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Immunochemical demonstration of plasminogen phenotypes using electroblotting

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Summary. The polymorphism of plasminogen (PLG) was analysed by isoelectric focusing on polyacrylamide gels, followed by electroblotting. For analysis, neuraminidase-pretreated sera were used. In a random sample of 500 unrelated individuals from east Westphalia 8 phenotypes were observed. The allele frequencies were: PLG*1 (A), 0.708; PLG*2 (B), 0.274; PLG*3 (A3), 0.013; PLG*V, 0.005. The family data (300 mother-child pairs) confirmed the hypothesis of autosomal codominant inheritance. PLG phenotypes could be determined in 1-year-old bloodstains (cotton and glass) that had been stored at +4°C and −20°C. The PLG phenotypes could clearly be demonstrated in sera diluted 1:4. The phenotype 1 (A) could still be detected in dilutions of 1:6. The theoretical exclusion rate was calculated to be 22.6%.

Key words: Plasminogen polymorphism – Electroblotting – Paternity PLG system

Zusammenfassung. Der Polymorphismus des Plasminogens wurde mit der isoelektrischen Fokussierung auf Polyacrylamidgelen und anschließendem Elektroblotting untersucht. Es wurden Neuraminidase vorbehandelte Serumproben verwendet. Bei einer Stichprobe von 500 nicht miteinander verwandten Personen aus dem ostwestfälischen Raum wurden 8 Phänotypen beobachtet. Die Genfrequenzen waren: PLG A = 0,708, PLG B = 0,274, PLG A3 = 0,013, PLG V = 0,005. Die Familiendaten (300 Mutter-Kind-Kombinationen) bestätigen die Hypothese des autosomal-kodominanten Erbganges. Der PLG-Phänotyp konnte bei Blutspuren (Glas und Baumwolle), die bei +4°C und −20°C gelagert wurden, eindeutig nachgewiesen werden. Mit Aqua dest. verdünnte PLG-Phänotypen waren bis zu einer Verdünnungsstufe von 1:4 bzw. 1:6 in Abhängigkeit vom Phänotyp ablesbar. Die theoretische allgemeine Vaterschaftsausschlußchance wurde mit 22,6% errechnet.

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Schlüsselwörter: Plasminogen-Polymorphismus – Elektroblotting – Vaterschaft-Plasminogen (PLG)

Introduction

The genetic polymorphism of plasminogen was first described in 1979 by two research groups. Hobart [1] and Raum et al. [8] postulated a 2-allelic model at an autosomal locus. Recent investigations from various groups [2–4] indicate the existence of 8 alleles, 4 of which have a frequency of more than 1%: PLG 1, 0.69, PLG 2, 0.26, PLG 3, 0.02, PLG 4, 0.01 (PLG 5, PLG 6, 0.03–0.005) [2].

During the 1986 PLG symposium an alphanumeric nomenclature was recommended [9]. This was based on isoelectric focusing with Western blotting and casein-overlay detection methods. The aim of this study was to introduce the immunoblotting technique for the determination of PLG phenotypes, because a much higher degree of sensitivity is required for forensic investigations, especially with regards to stains.

Material and methods

Serum samples were obtained from 500 unrelated donors and from 300 mother-child pairs from Westphalia. Serum samples were desialised by treating 10 µl serum with 5 µl CPN (*Clostridium perfringens* neuraminidase type V) and incubated overnight at +37°C.

Bloodstains were made on glass and cotton and stored at temperatures ranging from +37°C to −20°C. An additional series of stains was stored at +37°C under humid conditions.

Isoelectric focusing was carried out on thin-layer polyacrylamide gels (1 mm) with the following composition: 15 ml saccharose (20.5%); 1.515 g gelling agent in 10 ml aqua dest.; ampholine: 1.5 ml pH 6–8, 0.3 ml pH 3.5–10, 1.0 ml pH 5–7; 1.5 ml 1% ammonium persulphate; 100 µl TEMED.

Electrode solutions were 1 M H_3PO_4 for the anode and 1 M NaOH for the cathode. Pre-focusing was carried out for 30 min at 1200 V, 20 mA and 20 W at +5°C. The samples were applied using

Whatmann no. I filter-paper squares and focusing was carried out for a further 2.5 h. Transfer of the proteins to nitrocellulose membrane was carried out using electroblotting as previously described [6], followed by immunofixation (primary antibody: anti-PLG, 1:200, A 081 Dakopatts) and finally immune overlay (secondary antibody: alkaline phosphatase-conjugated swine anti-rabbit, 1:1000, P 217, Dakopatts) as described by Pflug [5] using 5-bromo-4-chloro-3-indolylphosphate as substrate.

Results and discussion

In the samples investigated 8 different phenotypes (Fig. 1) were observed. The different band patterns are diagrammatically represented in Fig. 2. The 2 homozygous types PLG*1 (A) and PLG*2 (B) each show 2 intensive main bands and a varying number of fainter bands. The heterozygous types are usually characterized by 4 equally intensive major bands, each pair reflecting the allele products of the respective homozygous types. Only the 4–2 phenotype shows a 3-band pattern, since 2 major bands overlap.

In the population sample investigated, no significant differences were found between the observed and expected numbers, so that equilibrium under Hardy-Weinberg conditions was present (Table 1). The investigation of 300 mother-child pairs demonstrated an autosomal codominant inheritance. No genetical incompatibility could be demonstrated in a total of 113 so-called critical mother-child pairs (Table 2).

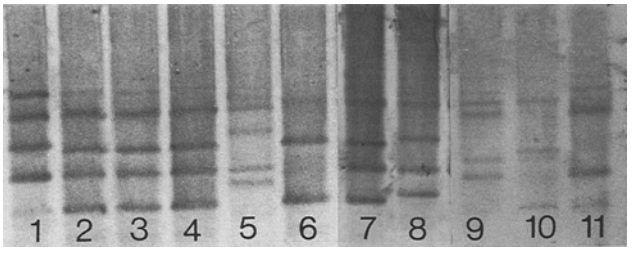


Fig. 1. Plasminogen (PLG) phenotypes after isoelectric focusing (pH 3.5–10). *Left* to *right: 1,* A3A; *2, AB; 3, AB; 4, AB; 5,* AM4; *6,* B; *7, AB; 8,* AM3; *9,* A1A; *10,* A1B; *11,* A

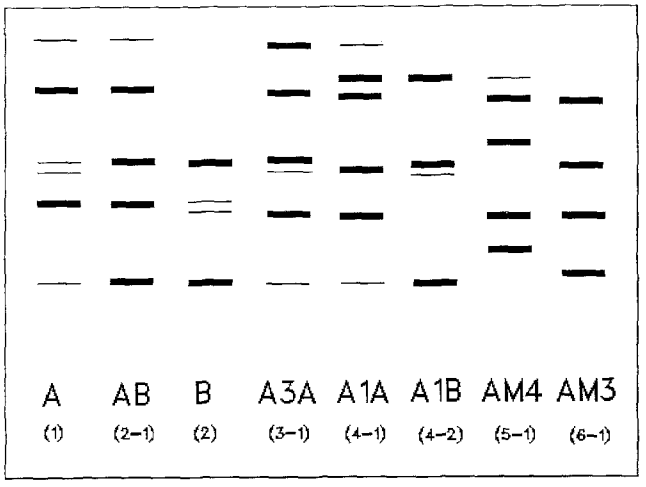


Fig. 2. Diagrammatic representation of PLG variants

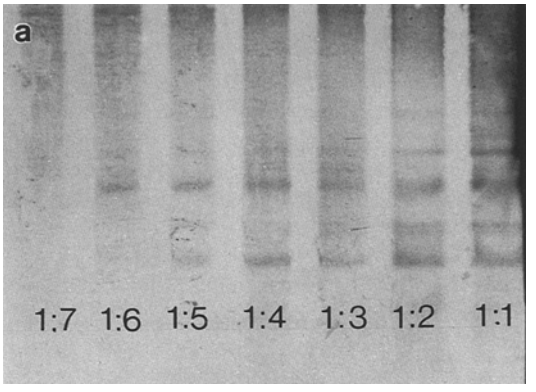
Table 1. Distribution of PLG phenotypes in east Westphalia

PLG	1–1	2–1	3–1	4–1	5–1	6–1	4–2	2–2
No. observed	248	195	13	2	1	1	1	39
No. expected	251	194	9	0.9	0.7	0.7	0.8	38
Percent observed	50	39	2.6	0.4	0.2	0.2	0.2	7.8
Percent expected	50	39	1.8	0.4	0.1	0.1	0.2	7.5

$\sum X^2 = 3.3848$

Table 2. Distribution of phenotypes in 300 mother/child pairs

Mother	Child								
	1	2–1	3–1	4–1	5–1	6–1	4–2	2	Total
1 (A)	93	45	3	–	–	–	–	–	141
2–1 (AB)	42	39	2	–	–	1	–	24	108
3–1 (A3A)	5	4	1	–	–	–	–	–	10
4–1 (A1A)	1	1	–	–	–	1	1	–	4
5–1 (AM4)	1	–	–	–	–	–	–	–	1
6–1 (AM3)	1	–	–	–	–	–	–	–	1
4–2 (A1B)	–	–	–	–	–	–	–	–	–
2 (B)	–	20	–	–	–	–	1	14	35



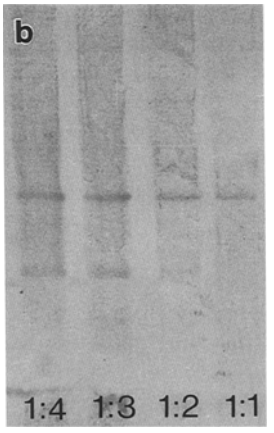


Fig. 3a, b. Serial dilutions of PLG phenotypes. **a** PLG A; **b** PLG B

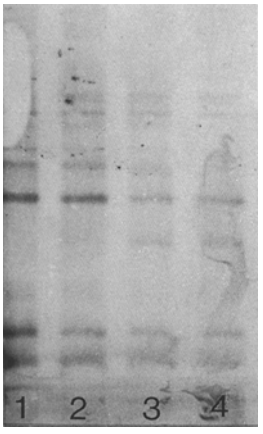


Fig. 4. PLG phenotyping of 5-month-old bloodstains on cotton after storage at 4°C. *Left* to *right: 1,2,* PLG A; *3,4,* PLG AB

Serial dilutions were made from serum samples of phenotypes 1, 2–1, 3–2 and 2 with aqua dest. The intensity of the bands was definitely attenuated at dilutions of 1:6 by phenotype 1 and by the other phenotypes at 1:4 (Fig. 3).

The sensitivity of detection of plasminogen using electroblotting was investigated on bloodstains with special reference to forensic application. Large series of experimental bloodstains were applied on glass and cotton and stored between +37°C and −20°C (Fig. 4) The 37°C series was also stored in a humid chamber. The time limits of the ability to detect distinct PLG patterns varied: 1 month (humid chamber, 37°C), 5 months (20°C) and at least 1 year (+4°C, −20°C).

The use of electroblotting combined with immunochemical visualization (immunochemophoresis) results in a definite increase in sensitivity compared with the casein-overlay technique described by other authors [3, 10], for which undiluted serum samples were used. Although the sensitivity of this method cannot compare with that reported for the Gc system [6], the stability of the protein in stored bloodstains indicates it's suitability for forensic use.

In this study, fresh serum samples and laboratory-made bloodstains were examined and no artefacts or additional bands were observed. This is in contrast to more recent findings [7], which have demonstrated that additional bands and changes in the band pattern can be observed in post-mortem blood samples and stored alcohol samples. Although this does not normally lead to false interpretation of the phenotypes it can cause some confusion and care must be taken when typing is carried out on samples such as these.

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