Int J Leg Med (1993) 106:1–4

International Journal of
Legal Medicine

Original articles

Human erythrocyte band 3 (EPB3) polymorphism: analysis of blood and bloodstains by an immunodetection method and frequency of the EPB3* Memphis variant

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Received November 18, 1992 / Received in revised form March 24, 1993

Summary. The erythrocyte band 3 (EPB3) variant, band 3 Memphis (EPB3*Memphis), was detected by immunoblotting with a monoclonal antibody to the 41 kDa cytoplasmic N-terminal domain of band 3 without protease treatment of erythrocytes. EPB3*Memphis was also detected by immunoblotting from 3-month-old bloodstains subjected to α-chymotrypsin treatment. A population genetic study using this method indicated that the EPB3 variant would be useful for forensic work in Japan, since the frequency of this variant in Japanese (Wakayama prefecture) is relatively high (0.159).

Key words: Band 3 Memphis – Erythrocyte band 3 – Genetic polymorphism – Monoclonal antibody

Zusammenfassung. Eine Variante der Erythrozyten-Bande 3 (EPB 3), nämlich die Memphis-Variante (EPB 3*Memphis), wurde mit Hilfe eines monoklonalen Antikörpers, welcher gegen die 41 kDa zytoplasmatische N-terminale Domäne der Bande 3 gerichtet ist, und des Immunoblottings ohne Proteasebehandlung der Erythrozyten nachgewiesen. EPB 3*Memphis wurde auch mit Hilfe des Immunoblottings aus 3 Monate alten Blutspuren nachgewiesen, welche einer α-Chymotrypsinbehandlung unterzogen wurden. Eine populationsgenetische Studie mit Hilfe dieser Methode zeigte, daß die EPB*3-Variante nützlich für forensische Arbeiten in Japan sein würde, da die Häufigkeit dieser Variante bei Japanern (Präfektur Wakayama) relativ hoch ist (0,159).

Schlüsselwörter: Bande 3-Memphis – Erythrozyten-Bande 3 – Genetischer Polymorphismus – Monoklonaler Antikörper

Introduction

Erythrocyte band 3, a transmembrane glycoprotein (Mr = 90–100 kDa) which acts as an anion exchanger and plays a key role in membrane stability, is one of the major components of the human erythrocyte membrane [1, 2]. The C-terminal domain of band 3 (Mr = 55–60 kDa) spans the membrane several times and is a functional domain for anion exchange. The cytoplasmic N-terminal domain of band 3 (Mr = 41 kDa), however, binds to cytoskeletal proteins and glycolytic enzymes, and is not essential for anion exchange [3, 4]. Band 3 is detected as a diffuse band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), since a highly heterogenous carbohydrate chain is linked to the asparagine residue at its C-terminal domain. Band 3 is polymorphic and several variants have been reported [5–9]. The locus for band 3 (EPB 3) has been assigned to chromosome 17q12–q21 [10] and EPB 3*Memphis [11] is a genetically determined variant at the EPB 3 locus. The alternative allele is inherited codominantly and has been found to occur at appreciable frequencies in all populations tested, the frequency being higher in Asians and Africans than in Caucasians [11]. No abnormalities in the function or shape of erythrocytes have been observed even in homozygotes [5]. The amino acid substitution (Lys 56→ Glu) responsible for this variant gives N-terminal proteolytic fragments with Mr = 63 kDa and 43 kDa instead of those with Mr = 60 kDa and 41 kDa found in normal band 3 by SDS-PAGE [12]. This variant can be detected only in the proteolytic fragments of band 3 and not in intact band 3 by SDS-PAGE, since it is masked by the highly heterogeneous carbohydrate chain [5].

In this paper, we describe the immunological detection of EPB 3*Memphis from erythrocytes and bloodstains, and we report the gene frequency for this variant in a Japanese population.

Materials and methods

Specimens. Blood samples were collected from healthy donors and prepared immediately. Outdated blood samples, used for the population genetic study of EPB 3 polymorphism, were obtained from the Wakayama Red Cross Blood Center. Bloodstains were

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made by dropping whole blood onto cotton gauze and stored at room temperature.

Preparation of samples for SDS-PAGE. a) 50 µl of packed erythrocytes were lysed with 5 m*M* phosphate buffer, pH 7.4 (lysis buffer). b) Intact erythrocytes were digested with α-chymotrypsin solution (1 mg/ml in PBS) at 37°C for 1 h [12] and then lysed with lysis buffer. The resultant ghosts were dissolved in sodium dodecyl sulfate sample buffer (SDS sample buffer final concentrations: 10 m*M* Tris-HCl, pH 6.8, 1% SDS, 1% 2-mercaptoethanol, 20% glycerol, 0.001% bromophenol blue) and boiled for 5 min.

α-Chymotrypsin treatment of bloodstains. Bloodstains (1 cm^2) were washed once with PBS for 5 min, digested with 100 µl of α-chymotrypsin solution (15 µg/ml in lysis buffer) for 1 h at 4°C and subsequently washed with PBS. The digested bloodstains were boiled in 30 µl of SDS sample buffer for 5 min.

SDS-PAGE and immunoblotting. SDS-PAGE was performed on 10% polyacrylamide gels in a mini-PROTEAN II cell (BIO-RAD Lab., CA, USA) according to the method of Laemmli [13] and stained with Coomassie brilliant blue (CBB). Immunoblotting was carried out by separating the proteins on a SDS-polyacrylamide gel and electroblotting onto a PVDF membrane (Millipore, MA, USA) as described by Towbin et al. [14]. After soaking in blocking buffer (10 m*M* Tris-HCl, pH 7.4 containing 0.3% gelatin and 1.5 *M* NaCl), the blots were incubated with a monoclonal antibody (mAb, P3–9H [15]) to the cytoplasmic domain of band 3 (10 µg/ml in 10 m*M* Tris-HCl, pH 7.4 containing 0.5 M NaCl, 0.3% gelatin and 0.2% Tween–20) for 1 h at room temperature. Bound mAb was identified with peroxidase-conjugated goat anti-mouse IgG (× 2,000 dil.; CALTAG Lab., CA, USA) using 4-chloro-1-naphthol as the substrate for color development.

Nomenclature. The corresponding genes for the common and Memphis variant types of band 3 were designated EPB 3*C and EPB 3*M, respectively.

Results and discussion

The EPB 3*Memphis (EPB 3*M) variant can only be detected by SDS-PAGE with Coomassie brilliant blue (CBB) staining after digestion with α-chymotrypsin as anomalous bands with 63 kDa and 43 kDa, corresponding to the N-terminal fragments of band 3 (Fig. 1A, B), since band 3 carries a highly heterogeneous carbohydrate chain on the asparagine residue in the 35 kDa C-terminal domain [5]. It is known that band 3 is degraded by intrinsic proteases during aging in vivo [16]. Although substantial amounts of the N-terminal fragments of band 3 must be present, even in intact erythrocytes, these fragments can not be identified by SDS-PAGE with CBB staining. However, these fragments can be identified in intact erythrocytes by immunoblotting with monoclonal antibody (mAb) P3–9H to the 41 kDa N-terminal fragment of band 3 [15]. Thus, the EPB 3*M variant was detected by immunoblotting using P3–9H without α-chymotrypsin digestion of erythrocytes (Fig. 2A). Additional 34 kDa variant and 32 kDa normal bands were identified in the immunoblotting of erythrocyte membranes digested with α-chymotrypsin (Fig. 2B). Fragments of 60 and 63 kDa are generated by α-chymotrypsin treatment and all fragments, namely 63, 60, 43, 41, 34 and 32 kDa, can be generated by intrinsic proteases in vivo and during sample preparation. The alignment of

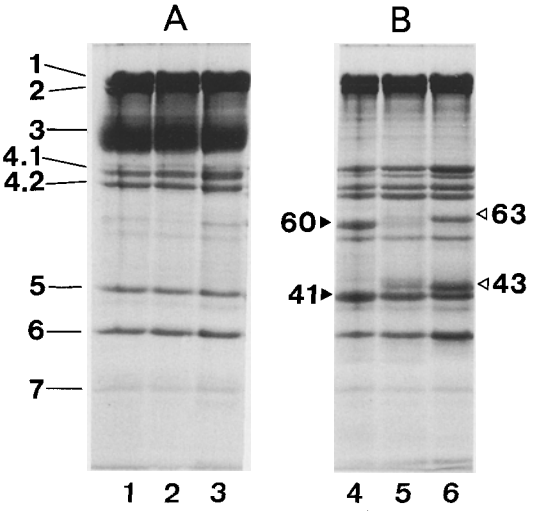

Fig. 1A, B. SDS-PAGE analysis of erythrocyte membrane band 3 Memphis. Membrane proteins of undigested (**A**) and α-chymotrypsindigested (**B**) erythrocytes were separated by SDS-PAGE on a 10% acrylamide gel and stained with CBB. Lanes 1 and 4, normal erythrocytes; lanes 2 and 5, heterozygotes for EPB 3*Memphis; lanes 3 and 6, homozygotes for EPB 3*Memphis. Closed arrowheads indicate fragment peptides derived from normal band 3. Open arrowheads indicate fragment peptides derived from band 3 Memphis. Proteins in the erythrocyte membrane are numbered according to the system of Fairbanks et al. [19]

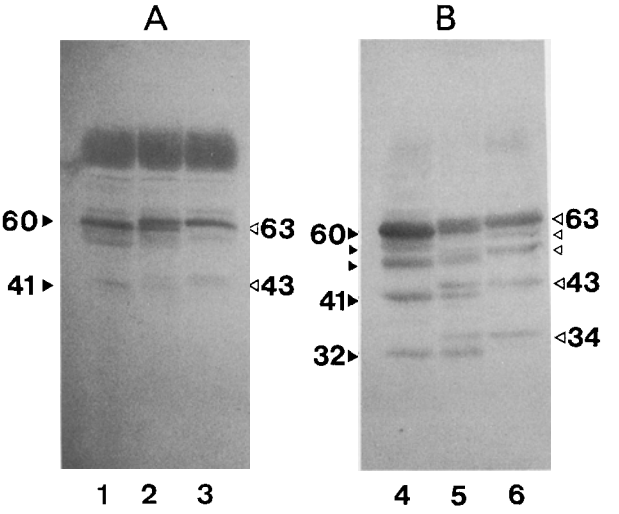

Fig. 2A, B. Immunoblot analysis of erythrocyte membrane band 3 Memphis. Membrane proteins of undigested (**A**) and α-chymotrypsindigested (**B**) erythrocytes were separated by SDS-PAGE on a 10% acrylamide gel, blotted electrophoretically onto a PVDF membrane and stained with mAb P3–9H to the cytoplasmic domain of band 3. Lanes 1 and 4, normal erythrocytes; lanes 2 and 5, heterozygotes for EPB 3*Memphis; lanes 3 and 6, homozygotes for EPB 3*Memphis. Closed arrowheads indicate fragment peptides derived from normal band 3. Open arrowheads indicate fragment peptides derived from band 3 Memphis

these proteolytic fragments and the amino acid substitution positions are shown in Fig. 3. Detection of the EPB 3*M variant in intact erythrocyte membranes by immunoblotting with P3–9H was much easier than detection by staining of protease-digested erythrocyte membrane proteins with CBB. The banding patterns on

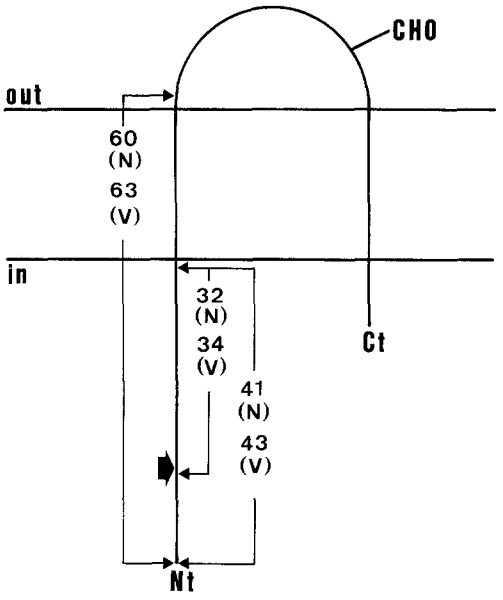

Fig. 3. Alignments of band 3 fragment peptides in normal (*N*) and variant (*V*) erythrocytes. The *arrow* and CHO indicate the position of the amino acid substitution and the carbohydrate chain, respectively. The molecular weights given are $\times 10^3$

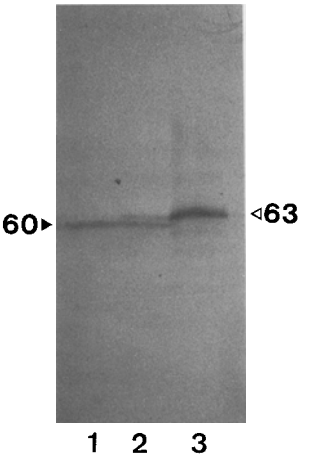

Fig. 4. Detection of band 3 Memphis from bloodstains by immunoblotting. The proteins in the bloodstains (1 cm^2) digested with α-chymotrypsin were separated by SDS-PAGE and blotted electrophoretically onto a PVDF membrane. The band 3 fragment peptides on the membrane were detected with P3–9H. Lane 1, bloodstain of normal erythrocytes; lane 2, bloodstain of heterozygotes for EPB 3*Memphis; lane 3, bloodstain of homozygotes for EPB 3*Memphis

Table 1. Frequency of EPB 3*Memphis in Japanese

Phenotype	Observed		Expected	
	No.	%	No.	%
MM	9	2.22	10.24	2.53
CM	111	27.41	108.31	26.74
CC	285	70.37	286.45	70.73
Total	405	100.00	405.00	100.00

Gene frequency (M): 0.159; $\chi^2 = 0.222$; $0.7 > P > 0.5$

the blots were much simpler and clearer than those on the CBB-stained gel.

A family study has supported the theory that EPB 3 polymorphism is controlled by two autosomal codominant genes, EPB 3*C and EPB 3*M [11]. The 2 homozygous phenotypes (CC or MM) consist of 2 major bands, whereas the heterozygous phenotype (CM) reveals a composite pattern of these components. Table 1 presents the results of a population study for EPB 3 polymorphism carried out on 405 unrelated Japanese living in the Wakayama prefecture of western Japan. The gene frequencies were calculated to be 0.841 and 0.159 for EBP 3*C and EBP 3*M, respectively. The observed distribution was in good agreement with the Hardy-Weinberg law. The paternity exclusion efficiency was estimated to be 11.6%. The frequency of EBP 3*M in this Japanese population (0.159) was signigicantly higher than that reported for other populations e.g. American Indians (0.098), African Americans (0.091), Filipinos (0.100) and Caucasians (0.040) [11]. Further population genetic studies of the EPB 3 polymorphism are needed to clarify why the frequency of EPB 3*M is significantly higher in Japanese than in other populations.

A single point mutation (A→G) in the first base of codon 56 of EPB 3*M leads to substitution of Lys-56 by Glu. This mutation is detected in SDS-PAGE as bands differing in molecular weight from common ones. The same variant bands have also been observed in Southeast Asian ovalocyte (SAO) and band 3 Tuscaloosa, which cause morphological abnormalities and spherocytic hemolytic anemia, respectively [17, 18]. SAO carries a deletion of codons 400–408 [17] and band 3 Tuscaloosa carries a point mutation (C→G) in the second base of codon 327 of EPB 3, in addition to the mutation in EPB 3*M [18]. However, it is unlikely that these variants contribute to the results in our population study of EPB 3*M in Japanese, since the distribution of SAO is restricted in southeast Asia and none of the subjects in our population study had anemia.

EPB 3 polymorphism from bloodstains was not detected by any method other than immunoblotting with P3–9H. However, for the detection of the variant from bloodstains by immunoblotting, α-chymotrypsin digestion was indispensable (Fig. 4) as the results were greatly affected by the conditions for protease digestion of bloodstains. Mild conditions (15–30 μg/ml α-chymotrypsin, for 1 h at 0°C) gave the best results. It was possible to type the band 3 variant from 1 cm^2 bloodstains even after 3 months.

In conclusion, the present method enabled EPB 3*M to be detected more easily and clearly from erythrocytes that were not subjected to protease digestion and from bloodstains that were so treated. The frequency of EPB 3*M is relatively high in Japanese, therefore, this polymorphism should be a useful marker for forensic investigations and paternity tests.

Acknowledgement. We would like to thank Dr. I. Yuasa for valuable comments and suggestions.

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