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# Somatic-Cell Selection Is a Major Determinant of the Blood-Cell Phenotype in Heterozygotes for Glucose-6-Phosphate Dehydrogenase Mutations Causing Severe Enzyme Deficiency

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## Summary

**X-chromosome inactivation in mammals is regarded as an essentially random process, but the resulting somatic-cell mosaicism creates the opportunity for cell selection. In most people with red-blood-cell glucose-6-phosphate dehydrogenase (G6PD) deficiency, the enzyme-deficient phenotype is only moderately expressed in nucleated cells. However, in a small subset of hemizygous males who suffer from chronic nonspherocytic hemolytic anemia, the underlying mutations (designated class I) cause more-severe G6PD deficiency, and this might provide an opportunity for selection in heterozygous females during development. In order to test this possibility we have analyzed four heterozygotes for class I G6PD mutations: two with G6PD Portici (1178G→A) and two with G6PD Bari (1187C→T). We found that in fractionated blood cell types (including erythroid, myeloid, and lymphoid cell lineages) there was a significant excess of G6PD-normal cells. The significant concordance that we have observed in the degree of imbalance in the different blood-cell lineages indicates that a selective mechanism is likely to operate at the level of pluripotent blood stem cells. Thus, it appears that severe G6PD deficiency affects adversely the proliferation or the survival of nucleated blood cells and that this phenotypic characteristic is critical during hematopoiesis.**

## Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common form of red blood cell (RBC) enzymopathy, affecting millions of individuals worldwide. The vast majority of subjects with G6PD deficiency experience significant hemolysis and anemia only under conditions of oxidant stress (Luzzatto and Mehta 1995). However, a small subset of G6PD-deficient subjects have overt chronic nonspherocytic hemolytic anemia, which is further exacerbated by oxidant stress. This condition is associated with specific G6PD variants (of which some 80 have been reported so far), which are designated as belonging to class I (Beutler and Yoshida 1988).

G6PD is a housekeeping gene, but the main phenotypic manifestations of G6PD deficiency are in RBCs. Presumably this is due to the relatively limited biochemical machinery of these cells, which lack any alternative to G6PD for the production of NADPH, a compound required for the detoxification of reactive-oxygen species. On the other hand, the pentose phosphate pathway, of which G6PD is the first and rate-limiting enzyme, is active in all cell types and in all organisms. G6PD "null" mutations have been seen in bacteria (Fraenkel 1968), yeast (Nogae and Johnston 1990; Thomas et al. 1991), and *Drosophila* (Gvozdev et al. 1976; Hughes and Lucchesi 1977) but never in the human species, suggesting that some function of G6PD may be indispensable in mammalian cells. Recently, it was found that a G6PD knockout mutation introduced in mouse cells makes these cells exquisitely sensitive to oxidative stress, indicating that this ubiquitous enzyme of the central metabolism has a major role in the defense against oxidative stress in eukaryotic nucleated cells, even those which may have other enzymes producing NADPH (Pandolfi et al. 1995).

Women who are heterozygous for G6PD deficiency are genetic mosaics as a result of X-chromosome inactivation. Mosaicism creates an opportunity for cell selection, but it is not clear whether this applies to G6PD alleles (Beutler 1964, 1986; Gartler and Linder 1964; Nance 1964; Brewer et al. 1967; Gaetani et al. 1990; Filosa et al. 1992). To test the hypothesis that, in cases of severe G6PD deficiency, selection favors somatic cells having the normal G6PD allele on their active X chromosome, we have analyzed systematically the mosaic composition of different blood-cell types in four women who are heterozygous for class I G6PD mutations.

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## Material and Methods

### DNA Extraction and Analysis

DNA was extracted, by standard techniques (Sykes 1983), from peripheral blood, hair bulbs, buccal mucosa desquamation cells present in the saliva, and urinary-tract cells present in the urine. The mosaicism of X-chromosome inactivation was analyzed by determining the electrophoretic pattern of allele-specific fragments obtained by PCR amplification of a CpG island associated with the HUMARA gene restricted with methylation-sensitive enzymes, as described by Allen et al. (1994), with the following modifications. The PCR fragments were restricted with *Hae*III to improve separation. Two microliters of digested mixture were loaded on 6% (29:1 acrylamide/bis-acrylamide gel) 8 M urea, 40% formamide, and 1 × Tris-borate EDTA (TBE), and the gel was vacuum-dried and exposed to Phosphor Imager screens (Molecular Dynamics). Area quantitation of scanned images (150 nm resolution, 16-bit image depth) was obtained by analyzing the pixel intensities along each gel lane, by the computer program ImageQuaNT (Molecular Dynamics).

### Cell Separations

Blood collected in EDTA (20 ml) was centrifuged at 1,000 rpm for 10 min, the platelet-containing supernatant was removed and was centrifuged at 3,000 rpm for 20 min, and the resulting platelet-containing pellet was resuspended in calcium/magnesium-free PBS and used to prepare RNA. The plasma-containing supernatant was added back to the first pellet, followed by addition of 4 ml of 3% Dextran T-500 in PBS and by subsequent sedimentation. The white blood cell (WBC)–enriched supernatant was mixed 1:1 with PBS, loaded on Ficoll-Paque, and centrifuged at 2,500 rpm for 20 min at room temperature. After centrifugation, granulocytes were purified from contaminant RBCs, which cosedimented at the bottom of the tube, by differential lysis with hypotonic erythrocyte lysis solution. Lymphocytes and monocytes banding at the interface of the Ficoll-Paque solution were washed in PBS, resuspended in RPMI 1640 with 10% FCS, loaded on 46% Percoll, and centrifuged at 1,800 rpm for 30 min. Monocytes banding at the interface were collected for RNA extraction. Lymphocytes sedimenting at the bottom were separated into B and T subpopulations by means of magnetic Dynabeads M-450 Pan-B (CD19) (Dynal) used as recommended by the manufacturer.

To prepare reticulocytes, three volumes of PBS were added to packed RBCs obtained from the aforementioned 3% dextran step, and the cell suspension was percolated through cotton wool to eliminate contaminating white blood cells (Colonna-Romano et al. 1985) and was loaded on a solution of 38% Percoll, 13% Selectografin, and 37 mM NaCl, to separate RBCs on the basis of cell age (C. Carestia, personal communication). The upper fractions containing reticulocytes were collected for RNA extraction.

Each fraction (platelets, granulocytes, monocytes, lymphocytes, and reticulocytes) was assessed as >90% pure by histological examination of slide smears. The age distribution of granulocytes was determined by assigning each of ⩾100 cells to one of five categories, defined by their nuclei being either nonsegmented or having one, two, three, four, or five lobes.

### RNA Extraction and Reverse-Transcriptase–PCR (RT-PCR)

Total cellular RNA was isolated from guanidium isothiocyanate–lysed cells and was purified by acidic phenol extraction (Chomczynski and Sacchi 1987). The RT-PCR was performed with the Perkin-Elmer RT-PCR kit, as recommended by the manufacturer. The PCR reaction was carried out in a 50-μl volume containing PCR buffer (50 mM KCl, 2 mM MgCl2, 10 mM Tris HCl pH 8.3, and 800 μM dNTP), oligo LEx9-10 (5′ATGAGAGGTGGGATGGGGTG3′; 100 ng), oligo REx11-10 (5′GAGCTTCACGTTCTTGTATCT3′; 100 ng), 0.25 μl of [α-$^{32}$P] dCTP (3,000 Ci/mmol; Amersham), and 1.25 U of *Taq* polymerase (Perkin Elmer). DNA was amplified by 31 cycles of PCR (each of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min). The amplimers utilized are not able to amplify genomic DNA. Restriction-enzyme analysis diagnostic for the mutation was performed with *Bbr*PI or *Alu*I on 20 μl of amplified DNA. The digested products were separated in 8% polyacrylamide gels in 1 × TBE, and the gels were vacuum-dried and exposed to Phosphor Imager screens (Molecular Dynamics). Area quantitation of scanned images was obtained as described above.

## Results

### Uneven X-Linked Mosaicism in Heterozygotes for G6PD Class I Mutants

Methylation of specific *Hpa*II sites in the first exon of the gene encoding the alpha subunit of the human androgen receptor, HUMARA, correlates with X-chromosome inactivation (Allen et al. 1992). These *Hpa*II sites are in close proximity to a highly polymorphic short tandem repeat (STR) sequence. We found that four women heterozygous for class I G6PD variants—two with G6PD Bari (subjects Bari 1 and 2) and two with G6PD Portici (subjects Portici 1 and 2)—are also heterozygous at the HUMARA STR. The first exon of the HUMARA gene was amplified by using, as template, DNA extracted from peripheral blood. When the DNA was subjected to amplification without prior *Hpa*II digestion, the bands corresponding to the two STR alleles

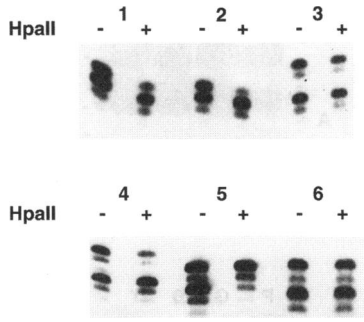


**Figure 1** X-chromosome inactivation analysis, on DNA extracted from blood, based on differential methylation of the HUMARA CpG island. The two strong bands in the undigested (−) samples and the corresponding bands after *Hpa*II digestion (+) are considered for quantitative analysis. Lanes 1, Subject Portici 1. Lanes 2, Subject Portici 2. Lanes 3, Normal female daughter of Portici 1. Lanes 4, Subject Bari 2. Lanes 5, Subject Bari 1. Lanes 6, Normal female daughter of Bari 1.

were easily resolved by size, and their intensity was approximately the same. However, if the DNA was subjected to *Hpa*II digestion before PCR amplification, the intensity of one allele-specific band was much stronger than that of the other (fig. 1). By contrast, in two normal subjects the bands were equally intense (table 1).

### *Uneven Levels of G6PD mRNA in Heterozygotes for Class I Mutants*

The results reported above clearly demonstrate a markedly skewed blood-cell mosaicism, but they did not tell us which G6PD allele is expressed in each cell population. In order to measure directly the expression of G6PD alleles, we proceeded to analyze the G6PD mRNA itself. The point mutation that we previously had identified in the G6PD Portici gene consists in a G→A transition in position 1178 in exon X, which creates a target site for the restriction enzyme *Bbr*PI. The point mutation that we previously had identified in the G6PD Bari gene consists in a C→T transition also in exon X in position 1187, which creates a target site for the restriction enzyme *Alu*I (fig. 2). Provided that these two G6PD mutations do not affect the steady-state mRNA level, the ratio of allele-specific RNAs will reflect the population ratio of cells having the mutation on the active X chromosome. Similar procedures have been utilized elsewhere (Curnutte et al. 1992; Prchal et al. 1993).

In order to obtain reference patterns, we first extracted RNA from lymphoblastoid cell lines derived either from a normal male or from a G6PD-Bari hemizygote, and we used it as template to amplify G6PD exon X by RT-PCR. To avoid interference from trace amounts of DNA that might be present as a contaminant, oligonucleotide primers belonging to two different exons were employed. RNA extracted from normal and G6PD-Bari cell lines was mixed in different ratios and used as template for RT-PCR amplification followed by *Alu*I restriction and electrophoresis (fig. 3, *top*). The ratio of bands specific for G6PD B and G6PD Bari closely reflects the ratio of RNAs present in the artificial mixture (fig. 3, *bottom*), thus demonstrating that the abundance of G6PD-specific mRNA was similar. In another set of experiments, artificial mixtures of G6PD-normal and G6PD-Bari cells were prepared before RNA extraction and RT-PCR analysis, with similar results; furthermore, the ratio of G6PD-specific to HPRT-specific bands produced by RT-PCR was the same for the two cell lineages analyzed over a range of input RNA concentrations (data not shown). This confirms that a normal level of G6PD-specific RNA is present in cells expressing the G6PD-Bari allele.

When the procedure described above was employed with RNA samples from two women heterozygous for G6PD Bari, we found a low level of PCR product deriving from the mutant allele (fig. 4, lanes 4–6). Similar results were obtained for G6PD Portici (data not shown, but see below). Thus, four G6PD class I–mutation heterozygotes have a marked predominance of blood cells having the normal allele on the active X chromosome.

### *Expression of Class I Mutant G6PD in Different Blood-Cell Types of Heterozygotes*

Since we presumed that the aforementioned findings might result at least in part from cell selection, it seemed

## Table 1

**Quantitation of Allele-Specific Bands after Methylation-Sensitive Restriction**

| | Pixel Value in Less-Intense Band (% above Background Level) | | | |
|---|---|---|---|---|
| Subject[a] | Unfractionated Blood Cells | Saliva | Urine | Hair Bulbs |
| G6PD Bari 1 | 16 | 35 | 52 | |
| G6PD Bari 2 | 20 | 37 | 30 | 50 |
| G6PD Portici 1 | 32 | | | |
| G6PD Portici 2 | 22 | | | |
| Normal 1 | 48 | | | |
| Normal 2 | 50 | | | |

[a] G6PD allele-specific bands are from figure 1.

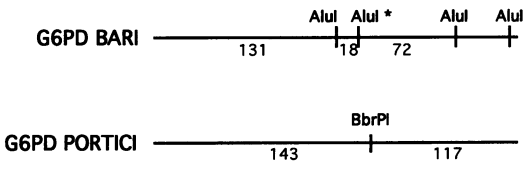


**Figure 2** Schematic representation of the DNA fragments obtained by RT-PCR. A star indicates the *Alu*I site created by the G6PD-Bari mutation.

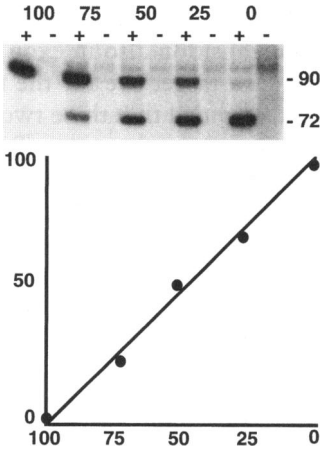


**Figure 3** Analysis of RNA extracted from normal and G6PD-Bari cell lines, mixed in different ratios and submitted to RT-PCR followed by allele-specific restriction. *Upper panel,* Autoradiography of an electrophoretic gel displaying DNA fragments obtained by RT-PCR amplification of G6PD-specific transcripts followed by digestion with *Alu*I. The numbers above the gel indicate the percentage of RNA extracted from G6PD-normal cells present in the artificial mixture of input RNA. The presence/omission of Reverse Transcriptase during sample manipulations is indicated (+/−). *Lower panel,* Percent of G6PD Bari bands, plotted against the percent of RNA extracted from normal cells present in the input mixture.

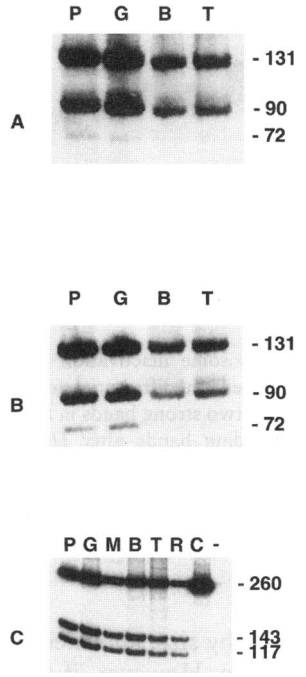


**Figure 5** Digitalized autoradiography of an electrophoretic gel displaying DNA fragments obtained by RT-PCR amplification of G6PD-specific transcripts followed by allele-specific digestion. Digestion was with either *Bbr*PI (*A* and *B*) or *Alu*I (*C*). In panels *A* and *B*, the 90-bp and the 72-bp bands respectively corresponding to normal G6PD and G6PD Bari were considered for quantitation; in panel *C*, the 143-bp and 117-bp bands are both generated by a G6PD Portici–specific *Alu*I cut, and they were quantitated with reference to the *Alu*I-uncut, G6PD-normal band of 260 bp. *A*, Subject Bari 1. *B*, Subject Bari 2. *C*, Subject Portici 1. P = platelets; G = granulocytes; B = B lymphocytes; T = T lymphocytes; M = monocytes; R = reticulocytes; C = normal control; H = Hela cells; and − = negative control.

of interest to determine whether this might differ in different blood-cell populations. For this purpose RNA was extracted and analyzed as before, from separately purified reticulocytes, granulocytes, B lymphocytes, T lymphocytes, monocytes, and platelets. The G6PD-normal allele is clearly much more represented than the mutant allele, in all these cell fractions (fig. 5), and quantitation of the ratios yielded remarkably uniform results (table 2).

The results reported above might be due to a shortened life span of the G6PD-deficient blood cells. However, we found that the age distribution of granulocytes (as assessed from lobe counts) was normal in the patient bearing G6PD Bari as well as in the two heterozygotes for the same mutation. Also, we did not notice in the same samples any increased average platelet size in association with the G6PD mutation, as would be expected if younger cells were more prevalent among G6PD-deficient cells.

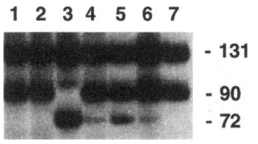


**Figure 4** Analysis of G6PD Bari–specific transcripts in RNA from unfractionated blood. Autoradiography of an electrophoretic gel displaying DNA fragments obtained by RT-PCR amplification of G6PD-specific transcripts followed by digestion with *Alu*I. The 90-bp and the 72-bp bands respectively corresponding to normal G6PD and G6PD Bari are indicated together with the common 131-bp band. A family segregating G6PD Bari was analyzed. Lane 1, Father. Lane 2, Sister (G6PD B homozygote). Lane 3, Propositus (G6PD 1187C→T hemizygote). Lane 4, Subject Bari 1 (mother). Lane 5, Subject Bari 2 (grandmother). Lane 6, Subject Bari 1 (second RNA preparation). Lane 7, HeLa cells.

### *Expression of Class I Mutant G6PD in Nonhematopoietic Cell Types in Heterozygotes*

In order to test whether the patterns of selection might be different in blood cells compared with other somatic cells, DNA was extracted from hair bulbs, from buccal mucosal cells present in saliva, and from urinary-tract cells present in urine and then was subjected to HUMARA STR analysis before and after digestion with *Hpa*II. In both of our subjects who are heterozygous for G6PD Bari we found in these DNA samples a ratio of the two alleles (fig. 6) that was closer to 50% than was the ratio that we previously had found in blood cells (table 1).

**Table 2**

**Quantitation of Allele-Specific RT-PCR Bands**

| | Pixel Value in Mutant Band[a] (% above Background Level) | | | | | |
|---|---|---|---|---|---|---|
| Subject | Platelets | Granulocytes | Monocytes | B Lymphocytes | T Lymphocytes | Reticulocytes |
| G6PD Bari 1 | 8 | 7 | | 6 | 7 | |
| G6PD Bari 2 | 17.0 | 17.0 | | 9.2 | 10.6 | |
| G6PD Portici 1 | 26.7 | 21.2 | 32.6 | 23.1 | 26.5 | 31.2 |

[a] Calculated from figure 5.

## Discussion

### *Random X-Chromosome Inactivation*

Females heterozygous at X-linked loci are mosaics by virtue of permanent inactivation of one X chromosome in each somatic cell early in embryogenesis (Lyon 1992). X inactivation is initially subject to imprinting, whereby the first cell types to differentiate—i.e., trophectoderm and extraembryonic tissues originating from the primitive endoderm—show selective inactivation of the paternal X chromosome. By the time X inactivation occurs in the embryonic ectoderm, from which virtually all tissues of the embryo proper are derived, the imprint has been lost—so that the maternal and paternal X chromosomes have an even chance of being inactivated, resulting in a relatively balanced mosaic organism.

### *Methodologies for the Quantitative Analysis of X-Linked Somatic-Cell Mosaicism*

In heterozygotes for G6PD variants with normal activity, it is reasonable to assume that the quantitative ratio between them is a measure of the respective cell populations with either allele on the active X chromosome (see Nance 1964). However, if one of the two allelic variants is severely deficient, it may be markedly unstable, and therefore this assumption cannot be made. A more recent method for studying X-chromosome inactivation in humans is based on differences between the active and inactive X chromosome in the DNA-methylation pattern of CpG islands, such as HUMARA; however, this method has failed to show strong correlation between different probes, which might be caused by incomplete methylation of some DNA sequences. On the other hand, in general there is no reason to expect that point mutations in the G6PD-coding region will affect the levels of mRNA products; and therefore their ratio may give us a good estimate of the ratio between the respective somatic-cell populations. This method already has been utilized to analyze X-chromosome mosaicism in hematopoiesis, by means of ligase-detected reaction (LDR) and ligase chain reaction (LCR) amplification of G6PD alleles carrying the conservative 1311 C→T substitution (Prchal et al. 1993). We have first demonstrated that the G6PD Bari allele does not affect the steady-state level of mRNA. Furthermore, in heterozygotes for G6PD Bari and G6PD Portici we were able to compare the levels of G6PD mRNA by PCR amplification of cDNA, thanks to the fact that the respective mutations introduce new restriction sites in the wild-type sequence. We also found substantial agreement between the RNA and methylated-DNA methods, although RNA is probably more reliable, because the intensity of some bands obtained from DNA may be seriously affected by spurious resistance to digestion of some restriction sites even when the DNA is unmethylated.

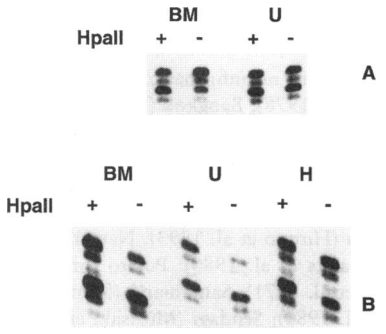


**Figure 6** X-chromosome inactivation analysis, on DNA extracted from buccal mucosa cells (BM), urinary-tract cells (U), and hair bulbs (H), based on differential methylation of the HUMARA CpG island. The strongest bands in the undigested (−) samples and their corresponding bands after *Hpa*II digestion were considered for quantitation. *A*, Bari 1. *B*, Bari 2.

### *Somatic-Cell Selection after X Inactivation*

In the human species, extremely unbalanced mosaicism is observed in certain tissues in females who are heterozygous for X-linked gene defects that may affect cell growth, such as those responsible for the Lesh-Nyhan syndrome (Nyhan et al. 1970), adrenoleukodystrophy (Migeon et al. 1981), and X-linked immunodefi-

ciencies (Allen et al. 1992, 1994). Markedly unbalanced mosaicism is also observed in an appreciable proportion of women without any known abnormal gene (Gale et al. 1991). Again, this might be due to pressure on an as yet unidentified selectable allele, as has been suggested elsewhere (Luzzatto 1979); to the small size of cell pools at the time of X inactivation (Fialkow 1973); or, in analogy with the Xce locus in mice (Cattanach and Williams 1972), to the effect of genetic elements acting on the mechanisms of X inactivation.

### *Somatic-Cell Selection in Heterozygotes for G6PD Deficiency*

In general, the distribution of the ratio of expression of the two G6PD alleles in heterozygotes for polymorphic variants is unimodal but quite wide, with ~1% of subjects having a phenotype indistinguishable from that of a homozygote (Rinaldi et al. 1976). By contrast, this does not seem to be the case in heterozygotes for G6PD mutations associated with chronic hemolysis (class I variants). From a survey of the literature on 42 class I variants (table 3) we retrieved the results of enzyme-assay values for the RBCs of heterozygotes for 12 such variants. In two of these (G6PD Barcelona and G6PD Chicago) the values are compatible with a relatively balanced mosaicism; in no case was the enzyme level in the hemizygous-deficient range; and in the majority of cases (10 of 12) the values were in the normal range. Although other possible reasons can be entertained (see Gaetani et al. 1990), the simplest and most likely explanation for these findings is that cell selection takes place in these G6PD class I heterozygotes.

### *Somatic-Cell Selection in Different Hematopoietic Lineages*

Since RBCs with a class I G6PD variant have, by definition, a shortened life span, the predominance of normal RBCs in heterozygotes for these variants might have been explained simply by their selective longer survival. On the other hand, there is little information re-

**Table 3**

**Previously Published Data on the Expression of G6PD Class I Variants in Heterozygotes**

| G6PD Variant[a] | G6PD Activity (% of Normal[b]) | | Reference |
|---|---|---|---|
| | Hemizygote(s) | Heterozygote(s) | |
| Albuquerque | 0, 0 | 90, 94 | Beutler et al. (1968) |
| Barcelona | 0 | 40[c] | Vives Corrons et al. (1982) |
| Bari | <1 | 100, 100 | Filosa et al. (1994) |
| Chicago | 9 | 28 | Beutler et al. (1968) |
| Duarte | 10 | 101 | Beutler et al. (1968) |
| Genova | 1.3 | 113[d] | Gaetani et al. (1990) |
| Harilaou | <1[e] | 82.1 | Town et al. (1990) |
| Nagano | 1.7 | 86 | Takahashi et al. (1982) |
| Portici | .86 | 89, 100 | Filosa et al. (1992) |
| San Francisco | 0 | 70, 63 | Mentzer et al. (1980) |
| Walter Reed | 7 | 87.2 | Beutler et al. (1986) |
| Wayne | 6, 10 | 80 | Ravindranath and Beutler (1987) |

[a] Literature on the following class I G6PD variants was also surveyed (but no information on G6PD activity in heterozygotyes was provided in these sources): Akita (Miwa et al. 1978); Bangkok (Talalak and Beutler 1969); Birmingham (Prchal et al. 1980); Cornell (Miller and Wollman 1974; Fairbanks et al. 1980); Den Haag (Rattazzi et al. 1971); Englewood (Rattazzi et al. 1971); Fukuoka (Fujii et al. 1984); Gifu (Fujii et al. 1984); Guadalajara (Vaca et al. 1982); Hong Kong (Wong et al. 1965); Hong Kong–Pokfulam (Chan et al. 1972); Hotel Dieu (Kahn et al. 1977); Huntsville (Hall et al. 1988); Huron (Beutler and Ravinrath 1987); Kobe (Fujii et al. 1981); Long Prairie (Johnson et al. 1977); Nara (Hirono et al. 1993); New York (Rattazzi et al. 1971); Ogikubo (Miwa et al. 1978); Pea Ridge (Fairbanks et al. 1980); Puerto Limon (Elizondo et al. 1982); Rennes (Picat et al. 1980); Rotterdam (Rattazzi et al. 1971); Santamaria (Saenz et al. 1984); Santiago de Cuba (Vulliamy et al. 1988); Sapporo (Fujii et al. 1981); Sendagi (Morisaki et al. 1983); Tepic (Lisker et al. 1985); Tripler (Engstrom and Beutler 1970); and Tsukui (Ogura et al. 1988).

[b] As reported in the reference cited. In our experience, the range of normal values is approximately the mean ± 25%. Where two subjects were reported to have the same variant, two values are given.

[c] Individual was said to have slight hemolytic anemia.

[d] Individual was found to have an intermediate NADPH:NADP ratio in RBCs, intermediate G6PD activity in mononuclear cells, and both phosphoglycerate kinase (PGK) alleles methylated.

[e] In fibroblasts.

garding the impact of severe G6PD deficiency on the growth and survival of nucleated cells, and similar considerations might have applied in this case also. However, we did not find any indication of decreased transit time for either RBCs or granulocytes. At the same time, the quantitative data that we obtained were remarkably similar among the individual blood-cell types. In a first approximation, these results suggest that the level of G6PD already is generally important in the erythroid-cell lineage, before RBCs are released in the bloodstream, and also is important in the myeloid- and lymphoid-cell lineages.

By contrast, relatively little imbalance was seen (table 1) in cells present in saliva, urine, and hair bulbs, suggesting that G6PD appears to be less important in epithelial cells. We note, however, that information from hair bulbs may be subject to sampling errors and that most values for cells derived from saliva and from the urinary tract are still suggestive of unbalanced mosaicism. Therefore, it is still possible that extreme G6PD deficiency results either in selection in the generality of tissues or (less likely) in an inactivation bias, and more data are necessary to firmly establish whether the imbalance observed in hematopoietic lineages reflects selection acting only in this tissue.

Overall, the findings reported in this paper indicate that the G6PD level does affect the proliferation and/or survival of nucleated precursors of blood cells of various lineages. Although we do not yet know the exact mechanism, it is likely that the ability to withstand oxidative damage is a major factor, as recently has been shown to be the case in various cell lines (Pandolfi et al. 1995; M. V. Ursini, A. Parrella, G. Rosa, and G. Martini, unpublished data) and as has been known, for a long time, with respect to mature RBCs. In addition, reactive-oxygen species may be involved in triggering programmed cell death, a highly regulated process that is required not only for the successful completion of embryonic development but also for several stages of hematopoiesis, including the ability of a multipotential hemopoietic cell line to survive in the absence of growth factors and to differentiate toward erythroid or myeloid lineages (Fairbairn et al. 1993; Efferth et al. 1995). In fact, the differentiation, in vitro, of murine embryonic stem cells into cell types of the hemopoietic system is exquisitely sensitive to the oxygen content of the surrounding atmosphere (Potocnick et al. 1994). That G6PD deficiency probably affects both the growth rate and the survival of hematopoietic-cell progenitors may be of importance in working out protocols for gene therapy for hematologic diseases.

*Note added in proof.*—Recently we have investigated a new patient from Israel, unrelated to either of the families reported here, in whom the G6PD Portici mutation has been identified in the laboratory of T. Vulliamy (personal communication). A quantitative G6PD assay performed in L.L.'s laboratory by M. Estrada once again gave a normal result on the mother's RBCs and WBCs.

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