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Factor B subtyping in sera and bloodstains by isoelectric focusing and immunoblotting

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Summary. The polymorphism of BF was investigated in 765 unrelated Japanese individuals by isoelectric focusing and immunoblotting. Besides five common subtypes three rare variants were observed. The allele frequencies were: BF*S = 0.8078, BF*FA = 0.1797, BF*FB = 0.0105, BF*Var. = 0.0020. The above method was successfully applied to subtyping BF in stored bloodstains. The determination limits were: at 4°C 8 weeks, at room temperature 2 weeks and at 37°C only 2 days after storage. The BF subtyping is of practical use in medicolegal individualization of unknown bloodstains.

Key words: Polymorphism, serum groups – Serum groups, BF – Isoelectric focusing and immunoblotting, BF subtypes – Bloodstains, BF subtyping

Zusammenfassung. Mittels isoelektrischer Fokussierung und Immunoblotting wurden die BF-Subtypen bei 765 nicht verwandten japanischen Individuen untersucht. Neben fünf häufigen Phänotypen wurden drei seltene Varianten beobachtet. Die Allelfrequenzen betrugen: BF*S = 0,8078, BF*FA = 0,1797, BF*FB = 0,0105, BF*Var. = 0,0020. Diese Methode wurde mit Erfolg zur BF-Subtypisierung an gelagerten Blutspuren angewandt. Die zeitlichen Nachweisgrenzen betrugen: bei 4°C 8 Wochen, bei Zimmertemperatur 2 Wochen und bei 37°C nur 2 Tage nach Lagerung. Die BF-Subtypisierung ist zur rechtsmedizinischen Individualisierung von unbekannten Blutspuren von praktischem Nutzen.

Schlüsselwörter: Polymorphismus, Serumgruppen – Serumgruppen, BF – Isoelektrofokussierung und Immunoblotting, BF-Subtypen – Blutspuren, BF-Subtypisierung

Introduction

The genetic polymorphism of human properdin factor B (BF) was first reported by Alper et al. (1972) using high-voltage agarose gel electrophoresis with the exis-

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tence of two common alleles, BF*F and BF*S, and two less common alleles, BF*F1 and BF*S1 (BF*S07). Subsequently, a number of rare variants have been identified and a nomenclature has been proposed by Mauff et al. (1978).

Recent isoelectric focusing studies have revealed the occurrence of subtypes of BF F (Teng and Tan 1982; Geserick et al. 1983; Abbal et al. 1985; Nakamura et al. 1987; Nishimukai et al. 1988; Segurado and Arnaiz-Villena 1989) and BF S (David et al. 1983; Weidinger et al. 1984). Using this technique and immunofixation, Wegener and Rummel (1986) demonstrated BF banding patterns in 2 week old bloodstains, but no available data were given on BF subtyping from bloodstains.

In the present study the polymorphism of BF was investigated in a Japanese population and BF subtyping was attempted from bloodstains by means of polyacrylamide gel isoelectric focusing and immunoblotting.

Materials and methods

Sera. Blood samples were collected from 765 unrelated Japanese individuals living in a central part of Japan, Yamanashi Prefecture. Serum samples were separated and stored at −20°C until use.

For BF typing, 5 µl of the serum was applied to a slot of the gel.

For BF subtyping, the serum was treated with neuraminidase according to Mauff et al. (1984): 9 µl of serum was added with 1 µl of 1 *M* potassium phosphate buffer (pH 7.0) containing 50 U/ml neuraminidase from *Clostridium perfringens* (Type V, Sigma, St. Louis, USA) and left overnight at room temperature. The mixture was applied to the gel using 5 × 6 mm filter paper strips (Whatman No. 3, UK).

Bloodstains. Venous blood from 20 donors with known phenotypes was dropped on filter paper (Whatman No. 3) and dried at room temperature. The bloodstains thus made were stored in a refrigerator at 4°C, at room temperature and in a thermostatic chamber at 37°C, and examined at different time intervals over a period of 10 weeks.

For BF typing, 5 × 5 mm pieces of the stains were soaked with 50 µl of distilled water overnight at 4°C and 5 µl of the extracts were applied to a slot of the gel.

For BF subtyping, the stains were cut in 5 × 6 mm pieces, moistened with 10 µl of 1 *M* potassium phosphate buffer (pH 7.0) containing 5 U/ml neuraminidase (Type V, Sigma), left overnight at room temperature and directly applied to the gel.

BF typing. BF types were determined by high-voltage agarose gel electrophoresis followed by immunofixation as described previously (Kido et al. 1988).

BF subtyping. The BF subtyping method of Nishimukai et al. (1988) was adopted in this series of investigations with slight modifications.

Isoelectric focusing was carried out with an LKB 2117 Multiphor apparatus (Bromma, Sweden). Polyacrylamide gel plates (230 × 110 × 0.5 mm) were composed of 20 ml of stock solution (5.25% acrylamide/0.25% N,N′-methylenebisacrylamide), 3 g of urea, 0.34 ml of Servalyt pH 3.5–5 (Serva, Heidelberg, FRG), 0.85 ml of Ampholine pH 5–8 (LKB), 0.24 ml of Pharmalyte pH 3–10 (Pharmacia, Uppsala, Sweden), 0.3 ml of 0.01% riboflavin and 2.5 g of sucrose. The electrode paper strips were soaked with 0.5 *M* phosphoric acid for the anode and with 0.5 *M* sodium hydroxide for the cathode. After prefocusing at Vmax 1200 V and Imax 10 mA for 40 min, the specimens were applied to the gel surface 2 cm from the anode. Electrofocusing was conducted at Vmax 1200 V and Imax 10 mA for 30 min, and at Vmax 1300 V for 3 h. The specimens were removed after 30 min of electrofocusing. During focusing the gel plate was cooled by circulating water at 4°C.

A sheet of nitrocellulose membrane (Bio-Rad, Richmond, USA) was placed onto the gel surface and left for 40 min without pressing. Thus, the focused proteins were passively transferred to the membrane. Following blotting the membrane was rinsed in 20 m*M* tris/500 m*M*

sodium chloride buffer, pH 7.2 (TBS), for 10 min and immersed in TBS containing 3% gelatin for 30 min. After one wash in TBS containing 0.05% Tween 20 (TTBS) for 15 min, the membrane was incubated for 60 min in goat anti-human BF serum (Atlantic Antibodies, Scaborough, USA) diluted 400-fold with TTBS. Then, the membrane was washed twice in TTBS for 20 min and incubated for 60 min in peroxidase-conjugated rabbit anti-goat IgG serum (Cappel, West Chester, USA) diluted 400-fold with TTBS. After two washes in TTBS for 20 min, the membrane was incubated in a freshly prepared reaction mixture (50 ml of TBS, 10 mg of 3,3′-diaminobenzidine and 1 ml of 30% hydroperoxide) for a few min.

Results and discussion

Figure 1 shows BF subtypes revealed by the present isoelectric focusing technique followed by immunoblotting. BF F is subdivided into two components: the faster BF FA and the slower BF FB. Thus, six common phenotypes associated with the BF*S, BF*FA and BF*FB alleles are classified. Our sample included

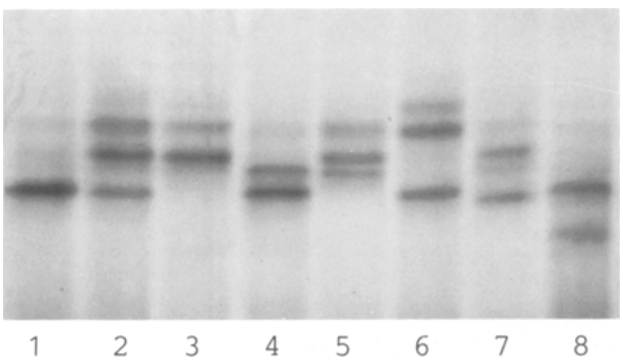

Fig. 1. Isoelectric focusing patterns of BF subtypes observed in the present study. 1: S; 2: FAS; 3: FA; 4: FBS; 5: FAFB; 6: F075S; 7: FAS045; 8: SS075. The anode is at the *top*

Table 1. Distribution of BF subtypes in a Japanese population

Subtype	No. observed	(%)	No. expected
S	500	(65.4)	499.2
FAS	221	(28.9)	222.1
FBS	13	(1.7)	13.0
FAFB	3	(0.4)	2.9
FA	25	(3.3)	24.7
FB	0	(0.0)	0.1
F075S	1	(0.3)	2.5
SS075	1		
FAS045	1	(0.1)	0.5
Total	765	(100.1)	765.0

Allele frequencies: BF*S = 0.8078; BF*FA = 0.1797; BF*FB = 0.0105; BF*Var. (the combined frequency of BF*F075, BF*S075 and BF*S045) = 0.0020

$\chi^2 = 0.713$, $df = 6$, $P > 0.99$

Table 2. Positive results for the determination limits of BF S, FAS, FBS and FA subtypes in 20 bloodstains stored at 4°C, room temperature and 37°C

Temperature	Subtype	Age of bloodstains											
		2 d	5 d	1 w	2 w	3 w	4 w	5 w	6 w	7 w	8 w	9 w	10 w
4°C	S	8	8	8	8	8	8	8	8	8	8	6	5
	FAS	6	6	6	6	6	6	6	6	6	6	2	2
	FBS	3	3	3	3	3	3	3	3	3	3	1	0
	FA	3	3	3	3	3	3	3	3	3	3	3	3
Room temperature	S	8	8	8	8	6	5	0					
	FAS	6	6	6	6	6	5	1	0				
	FBS	3	3	3	3	3	0						
	FA	3	3	3	3	3	3	3	2	0			
37°C	S	8	5	0									
	FAS	6	4	0									
	FBS	3	2	0									
	FA	3	3	0									

d: Days, w: weeks

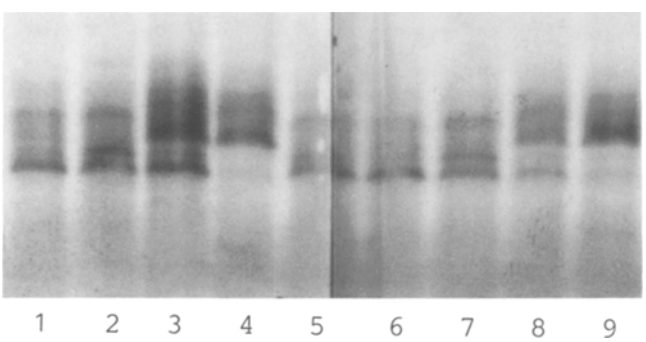

Fig. 2. Isoelectric focusing patterns of BF subtypes in bloodstains stored at room temperature for 2 weeks. 1: S; 2: FBS; 3: FAS; 4: FA; 5: S; 6: S; 7: FBS; 8: FAS; 9: FA. The anode is at the *top*

no BF FB type. The other three types of rare variant were further determined by agarose gel electrophoresis as F075S, FAS045 and SS075. The results for the distribution of BF subtypes in 765 Japanese are given in Table 1. There was good agreement between the observed and expected numbers of phenotypes according to the Hardy-Weinberg law. The allele frequencies are similar to those for another Japanese subpopulation (Nishimukai et al. 1988). The results provide supporting evidence for the reliability of the method.

At first we tried to demonstrate BF types from bloodstains using high-voltage agarose gel electrophoresis followed by immunofixation. However, the banding patterns were so faint and diffuse that the typing was no longer possible a few days after stain formation.

The present isoelectric focusing technique and subsequent immunoblotting yielded fairly clear BF patterns from dried bloodstains though the bands became

fainter and more indistinct with increasing age of stains. There were no remarkable differences in loss of intensity among the allelic products. Table 2 shows the results for the determination limits of BF subtypes in bloodstains stored at 4°C, room temperature and 37°C. All the bloodstains examined were subtyped for BF at 4°C for periods of up to 8 weeks, at room temperature for periods of up to 2 weeks and at 37°C for periods of up to only 2 days. Our results at 4°C and room temperature are superior to those of Wegener and Rummel (1986) at 4–25°C. The BF subtype patterns in bloodstains stored at room temperature for 2 weeks are shown in Fig. 2.

The method of Nishimukai et al. (1988) also permits reliable BF subtyping from dried and stored bloodstains. The above limits of determination imply that this protein is stable enough to be used for forensic purposes, but unsuitable to be used in summer or in tropical countries. The BF subtype system would offer a new useful genetic marker for the medicolegal grouping of bloodstains provided that polyacrylamide gel isoelectric focusing combined with immunoblotting is used.

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