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Molecular Genetics of the Glucose-6-Phosphate Dehydrogenase (G6PD) Mediterranean Variant and Description of a New G6PD Mutant, G6PD Andalus^{1361A}

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Summary

Glucose-6-phosphate dehydrogenase (G6PD; E.C.1.1.1.49) deficiency is the most common human enzymopathy; more than 300 different biochemical variants of the enzyme have been described. In many parts of the world the Mediterranean type of G6PD deficiency is prevalent. However, G6PD Mediterranean has come to be regarded as a generic term applied to similar G6PD mutations thought, however, to represent a somewhat heterogeneous group. A C→T mutation at nucleotide 563 of G6PD Mediterranean has been identified by Vulliamy et al., and the same mutation has been found by De Vita et al. in G6PD Mediterranean, G6PD Sassari, and G6PD Cagliari. The latter subjects had an additional mutation, at nucleotide 1311, that did not produce a coding change. We have examined genomic DNA of five patients—four of Spanish origin and one of Jewish origin—having enzymatically documented G6PD Mediterranean. All had both the mutation at nucleotide 563 and that at nucleotide 1311. A sixth sample, resembling G6PD Mediterranean kinetically but with a slightly rapid electrophoretic mobility, was designated G6PD Andalus and was found to have a different mutation, a G→A transition at nucleotide 1361, producing an arginine-to-histidine substitution. These studies suggest that G6PD Mediterranean is, after all, relatively homogeneous.

Introduction

Among human enzymes, glucose-6-phosphate dehydrogenase (G6PD) is the one with the greatest known variability. For this reason an expert committee of the World Health Organization standardized procedures for purification and characterization of G6PD variants (Betke et al. 1967). The use of these recommended techniques has resulted in the description of more than 300 variants of G6PD that are regarded as being biochemically distinct (Beutler and Yoshida 1988). However, even with standardized methods, it is difficult to ensure that G6PD variants that seem different are not actually the same (Fairbanks et al. 1980). Conversely, variants that seem to be the same can be different. With the cloning and sequencing of G6PD variants (Martini et al. 1986; Persico et al. 1986) we can now examine directly the mutations that cause the common types of G6PD deficiency. Such studies have already shown that G6PD A-, previously regarded as being homogeneous, is the result of several different mutations (Hirono and Beutler 1988; Beutler et al. 1989*a*).

G6PD Mediterranean is the most common G6PD-deficient variant in many parts of the world (Miwa et al. 1977; Luzzatto and Testa 1978; Beutler 1983; Bhattacharya and Mitra 1984; Luzzatto and Mehta 1990). This mutant is characterized by very low activity in erythrocytes, an electrophoretic mobility that is indistinguishable from that of normal G6PD, and a characteristic set of kinetic abnormalities, first described by Kirkman et al. (1964). These latter include low K_m values for the substrates NADP and glucose-6-phosphate, a biphasic pH optimum curve, thermal lability, and increased utilization of 2-deoxy glucose-6-phos-

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phate. However, more detailed biochemical characterization of G6PD Mediterranean from different subjects has led to the perception that G6PD Mediterranean is not a single genotype but is the result of several different mutations giving rise to biochemically very similar mutant enzymes (Kirkman et al. 1965; Stamatoyannopoulos et al. 1971; Vives-Corrons and Pujades 1980). The sequencing of a very limited number of variants resembling G6PD Mediterranean has now suggested that this deficiency may be genetically more homogeneous than had previously been thought. Vulliamy et al. (1988) have reported a C→T transition, at nucleotide 563, that causes a serine-to-phenylalanine substitution in a patient with G6PD Mediterranean. De Vita et al. (1989) subsequently demonstrated that both an additional sample of G6PD Mediterranean and two other G6PD variants from the Mediterranean region, which had been designated Cagliari and Sassari, had the same mutation. These variants all contained an additional mutation—a C→T at nucleotide 1311—which, however, did not change the amino acid sequence. To establish whether subjects with G6PD variants of the Mediterranean type from other than Italian populations have the same mutations, we have studied the DNA of additional G6PD-deficient males, determining also whether the C→T mutation was present at nucleotide 1311.

Material and Methods

Blood samples were obtained from five Spanish males who had come to medical attention because of an attack of favism and from one Ashkenazi Jewish male from the United States. Complete biochemical characterization of these samples was performed (see below).

Biochemical Characterization

Blood from the patients and normal controls was collected in heparin or in citrate-phosphate-dextrose anticoagulant and was stored at 4°C prior to purification. Leukocytes were isolated from whole blood by using the method of Boyum (1968). G6PD assays were performed by standard methods on freeze-thaw hemolysates (Beutler 1984). In the case of the Ashkenazi Jewish sample the first method recommended by the WHO scientific group (Betke et al. 1967) was employed. G6PD from the Spanish samples was partially purified from a leukocyte-enriched red cell suspension according to the method of Rattazzi (1969). This purification procedure is identical to the second method of the WHO scientific group, except that DEAE-Sephadex A-50 is used instead of DEAE-cellulose in order to obtain a better yield.

Characterization of partially purified enzyme was performed according to standard methods (Betke et al. 1967). Isoelectric focusing was carried out by a modification of the method of Basset et al. (1978) by using a pH gradient of 3.5–10.

Restriction-Enzyme Analysis of Genomic G6PD

DNA was purified from whole-blood samples or leukocytes (Kan and Dozy 1978). The relevant segment of DNA was amplified using the polymerase chain reaction (PCR) (Saiki et al. 1985) with oligonucleotides 7 and 8 (Hirono and Beutler 1989) and was tested for the presence of the 563 C→T mutation by using the restriction endonuclease *Mbo*II. The amplified fragment is 127 bp in size. It is cut into 54- and 73-bp pieces when the restriction site is present.

The mutation at nucleotide 1361 (see below) destroys an *Fsp*I restriction-endonuclease site. Amplification of the relevant fragment by PCR was performed using the oligonucleotides amplifying exons 11 and 12 (Beutler et al. 1989*c*), producing a fragment 400 bp in size. The normal sequence has a restriction site that results in formation of 120- and 280-bp fragments after treatment with *Fsp*I, while the mutant G6PD remains uncut after treatment.

Sequence Analysis

In the case in which the mutation at 563 was not present, all of the exons in the G6PD gene were amplified by PCR using primers designed from intron sequences as published previously (Beutler et al. 1989*c*). The amplified fragments were sequenced by the chemical cleavage method (Maxam and Gilbert 1977) and were compared with the known sequence of G6PD cDNA (Persico et al. 1986; Beutler and Yoshida 1988).

Identification of nucleotide 1311 was achieved by sequencing single-stranded DNA prepared from genomic DNA by using PCR (Hirono et al. 1989). The flanking oligonucleotides were 5′-ACTCCACATGGTGCAGGCAG and 5′-AATGTGCAGCTGAGGTCAAT, amplifying exons 11, 12, and part of 13. The nested oligonucleotide that made the sense strand was 5′-TGGCATCAGCAAGACACTCT. The sequencing primer was 5′-ATAGCCCACAGGTATGCAGG, which primes the antisense sequence of exon 11.

Results

The biochemical characteristics of the G6PD vari-

Table 1

Biochemical Characteristics of Six Characterized G6PD Variants

Patient	Clinical Manifestation	G6PD Activity (% normal)		Electrophoretic Mobility (% normal)			K_m G6P (μM)	K_m NADP (μM)	Substrate Analogue Utilization (%)		pH Optimum	Thermostability (% activity after 20 min incubation)	Ethnic Origin
		RBC	WBC	TEB pH 8.0	Phosphate pH 7.0	Tris pH 8.0			2dG6P	dNADP			
1	Favism	0	25	100	100	93	17.8	3.9	20	257	6.5 and 10	3	Catalonia
2	Favism	2.6	11	100	100	. . .	12.9	2.6	25	273	7 and 10	5.5	Catalonia
3	Favism	0	20	100	. . .	. . .	17.0	. . .	32	273	6.5 and 10	10	Catalonia
4	Favism	.5	27	100	100	100	20.1	1.4	23	250	6.5 and 10	5	Southern Spain
5		1.2	. . .	100	100	100	14.2	3.8	32	288	6.5 and 10	Labile	Ashkenazi Jew
6	Favism	.1	10	100	102	105	14.2	1.2	44	311	6.5 and 10	4.5	Southern Spain
Mediterranean	Favism	.7	Decreased	100	100	100	17 ± 4	2 ± .5	28.5 ± 8.5	261 ± 33	6.5 and 10	3.5–6	Mediterranean and elsewhere
G6PD B+	None	100	100	100	100	100	60 ± 9	8.6 ± 6	2.3 ± .5	48 ± 2	Truncate	80–90	Worldwide

ant studied are summarized in table 1, along with values obtained from blood samples obtained from 11 Spanish G6PD Mediterranean subjects and from 50 Spanish G6PD B subjects. In five patients (patients 1–5) the biochemical properties of the residual G6PD were identical to those described in G6PD Mediterranean. The enzyme from patient 6 has kinetic properties indistinguishable from those of the other variants, except for a slightly higher utilization of the substrate analogues 2-deoxy glucose-6-phosphate and deamino NADP. Moreover, the electrophoretic mobility of the enzyme both in the phosphate and Tris buffer systems was slightly faster than that of G6PD B at 102% and 105% of normal, respectively. A distinctly lower isoelectric point was also documented (fig. 1). The new variant was designated G6PD Andalus. In the first five cases the restriction site formed by the 563 C→T mutation was present, but it was absent from the DNA of the sixth patient. Sequencing of the exons of G6PD of this subject revealed the presence of a new mutation—G→A at nucleotide 1361—producing an arginine-to-histidine amino acid substitution. The existence of this mutation was confirmed by amplifying genomic DNA and showing that the normally present *FspI* site had been lost. All five subjects with the mutation at 563 were also found to have the C→T transition at nucleotide 1311 that had been described by De Vita et al. (1989) as being in association with the mutation at 563.

Discussion

Until the cloning of the G6PD gene by Luzzatto and his colleagues (Martini et al. 1986; Persico et al. 1986), polymorphic variants of G6PD were distinguished from each other only by painstaking biochemical character-

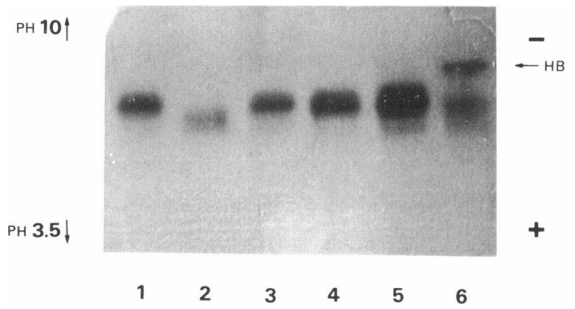

Figure 1 Isoelectric focusing patterns of G6PD. Lanes 1 and 3, G6PD from leukocytes from patients 4 and 3, respectively. Lane 2, G6PD from leukocytes of patient 6 (G6PD Andalus^{1361A}). Lanes 4 and 5, Normal G6PD from white blood cells. Lane 6, Normal G6PD from red blood cells.

ization. Although in most cases investigators have cooperated by using the standardized methods for the characterization of G6PD that were published more than 20 years ago (Betke et al. 1967), biochemical characterization has left much to be desired. Indeed, with examination at the DNA level, variants regarded as being identical have proved to be heterogeneous. For example, what was known as G6PD A- is now known to consist of at least three different genotypes (Beutler et al. 1989*a*). Conversely, variants that were thought to be different have proved to be the same. Thus, examples of G6PD Tepic, Castilla, Betica, Matera, Distrito Federal, and Alabama have all proved to be identical to G6PD A-$^{202A/376G}$ (Beutler et al. 1989*b*; E. Beutler and W. Kuhl, unpublished data).

In many parts of the world the most common type of G6PD is G6PD Mediterranean, originally designated G6PD B(−) because of its normal electrophoretic mobility. This variant is characterized by both very low residual erythrocyte G6PD activity and a number of abnormal kinetic properties. Until recently, G6PD Mediterranean had come to be regarded as somewhat of a generic term that applied to a group of very similar but probably heterogeneous variants found in the Mediterranean region (Kirkman et al. 1965; Stamatoyannopoulos et al. 1971; Vives-Corrons and Pujades 1980). After the original report of a C→T transition at nucleotide 563 of G6PD Mediterranean (Vulliamy et al. 1988), the same mutation was found in other, similar variants from the Mediterranean region. We now find that all five electrophoretically normal G6PD Mediterranean variants that we have examined by restriction-endonuclease analysis of PCR-amplified genomic DNA have the same mutation, even though they were drawn from two different population groups, i.e., Spanish and Ashkenazi Jewish. Only a G6PD Mediterranean–like sample with slightly increased electrophoretic mobility was found to lack the mutation at nucleotide 563. Instead, a substitution leading to an arginine-to-histidine substitution was found at nucleotide 1361. The loss of the positive charge of arginine would account for the slightly rapid electrophoretic mobility and lower isoelectric point. Although this G6PD closely resembles a variant—G6PD Songkhla (Panich and Na-Nakorn 1980)—described in a Thai subject, it was accorded a separate name, G6PD Andalus^{1361A}, because of the very different ethnic origin of the propositus.

It is becoming apparent that the mutation in nucleotide 563 is common in Mediterranean populations with G6PD deficiency and represents the prototypic G6PD Mediterranean. This mutation appears to be more homogeneous than had previously been assumed, but it will be of interest to study samples of subjects from other parts of the world who have also been reported to have this phenotype.

Of particular interest is the occurrence of a silent mutation at nucleotide 1311 in most of the subjects with G6PD Mediterranean. De Vita et al. (1989) characterized patients with G6PD Mediterranean and with two mutants, Sassari and Cagliari, that were thought to be distinct. They found that, in addition to the mutation previously described in G6PD Mediterranean at nucleotide 563, a silent mutation was present also at nucleotide 1311. Our studies show that this second mutation is found in all of the G6PD Mediterranean subjects we studied. The change at nucleotide 1311 appears to be uncommon: it has not been reported in any other sample of G6PD sequenced in our laboratory or elsewhere. Thus, it seems possible that the original mutation at 563 arose in an individual who happened to have this silent mutation and that most individuals with the common G6PD Mediterranean genotype are descendants of a single individual.

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