Z Rechtsmed (1987) 99:55–64

Zeitschrift für
Rechtsmedizin

Originalarbeit / Original Work

Identification in Blood Stains Through DNA Typing with C4 and HLA-DR Probes

B. Olaisen[1], B. Mevåg[1], R. Jonassen[1], G. Paulsen[2], E. Thorsby[2], and P. Teisberg[3]

[1] Institute of Forensic Medicine, [2] Institute of Transplantation Immunology,
[3] Medical Department, University of Oslo, Rikshospitalet, N-0027 Oslo, Norway

Summary. A restriction fragment length polymorphism analysis using double digestion of DNA preparations with *XbaI* and *BglII* restriction enzymes and hybridization with C4 and HLA-DR probes is described. The typing conditions selected reveal extensive individual variation in both *C4* and *DR* gene regions. In our panel of 46 unrelated individuals, 37 different phenotypic patterns were recognized when both probes were used, and preliminary discriminative power values of 0.865 and 0.914 were calculated for C4 and DRβ, respectively. The probability of a chance match using both systems is probably about $1.5 \cdot 10^{-2}$.

The potential of this method for individual identification of blood stains was demonstrated on DNA prepared from 6-month-old dried blood stains from seven panel individuals. The seven individuals were all identified when comparing stain DNA patterns with panel control patterns. No RFLP pattern changes were observed following storage of blood stains. Based on these experiments with C4 and DRβ DNA typing under laboratory conditions, it is concluded that DNA typing with such probes may become a powerful tool in future stain identification analyses.

Key words: DNA-typing – Complement component C4 – HLA-DR – Blood stain identification

Zusammenfassung. Es wird die Analyse des Polymorphismus der Länge eines Restriktionsfragments (RFLP, restriction fragment length polymorphism) beschrieben, wobei eine zweifache Spaltung von DNA-Präparaten mit *XbaI* und *BglII* Restriktionsenzymen und Hybridisierung mit C4 und HLA-DR angewandt wird. Bei der gewählten Untersuchungstechnik lassen sich zahlreiche individuelle Variationen in den *C4* und *DR* Genregionen nachweisen. In einem Untersuchungsgut von 46 nicht verwandten Personen wurden in beiden Systemen 37 verschiedene Phänotypen-Muster gefunden,

Offprint requests to: B. Olaisen (address see above)

wobei die vorläufigen Werte des Diskriminations-Indexes mit 0.865 für C4 und mit 0.914 für DRβ berechnet wurden. Die Wahrscheinlichkeit einer Übereinstimmung bei Anwendung beider Systeme dürfte etwa bei 1.5×10^{-2} liegen. Die Brauchbarkeit dieser Methode zur Identifizierung individueller Blutspuren wurde anhand der DNA demonstriert, die aus 6 Monate alten, getrockneten Blutspuren von sieben Personen des Untersuchungskollektivs gewonnen wurde. Alle Proben konnten durch Vergleich der DNA-Muster mit denen der zuvor erstellten den entsprechenden Personen eindeutig zugeordnet werden. Es zeigten sich im DNA-Muster der Blutspuren keine u. U. lagerungsbedingte Veränderungen. Aus dieser unter Laborbedingungen durchgeführten D4 und DRβ DNA-Untersuchung kann geschlossen werden, daß die DNA-Hybridisierungstechnik zukünftig als wichtiges Hilfsmittel bei der Spurenanalyse eingesetzt werden kann.

Schlüsselwörter: DNA-Typisierung – Komplement-Komponente C4 – HLA-DR – Blutspuren, Identifizierung

Introduction

The genes in the HLA region of the short arm of chromosome 6 exhibit extensive genetic polymorphism. Serologically defined individual variation has been shown to be considerable in the HLA class 1 *(A, B, C)* and class 2 *(DR, DQ, DP)* loci as well as in complement loci *Bf, C2,* and *C4 (A, B).*

In C4 genes, allele variation is partly due to single base-pair differences. Variation in gene number (hetero- and homoduplications as well as deletions of both *C4A* and *C4B* genes) have, however, also been shown to be common features (see, e.g., Schneider et al. 1986).

In HLA class I and -II genes, the differences in amino acid sequences between serologically detectable allele specificities may be extensive and often involve as many as 5%–10% of the amino acid residues. Furthermore, the number of β-genes in the DR subregion seems to vary depending on the haplotype (for review see Thorsby 1987).

These kinds of individual variation between different alleles or haplotypes do, of course, substantially increase the possibilities of finding restriction fragment length polymorphisms (RFLP).

If an appropriate choice of restriction enzymes is made, different genetic regions may be evaluated either simultaneously or one by one on the same filter.

This will be of major importance when the amount of DNA available is limited.

Recently published reports have shown that DNA typing may also be possible in blood stains, and different kinds of polymorphic genetic systems have been studied (Kanter et al. 1985; Gill et al. 1985). The minisatellite polymorphisms described by Jeffreys et al. (1985a) are characterized by inherent variation in DNA chain length, and have been shown to be extremely promising for different identification purposes (Gill et al. 1985; Jeffreys et al. 1985a, b; Dodd 1985).

In this study we present the results of RFLP studies in DNA extracted from blood and blood stain material using C4 and HLA-DRβ probes[1].

Materials and Methods

Population Panel

An institute panel of 46 unrelated adults constitutes the basis for studies of RFLP pattern distribution in Norwegians.

For correlation of DR serologic specificities with DR RFLP patterns, a panel of cells from individuals with known serologically homozygous DR genotypes (HLA workshop panel) has been included. Information on origin and specificities of these cell lines was given by Grosse-Wilde et al. (1984).

Blood Stain Material

Blood stains from seven randomly chosen individuals in our blood donor panel were made by pouring 10-ml aliquots of freshly drawn whole blood onto pieces of cotton shirt. The material was then air-dried (on the desk) and stored in laboratory drawers for 6 months at room temperature and humidity.

Preparation of DNA

Genomic DNA from *blood samples* was prepared from 10 ml of ACD blood as described by Kanter et al. (1985), except that dialysis prior to ethanol precipitation was omitted. DNA pellets were air-dried and resuspended in 0.01 *M* Tris/HCl-0.001 *M* EDTA buffer (TE), pH 7.6 to a final concentration of 0.5 mg/ml.

Blood stain DNA (from approximately 500 µl blood) was isolated by the method of Gill et al. (1985). The blood-stained cotton cloth was cut into small strips and incubated in 200-µl aliquots of lysis buffer overnight, whereafter the eluate was spun out of the cotton material. Following ethanol precipitation pelleted DNA was washed, air-dried, and dissolved in 20µl TE (pH 7.6).

DR homozygous cell line DNA was prepared as described earlier (Paulsen et al. 1984).

Probes

The two probes employed in this study were the inserts of the C4 specific probe pAT-F (Carroll et al. 1985b) and an HLA-DRβ specific pII-β-4 probe (Gustafsson et al. 1984).

RFLP Typing Procedure

For RFLP analysis, double digestions of DNA were carried out with *XbaI* (20 U) and *BglII* (20 U) as described by the manufacturer (Amersham). For each digest, approximately 7.5 µg whole blood and cell line DNA were used, from blood stain samples the whole pellet. Blood stain DNA digestion was performed in the presence of 4 m*M* spermidine trichloride (Gill et al. 1985). Restriction fragments were separated by electrophoresis in 0.7% agarose gels at 40 V overnight and blotted onto nylon membranes (Hybond-N, Amersham) by ordinary Southern procedure. Prehybridization and hybridization (nick translated labeled cDNA probes (Amersham International)), were performed essentially as described earlier (Anderson et al. 1984). After hybridization, filters were washed once for 5 min at room temperature in 2 × SSPE (1 × SSPE = 0.18 *M* NaCl, 0.01 *M* NaH_2PO_4, 1 m*M* EDTA), twice for 15 min at

[1]Preliminary results were presented at the Hague meeting of the English Speeking Group of the European Society of Forensic Haemogenetics (October 1986).

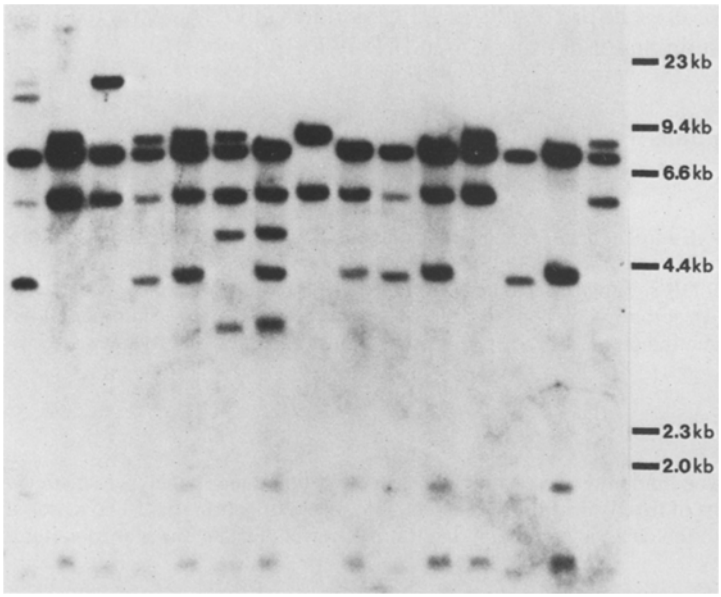

Fig. 1. C4 DNA patterns in 15 panel members using combined *BglII/XbaI* digestion and hybridization with the pAT-F-probe. Position and size of marker fragments (λ_{HIII}) are shown *on the right*

65°C in 2 × SSPE, 1% SDS, and once for 30 min at room temperature in 0.1 × SSPE. Filters were then autoradiographed at −70°C using intensifying screen.

Results

C4 DNA Polymorphism

The presently chosen selection of restriction enzymes, and the use of a nearly full length C4 c-DNA probe, allow the identification of the *XbaI* polymorphism near the 3′ end of the C4 gene as described by Whitehead et al. (1984) and the *BglII* polymorphism upstream to the C4d region as described by Pálsdottir et al. (1983). In addition, the double digestion reveals a *BglII* RFLP with an 8 kilobase fragment characteristic for the short C4 gene as defined by Carroll et al. (1985a, b) and Schneider et al. (1986). A more comprehensive description of C4 genetics, including different kinds of gene dosage effects further increasing the discriminative power of C4, is given elsewhere (Teisberg et al., in preparation).

Figure 1 shows C4 patterns illustrating rather extensive individual variation with a least ten different phenotypic patterns in 15 unselected panel members.

In the 46 individuals, 12 different phenotypes were encountered of which one was shared by nine individuals, one by eight individuals, two by seven individuals, one by five, one by three, and one by two individuals. The remaining five types were each found in one individual only. When calculated from phenotype frequencies in this material, a discriminative power of this C4 typing method of 0.865 was obtained.

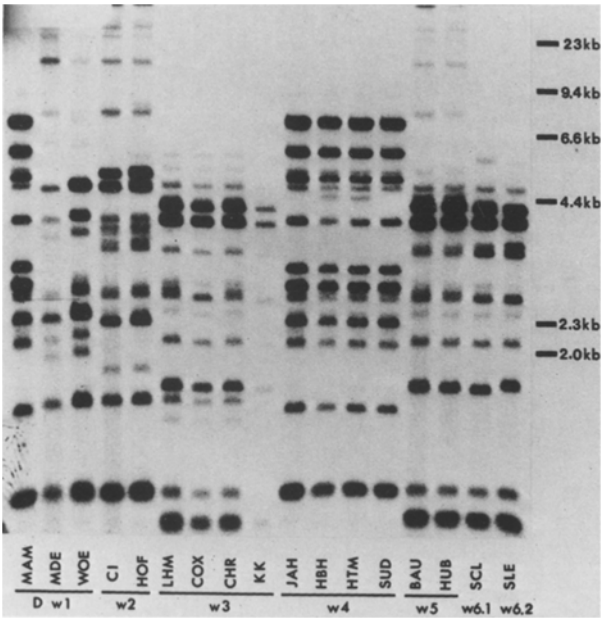

Fig. 2. DRβ DNA patterns using *BglII/XbaI* digestion and hybridization with DRβ pII-β-4 probe. The serologic DR types are given *at the bottom*, while positions and size of marker fragments (λ_{HIII}) are indicated on *the right*

*HLA-DR*β *Polymorphism*

Figures 2 and 3 show DR patterns after *XbaI/BglII* double digestion in the panel of DR homozygous test cells. It is evident that it is possible to discriminate between most, if not all, serologically defined specificities, and there may be indications of "splits" or variations of some specificities like DR3 and DR4. However, since probing with DQ and DP probes was not performed, it cannot be excluded that the "splits" may be due to cross-hybridization with *DQ* or *DP* genes.

Figure 4 shows DRβ patterns in 16 of the panel members after dehybridizing and rehybridizing with the DRβ probe.

In the material of 46 individuals, 23 different DR DNA phenotypes were observed: One type was shared by ten individuals, two by four, three by three, and two by two individuals, while the remaining 15 phenotypes were each observed in one individual only.

Phenotype frequencies in this material gave a DRβ DNA type discriminative power of 0.914.

*Typing of C4 and DR*β *Simultaneously*

Figure 5 shows the DNA pattern of unselected panel members after hybridizing simultaneously with C4 and DRβ probes. A rather complex pattern of radiolabeled fragments was achieved, allowing distinct differences in band patterns to be found between all 19 individuals.

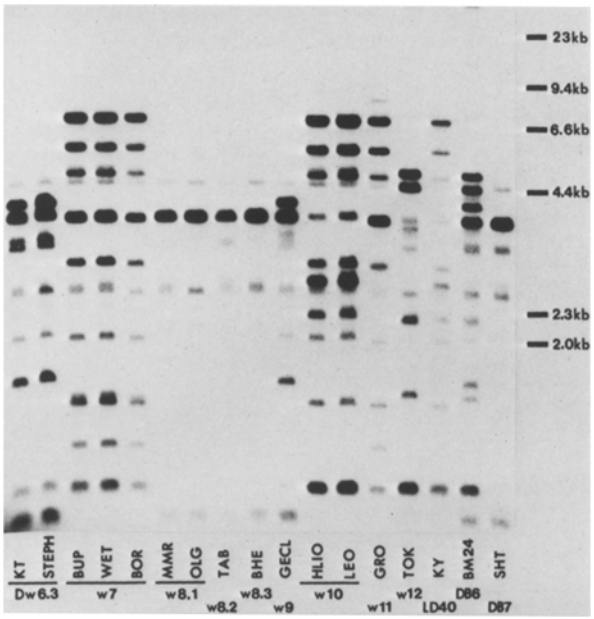

Fig. 3. DRβ DNA patterns using *BglII/XbaI* digestion and hybridization with the DRβ pII-β-4 probe. The serologic DR types are given *at the bottom*, while positions and size of marker fragments (λ_{HIII}) are indicated *on the right*

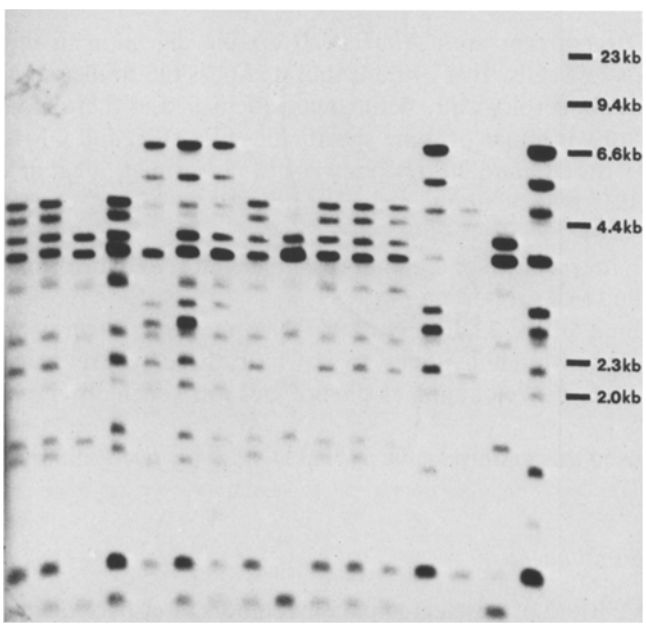

Fig. 4. DRβ DNA patterns in blood samples from 16 panel members using combined *BglII/XbaI* digestion and hybridization with the DRβ pII-β-4 probe. Position and size of marker fragments (λ_{HIII}) are indicated

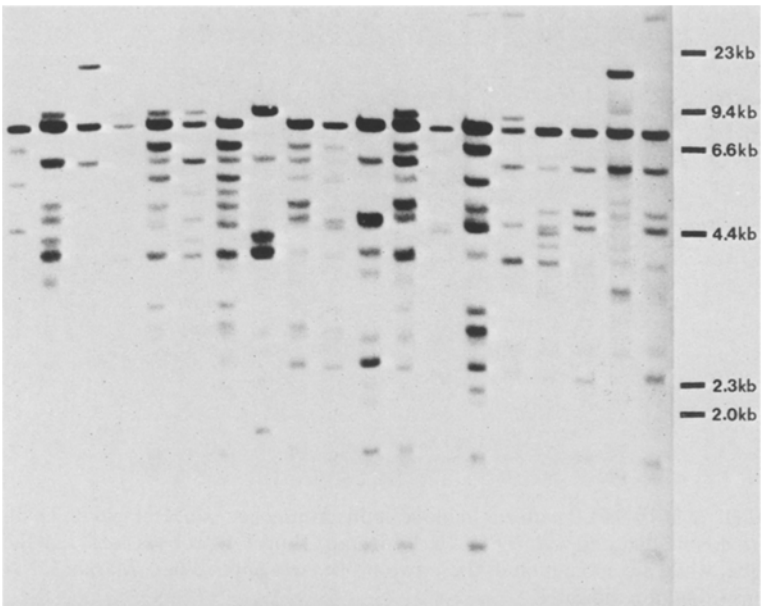

Fig. 5. Combined C4 and HLA-DRβ DNA patterns as visualized after *BglII/XbaI* double digestion and hybridization with a mixture of C4 pAT-F and DRβ pII-β-4 probes. Marker DNA fragment positions (λ_{HIII}) are indicated *on the right*

In the total panel material of 46 individuals, 37 different phenotypes were observed when using both probes. The most common phenotype was observed in five individuals, two types were encountered in three individuals each, and one type was shared by two individuals. The remaining 33 types were each found in one individual only.

When calculations of the discriminative power of the combined probes were based on phenotype frequencies observed in this material, a value of 0.962 was achieved. If, however, calculations were based on the phenotype frequencies for each of C4 and DRβ, and the two systems were presumed independent, a value of 0.988 was encountered.

Typing of Blood Stains

Blood stain DNA preparations contained mainly high molecular DNA as revealed by agarose electrophoresis. Typable C4 and DRβ patterns were usually obtained within 2 days of exposure.

C4 DNA patterns of the blood stains from the seven panel members are shown in Fig. 6a. There were no signs of incomplete restriction enzyme digestion. When comparing individual blood stain patterns with those of their ordinary blood sample DNA, no pattern changes were observed.

In the seven individuals, five different phenotypic C4 DNA patterns were observed when the evaluation was based on presence or absence of fragments, while there were six different patterns when band intensity differences within

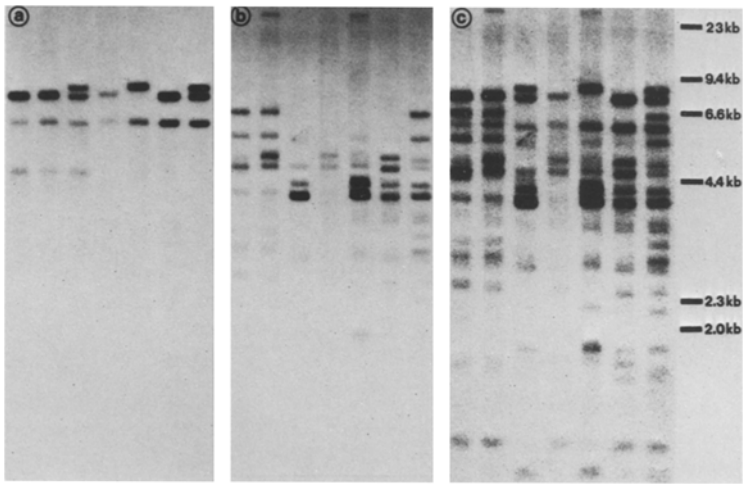

Fig. 6a-c. C4 and HLA-DRβ RFLP patterns in blood stains from seven panel members. DNA preparations were double-digested with *BglII/XbaI*. In **a** the C4 pAT-F probe is used, in **b** the DRβ pII-β-4 probe, while a combination of these two probes was applied in **c**. Marker DNA fragment positions (λ_{HIII}) are indicated

individual patterns (gene dosage effects) were considered as well (individuals no. 1 and 2 from the left have the same fragments, but fragment intensity patterns were clearly different).

HLA-DRβ DNA patterns of the blood stains from the same seven individuals are shown in Fig. 6b. In the stain DNA samples, a slight degree of background staining did not influence typing. Based on presence or absence of bands only, all seven showed distinctly different DR DNA patterns.

Combined C4 and HLA-DRβ DNA patterns of the stains are presented in Fig. 6c. An increased band pattern complexity was obtained, representing the sum of C4 and HLA-DRβ DNA bands encountered in Fig. 6a, b.

All seven blood stain DNA pattern are individually different, and when comparing each with their control blood DNA pattern, no pattern changes were observed.

When comparing with the blood DNA patterns of the 46 panel members (blind test), each of the seven blood stain panel members was easily identified.

Discussion

We have described RFLP analysis using combined *BglII/XbaI* digestion of DNA samples disclosing extensive restriction fragment length polymorphisms at both the *C4* and *DRβ* gene loci. C4 and DRβ DNA patterns may be typed consecutively or simulatenously on the same filter, thus limiting the need for typing material.

We have shown that C4 as well as DRβ DNA RFLP patterns may be demonstrated in blood stains produced and stored under laboratory conditions. As

shown in the present, small population material the discriminative power of the combined use of these two RFLPs is high. The discriminative power of a system indicates the overall probability that two freely chosen individuals show different phenotypic patterns. The calculations of combined discriminative power based on the observation in 46 individuals, including a total of 37 different phenotypes, are, of course, very conservative, the theoretical number of possible phenotype combinations calculated from the 12 and 23 observed phenotypes in each of C4 and DRβ being 276. Even this is a minimum estimate since these numbers of C4 and DRβ phenotypes are definitely underestimates due to the small population materials. On the other hand, it is well known that linkage disequilibrium exists between C4 and DR protein polymorphisms (Olaisen et al. 1983). This makes discriminative power values based on C4 and DRβ phenotype frequencies to be overestimates.

Taking these arguments into account, a combined discriminative power of about 0.985 seems likely. Thus, the overall probability that two freely chosen individuals have the same combined C4/DRβ DNA patterns is about 1.5% or $1.5 \cdot 10^{-2}$.

Such a probability of a chance match is, of course, substantially higher than that of $3 \cdot 10^{-11}$ calculated by Gill and Jeffreys (1985) using one minisatellite probe. The presently described procedure would nevertheless imply a major contribution to forensic stain analyses provided it can be proven useful in practical forensic work. Both C4 and DR genes have been studied extensively. In contrast to what has already been reported in minisatellite polymorphisms (Jeffreys et al. 1985b), no cases of "mutational" events have to our knowledge been reported for C4 or HLA-DR.

The presently described studies of DNA typing in blood stains are in good accordance with similar studies using probes recognizing other parts of the human genome (Kanter et al. 1985; Gill et al. 1985). At present, the impression is that blood stain DNA is reasonably well preserved for years, and that RFLP typings may be achieved when sufficient stain material is at hand. Still, the sensitivity of the methods seems insufficient for perhaps a majority of practical forensic cases. Much work pertaining to problems like sensitivity enhancement, e.g. by DNA amplification, and to the influence of different external conditions on stain DNA degradation, remains to be done.

References

Andersson M, Bohme J, Andersson G, Moller E, Thorsby E, Rask L, Peterson PA (1984) Genomic hybridization with class II transplantation antigen cDNA probes as a complementary technique in tissue typing. Hum Immunol 11:57–67

Carroll MC, Belt KT, Pálsdottir A, Yu Y (1985a) Molecular genetics of the fourth component of human complement and steroid 21-hydroxylase. Immunol Rev 87:39–60

Carroll MC, Pálsdottir A, Belt KT, Porter RR (1985b) Deletion of complement C4 and steroid 21-hydroxylase genes in the HLA class III region. EMBO J 10:2547–2552

Dodd BE (1985) DNA fingerprinting in matters of family and crime. Nature 318:506–507

Gill P, Jeffreys AJ, Werrett DJ (1985) Forensic application of DNA "fingerprints". Nature 318:577–579

Grosse-Wilde H, Doxiadis I, Brandt H (1984) Definition of HLA-D with HTC. In: Albert ED, Baur MP, Mayr WR (eds) Histocompatibility testing 1984. Springer, Berlin Heidelberg New York, pp 249–265

Gustafsson K, Wiman K, Emmoth E, Larhammar D, Bohme J, Hylding-Nielsen JJ, Ronne H, Peterson PA, Rask L (1984) Mutations and selection in the generation of class II histocompatibility antigen polymorphism. EMBO J 3:1655–1661

Jeffreys AJ, Brookfield JFY, Semeonoff R (1985a) Positive identification of an immigration test-case using human DNA fingerprint. Nature 317:818–819

Jeffreys AJ, Wilson V, Thein SL (1985b) Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73

Kanter E, Baird M, Shaler R, Balazs I (1985) Analysis of restriction fragment length polymorphisms in deoxyribonucleic acid (DNA) recovered from dried bloodstains. J Forensic Sci 31:403–408

Olaisen B, Teisberg P, Jonassen R, Thorsby E, Gedde-Dahl T Jr (1983) Gene order and gene distances in the HLA region studied by the haplotype method. Ann Hum Genet 47: 285–292

Pálsdottir A, Cross SJ, Edwards JH, Carroll MC (1983) Correlation between a DNA restriction fragment length polymorphism and C4A6 protein. Nature 306:615–616

Paulsen G, Rask L, Peterson RA, Bohme J, Grosse-Wilde H, Moen T, Thorsby E (1984) DNA restriction fragment length polymorphism of some ninth workshop homozygous typing cells using a cDNA probe specific for DR. In: Albert ED, Baur MP, Mayr WR (eds) Histocompatibility testing 1984. Springer, Berlin Heidelberg New York, pp 286–288

Schneider PM, Carroll MC, Alper CA, Rittner C, Whitehead AS, Yunis EJ, Colten HR (1986) Polymorphism of the human complement C4 and steroid 21-hydroxylase genes. J Clin Invest 78:650–657

Thorsby E (1987) Structure and function of HLA. EOS Riv Immunol Immunofarmacol (in press)

Whitehead AS, Woods DE, Fleischnick E, Chin JE, Yunis EJ, Katz AJ, Gerald PS, Alper CA, Colten HR (1984) DNA polymorphism of the C4 genes. A new marker for analysis of the major histocompatibility complex. N Engl J Med 310:88–91

Received March 2, 1987