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Association between ACP_1 genetic polymorphism and favism

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ABSTRACT. An association between favism (a hemolytic reaction to consumption of fava beans), glucose-6-phosphate dehydrogenase deficiency ($G6PD^-$) and acid phosphatase locus 1 (ACP_1) phenotypes has been reported; the frequency of carriers of the p^a and p^c ACP_1 alleles was found to be significantly higher in $G6PD^-$ individuals showing favism than in the general population. Here, we investigated the hypothesis that favism is caused by toxic *Vicia faba* substances, which in some ACP_1 phenotypes cause increased phosphorylation and consequently increased glycolysis, with strong reduction in reduced glutathione production, resulting in hemolysis. It has been demonstrated that ACP_1 *f* isoforms have physiological functions different from those of *s* isoforms and are responsible for most of the phosphatase activity, in addition to being less stable in the presence of oxidizing molecules. Thus, the C, CA and A phenotypes, characterized by lower concentrations of *f* isoforms, could be more susceptible to damage by oxidative events compared to the other phenotypes. To test this hypothesis, the (*f*+*s*) enzymatic activity of different ACP_1 phenotypes with and without added *V. faba* extract was analyzed. Enzymatic activities of ACP_1 A, -CA, -C groups (low activity) and -B,

-BA, -CB groups (high activity) were significantly different after addition of *V. faba* extract. Phenotypes A, CA and C had extremely low enzymatic activity levels, which would lead to low levels of reduced glutathione and bring about erythrocyte lysis.

Key words: Acid phosphatase locus 1; Genetic polymorphism; Favism; Glucose-6-phosphate dehydrogenase deficiency; LMW protein tyrosine phosphatases

INTRODUCTION

An association between hemolytic favism and acid phosphatase locus 1 (ACP_1) genotype was described over thirty years ago (Bottini et al., 1971a). Upon ingesting *Vicia faba* beans, approximately 30% of the subjects deficient in glucose-6-phosphate dehydrogenase ($G6PD^-$) (EC 1.1.1.49) underwent a hemolytic crisis. The susceptibility to favism was shown to be correlated to the ACP_1 genotype in the order BB < AB,BC < AA,AC. At the time, it was not possible to propose a satisfactory explanatory mechanism.

More recently, two hypotheses have been formulated to explain the association between favism, $G6PD^-$ deficiency and ACP_1 alleles. Starting with the notion that reduced levels of reduced glutathione (GSH) are responsible for red blood cell lysis (Beutler, 1969; Johnson et al., 1994), the hypotheses differ in the mechanism leading to a reduction of GSH levels.

1) The ACP_1 enzyme (EC 3.1.3.2) dephosphorylates flavin mononucleotide to riboflavin (Fuchs et al., 1992). By modulating the cellular concentration of flavin mononucleotide and thereby flavin adenine dinucleotide, ACP_1 may influence the activity of glutathione reductase (EC 1.8.1.7), where flavin adenine dinucleotide acts as a coenzyme. This hypothesis, however, does not fully explain the biochemical mechanism because the ACP_1 genotypic order of susceptibility to favism BB < AB,BC < AA,AC is different from that based on enzymatic activity, which is AA < AB < BB,AC < BC.

2) An alternative biochemical basis for favism was also suggested (Dissing, 1993; Bottini et al., 1997). ACP_1 isoforms were shown to dephosphorylate band 3 protein (B3P) (Boivin and Galand, 1986). When one or two tyrosines in the amino terminal portion of this protein are phosphorylated, binding of glycolytic enzymes such as aldolase (EC 4.1.2.13), phosphofructokinase (EC 2.7.1.11) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) are inhibited.

Thus, ACP_1 would modulate the glycolytic activity through its phosphatase activity, which determines the phosphorylation level of B3P. A decreased phosphatase activity leading to enhanced phosphorylation of B3P would lead to enhanced glycolysis, causing a decreased nicotinamide adenine dinucleotide phosphate (NADPH) level, which would ultimately block GSH production and cause red blood cell lysis. Each ACP_1 allele encodes a different amount of the *f* and *s* isoforms (Dissing, 1993). It was suggested that the inhibition of ACP_1 *f* isoforms, caused by toxic substances contained in *V. faba*, could cause damage in the C, CA and A phenotypes, constitutionally with lower concentration of *f* isoforms, more than in the other phenotypes (Bottini et al., 1997).

To throw light on the mechanism leading to favism, a *V. faba* extract (*Vfe*) was pre-

pared, its oxidation capacity on GSH tested and the enzymatic activity associated with 119 ACP_1 phenotypes was determined in the absence and presence of *Vfe*.

MATERIAL AND METHODS

Subjetcs

A total of 119 whole blood samples from anonymous individuals were obtained from the Macerata Hospital Transfusion Center, Macerata, Italy. Ethics approval and patient consent statements were not required in this case.

Vicia faba extract

Vfe was prepared as described elsewhere (Bottini et al., 1970; Beutler, 1975) with slight modifications. A total of 450 g *V. faba* beans were homogenized and added to an equal weight of water and a chloroform-methanol mix (chloroform-methanol, 1:1). The resulting mixture was stirred for 20 h at room temperature. The liquid phase was then separated from the solid residue by two sequential filtrations on a filter funnel and Whatman 3MM paper. The liquid phase was separated using a separation funnel into an organic phase that was discarded, and an aqueous phase, which was concentrated by roto-evaporation at 37°C until a solid residue, was obtained. This was resuspended in distilled water. After clarification by centrifugation at 257,000 *g* for 1 h, the resulting supernatant (50 mL) was used to carry out glutathione oxidation experiments and ACP_1 activity assays.

GSH assay

To evaluate the oxidative properties of *Vfe*, GSH oxidation was determined as described by Beutler (1975) with slight modifications (Figure 1).

The oxidative effect of *V. faba* bean extract was measured using 5,5' dithiobis-2-nitrobenzoic acid (DTNB), which reacts with GSH to generate glutathione disulfide and 2-nitro-5-thiobenzoic acid, a yellow-colored product. The GSH concentration was determined by measurement of the absorbance at 410 nm. The reaction mixtures, in a final volume of 5 mL, containing 0.1 M phosphate buffer, pH 7.4, 0.02 M EDTA, and 0.005 M GSH, with and without *V. faba* bean extract, were incubated at 37°C. After the incubation time, an aliquot of the mixture was added to DTNB and the absorbance was measured against the blank.

ACP_1 genotyping

DNA was extracted from whole blood samples using standard procedures. ACP_1 genotyping of single nucleotide polymorphisms characterized by a silent C-T transition at codon 41 within exon 3 and an A-G transition at codon 105 within exon 6 was performed by restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR), essentially as described elsewhere (Bottini et al., 2002). Following PCR amplification of the regions of interest, the resulting 341- and 299-bp amplicons were digested with restriction endonucleases *Cfo*I and *Taq*I, respectively, and analyzed on 1.8% agarose gels. *Cfo*I digestion of the 341-bp

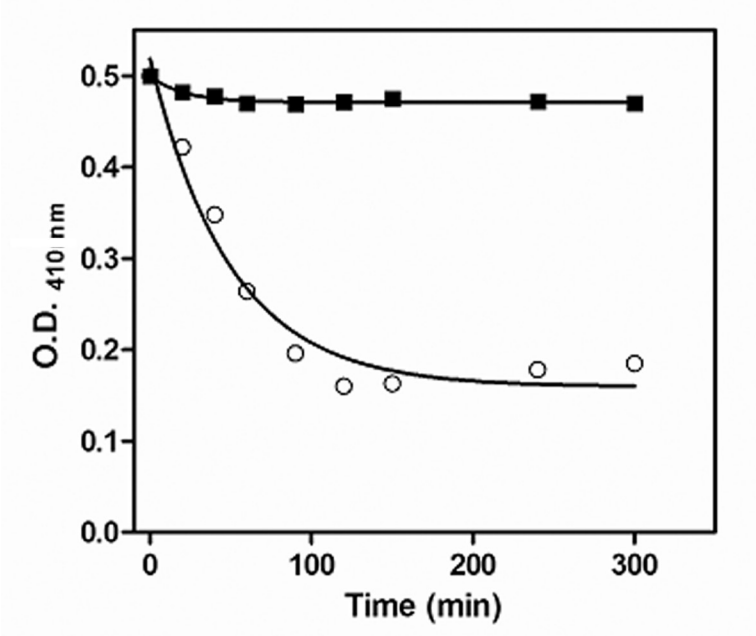

Figure 1. Oxidative effect of *Vicia faba* bean extract on GSH. Squares = reaction mixture without *V. faba* bean extract; circles = reaction mixture with *V. faba* bean extract.

exon 3 amplicon yielded 2 fragments of 255 and 86 bp for the ACP_1*A and ACP_1*B alleles, while the ACP_1*C allele was not cut. Similarly, the *Taq*I digested a 299-bp amplicon and generated 2 fragments of 100 and 199 bp for the ACP_1*A allele, but not for the *B and *C alleles. Thus, comparative analyses of the two RFLPs allowed us to determine the two haplotypes and from that the ACP_1 genotypes.

The ACP_1 genotypic distribution determined in our sample population was: BB = 41; BA = 42; CB = 15; AA = 11; CA = 9; CC = 1. The distribution was in Hardy-Weinberg equilibrium ($P_{3d.f.}$ = 0.8).

ACP_1 enzymatic activity assay

ACP_1 activity, with and without *Vfe*, was performed as reported previously by us (Lucarini et al., 1989). ACP_1 activity was determined for each sample with and without *Vfe* (50 µL) in a 500-µL reaction mixture. Total protein was determined for each sample with the Bradford method (Bradford, 1976). A unit of activity was expressed as 1 µmol p-nitrophenol produced per minute per gram protein at 37°C.

Statistical analysis

The Student *t*-test and analysis of variance were performed using the SPSS program (Nie et al., 1975).

RESULTS

The results obtained are presented in Table 1, which lists the total activity (*f*+*s*) of the different ACP_1 phenotypes found in the absence and presence of *Vfe*. We also reported the *f* isoforms' mean activity of ACP_1 phenotypes calculated according to Dissing et al. (1993).

Table 1. Enzymatic activity of human ACP_1 in hemolysates of different phenotypes with and without *Vicia faba* bean extract (*Vfe*).

ACP_1 phenotype	Proportion of *f* and *s* activity (%)[a]		Total (*f*+*s*) activity without *Vfe*[b]	*f* activity without *Vfe* calculated as in *a*	Total (*f*+*s*) activity with *Vfe*[b]	*f* activity with *Vfe* calculated as in *a* and *c*
	Isoform	Activity				
B (N = 41)	*f*	75.7	25.86 (6.42)	19.57	16.36 (6.79)	12.34
	s	24.3				
BA (N = 42)	*f*	69.4	20.21 (4.66)	14.02	14.34 (3.10)	9.95
	s	30.6				
A (N = 11)	*f*	60.1	16.54 (4.94)	9.88	10.68 (4.07)	6.42
	s	39.9				
CB (N = 15)	*f*	39.3	39.13 (8.83)	15.37	24.32 (6.97)	9.57
	s	60.7				
CA (N = 9)	*f*	29.2	22.54 (4.02)	6.58	12.47 (4.20)	3.64
	s	70.8				
C (N = 1)	*f*	16.7	46.23	7.72	30.06	5.00
	s	83.3				

[a]As reported by Dissing et al. (1993). [b]Activity is calculated as µmol p-nitrophenol produced per minute per gram of protein at 37°C (SD in parentheses). [c]Values assuming that the activity of both *f* and *s* isoforms is reduced by the same percentage.

As seen in Table 1, the total (*f*+*s*) activities associated with different ACP_1 phenotypes are much lower in the presence than in the absence of *Vfe* [Student *t*-test (d.f. 118) = 12.466, P = 2.85 x 10^{-23}]. In the absence of *Vfe*, the differences between enzymatic activities associated with ACP_1 A, -CA, and -C phenotypes (mean = 19.46, SD = 8.04) and those associated with -B, -BA, -CB phenotypes (mean = 23.88, SD = 9.04) show a statistically significant albeit minor difference (d.f. = 1, F = 4.007, P = 0.048). On the other hand, in the presence of *Vfe*, the difference between the two phenotype groups (mean = 12.04, SD = 5.65 and mean = 16.71, SD = 6.44, respectively) reaches a highly significant level (d.f. = 1, F = 9.466, P = 0.003). In particular, assuming that the *f* and *s* isoform activities are reduced by the same proportion, the distribution of phosphatase activity values associated with *f* isoforms in the presence of *Vfe* (that is: B*f* = 12.34; A*f* B*f* = 9.95; A*f* = 6.42; B*f* C*f* = 9.57; A*f* C*f* = 3.64; C*f* = 5.00) shows that the activity of isoform *f* of ACP_1 reaches much lower values in C, CA, and A phenotypes than in B, BA, BC phenotypes.

DISCUSSION

ACP_1 enzymatic activity is determined by two types of isoforms (*f* and *s*) (Dissing, 1993), and each ACP_1 phenotype expresses different amounts of the *f* and *s* isoforms. These two isoforms have different biochemical properties. In fact, when the effect of oxidizing agents and heat treatment on the electrophoretic patterns of hemolysates was analyzed, the ACP_1 *s* isoforms were found to be endowed with a higher stability compared to the *f* isoforms (Luffman and Harris, 1967; Fisher and Harris, 1971) and to be more stable under the action of oxidized glutathione and

acetylphenylhydrazine (Bottini et al., 1971b). Furthermore, results obtained with the pure A*f*, A*s*, B*f*, B*s*, C*f*, and C*s* isoforms confirmed that *s* isoforms are more stable than *f* isoforms *vis-à-vis* urea denaturation and heat treatment (Fisher and Harris, 1971; White and Butterworth, 1971).

These findings, together with the different behavior of *f* and *s* isoforms towards inhibitors and activators (Dissing et al., 1993), strongly suggest that the two isoforms, which are expressed at the same time in different tissues (Fujimoto et al., 1988), perform different biological functions *in vivo* (Dissing and Svensmark, 1990; Stefani et al., 1993) and that *f* isoforms are the main ones responsible for phosphatase activity of the ACP_1 gene, rather than *s* isoforms. Furthermore, it has been demonstrated (Stefani et al., 1993) that the 5-16 phosphotyrosines present in the N-terminal peptide of human B3P are more efficiently dephosphorylated by *f* isoforms than by *s* isoforms [K_m (mM) 1.4→0.4; V_{max} (μmol·min^{-1}·mg^{-1}) 68→10.5].

In light of that mentioned above, our data show that *f* isoforms, which are more sensitive to oxidative damage compared to *s* isoforms (Luffman and Harris, 1967; Fisher and Harris, 1971; Bottini et al., 1971b), are likely to undergo inhibition by *Vfe* more than that reported in the last column of Table 1, where the *f* activity is calculated reducing it by the same proportion of *s* as reported by Dissing et al. (1993) and where the much higher inhibition of *f* isoforms compared to *s* isoforms is not taken into consideration. Therefore, considering that the values of *f* isoform activities in the presence of *Vfe* will be certainly lower than those that we have calculated here (i.e., AA = 6.42; CA = 3.64; CC = 5.00), the real *f* activity associated with ACP_1 AA, ACP_1 CA, ACP_1 CC genotypes will reach extremely low values. These phenotypes could have an increased phosphorylation of B3P tyrosines, an increased glycolysis, a consequent reduction of glucose-6-phosphate, and a further loss of NADPH in $G6PD^-$ subjects. In turn, this would be associated with levels of GSH so low as to be harmful to erythrocyte integrity.

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