw1 could be considered as cross-reacting anti-DR1 × DR2 × DRw6 × DRw10).

The most frequent cross-reactions involving gene products of one locus are (for details see Mayr 1986):
HLA-A: A1 × Aw36, A2 × A28, A3 × A11, A10 × Aw19, A10 × Aw34 × Aw66,
HLA-B: B5 × B35 × Bw53, B7 × B27 × B40 × Bw22 × Bw42 × Bw73, B8 × B14, B8 × Bw59, B12 × B21, B13 × B40 × Bw47,
HLA-C: Cw4 × Cw6, Cw5 × Cw8,
HLA-DR: DR1 × DRw10, DR2 × DRw6, DR3 × DRw6, DR4 × DR7 × DR9, DR5 × DRw8.

The reactivity of the "broad" antisera against Bw4 and Bw6 can also be explained by cross-reactivity, as Bw4 and Bw6 are epitopes which are present on many HLA-B gene products: HLA-Bw4 is carried by the gene products of B5, B13, B17, B27, B37, B38, B44, Bw47, B49, Bw53, Bw59, Bw63, and Bw77, while Bw6 is present on the molecules defined by B7, B8, B14, B18, Bw22, B35, B39, B40, Bw41, Bw42, B45, Bw46, Bw48, Bw50, Bw62, Bw67, Bw70, Bw73, Bw75, and Bw76.

The cross-reactivity of human alloantisera between gene products of different loci is rare; known examples are A9 × Bw4, A11 × Bw6, and A2 × B17. Such an inter-locus cross-reactivity can be found more frequently when using monoclonal antibodies.

The analysis of cross-reacting antibodies is often complicated by the fact that some antisera contain specific as well as cross-reacting antibodies and that the reaction pattern of the specific antibody is included in the pattern of the supertypic cross-reacting serum.

Further difficulties can arise when two molecules are coded for by two genes in an extreme linkage disequilibrium, e.g., *B14* and *Cw8*. In such cases, cells being B14+, Cw8− or B14−, Cw8+ are necessary to define the specificity of an antibody which could be an anti-B14, an anti-Cw8, or a mixture of both; co-capping experiments, however, with well defined anti-B14 and anti-Cw8 sera are also able to clarify such problematic situations.

Possible cross-reactivities cannot be excluded by testing the antisera only in the lymphocytotoxic assay; due to the existence of the "CYNAP"-phenomenon (cytotoxicity negative, absorption positive), all sera have to be analyzed in absorption techniques to fix their specificity and to demonstrate or exclude cross-reactivities. The use of monoclonal antibodies does not solve this problem as these antibodies, due to their higher affinity, show much more cross-reactions (in the classical sense) than human alloantisera.

Because of these reasons, it is absolutely necessary to check all the antisera used in absorption tests for the detection of HLA-ABC factors in bloodstains in this technique. Such a preliminary investigation represents, of course, a very

work and time-consuming procedure. However, without this information, it is not possible to obtain valuable results. Analogous considerations apply to the use of the complement-fixation test in order to detect the absorbing capacity of bloodstains.

A few attempts have been made to use a direct test for HLA-ABC typing bloodstains. In a series of preliminary investigations, Rittner and Walger (1986) showed that an immuno-dot blot test was not usuable for this purpose, while the use of an ELISA-method gave better results. However, in this latter technique, cross-reactions of the monoclonal antibodies which have been used produced yet unsolved difficulties.

Besides the problems arising from the cross-reactivity, it is not clear for how long the HLA-ABC antigens are stable in bloodstains. It is conceivable that a dissociation of β2-microglobulin from the class I gene product changes the conformation of many alloantigenic epitopes and hence reduces the absorptive power of the blood stains. This disintegration of the complete HLA-ABC molecule could also change the epitopes in a way which increases the various cross-reactivities. These problems have to be analyzed by further detailed investigations taking into account various storage conditions of the bloodstains and many HLA antisera.

In conclusion, it can be remarked that the HLA-ABC typing of bloodstains is for the moment not a method that can be used in routine. Several further preliminary investigations have to be performed to find truly monospecific HLA-ABC antisera or monoclonal antibodies which show no cross-reactions (according to the present knowledge, it is very difficult to find such reagents) and which can be used in absorption tests; besides these indirect tests, ELISA-methods with bloodstains or immunochemical methods in which HLA-ABC alloantigens are characterized by isoelectric focusing should be tried. Furthermore, systematic studies on the stability of the HLA-ABC alloantigens should be performed. It seems probable that a standardized technique for the detection of class I antigens in well preserved blood stains will become available, provided that antisera showing no cross-reactivity are found. Concerning the class II antigens, the situation must be seen in a much more pessimistic way: due to the small number of B-lymphocytes and monocytes in blood, the absorptive power of blood stains is minimal; for this reason, direct tests to define these alloantigens have to be developed.

In the future, the use of DNA extracted from bloodstains (if necessary, amplified by the polymerase chain reaction) to detect restriction fragment length polymorphisms corresponding to HLA phenotypes seems much more promising for identification purposes than the demonstration of HLA gene products.

References

Bodmer WF, Albert E, Bodmer JG, Dupont B, Mach B, Mayr W, Sasazuki T, Schreuder GMT, Svejgaard A, Terasaki PI (1988) Nomenclature for factors of the HLA system 1987. In: Dupont B (ed) Histocompatibility testing 1987. Springer, Berlin Heidelberg New York (in press)

Mayr WR (1986) Leukozytäre und thrombozytäre Merkmale. In: Prokop O, Göhler W (Hrsg) Die menschlichen Blutgruppen, 5. Aufl. Fischer, Jena, S 145–170

Mayr WR (1988) Das HLA-System. In: Mueller-Eckhardt C (Hrsg) Transfusionsmedizin. Springer, Berlin Heidelberg New York, S 173–193

Newall P (1985) Determination of the antigens of the HLA system in bloodstains. In: Lee HC, Gaensslen RE (eds) Advances in forensic science. Biomedical Publications, Foster City, pp 205–234

Rittner Ch, Walger H (1986) HLA-Typisierung in Blutspuren: Untersuchungen mit Immuno-Dotblotting und einer ELISA. HAIMA 1:29–33

Tanigaki N, Tosi R (1982) The genetic control of human Ia alloantigens: A three-loci model derived from the immunochemical analysis of "supertypic" specificities. Immunol Rev 66:5–37

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