Molecular Heterogeneity of Glucose-6-Phosphate Dehydrogenase A

By E. Beutler, W. Kuhl, J.-L. Vives-Corrons, and J.T. Prchal

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is probably the most common disease-producing genetic polymorphism of humans. Virtually all G6PD-deficient Africans show the G6PD A" phenotype, an electrophoretically rapid, deficient enzyme. The recently acquired ability to identify the point mutations producing the different variants has given us new insights into the population genetics of G6PD variants. Twenty-nine males with the G6PD A" phenotype were studied. They were of African, Mexican, Spanish, and US white ethnic origin. All had the A→G transition at nucleotide 376 characteristic of G6PD A. In each case, one of three additional mutations was present, at nucleotides 202, 680, or 968. That in this population second mutations producing G6PD deficiency occurred only on the genetic background of G6PD A suggests that G6PD A was at one time the most common type of G6PD in Africa. However, the nucleotide sequence of the chimpanzee (Pan troglodytes) G6PD indicates that the primordial human type of G6PD was G6PD B.

MATERIALS AND METHODS

Blood and DNA samples. Blood samples were obtained from 29 men with G6PD deficiency in whom the residual enzyme was indistinguishable from G6PD A when studied by starch gel electrophoresis in phosphate buffer. Enzyme characterization was performed by standard methods. Included were 20 American blacks, two subjects of Mexican and two of Puerto Rican origin, one American white of Northern European origin, and four Spaniards previously diagnosed as having G6PD Bética, a variant that we now realize is identical to the African G6PD A" variant. Blood was obtained from three male Pan troglodytes (chimpanzees) from the San Diego Zoo and the Yerkes Regional Primate Research Center (Atlanta, GA). DNA was purified from each sample by standard methods.

Amplification with the polymerase chain reaction and endonuclease cleavage. In each case, the DNA of the portion of exon 5 surrounding nucleotide 376 was amplified with the polymerase chain reaction (PCR). With methods and primers described previously, we determined whether the FokI restriction endonuclease (New England Biolabs, Beverly, MA) could cleave the fragment. Similarly, we examined the sequence in the area of nucleotide 202 in exon 4 by PCR amplification and digestion with the NlaIII restriction endonuclease (New England Biolabs) to demonstrate the G→A transition. The mutations found at nucleotides 680 and 968 could also be identified by restriction endonuclease digestion of genomic DNA fragments amplified by PCR. The primers used and fragments obtained are summarized in Table 1.

Sequence analysis. In cases in which the G→A transition at nucleotide 202 was not present, all exons were amplified by PCR. The primers used to amplify exons 3 through 10 were designed from the intron sequences that we had determined on genomic clones. The flanking sequences surrounding exons 1 and 2 were obtained from Dr Akira Yoshida. The amplified fragments were sequenced using the chemical cleavage method, and were compared with the known cDNA sequence.

The G6PD of RBCs from three male Pan troglodytes (chimpanzees) were studied by starch gel electrophoresis. DNA was purified from the blood of one chimpanzee whose G6PD had fast electrophoretic mobility and from the blood of one with slow electrophoretic mobility. Exon 5 (containing nucleotide 376) was amplified using the same flanking primers used to amplify the human enzyme. Sequencing was performed by the chain termination method, using Taq polymerase (Promega, Madison, WI) after the appropriate band was reamplified.

RESULTS

All 29 subjects studied had the A→G mutation at nucleotide 376, as revealed by digestion of the amplified genomic DNA fragment with FokI. Twenty-six of the subjects also had a G→A mutation at nucleotide 202, the mutation we
had previously found in four of five men with the G6PD A− phenotype (Fig 1). As shown in Fig 2, two other mutations were found, a T→C change at nucleotide 968 producing a Leu→Pro substitution, and a G→T at nucleotide 680 causing an Arg→Leu substitution. Because these changes produced new restriction sites, their presence could be confirmed by amplifying the relevant portion of the DNA and cleaving with the appropriate restriction endonuclease (Table 1 and Figs 3 and 4). Distribution of the three mutations is summarized in Table 2.

In some cases, enough blood could be obtained to permit biochemical characterisation of the sample. The results of these studies are summarized in Table 3. Although the Spanish sample (G6PD Betica) with the mutation at nucleotide 968 had characteristics indistinguishable from those observed when the mutation was at nucleotide 202, the G6PD obtained from the black subject with this mutation had previously been considered to be quite homogeneous, and the molecular variability we found is surprising. It is not that different mutations are found in different population groups. Indeed, the limited sample that we examined shows similarity between the Spanish and the African groups, and the mutation at nucleotide 202 was also reported to occur in an Italian male. Rather, within each group several different variants were found, all three in blacks.

Whether these variants differ kinetically is not clear. We

### Table 1. Detection of Mutations by Endonuclease Cleavage After PCR

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Restriction Endonuclease</th>
<th>Fragment Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>202 G→A</td>
<td>5'-GTCGGCTGTTCCGAGTGCTTCTTG</td>
<td>3'-GGTGTCTCACTCGGAAGAAGTC</td>
<td>NdeII</td>
<td>109 109 63, 46</td>
</tr>
<tr>
<td>376 A→G</td>
<td>5'-CGCCAGACGACATGAGGCAG</td>
<td>3'-GTCGGAGAAATGAGACGCC</td>
<td>FokI</td>
<td>90 90 58, 32</td>
</tr>
<tr>
<td>680 G→T</td>
<td>5'-GACATGGCGGAAGCTTGCACC</td>
<td>3'-GGGAGCGGGATCGTCCAG</td>
<td>BstNI</td>
<td>242 213, 29 98, 115, 29</td>
</tr>
<tr>
<td>968 T→C</td>
<td>5'-TCCGGAGAACCTGCTTCACC</td>
<td>3'-CGGGTGCGGGATCGTCCAG</td>
<td>NcoI</td>
<td>282 282 162, 120</td>
</tr>
</tbody>
</table>

G6PD A− has previously been considered to be quite homogeneous, and the molecular variability we found is surprising. It is not that different mutations are found in different population groups. Indeed, the limited sample that we examined shows similarity between the Spanish and the African groups, and the mutation at nucleotide 202 was also reported to occur in an Italian male. Rather, within each group several different variants were found, all three in blacks.

DISCUSSION

G6PD A− has previously been considered to be quite homogeneous, and the molecular variability we found is surprising. It is not that different mutations are found in different population groups. Indeed, the limited sample that we examined shows similarity between the Spanish and the African groups, and the mutation at nucleotide 202 was also reported to occur in an Italian male. Rather, within each group several different variants were found, all three in blacks.

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Fig 2. Autoradiograph of sequencing gels of normal and mutant DNA sequenced after PCR of genomic DNA followed by chemical cleavage (described in text). The nucleotide mutation at 680 is shown in the antisense strand reading from nucleotide 678 to 685, therefore showing the mutation as C→A. The T→C mutation at nucleotide 968 is shown in the sense strand reading from nucleotide 970 to 963. *indicates the site of the mutation.

Fig 3. NcoI cleavage of the 282 bp PCR fragment of G6PD containing exon 9. A new NcoI site is created by the T→C mutation at nucleotide 968, resulting in two fragments of 120 and 162 bp. Channel 1, pBR322/MspI size markers; channel 2, G6PD A−3790/3790MC, uncut; channel 3, G6PD A−3790/3790MC, cut; channel 4, G6PD B, uncut; channel 5, G6PD B, cut.

Fig 4. BstNI cleavage of the 242 bp PCR fragment that includes exon 7 with the G→T mutation in nucleotide 680. Channel 1, G6PD 3790/3790MT, uncut; channel 2, G6PD A−3790/3790MT, cut; channel 3, G6PD B, cut; channel 4, G6PD B, uncut.

originally reported unique kinetic properties in the variant lacking the mutation at nucleotide 202.7 This black man (designated as having G6PD Selma) is included in the present study and had the mutation at nucleotide 968. However, a subject who had been characterized previously as having G6PD Betica had the same mutation. The latter
ently identical variants is unexplained. It could result from the X-chromosomes of modern-day Africans contain the sequence of an undetected sequencing error, having missed yet a third difference of 0.2, the probability that this is a remote possibility. Given the modern gene frequency of G6PD A mutation. There is no reason to suppose that the trimerization.

Clearly the G6PD A mutation at nucleotide 376 antedated the appearance of the deficiency mutations. Only about 20% of the X-chromosomes of modern-day Africans contain the G6PD A mutation. There is no reason to suppose that the mutations found at nucleotides 202, 680, and 968 would not have been tolerated in the normal B-type enzyme, although this is a remote possibility. Given the modern gene frequency of 0.2, the probability that the second mutations occurred each time in a G6PD A individual by chance is less than 1% (ie, 0.22), and probably when these mutations arose G6PD A was considerably more prevalent than it is today.

When in the evolution of homo sapiens did the mutation at nucleotide 376 appear? Was G6PD A perhaps the "normal" genotype of primitive humans? An electrophoretically rapid G6PD exists at polymorphic frequencies in Pan troglodytes and Homo sapiens: The nucleotide sequence of exon 5 of G6PD B of humans and both the rapid and slow chimpanzee G6PD are the same except for three substitutions, none of which affect the amino acid sequence (Fig 5). These findings suggest that the G6PD of early humans was G6PD B. G6PD A presumably arose in Africa, possibly after the human race had begun to disperse to Europe and Asia.20

Although G6PD B appears to have been the predominant type in early hominids, probably at one time in human evolution in Africa G6PD A may have been the most common genotype. This enzyme type might have provided some type of selective advantage in the African environment, or its high frequency might have occurred merely by chance. It appears to have been the predominant genotype at the time G6PD-deficient mutations became advantageous with respect to resistance against malaria,6 explaining the fact that the deficiency mutations appear to have arisen in genes that have guanine at nucleotide 376. But as the human species evolved in Africa and selection favored the G6PD A gene, the G6PD A gene appears to have been markedly disfavored, with its frequency declining to the current level.

The mutation at nucleotide 376 is not unique to Africa. It has been detected in Spanish and Italian populations. Electrophoretically fast variants that could represent the same mutation have been noted occasionally in white populations in Brazil21 and Cuba22,23 and in various Middle Eastern and Asian populations.24 However, the relative rarity of such mutations is evident from our recent examination of the RBCs of 193 phenotypically white male and female subjects, representing 305 G6PD alleles: not a single sample of G6PD A was found.

Today one can only speculate about the evolutionary forces that have shaped the shifting population frequencies of the G6PD A and G6PD B allele in humans. Possibilities may include infections such as malaria, factors that have played such a major role in selection of a variety of other genes that affect the RBC.25

### Table 2. Ethnic Origin of G6PD A− Subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>376</th>
<th>202</th>
<th>680</th>
<th>968</th>
</tr>
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<tbody>
<tr>
<td>Nucleotide change</td>
<td>A→G</td>
<td>G→A</td>
<td>G→T</td>
<td>T→C</td>
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<tr>
<td>Amino acid change</td>
<td>Asn→Asp</td>
<td>Val→Met</td>
<td>Arg→Leu</td>
<td>Leu→Pro</td>
</tr>
<tr>
<td>American black (n = 20)</td>
<td>20</td>
<td>18</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mexican (n = 2)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Puerto Rican (n = 2)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spanish (n = 4)</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>American white (n = 1)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of total</td>
<td>100</td>
<td>89.7</td>
<td>6.9</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Table 3. Biochemical Characteristics of G6PD A Variants With Mutations 202A/376G and 202A/680T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>202A/376G (mean ± 1 SD, n = 8)</th>
<th>376G/680T Selma</th>
<th>376G/680T Betica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic mobility*</td>
<td>Tris 111.0 ± 2.4</td>
<td>117</td>
<td>110</td>
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<tr>
<td>Phosphate</td>
<td>109.7 ± 3.3</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>Activity (% of N)</td>
<td>113.6 ± 4.2</td>
<td>118</td>
<td>116</td>
</tr>
<tr>
<td>kₙ, G6P (μmol/L)</td>
<td>11.6 ± 5.4</td>
<td>9</td>
<td>11.0</td>
</tr>
<tr>
<td>kₑ, NADP (μmol/L)</td>
<td>51.9 ± 9.5</td>
<td>40</td>
<td>56</td>
</tr>
<tr>
<td>Utilization of 2-deoxy G6P (% of N)</td>
<td>7.0 ± 2.3</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Utilization of deaminase NADP (% of N)</td>
<td>3.0 ± 1.2</td>
<td>0</td>
<td>3.3</td>
</tr>
<tr>
<td>Heat stability</td>
<td>Normal</td>
<td>Slightly decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>pH optimum</td>
<td>Normal</td>
<td>Optimum at 9.5–10</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*In starch gel using standard buffers, expressed as percentage of normal.
Hum G6PD A | gggctgacat ctg | tctgtgtgct tggctgtcgc tgtctccacg
Hum G6PD A | gggctgacat ctg | tctgtgtgct tggctgtcgc tgtctccacg
Chimp G6PD B-like | ctgctgttct tggctgtcgc tgtctccacg
Chimp G6PD A-like | ctgctgttct tggctgtcgc tgtctccacg
Hum G6PD B | GCCACCCAG AGGAGAAGCT CAAGCTGAG GACTTCTTGC CCCGCAACTC
Hum G6PD A | GCCACCCAG AGGAGAAGCT CAAGCTGAG GACTTCTTGC CCCGCAACTC
Chimp G6PD B-like | AGGAGAAGCT CAAGCTGAG GACTTCTTGC CCCGCAACTC
Chimp G6PD A-like | AGGAGAAGCT CAAGCTGAG GACTTCTTGC CCCGCAACTC
Hum G6PD B | CTGCTGCTGC GCCGCAAGGC ATGATGCAAG CTCCTACGAG CGCCTGAACA
Hum G6PD A | CTGCTGCTGC GCCGCAAGGC ATGATGCAAG CTCCTACGAG CGCCTGAACA
Chimp G6PD B-like | GCCGCAAGGC ATGATGCAAG CTCCTACGAG CGCCTGAACA
Chimp G6PD A-like | GCCGCAAGGC ATGATGCAAG CTCCTACGAG CGCCTGAACA
Hum G6PD B | CTCCTACGAT AGGGAGATg taaggcttgcg tgtgctccacg ttgctcctcc tttcgtgcgt
Hum G6PD A | CTCCTACGAT AGGGAGATg taaggcttgcg tgtgctccacg ttgctcctcc tttcgtgcgt
Chimp G6PD B-like | AGGGAGATg taaggcttgcg tgtgctccacg ttgctcctcc tttcgtgcgt
Chimp G6PD A-like | AGGGAGATg taaggcttgcg tgtgctccacg ttgctcctcc tttcgtgcgt
Hum G6PD B | ccaaagactgc ccaagcactg ctc
cHum G6PD A | ccaaagactgc ccaagcactg ctc
Chimp G6PD B-like | ccaagcactg ctc
Chimp G6PD A-like | ccaagcactg ctc

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