Orosomucoid polymorphism: Determination by Separator Isoelectric Focusing and Demonstration of ORM*F subtypes.

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Genetic polymorphism of Orosomucoid (ORM=alpha$_1$-acid glycoprotein) was demonstrated already twenty years ago, and appeared to be based on two allelic genes (Schmid et al.1965). The only population study published comprised 220 Caucasians living in U.S.A. and the frequencies of the two alleles, S and F, were 0.36 and o.64, respectively (Johnson et al. 1969). Linkage on chromosome 9 of ORM to ABO , AK$_1$(Adenylate kinase), and ALADH (delta-Amino-levulinate dehydrase) was recently demonstrated (Eiberg et al. 1982). Based on the above frequencies the discriminative power of Orosomucoid is 60% and the theoretical chance of exclusion of non-fathers in paternity testing 18%. As application to Forensic Hemo genetics seems obvious, it was decided to reinvestigate the Orosomucoid polymorphism using modern techniques.

Materials

172 serum samples from unrelated Danes and sera from 33 families with 82 children were examined. Half of the family material originated from staff members with relatives, the other half was families from North Zealand. A great deal of the sera has been stored at - 20^{0}C up to four years.

Methods

Treatment of serum

6 drops of serum were dialysed 30 min. against 50 ml 0.1M CH_3COONa pH 5.5 using Millipore Filters (VSWP 02500). 25 µl dialysate was then incubated 16-18 hours at 37^{0}C with 15 µl neuraminidase (Clostridium perfringens, Boehringer, Mannheim, 1U/mg. 1mg of the enzyme was dissolved in 2.5 ml 0.1M CH_3COONa).

Electrophoresis

Conventionel agarose electrophoresis was performed ad modum Alper & Propp 1968 using a discontinuous buffer system (Ashton & Braden 1961). Wessel-buffer pH 8.6: LiOH 1.2g/l, H_3Bo_3 11.8g/l. Gel-buffer pH 8.4: 1 volume wessel-buffer, 9 volumes of a buffer containing Tris 6.2g/l, citric acid 1.6g/l.

Isoelectric focusing

The gel (0.5x110x205mm) contained 0.62% agarose (Isogel,LKB), 10.5% sucrose, 0.4% MES (2(N-morpholino)-ethane sulfonic acid,Sigma) 1.1% ACES (N-(2-acetamido)-2-aminoethane sulfonic acid,Sigma) 1.9 v/v % Ampholine pH 3.5-5 (LKB), and 5.5 v/v%

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Ampholine pH 4-6 (LKB). Isoelectric focusing was performed at 6°C for 30 min. with 1500 V as maximum and using a constant power mode adjusted after 0, 10 and 20 min. to 10, 20 and 25 W, respectively. Samples were applied on Whatman paper no 1 (4x5mm) at the cathodic side of the gel. Electrode solutions were 0.5M NaOH for the anode, 0.5M CH_3COOH for the cathode.

Immunofixation

After conventional electrophoresis the ORM phenotypes were identified by immunofixation. Unfractionated, undiluted rabbit anti-serum against orosomucoid were applied directly on to the surface of the gel. The specific antibody was raised in rabbits ad modum Johnson et al. 1969.
After isoelectric focusing immunoprinting (10min) was carried out on Cellulose Acetate (Satorius 25G 11200) using undiluted anti-orosomucoid (Dakopatt a/s) and staining at 70°C for 15 min with 0.12% Kenacid Blue R (BDH) dissolved in ethanol:acetic acid:water (25:8:67).

Immunoblotting

The technique used was mainly according to Bjerrum et al. 1983. NC-membrane: Nitrocellulose BA 85-SB (Schleicher & Schuell). Protein tranfer: capillary pressure for 10 min. The NC membrane, presoaked in water,was smoothed on the gel surface followed by a piece of wet filter paper, a stack of paper towels and a glass plate (470 g). After air drying (30-60 min) the additional binding sites were blocked by agitating the membrane 15min in 15 ml 2% Tween 20 (polyoxyethylene sorbitan monolaurate) in washing-buffer (0.5% Tween 20, 50 mM Tris, 150 mM NaCl, pH 10.5), followed by 1x5min washing in the same buffer. Reaction with the primary antibody was performed overnight by agitating the membrane at 20°C with anti-orosomucoid (Dakopatt a/s) diluted 1/250 or 1/500 with washing buffer. The following morning the membrane was agitated 3x5 min in washing-buffer. The reaction with 15 ml of the secondary antibody (swine anti-rabbit immunoglobuline conjugated with alkaline phosphatase (Dakopatt a/s)) diluted 1/250, 1/500 or 1/1000 was performed for 1 hour, 20°C. Finally the membrane was washed three times (2x5 min in washing-buffer, 1x5min in 0.1M ethanolamine buffer pH 9.6) before development of the phenotypes with 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium (Blake et al. 1984).The reaction was stopped by washing with destilled water.

Results and Discussions

By isoelectric focusing it was found, that the electrophoretic F-band was subdivided as two bands with different pI values. The most acidic band was named F1, the other F2, and five phenotypes (F1, F1F2, F1S, F2S, and S) were observed (figure 1). Only the most infrequent of the expected six phenotypes , the homozygote F2, still has to be demonstrated. 182 neuraminidase treated samples were examined both by conventional electrophoresis and by isoelectric focusing,

and within the main groups F, FS, and S all the results agreed.
The distribution of the phenotypes observed in 215 Danes, and the frequencies of the three genes, calculated by gene counting, is given in table 1. The material was found to be in Hardy Weinberg equillibrium, and the total frequency of F1 plus F2, and the frequency of S, is in accordance with the results obtaind by conventional electrophoresis (Johnson et al. 1965, Eiberg unpublished).
The results of the examination of 33 families with 82 children are given in table 2, and 11 of the expected 21 mating classes were observed. In each combination group of parental types, the segregation of phenotypes in the children is in accordence with the assumtion of autosomal, codominant inheritance.
The method described using immunoprinting for identification of the ORM-subtypes is rather expensive, as the specific antibody has to be used undiluted, in order to obtain sharp and well-defined F-bands. Therefore, experiments with development of the ORM-types by immunoblotting was initiated. Both the primary antibody (anti-orosomucoid) and the phosphatase conjugated secondary antibody was used in high dilutions (1/250 - 1/1000), and the "anti-antibody" was visualised by a very sensitive histochemical staining method. 5-bromo-4-chloro-indoxyl phosphate was used as substrate and staining was performed with nitroblue tetrazolium (Blake et al 1984), but compared to the immunoprinting technique the pattern of the ORM-subtypes were more diffuse, and further experiments with the immunoblotting method is planned.

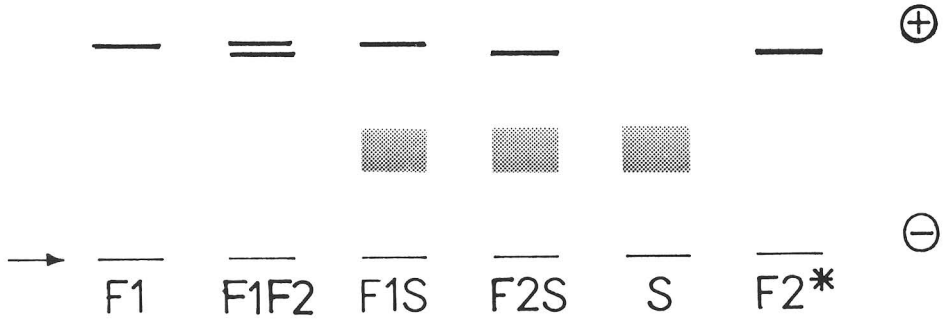
In summary, determination of Orosomucoid by isoelectric focusing extends the number of common phenotypes from three six, and the theoretical chance of exclusion of non-fathers from 18% to 21%. However, before application to paternity testing can be recommended analysis of a larger material, including mother/child pairs, should be performed.

References

Alper, C.A. and Propp, R.P.: Genetic polymorphism of the third component of human complement (C3) J. Clin. Invest:47 2181 (1968).
Ashton, G.C. and Braden, A.W.H.: Serum beta globulin polymorphism in mice. Aust J Biol Sci 14:248-253 (1961).
Bjerrum, O.J.,Larsen, K.P. and Wilken, M.: Some recent developments of the electroimmunochemical analysis of menbrane proteins. Application of Zwittergent, Triton X-114 and Western blotting technique.; in Tschesche, Modern Methods in Protein Chemistry, pp.79-124 (W.Gruyter Co.,Berlin 1983).
Blake, M.S., Johnston, K.H., Russell-Jones, G.J., Gotschlich, E.C.: A rapid, sensitive method for detection of Alkaline

Phosphatase conjugated anti-antibody on Western blots. Anal. Biochem. 136:175-179 (1984).
Johnson, A.M., Schmid, K., Alper, C.A.: Inheritance of human alpha$_1$- Acid Glycoprotein (Orosomucoid) variants. J.Clin.-Invest. 48:2293-2299 (1969).
Eiberg, H., Mohr, J., Nielsen, L.S.: Linkage of Orosomucoid (ORM) to ABO and AK$_1$. Cytogenetics and Cell Genetics 32:272 (1982).
Schmid, K., Tokita, K., Yooshizaki, H.: The alpha$_1$-Acid Glycoprotein Variants of Normal Caucasian and Japanese Individuals. J. Clin. Invest. 44:1394-1401 (1965).

ORM-phenotypes as demonstrated either by immunoprinting (cellulose acetate) or by immunoblotting (nitrocellulose)



* hypotetic pattern
origin

Distribution of ORM-phenotypes in 215 Danes determined by Isoelectric Focusing

	F1	F2	S	F1F2	F1S	F2S
obs	77	0	33	5	91	9
exp	72.7	0.2	32.0	8.1	96.5	5.4

chi^2= 4.4315 d.f.2 0.1<p<0.2

Gene frequencies:	ORM*F1	ORM*F2	ORM*S
This study :	0.581	0.033	0.386
Eiberg et al. n=1679 (unpublished) :	0.611		0.389

Segregation of ORM-phenotypes in 33 families with 82 children.

Matings	n	Children (n=82) F1	S	F1F2	F1S	F2S
F1 x F1	5	11	-	-	-	-
F1 x S	4	-	-	-	10	-
F1 x F1F2	1	0	-	1	-	-
F1 x F1S	9	12	-	-	13	-
F1F2 x F1S	2	2	-	0	2	3
F1F2 x S	1	-	-	-	1	1
F1S x F1S	5	3	3	-	6	-
F1S x F2S	1	-	2	0	1	0
F1S x S	2	-	2	-	4	-
F2S x S	1	-	1	-	-	1
S x S	2	-	3	-	-	-
Total	33	28	11	1	37	5