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GENETIC VARIANTS OF THE HUMAN DIAPHORASE DIA_3 IN JAPANESE: REPORT OF A NEW RARE ALLELE, DIA_3^4

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Summary Sperm-lysates from 264 unrelated Japanese males were tested for the polymorphism of the human diaphorase DIA_3 by isoelectric focusing in thin-layer polyacrylamide gel over pH range 3.5–9.5 gradient. The occurrence of a new rare allele, DIA_3^4, in addition to the previously described three alleles in German and British populations; has been demonstrated in our population. The estimated allele frequencies of the commonly encountered phenotypes, *i.e.* $DIA_3$1-1, 2-1, 3-1, 2-2 and 3-2, were: $DIA_3^1 = 0.837$, $DIA_3^2 = 0.143$ and $DIA_3^3 = 0.020$.

INTRODUCTION

Electrophoretic variants of the human diaphorase (DIA_3) were first demonstrated by Caldwell *et al.* (1976) using electrophoresis in polyacrylamide gel. In a population sample from USA, they described three electrophoretic patterns as 'sperm specific' which are determined by two common autosomal alleles. Subsequently, using two different techniques, polyacrylamide gel electrofocusing and agarose electrophoresis, for investigating the diaphorase (DIA_3) polymorphism, Kühnl *et al.* (1977) reported the existence of an additional third common allele in Germans beside the previously observed two in the USA population. Diaphorase (DIA_3) activity was also observed in extracts of the ovary, oviduct and uterus; so the term 'gonadal diaphorase' was proposed instead of 'sperm diaphorase' which was given by Caldwell *et al.* (1976). Similarly, Edwards *et al.* (1979) identified the three common diaphorase (DIA_3) genes in a survey of the British population, when a modification of the starch gel electrophoresis method used by Fisher *et al.* (1977) was employed in their study. On the other hand, Fisher *et al.* (1977) applied the notation 'diaphorase DIA_3' as the activity was observed not only in the sperm and female

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reproductive tract tissues but also in several others such as foetal tissues including placenta and adult brain extracts. Their notation was adopted here.

This study deals with the polymorphism of the human diaphorase (DIA_3) in the Japanese.

MATERIALS AND METHODS

Samples. Semen samples were collected from 264 unrelated Japanese males (16 volunteers and 148 outpatients) from Miyagi and Yamagata prefectures, Japan. Seminal plasma was separated by high speed refrigerated centrifugation at 12,000 rpm, and sperm-lysates were prepared according to the method of Black and Sensabaugh (1978) and analysed fresh or kept frozen at $-20°C$ until tested within few days.

Isoelectric focusing. The run was performed in the LKB Multiphor 2117 electrofocusing apparatus (Bromma, Sweden) in conjunction with the auto-conversion power unit, Model 2000-200 Auto Deluxe KPI (Kanagawa, Japan). Polyacrylamide gels of 0.3 mm thickness were prepared as described elsewhere (Sebetan and Akaishi, 1981), providing an gel concentration (T)=5% and degree of cross linkage (C)=7.5%. A 3.5% mixture of LKB ampholine carrier ampholytes over pH ranges 3.5–9.5 and 5–8 (6 : 1, v/v). After polymerization of the gel in the presence of riboflavin and UV light was completed, the mould was kept in the refrigerator overnight before use. Paper strips were saturated with 1 M phosphoric acid and 1 M ethanolamine and used at the anode and cathode, respectively. Pieces of filter paper 5×7 mm (Toyo No. 1) were placed on the gel surface at a distance of 2 cm from the anodal electrode strip and 10 μl of sperm-lysates were added. The power unit was adjusted to supply initial voltage of 300 V and maximum of 1,250 V. The total focusing time was about 3 hr. A cooling system with circulating water at 2°C was used during running. Visualization of the isozyme band patterns was accomplished by the following mixture; 5 mg of NADH, 0.1 mg of 2,6-dichlorophenolindophenol sodium and 2.5 mg MTT were dissolved in 2 ml of 0.2 M Tris-NCL buffer (pH 8.4), then added to 1% melted agar in 8 ml of the same buffer. The mixture was poured on the surface of the gel and the isozyme pattern could be seen after the gel was incubated at 37°C for about 20 min. Preservation of the gel was carried out as described previously (Sebetan *et al.*, 1982).

RESULTS AND DISCUSSION

Electrofocusing pattern obtained from the five common diaphorase DIA_3 phenotypes encountered in our population sample is illustrated in Fig. 1. The homozygous phenotypes are represented by major cathodal zone of two close isozymes with corresponding minor anodal pattern. The heterozygotes showed composite pattern consisting of two major and two minor zones, except for the heterozygote phenotype DIA_3 3-1 with three isozyme zones, since the isoelectric points of the

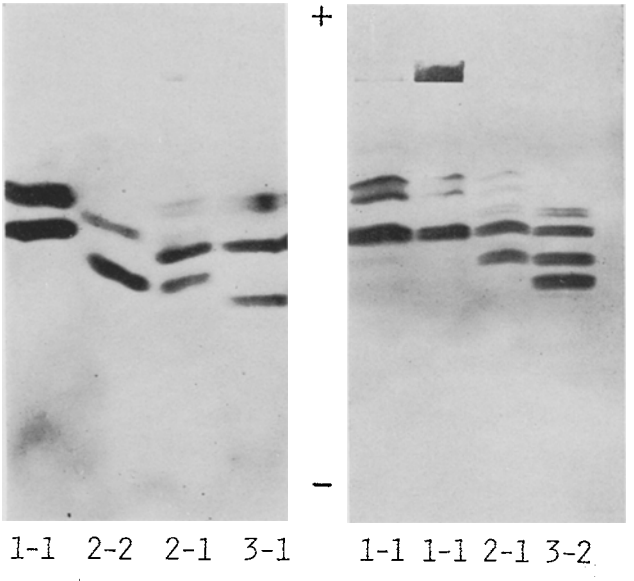

Fig. 1. Electrofocusing pattern showing five of the six common DIA_3 phenotypes (pH 3.5–9.5).

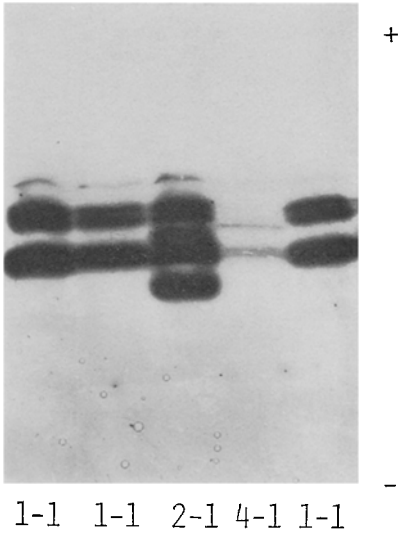

Fig. 2. Electrofocusing pattern showing the new variant phenotype (pH 3.5–9.5).

DIA_3^1 major isozymes and DIA_3^3 minor isozymes are exactly identical. The complete pattern of DIA_3^3 isozymes was clearly visible in a case of the heterozygote phenotype DIA_3 3-2. Besides the five common phenotypes, a new rare variant, designated as DIA_3 4-1, was detected in our population. The major isozyme of the

variant allele was found to be electrofocused slightly cathodal to the minor zone of the common allele DIA_3^1, while the minor isozyme was anodal to the minor zone of the DIA_3^1. The isozymes of the rare allele differ from that of the common ones in that they are represented by a single band as shown in Fig. 2. The staining intensity of the major isozymes was higher than the minor ones in all identified alleles. In addition to the above mentioned isozymes, two anodal bands were also observed with all the phenotypes. The isoelectric points of these bands were similar in all the common phenotypes and the variant one, which may suggest existence of an additional diaphorase gene locus in the sperm. Diagrammatic representation of the observed phenotypes pattern is given in Fig. 3.

The distribution of the common phenotypes is presented in Table 1. The calculated allele frequencies of our population sample were found to be: $DIA_3^1=0.837$, $DIA_3^2=0.143$ and $DIA_3^3=0.020$. The observed and expected values provide a good fit to the Hardy-Weinberg equilibrium ($\chi_0^2=1.05$, $0.75<p<0.80$, d.f.$=3$).

The published data on the diaphorase DIA_3 polymorphism among the different

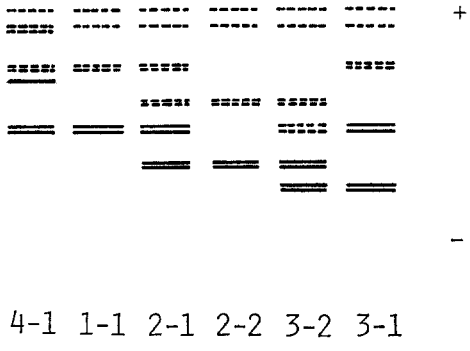

Fig. 3. Diagram showing the electrofocusing pattern in various DIA_3 phenotypes (pH 3.5–9.5).

Table 1. Distribution of the human diaphorase (DIA_3) phenotypes in Japanese.

Phenotypes	Number observed	Frequency observed (%)	Number expected	Allele frequencies
1-1	185	70.3	184.2	$DIA_3^1=0.837$
2-1	60	22.8	63.0	$DIA_3^2=0.143$
3-1	10	3.8	8.8	$DIA_3^3=0.020$
2-2	7	2.7	5.4	
3-2	1	0.4	1.5	
3-3	0	0	0.1	
Total	263	100.0	263	

Table 2. Comparison of the reported gene frequencies of the human diaphorase (DIA_3) phenotypes among different populations.

Population	N	Allele frequencies			References
		DIA_3^1	DIA_3^2	DIA_3^3	
USA	52	0.71	0.29	—	Caldwell *et al.* (1976)
German	141	0.76	0.22	0.02	Kühnl *et al.* (1977)
British	145	0.80	0.20	—	Fisher *et al.* (1977)
	346	0.76	0.23	0.01	Edwards *et al.* (1979)
Japanese	51	0.84	0.16	—	Suyama *et al.* (1979)
	263	0.837	0.143	0.020	This study

populations are summarized in Table 2. The frequencies obtained from our population sample are also given for comparison. Generally a marked difference was found among Japanese and Europeans or USA population samples. Our materials had a DIA_3^1 allele frequency markedly higher and DIA_3^2 allele frequency lower than those of Europeans or Americans. The third allele frequency was similar to or slightly higher than those of German or British, respectively. The differences between the previously reported data in Japanese (Suyama *et al.*, 1979), could be interpreted by better resolution in the present condition.

The probable correlation between the high frequency of DIA_3^1 and a reduced fertility stated by Kühnl *et al.* (1977) was not confirmed here, as the DIA_3^1 frequency of the healthy sample tested by Suyama *et al.* (1979) was exactly similar to that of our sample (16 healthy and 147 patients).

The considerable differences among the ethnic groups studied will make this genetic marker a valuable tool in population genetic investigations and will be useful for individualization of semen in cases of sexual offences.

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REFERENCES

Black, E.T. and Sensabaugh, G.F. 1978. Genetic markers in human Semen. 11. Quantitation of polymorphic proteins. *J. Forensic Sci.* **23**: 717–729.

Caldwell, K., Black, E.T., and Sensabaugh, G.F. 1976. Sperm diaphorase: Genetic polymorphism of a sperm-specific enzyme in man. *Science* **191**: 1185–1187.

Edwards, Y.H., Potter, J.E., and Hopkinson, D.A. 1979. A comparison of biochemical properties of the human diaphorase (DIA_3) isozymes determined by the common alleles DIA_3^1, DIA_3^2 and DIA_3^3. *Ann. Hum. Genet.* **42**: 293–302.

Fisher, T.A., Edwards, Y.H., Putt, W., Potter, J., and Hopkinson, D.A. 1977. An interpretation of

human diaphorase isozymes in terms of three gene loci DIA_1, DIA_2 and DIA_3. *Ann. Hum. Genet.* **41**: 139–149.

Kühnl, P., Langanke, U., Spielmann, W., and Neubauer, M. 1977. Investigations on the polymorphism of sperm diaphorase in man.—Evidence for a third common allele, SD^3. *Hum. Genet.* **40**: 79–86.

Sebetan, I.M. and Akaishi, S. 1981. Alpha-1-antitrypsin (PiM) subtypes in Japanese. *Forensic Sci. Int.* **18**: 155–159.

Sebetan, I.M., Kudo, T., Hiraiwa, K., Ohno, Y., Kuroda, F., and Akaishi, S. 1982. Isoelectric focusing studies on the PGM_1 subtypes in the northern Japanese population. *Z. Rechtsmed.* in press.

Suyama, H., Nakasono, I., and Ohya, I. 1979. The distribution of common phenotype of sperm diaphorase in the Japanese. *Forensic Sci. Int.* **13**: 125–127.