G6PD Deficiency as Protection Against *falciparum* Malaria: An Epidemiologic Critique of Population and Experimental Studies

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**ABSTRACT** Several recent reviews in the medical literature maintain that only heterozygous G6PD deficient females are relatively protected against *falciparum* malaria. However, a number of population studies provide compelling evidence that both the hemizygous G6PD deficient male and homozygous G6PD deficient female are also relatively protected against *falciparum* parasitization. An epidemiologic critique of a sample of these field investigations points out the methodological problems that underlie some of the negative findings. In vitro studies also provide compelling evidence that erythrocytes from all G6PD deficient genotypes are relatively protected against *falciparum* infestation, and that this protection is enhanced by oxidant substances derived from a number of food crops such as fava beans.

It is suggested that "quine" taste sensitivity reflects taste sensitivity to bitter-tasting, naturally occurring antimalarial substances of plant origin, and that the G6PD polymorphism and the genetic loci coding for "quine" taste sensitivity have co-evolved in human populations. It appears that adaptation at the G6PD locus in human populations reflects an intricate web of interactions between a large number of different G6PD deficient alleles which have reached polymorphic frequencies and a variety of food crops from which oxidant substances may be derived.

The specific goal of this article is to review the evidence indicating that all glucose-6-phosphate dehydrogenase (G6PD) deficient genotypes are relatively protected against *Plasmodium falciparum* malaria. A discussion of this issue is timely and necessary because several influential reviews of G6PD and G6PD deficiency in the medical literature over the past few years have reiterated the position that only G6PD deficient heterozygotes are protected against *falciparum* parasitization, while G6PD deficient hemizygotes and homozygotes are not (Luzzatto and Mehta, 1989; Luzzatto and Battistuzzi, 1985; Usanga and Luzzatto, 1985). However, this position is based on a single study (Bienzle et al., 1972), while a number of other field investigations support the view that all G6PD deficient genotypes are relatively protected against *falciparum* parasitization (Allison and Clyde, 1961; Gilles et al., 1967; Butler, 1973; Kar et al., 1992). The inaccuracy of the "heterozygote" position seriously obscures our understanding of how selection operates at the
G6PD locus and clouds any discussion of the relative fitnesses of the various G6PD genotypes in areas where *falciparum* malaria is endemic.

The first portion of the article will review evidence from population studies that have evaluated the relative protection provided by the various G6PD genotypes against parasitization with *Plasmodium falciparum*. The data from these investigations are conflicting; therefore, this review will present an in-depth evaluation, from an epidemiologic perspective, of the designs of the various studies in order to elucidate their relative strengths and weaknesses. A detailed epidemiologic critique of these population studies also provides a useful guide to future investigators with respect to a number of important issues encountered in such biomedical population studies—whether the investigations are concerned with G6PD deficiency or with other topics.

The second portion of the article reviews in vitro studies evaluating the relative protection provided G6PD deficient erythrocytes against *falciparum* parasitization. These investigations are also reviewed in some detail so as to provide the reader with a sense of the process of epidemiologic enquiry, rather than merely presenting a summary of findings that are frequently somewhat equivocal.

The third goal of this article is to reiterate and expand a biocultural perspective developed by Katz and others (Katz and Schall, 1979, 1986; Etkin, 1979; Golenser et al., 1983, 1988). It stresses the point that adaptation at the G6PD locus involves an interaction between genetic adaptation and cultural adaptation mediated through dietary consumption of foods containing naturally occurring antimalarial substances of plant origin. This viewpoint maintains that we can only come to an appreciation of the web of interacting selective forces that underlie population adaptation at the G6PD locus through an understanding of how cultural adaptation acts upon and modifies biological adaptation. This section presents a brief discussion of an hypothesis that there has been co-evolution at the G6PD locus and the genetic locus coding for "quinine" taste sensitivity and some preliminary data in support of this suggestion.

Again, the article focuses on a single narrow issue. That issue is whether the epidemiologic and experimental evidence indicates that all G6PD deficient genotypes are relatively protected against *falciparum* parasitization. Considerable attention is paid to epidemiologic detail in order to provide a fuller picture of the process of enquiry and to indicate some of the pitfalls encountered in such investigations. However, this article is not a comprehensive review of the selective factors influencing the G6PD polymorphism. Such a review will be presented elsewhere (LS Greene, in preparation).

**GLUCOSE-6-PHOSPHATE DEHYDROGENASE**

The role of glucose-6-phosphate dehydrogenase in the metabolism of the erythrocyte

G6PD is a "housekeeping" enzyme that performs vital functions within all cells of the body. However, within the erythrocyte, which lacks a nucleus, mitochondria, and other organelles, there are certain constraints on metabolism and this enzyme has a particularly important role. G6PD catalyzes the first step of the pentose phosphate pathway (hexose monophosphate pathway), a series of side reactions off the main glycolytic pathway in the erythrocyte and in all cells of the body. G6PD initiates this pathway by catalyzing the oxidation of glucose-6-phosphate to 6-phosphogluconolactone by the co-enzyme nicotinamide-adenine dinucleotide phosphate (NADP+), which is reduced to NADPH. The 6-phosphogluconolactone hydrolyzes spontaneously to 6-phosphogluconate. This serves as a substrate for 6-phosphogluconate dehydrogenase and NADP+. The second enzymatic step in this pathway is also associated with the reduction of NADP+ to NADPH. The NADPH produced as a consequence of these reactions reduces oxidized glutathione (GSSG) to reduced glutathione (GSH) in a reaction catalyzed by glutathione reductase. GSH then reduces hydrogen peroxide, a powerful oxidant produced in the course of cellular metabolism and as a consequence of the inflammatory response,
and other endogenous and exogenous oxidants, in a reaction catalyzed by glutathione peroxidase (Newsholme and Leech, 1983; Beutler, 1983; WHO Working Group, 1989; Luzzatto and Mehta, 1989). The first part of the pentose phosphate pathway is outlined in Figure 1.

The main function of the pentose phosphate pathway is the generation of reducing capacity through the production of NADPH and ultimately GSH. This is the only mechanism available to the erythrocyte for generating reducing capacity and is thus essential for cell survival, while in other cells of the body alternative means of NADPH production exist and the pentose phosphate pathway accounts for only 60% of NADPH production (Newsholme and Leech, 1983; Beutler, 1983; WHO Working Group, 1989; Luzzatto and Mehta, 1989).

GSH generated through the pentose phosphate pathway, as outlined above, is the only defense against oxidant stress in the red blood cell (Friedman, 1979; WHO Working Group, 1989). In the unstressed G6PD normal erythrocyte, G6PD activity is only about 2% of total capacity (WHO Working Group, 1989). This is increased greatly to meet the challenge of an oxidant stress and GSH is maintained at stable levels. However, the G6PD deficient erythrocyte has greatly reduced G6PD activity (10 to 20% of normal in G6PD A (−) and 0 to 10% of normal in G6PD Mediterranean and many similar variants) (Luzzatto and Mehta, 1989). An increased oxidant stress can lead to a marked depletion of GSH as the ability of the deficient G6PD to generate NADPH is exceeded by a high rate of GSH loss (Beutler, 1983; Eckman and Eaton, 1979).

Uncompensated oxidant stress in the normal erythrocyte (or more easily in the G6PD deficient erythrocyte) results in the oxidation of hemoglobin to methemoglobin, Heinz body formation, and membrane damage (Beutler, 1983). If extreme this will result in hemolysis, while a less severe but uncompensated oxidant stress will decrease the distortability of the erythrocyte and increase the likelihood that it will be removed from the circulation within the reticuloendothelial system (Johnson et al., 1986; Beutler, 1983; Arese et al., 1986). With the consequent loss of red cells hematopoiesis is increased as the body attempts to maintain normal vascular functioning, and there is a shower of reticulocytes (young erythrocytes
TABLE I. Classification of G6PD variants

<table>
<thead>
<tr>
<th>Class</th>
<th>Description</th>
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<tbody>
<tr>
<td>I</td>
<td>Enzyme deficiency with chronic nonspherocytic hemolytic anemia.</td>
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<tr>
<td>II</td>
<td>Severe enzyme deficiency. Less than 10% of the activity of G6PD B.</td>
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<tr>
<td>III</td>
<td>Moderate to mild enzyme deficiency. Ten percent to 60% of the activity of G6PD B.</td>
</tr>
<tr>
<td>IV</td>
<td>Normal activity. Sixty percent to 150% the activity of G6PD B.</td>
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<tr>
<td>V</td>
<td>Increased activity. More than 150% the activity of G6PD B.</td>
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GLUCOSE-6-PHOSPHATE DEHYDROGENASE VARIANTS

G6PD B

G6PD is a cytoplasmic enzyme that is found in all cells of the body. The active form of the enzyme is either a dimer or a tetramer consisting of identical subunits. The primary structure of a single subunit polypeptide chain consists of 515 amino acids (Luzzatto and Mehta, 1989). G6PD B is the enzyme found in most people worldwide and it is identical to that of the chimpanzee and gorilla (Luzzatto and Battistuzzi, 1985). In populations outside of tropical and semitropical regions it is virtually the only G6PD isoenzyme, except for rare private mutations, as no variants have reached polymorphic frequencies (> .1%) in populations in these areas (Luzzatto and Battistuzzi, 1985). Of all human loci, the G6PD locus is the most polymorphic. Over 300 variants have been fully described (Luzzatto and Battistuzzi, 1985; Beutler, 1990; Luzzatto and Mehta, 1989). In their comprehensive review, Luzzatto and Mehta indicate that 77 of these variants have reached polymorphic frequencies (> .1%). Eleven of these polymorphic alleles have normal activity and 66 of the variants have decreased activity and are called deficient variants (Luzzatto and Mehta, 1989). In a more recent overview Beutler (1992) states that over 400 variants have now been described, but does not provide an indication of how many of these variants have reached polymorphic frequencies.

Enzyme variation has traditionally been classified by means of electrophoretic mobility determined by starch gel electrophoresis and as a function of enzyme variant activity compared to the activity of G6PD B. The standard for activity is the rate at which NADP⁺ is reduced by glucose-6-phosphate with G6PD B as the catalyst. On this basis, enzyme variants are classified as fast, normal, and slow in terms of electrophoretic mobility, and as Classes I—V based on enzyme activity relative to G6PD B (Betke et al., 1967; Luzzatto and Mehta, 1989; Beutler, 1983, 1990).

Table 1 shows the classification of enzyme variants by activity level. Enzyme variants which fall into each activity class are further characterized in terms of electrophoretic mobility and unique variants are given specific names. The Class I variants are very rare and code for kinetically unstable enzymes that produce severe G6PD deficiency with variable degrees of hemolytic anemia (Beutler, 1983). These variants have not reached polymorphic frequencies and are not relevant to our discussion. Class II variants are severely deficient variants with less than 10% of the activity of G6PD B, while Class III variants are more moderately deficient with 10 to 60% of the activity G6PD B. Forty-four of the 109 Class II variants (40.4%) and 22 of the 75 Class III variants (29.3%) listed by
Luzzatto and Mehta (1989) have reached polymorphic frequencies of >.1% in various human populations and are associated with adaptation to *falciparum* malaria (see below).

**Some important regional polymorphic variants**

G6PD A is a normal variant (Class IV) that has 90% of the activity of G6PD B (Luzzatto and Mehta, 1989). The allele coding for this variant is probably the most common variant worldwide as it reaches frequencies of approximately .20 in sub-Saharan African populations (Beutler, 1990:152). Vulliamy and co-workers (1988) indicate that this variant derives from a single amino acid substitution of aspartic acid for asparagine at amino acid number 126, and that this was the result of an adenine to guanine substitution at nucleotide number 376.

G6PD A (−) is a Class II deficient variant which has 10 to 20% of the activity of G6PD B and which reaches allele frequencies of about .15 in sub-Saharan African populations (Luzzatto and Mehta, 1989). It appears that this variant has arisen from the G6PD A variant as both have aspartic acid substitutions at amino acid number 126. The G6PD A (−) variant has an additional guanine to adenine substitution at nucleotide 202 at the genetic level leading to a valine to methionine substitution at the protein level at amino acid number 67 (Vulliamy et al., 1988). In actuality, there is some molecular heterogeneity in G6PD A (−), with the nucleotide 202 substitution being the most common, but with alternative substitutions occurring at nucleotides 680 and 968 (Beutler et al., 1989).

G6PD Mediterranean is a Class II deficient variant with enzyme activity that is less than 10% of the activity of G6PD B; it reaches allele frequencies of between .10 and .25 or higher in many populations in the circum-Mediterranean region and the Near and Middle East (Luzzatto and Mehta, 1989). This variant is a consequence of a cytosine to thymine substitution at nucleotide number 563 resulting in a serine to phenylalanine substitution at amino acid number 188 (Vulliamy et al., 1988). There are many similar Class II variants that have reached polymorphic frequencies in the circum-Mediterranean region (Cagliari, Sassari, El Fayoum), South Asia (Hong Kong, Canton, Mahidol), and elsewhere. Most appear to be the consequence of point mutations resulting in single amino acid substitutions that have variable effects on activity and electrophoretic mobility. The characterization, classification, and distribution of these variants have been described in detail in a number of reviews (Luzzatto and Battistuzzi, 1985; Luzzatto and Mehta, 1989; Beutler, 1990, 1991, 1992).

**The effect of red cell age on erythrocyte G6PD activity**

G6PD is an age-dependent enzyme. Within normal G6PD B red blood cells the activity of G6PD declines exponentially, with a half-life of 62 days. However, despite this loss of enzyme activity older G6PD B red cells contain sufficient G6PD activity to maintain GSH levels in the face of an oxidant stress and the life span of the average G6PD B red cell is 100 to 120 days.

In erythrocytes with G6PD A (−) the defect is due to greater enzyme instability. Newly formed G6PD A (−) red cells have the same enzymatic activity as newly formed erythrocytes from G6PD B individuals. However, the G6PD activity of these cells declines rapidly. The half-life of G6PD A (−) erythrocyte is only 13 days, and in the G6PD A (−) individual the red cell population is composed of a mixture of erythrocytes of continuously decreasing levels of activity.

In the G6PD Mediterranean individual there is an even greater enzyme instability as the half-life of these erythrocytes is only about 8 days. Reticulocytes that are released into the circulation in G6PD Mediterranean individuals already have reduced levels of G6PD and mature erythrocytes have enzyme levels usually below 1% normal activity (Piomelli et al., 1968; Piomelli, 1986; Luzzatto and Testa, 1978; Kirkman and Gaetani, 1986).
TABLE 2. Drugs and chemicals associated with significant hemolysis in individuals with G6PD deficiency

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Definite association</th>
<th>Possible association</th>
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<tr>
<td>Antimalarials</td>
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<tr>
<td>Primaquine</td>
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<td>Chloroquine</td>
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<td>Pamaquine</td>
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<td>Pentaquine</td>
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<tr>
<td>Sulfonamides</td>
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<tr>
<td>Sulfanilamide</td>
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<td>Sulfamethoxypyridazine</td>
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<td>Sulfacetamide</td>
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<td>Sulfadimidine</td>
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<td>Sulfapyridine</td>
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<td>Sulfamethoxazole</td>
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<td>Sulfones</td>
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<tr>
<td>Thiazolesulfone</td>
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<tr>
<td>Diaminodihydroxyphenylsulphone (DDS, dapsone)</td>
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<td>Nitrofurans</td>
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<tr>
<td>Nitrofurantoin</td>
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<td>Antipyretic/analgesic</td>
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<tr>
<td>Acetanalid</td>
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<td></td>
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<tr>
<td>Others</td>
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<tr>
<td>Nalidixic acid</td>
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<td>Chloramphenicol</td>
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<td>Naphthalone</td>
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<td>Vitamin K analogues</td>
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<td>Nitrazole</td>
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<td>Phenylhydrazine</td>
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<td>Toluidine blue</td>
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<tr>
<td>Trinitrotoluene (TNT)</td>
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<tr>
<td>Methylene blue</td>
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<tr>
<td>Phenazopyridine</td>
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After Luzzatto and Mehta (1989).

Hemolysis in G6PD deficient individuals

Because the G6PD deficient erythrocyte only has a limited ability to deal with an increased oxidant stress (see above), G6PD deficient individuals may experience hemolysis (which at times is explosive and severe) under a variety of circumstances: when exposed to a number of drugs and chemicals, including several antimalarial drugs (see Table 2); during infections; during diabetic acidosis; upon consumption of fava beans (Vicia faba) in G6PD Mediterranean and other Class II deficient individuals; and as the result of a number of common exposures to environmental chemicals (Luzzatto and Mehta, 1989; Beutler, 1983; Belsey, 1973; Arese et al., 1986; Chevion et al., 1982; Calabrese, 1984). This hemolysis produces a significant degree of morbidity and mortality in G6PD deficient individuals. However, the increased vulnerability of the G6PD deficient erythrocyte to oxidant stress is the mechanism underlying its relative protection against falciparum parasitization (see below).

GENETIC BASIS

The genetic locus for G6PD in humans and all mammals is located on the telomeric region of the long arm of the X-chromosome (band Xq28) distal to the fragile site Xq27.3. It is closely linked to the loci for color blindness, hemophilia A, and adrenoleukodystrophy. These linkage groups are very stable and are the same in all mammals. Interestingly, the G6PD of Drosophila melanogaster is also sex-linked (Beutler, 1983, 1990; Luzzatto and Battistuzzi, 1985; Luzzatto and Mehta, 1989; WHO Working Group, 1989). Since it is sex-linked there are five possible genotypes in populations in which the G6PD locus is polymorphic. The frequency of the deficient condition is higher in males than females since males, being hemizygous, only need one copy of the allele to express the full deficient condition while females need two deficient alleles.

Hemizygous deficient males and homozygous deficient females express the same degree of enzyme deficiency, while hemizygous normal males and homozygous normal females also have comparable enzyme levels. Due to the random deactivation of one X-chromosome during embryological development in the female, heterozygous females actually have two populations of red cells (G6PD normal and
G6PD deficient) (Lyon Mosaic Effect) with varying total G6PD activity depending on the relative proportions of these two groups of erythrocytes. Total G6PD activity in heterozygous females may range from near-normal to near-deficient (Luzzatto and Battistuzzi, 1985; Beutler, 1983).

MALARIA

Malaria is a febrile illness caused by sporozoa of the genus Plasmodium, four species of which infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. The malarial parasites undergo a developmental cycle in the female anopheline mosquito, which is the vector. They are transmitted to the human host following a bite by the mosquito, rapidly enter the liver where they undergo a developmental phase of varying duration among the four species (pre-erythrocytic phase), and then enter the red blood cell (intra-erythrocytic phase) where they continue their multiplicative cycle. The asexual erythrocytic parasite is the stage in the life cycle that causes disease. This is characterized by fever, chills and sweats (which vary in periodicity among the different species), anemia, and enlargement of the liver and spleen (Woodruff, 1978; Miller, 1985; Strickland, 1991).

*P. falciparum* (malignant tertian) is the most severe form of malaria and causes the greatest degree of morbidity and mortality. It is found in warm, moist climates and is distributed over most of tropical Africa, parts of India and Pakistan, South-East Asia including Indonesia and New Guinea, and Central and South America. *P. vivax* (benign tertian) malaria is widespread in the tropics and subtropics and in some temperate regions. *P. ovale* (ovale tertian) is uncommon and is irregularly distributed in Africa and South America. *P. malariae* (quartan) is also less common and is found in the tropics of East and West Africa, India, and Central and South America.

Immunity to malaria is primarily directed against the asexual erythrocytic parasite and only develops after prolonged and repeated infections. Immunity usually does not prevent re-infection, but reduces the severity of the disease or leads to an asymptomatic infection. Immunity is species-specific, and in *P. falciparum* is usually strain-specific.

*P. falciparum*, *P. vivax*, and *P. ovale* invade reticulocytes (immature erythrocytes) preferentially, but *P. falciparum* can invade erythrocytes of all ages. This is a significant phenomenon since G6PD activity is markedly reduced in older erythrocytes. This renders mature erythrocytes more vulnerable to the oxidant stress produced by parasitization, and is the mechanism of protection produced by the G6PD deficient condition (see below). *P. malariae* tends to infect mature erythrocytes (Woodruff, 1978; Miller, 1985; Strickland, 1991).

The following sections present a discussion of the population and in vitro studies evaluating the relative protection of the G6PD deficient erythrocyte against *falciparum* parasitization and the mechanism through which this protection takes place. This review covers a large sample of the most informative studies, but is not all-inclusive. Again, the goal of this review is to present the evidence indicating that all G6PD deficient genotypes are relatively protected against *falciparum* malaria, and to illustrate some of the difficulties involved in such epidemiologic investigations.

THE GEOGRAPHICAL DISTRIBUTION OF POLYMORPHIC FREQUENCIES OF G6PD DEFICIENCY RELATIVE TO THE DISTRIBUTION OF FALCIPARUM MALARIA

The G6PD locus is the most highly polymorphic human genetic locus with over 300 known genetic variants of which at least 77 have reached polymorphic frequencies (Luzzatto and Mehta, 1989; Luzzatto and Battistuzzi, 1985). All of the polymorphic alleles occur in populations living in areas where *falciparum* malaria is or was endemic or in populations derived from these areas, and 66 of the 77 alleles that have reached polymorphic frequencies code for variable degrees of G6PD deficiency. The large number of G6PD deficient alleles that have reached polymorphic frequencies in tropical and subtropical environments where malaria
has been endemic and the total absence of polymorphic frequencies of these alleles at more extreme latitudes suggest a relationship between G6PD deficiency and malaria.

Stronger support for the relationship between G6PD deficiency and adaptation to *falciparum* malaria comes from the observation that there is a general covariation in distribution of G6PD deficiency and *falciparum* malaria worldwide and the more specific covariation in the distribution of the G6PD A (–) allele and the allele for hemoglobin S in sub-Saharan Africa (Luzzatto and Battistuzzi, 1985). The within-population variation in G6PD Mediterranean allele frequency with changes in altitude and with the historical pattern of malaria distribution in Sardinia (Siniscalco et al., 1966) lends further support to the view that G6PD deficiency is associated with adaptation to *falciparum* malaria, although Brown (1981) has questioned these relationships as have Gloria-Bottini et al. (1980). However, these relationships would be more meaningful and precise if they could be viewed within the context of population differences in consumption of food crops containing naturally occurring antimalarial substances of plant origin (e.g., fava beans) (see below). Unfortunately, large data sets that evaluate the relationships between quantitative measures of historical malarial morbidity (which distinguish between *falciparum* and *vivax* infection), G6PD deficiency as determined by quantitative assay, and population differences in the consumption of food crops high in antimalarial activity do not exist.

Some critiques of the relationship between G6PD deficiency and *falciparum* malaria are unfounded and are based solely on the fact that these relationships are imperfect. An oft-cited article by Kidson and Gorman (1962) is an example. They questioned the relationship between interpopulation variation in G6PD deficient allele frequency and *falciparum* malaria in New Guinea and New Britain based on the observation that there are somewhat elevated frequencies of G6PD deficiency in several highland populations where malaria is rare and relatively low frequencies of G6PD deficiency in some coastal populations where malaria is endemic. However, when the data they presented are pooled for all of their highland populations and compared to similarly pooled data for all of their lowland populations, the overall allele frequency for G6PD deficiency is 5.3% in the malarious lowlands and 0.93% in the non-malarious highlands. This is entirely consistent with the G6PD deficiency-malaria hypothesis, given the fact that there must be some gene flow between these adjacent regions.

**POPULATION STUDIES**

*Population studies supporting a protective effect of G6PD deficiency in hemizygous males and homozygous females*

**Africa**

Allison and Clyde (1961). An early field investigation by Allison and Clyde (1961) was carried out on 532 children from 4 months to 4 years of age in present-day Tanzania in an area where *falciparum* malaria was holoendemic. Two-thirds of the subjects were recruited from village surveys that were done on virtually all of the age-appropriate children. The remaining one-third of the sample was drawn from infant welfare and out-patient clinics. Parasite rates and densities were significantly lower in G6PD (–) males, but not females, although significant differences were almost reached in the female group. Parasite rates and densities were approximately equivalent in G6PD (–) males and subjects with sickle cell trait, and both were significantly lower than observed in children who were not G6PD deficient or who did not have sickle cell trait.

The strength of this study lies in the fact that the children observed were fairly young and thus the development of immunity to malaria was not complete (Brown, 1969; Jarra, 1983). The acquisition of relative immunity with age is a factor which greatly confounds the interpretation of the results of studies of older children and
adults. A second strength of this study lies in the fact that most of the subjects were sampled from village surveys rather than from among febrile hospitalized individuals and that the survey sample comprised almost the entire population of age-appropriate children. Such a study sample is much less likely to show selection bias than a sample of hospitalized children (see below). Finally, although the rate of malarial parasitization was found to be elevated in G6PD normal subjects, perhaps the more significant finding is the increase in parasite densities in the normals as there is a well-established relationship between parasite density and mortality from malaria (Allison, 1957; Brown, 1969).

Gilles et al. (1967). Gilles et al. (1967) also reported a protective effect of G6PD deficiency against *falciparum* malaria among 100 Nigerian children 4 months to 4 years of age. These children represented a sample of children admitted over a 3-year period to the pediatric emergency room at University College Hospital, Ibadan, who were found to have severe *falciparum* malaria with parasite counts over 100,000 per mm$^3$. A group of similarly aged children attending the same clinic for different reasons served as a control group. The frequency of G6PD deficiency among subjects with severe malaria was significantly lower among both males and females compared to the frequency of the trait in the control population. The protective effect of G6PD deficiency was independent of sickle cell trait status, which was also evaluated and shown to have a protective effect against *falciparum* parasitization.

The strengths of this study are that the subjects are young enough so that the development of immunity is not complete; the degree of *falciparum* parasitization is at levels at which mortality normally occurs, and thus differences in the frequency with which G6PD deficient and normal subjects experience these parasite densities reflect differential exposure to significant selective pressures; and there was no confounding of the protective effect of G6PD deficiency and sickle cell trait.

Asia

Butler (1973). A study by Butler (1973) suggests that G6PD deficiency provides protection against *falciparum* malaria among non-immune adults. This work was carried out on a sample of 277 African-American soldiers in Vietnam. Subjects represented virtually all African-American patients on a ward or attending a clinic on a particular study day. "Most subjects" were taking an antimalarial prophylactic regimen of chloroquine and primaquine. Subjects having a history of malaria in Vietnam were considered malaria patients and parasitological information from the previous hospitalization was taken from the hospital charts.

The G6PD deficient subjects (13.7%) had a significantly lower incidence of malaria (21%) than the G6PD normal group (39%), although the parasite densities did not differ. Approximately 80% of cases were *falciparum* malaria. These findings are particularly interesting in that they evaluate the relationship between G6PD deficiency and malaria incidence in a group of adults who have no exposure, and thus absolutely no degree of immunity, to this disease. This study thus avoids the possibility of sampling bias that occurs in studies carried out on children who have lived their entire lives in endemic areas and who are sampled in population surveys or when they present at hospital. In such studies the G6PD normal and deficient groups may have significantly different degrees of acquired immunity to *falciparum* malaria due to chance, despite the fact that they may be closely matched for age, and this factor could obscure the relationship between G6PD deficiency and protection against *falciparum* malaria in a random fashion from study to study.

That the G6PD deficient subjects showed lower infection rates despite the widespread use of antimalarial prophylaxis is particularly impressive and is consistent
with the findings of in vitro studies which indicate that divicine and isouramil (the naturally occurring antimalarial substances in fava beans) have a more profound antimalarial effect in G6PD deficient than normal erythrocytes (Golenser et al., 1983, 1988) (see below). These African-American servicemen are relatively resistant to P. vivax due to their high frequency of Duffy negative phenotype which conveys almost complete protection against vivax parasitization (Livingstone, 1984). This circumstance removes a confounding factor that occurs in many Asian studies in which it is difficult to evaluate the relative protection of G6PD deficient genotypes against falciparum malaria due to their possibly greater vulnerability to vivax parasitization (see below).

Although these findings are provocative there are several limitations to this study. One is that the subjects were not randomly recruited into the sample. Therefore, there may have been selection bias, although 95% of the hospitalized subjects approached agreed to participate in the study. Also, the author reports that "most subjects" were taking malaria prophylaxis of chloroquine and primaquine. Such imprecise assessment of drug use makes it impossible to assess differential antimalarial exposure between G6PD deficient and G6PD normal subjects. Nevertheless, despite these limitations this study is the only field investigation that has evaluated the possible protective effect of G6PD deficiency against falciparum malaria in a population where the immune status of the subjects was not a confounding factor.

Kar et al. (1992). Kar et al. (1992) evaluated the relationship between erythrocyte G6PD status and the prevalence of Plasmodium vivax and P. falciparum parasitization in a "random sample" of 708 Ao Nagas subjects of both sexes from Nagaland in northeast India. The age of the subjects was not given. Of these individuals, 324 (45.8%) had malarial parasites and constituted the malarial sample and 384 (54.2%) were uninfected and served as a control sample.

Both P. vivax and P. falciparum parasitization rates were significantly greater in G6PD normal than G6PD deficient males, and parasitization rates with both species were also greater among G6PD normal females than heterozygous G6PD deficient females. There were no significant differences in malarial parasitization between homozygous G6PD deficient females and G6PD normal subjects, but the number of homozygous G6PD deficient females was quite small.

The major strength of this study is that the subjects were "randomly selected" by sex so that there should not be a selection bias, although the authors provide no information about the source of the subjects and the precise manner in which they were sampled. Sample size is also quite large.

The main weakness of this study is that there is no indication whatsoever of the age of the subjects. Since acquired relative immunity is established by about 5 years of age, it is highly preferable that subjects in such studies be younger than 5 (see above). The period prior to the acquisition of immunity is the time during which a biologic trait like G6PD deficiency, or sickle cell trait, should convey its maximal relative protection, and when selection against G6PD normal individuals should be most intense.

One must assume that the sample is composed of adults. If this is the case, then these are even more important findings since they indicate that both G6PD deficient males and heterozygous deficient females have much lower parasite rates than G6PD normal subjects in an area where malaria prevalence is extremely high (45.8%). This finding suggests that the G6PD deficient condition still confers relative protection in the adult, even after immunity is acquired.

Another important finding of this study is the fact that the relative protection of the G6PD deficient condition extends to both P. vivax and P. falciparum, and in both sexes. P. vivax actually accounts for 56.8% of the cases of malaria, so it is more common that falciparum in this region and possibly a significant selective vector (although it is invariably a less severe form of malaria).
Population studies demonstrating a protective effect of G6PD deficiency in heterozygous females

Africa

Luzzatto et al. (1969). Luzzatto et al. (1969) presented an elegant demonstration of the protective effect of G6PD deficiency against *falciparum* malaria by evaluating differential parasitization of deficient and non-deficient red blood cells in heterozygous females. Subjects were 1- to 5-year-old Nigerian children with acute *falciparum* malaria. Due to the Lyon Mosaic Effect, approximately one-half of the erythrocytes of heterozygous females contain normal levels of G6PD while the other half are severely deficient. Normal and deficient cells can be distinguished microscopically using the methemoglobin elution technique. In the 20 heterozygous subjects studied, the rate of parasitization was two to 80 times greater in the G6PD normal than in the G6PD deficient erythrocytes in the same individual. Comparison of parasite rates in the G6PD deficient red cells and the small number of G6PD normal erythrocytes in homozygous females \((n = 5)\) led to similar findings. Between 1 and 15 percent of erythrocytes had normal G6PD activity in the hemizygous deficient males and homozygous deficient females with *falciparum* malaria. These were probably young reticulocytes. In these subjects the parasite rates in the G6PD normal erythrocytes were between six to 81 times greater than the rate of parasitization in the G6PD deficient erythrocytes in the same individuals.

This study is particularly convincing because it demonstrates differential levels of parasitism in G6PD deficient and non-deficient red cells *in the same individual*. The confounding effect of age differences among subjects and differential immunity is thus neatly avoided as is the possible problem of differences in intake of antimalarial substances of dietary origin among the different G6PD genotypes (see below).

Bienzle et al. (1972). In another series of studies in Nigeria, Bienzle, Luzzatto, and co-workers (Bienzle et al., 1972, 1979; Guggenmoos-Holzmann et al., 1981; Luzzatto and Bienzle, 1979) presented evidence suggesting that the G6PD deficient heterozygous females were relatively protected against *falciparum* malaria, but not the more severely deficient hemizygous males and homozygous females. These studies are discussed more fully below in the section on negative findings for a protective effect of G6PD deficiency in hemizygous and homozygous deficient subjects.

Asia

Kar et al. (1992). See above.

Population studies indicating no relative protection of hemizygous and homozygous G6PD deficient individuals against falciparum malaria

Africa

Bienzle et al. (1972). One of the most comprehensive and frequently cited field studies of the relationship between G6PD deficiency and *falciparum* malaria is that of Bienzle et al. (1972) in Nigeria on 702 children aged 9 months to 6 years presenting at hospital with acute febrile illness and 189 healthy young adults aged 14 to 20 years. Sixty-six percent of the children were found to have *falciparum* parasitemia.

G6PD genotypes were determined by a combination of quantification of the enzyme activity, qualitative analysis by starch-gel electrophoresis, and cytochemical localization of enzyme activity in individual cells. The authors reported that
among the children with malaria, G6PD A males and G6PD A/G6PD B females were less frequent compared to the group of children who did not have malaria, but these differences did not reach statistical significance. These genotypes also showed a lower frequency of high parasite counts compared to the other genotypes, but it is unclear from the report whether these findings reached statistical significance.

In a later reanalysis of the same data, Bienzle et al. (1979) reported that the malaria infection rates in the children up to 6 years of age were highest in hemizygous and homozygous G6PD deficient subjects at all ages, with the G6PD normal subjects having the next highest infection rate, and the G6PD A/G6PD B heterozygote females having the lowest infection rates at all ages. Their re-analysis of these data showed that the G6PD A/G6PD B subjects had the lowest mean, median, 90th percentile, and maximum parasite densities compared to the G6PD normal and the combined hemizygous and homozygous deficient group and that these differences were significant ($P = .025$). These data were re-analyzed further by Guggenmoos-Holzmann et al. (1981) with similar conclusions.

The viewpoint that emerged from the various publications emanating from this single study is "that there is no protection against malaria in G6PD deficient hemizygous (Gd A-) boys or homozygous (Gd A~/Gd A-) girls" (Luzzatto and Bienzle, 1979:1183) and that "epidemiologic data indicate strongly that G6PD deficiency can confer relative resistance against Plasmodium falciparum malaria, and clinical data indicate that this is confined to heterozygous females" (Luzzatto and Mehta, 1989:2237). This position is reiterated by Luzzatto and Battistuzzi (1985) and Usanga and Luzzatto (1985).

This field investigation is the most often-cited study in the current literature on the relationship between G6PD deficiency and malaria, probably in consequence of: 1) the fairly large sample; 2) the great authority of Bienzle and Luzzatto relative to the determination of enzyme activity and the precise genotypic characterization of the subjects; and 3) Luzzatto's frequent reviews which refer to this investigation. There are many strengths to the study and the findings of relative protection of G6PD A~/G6PD B heterozygotes from falciparum malaria seem well-established. Nevertheless, there are aspects of the study design that suggest the possibility of significant selection bias which may undermine the investigation's ability to detect any relative protection afforded to the hemizygous and homozygous deficient genotypes.

The major methodological problem with this study, and all those that are based on acutely ill subjects seen at hospital, is the fact that hemizygous and homozygous G6PD deficient individuals may be less likely to become infected with falciparum malaria (Allison and Clyde, 1961; Gilles et al., 1967; Butler, 1973; Kar et al., 1992), but that when they do become infected with malaria they are more likely to be brought to hospital. This is due to a more profound, malaria-associated, hemolysis as a consequence of the greater vulnerability of the G6PD deficient erythrocyte to the oxidant stress of malarial parasitization (see below). Therefore, the question is whether the malarial parasitization rate in the G6PD deficient subjects in this study by Bienzle et al. (1972) is representative of the parasitization rate among G6PD deficient subjects of this age in the community at large, or whether, for the above reasons, it is a sample of G6PD deficient subjects biased in the direction of greater parasitization rates because G6PD deficient subjects are more likely to go to hospital when they have malaria (more hemolysis) than G6PD normal subjects.

It is also possible that when falciparum parasitization does become established in G6PD deficient children it may present in a more severe form (even though it may be less frequent) if they have had fewer malarial infections due to the protection of the G6PD deficient condition and thus have not developed the same degree of acquired immunity as age-matched G6PD normal subjects.

Under these circumstances, this selection bias could only be avoided with a study design that samples differential falciparum parasitization among all children in a
community, not just those who are brought to hospital. This was the design employed by Allison and Clyde (1961), which is, unfortunately, the only population study that clearly employed such an approach.

Martin et al. (1979). In another study in Nigeria, Martin et al. (1979) evaluated the relative frequency of G6PD deficiency in Nigerian children with malaria and without malaria. Subjects were 30 children with a mean age of 3.6 years who were brought to hospital with convulsions and who had P. falciparum densities greater than 100,000/µl and 38 children with a mean age of 3.4 years without parasites in their blood smears who had been brought to the hospital because of convulsions from causes other than malaria. The authors reported that 20% of the subjects with malaria were G6PD deficient compared to 21% of the controls and concluded that G6PD deficiency does not protect against falciparum malaria.

Unfortunately, there are several methodological problems that greatly limit the usefulness of these findings. First, there could be significant sampling bias, as noted in the discussion of Bienzle et al. (1972) above, since G6PD deficient individuals with malaria hemolyze more readily than G6PD normal subjects and thus may be brought to hospital more frequently. A second difficulty is that both the experimental and control groups were chosen from among children presenting at hospital with convulsions, which is likely to produce a highly biased sample of subjects. This is a consequence of the well-established fact that G6PD deficient newborns experience a higher rate of neonatal jaundice, more intense hyperbilirubinemia, and a higher prevalence of kernicterus than do non-deficient subjects (Piomelli, 1986; Valaes et al., 1985). A greater prevalence of minimal cerebral dysfunction and various degrees of brain damage is also seen in G6PD deficient newborns (Meijer, 1984; Piomelli, 1986; Singh, 1986). A group of children sampled on the basis of having convulsions associated with falciparum malaria is much more likely to include a disproportionate number of G6PD deficient individuals, because G6PD deficient children are more likely to have minimal cerebral injury which will be expressed only under conditions of stress such as malaria. A sample of children experiencing convulsions unassociated with malaria is more likely to be composed primarily of individuals with more severe neurological defects that are not associated with G6PD deficiency. Therefore, a study sampling on the basis of presentation with convulsions is likely to produce spurious results. Given these limitations and the very small sample size, this study cannot be given much weight in evaluating the question of the relative protection of the various G6PD genotypes against falciparum parasitization.

Asia

Kruatrachue et al. (1962). Another negative finding on the protective effect of G6PD deficiency is a study by Kruatrachue et al. (1962) on 519 boys between 1 and 9 years of age in Thailand. Among the 203 boys aged 1 to 3 years, falciparum malaria was more common in G6PD deficient boys than in the G6PD normal subjects (23.7% to 19.4%) as was vivax malaria (39.5% to 21.9%) and the difference between the two groups for vivax infestation was statistically significant.

It is not clear from the report how the subjects were sampled. The authors only state that two areas of endemic malaria (32%, 51%) were chosen and state at one point that “most of our patients who had “fever” (1962:1186), suggesting that these were hospital or clinic patients. If the sampling took place in a hospital setting, then this study is open to the same criticism of all such studies (see above) in that there may be significant selection bias because of the greater likelihood that G6PD deficient subjects will hemolyze, and thus be brought to hospital, if they contract malaria.

If the subjects had been sampled randomly, the findings of this investigation would have greater authority and would undermine the view that G6PD deficiency protects against falciparum parasitization. However, this study may help answer
a part of the puzzle rather than erode the well-supported finding that G6PD deficiency provides relative protection against *falciparum* infection. The higher rate of malarial parasitization of G6PD deficient boys in this investigation is a consequence of *vivax* malaria (39.5% in deficients, 21.9% in G6PD normals) whereas the *falciparum* parasitization rates of children 1 to 3 years of age were not significantly different (23.7% in deficients, 19.4% in G6PD normals).

As the authors of this report note, *P. vivax* tends to parasitize preferentially young red blood cells which exist in elevated numbers in G6PD deficient subjects due to recurrent self-limited hemolysis and some associated reticulocytosis. If G6PD deficient subjects are more susceptible to *vivax* malaria this probably is not a major selective disadvantage as the infection is likely to be confined to the small number of reticulocytes, and *P. vivax* is self-limited and generally not life-threatening.

In areas where both *vivax* and *falciparum* malaria are endemic, such as South Asia, a greater susceptibility of G6PD deficient individuals to a mild *vivax* infection that is limited to reticulocytes may actually be advantageous if it decreases the likelihood that those same G6PD-rich erythrocytes will be invaded by the more virulent *P. falciparum*. Following this logic, the lower reticulocyte levels in G6PD normal individuals would render them less susceptible to *vivax* and thus more open to *falciparum* infection. Although these data of Kruatrachue et al. (1962) from Thailand show a higher degree of *vivax* parasitization of G6PD deficient subjects, they do not demonstrate a concomitantly lower level of *falciparum* parasitization in these individuals. However, there may be seasonal variability in such a phenomenon with the protective effect occurring only during very peak periods of *vivax* endemicity. Under these circumstances, the relative advantage of the G6PD deficient genotypes could only be assessed by evaluating infection rates for both species in all genotypes throughout an annual cycle.

*Vivax* malaria does not exist in sub-Saharan Africa because sub-Saharan African populations have virtually 100% (1.0) frequency of the Duffy negative allele which conveys almost total protection against *vivax* parasitization (Livingstone 1984). Therefore, it should be quite a bit more straightforward to evaluate the relative protection of G6PD deficient genotypes against *falciparum* malaria in studies carried out in this region.

**North America**

*Powell and Brewer (1965).* Powell and Brewer (1965) carried out an experimental study of susceptibility to induced malaria in 16 volunteer subjects who were African-American males in prison. Eight subjects were G6PD deficient with a mean age of 32 years and eight had normal G6PD activity and a mean age of 33 years. None of the subjects had a prior history of malaria, or a disease suggesting malaria, or had visited an area where malaria was common. Body weights were comparable in the two groups and none of the subjects had hemoglobin variants.

The subjects were exposed to *Anopheles stephensi* mosquitoes infected with the McLendon strain of *P. falciparum* and all subjects except one G6PD normal individual developed patent *falciparum* infections. Mean length of the prepatent period and mean levels of parasitemia did not differ between the two groups and chloroquine antimalarial therapy was initiated when parasite levels first exceeded 5,000 per mm$^3$. Therefore, this investigation appears to provide strong experimental evidence indicating that G6PD deficiency does not protect against *falciparum* infection.

However, the major weakness of this study is that it is experimental rather than naturalistic. Subjects were bitten by ten falciparum-infected *Anopheles stephensi* mosquitoes resulting in a massive parasite dose that is uncommon or non-existent under natural conditions. Livingstone (1958:536) states that in West Africa "the average number of infective bites per person per year is always greater than about
five, and in some areas ranges up to 100 or more." It is likely that the biochemical advantage of the G6PD deficient condition evolved in response to something like this modest infection rate and not as an adaptation to massive artificial levels of parasitization that would result as a consequence of an individual being exposed to ten infected mosquitos simultaneously. Also, in vitro studies have suggested that maximal antimalarial protection is achieved when G6PD deficient erythrocytes are treated with naturally occurring antimalarial substances from food sources (Golenser et al., 1988). Such data suggest that experimental studies like the one carried out by Powell and Brewer do not reproduce the natural conditions under which selection has taken place (natural levels of exposure and concomitant consumption of traditional dietary items), and provide further support for the view that community studies represent the optimal study design for evaluating the relative susceptibility of the various G6PD genotypes to malarial parasitization. An experiment such as the one carried out by Powell and Brewer is somewhat akin to an hypothetical study in which eight lightly pigmented and eight darkly pigmented individuals would be exposed to intense artificial ultraviolet radiation resulting in skin cancer in all of the subjects, with the conclusion that darkly pigmented skin does not provide relative protection against the carcinogenic effects of ultraviolet radiation.

IN VITRO STUDIES OF MALARIAL PARASITE GROWTH IN G6PD DEFICIENT ERYTHROCYTES

With the development of techniques for the continuous culture of human malarial parasites (Trager and Jensen, 1976) it became possible to experimentally evaluate whether there is a protective effect of G6PD deficient erythrocytes against P. falciparum parasitization. Clark et al. (1989) point out that it took decades to develop these techniques largely because it was not realized that the parasites only tolerated low levels of oxygen in vitro. Thus, from early on there were clues indicating that malarial parasites were vulnerable to oxidant stress. These observations suggested that genetic factors, such as G6PD deficiency, which increase the likelihood of uncompensated oxidant stress to the red cell, might provide some degree of antimalarial protection for the deficient erythrocytes. This led to a series of in vitro investigations which illuminated how malarial parasites are vulnerable to oxidant stress and how this phenomenon contributes to the protective effect provided by G6PD deficient red cells against falciparum malaria.

As noted above, certain aspects of the life cycle of the erythrocyte also have an influence on malarial parasitization. Since it lacks a nucleus and mitochondria, the normal erythrocyte has a life span of about 120 days. The level of activity of G6PD in erythrocytes declines with age, and the half-life of normal G6PD B is about 62 days. However, G6PD in the erythrocytes of deficient individuals is much less stable and the activity level decreases dramatically. Thus, the half-life of G6PD A (–) is only 13 days and that of G6PD Mediterranean is just 8.5 days (Luzzatto and Testa, 1978). Therefore, older G6PD deficient erythrocytes have very low G6PD levels and are extremely vulnerable to oxidant stress, while reticulocytes and young erythrocytes have much higher G6PD activities and are far less susceptible to oxidant damage.

In early field investigations it was observed that in the normal course of parasitization P. falciparum and P. vivax both preferentially invade younger red cells that are rich in G6PD (Allison and Clyde, 1961; Kruatrachue et al., 1962). That observation raised the possibility that the low G6PD levels of G6PD deficient red cells may be protective against Plasmodium parasitization. These observations had informed the direction of the field studies evaluating the possible protective effect of the G6PD genotypes against falciparum parasitization, and they also informed the direction of the in vitro studies that were carried out beginning in the latter half of the 1970s.
In vitro studies demonstrating a protective effect of G6PD deficiency against Plasmodium parasitization

Eckman and Eaton (1979) observed a twofold increase in GSH level in erythrocytes of Swiss white mice that had been infected with *Plasmodium berghei*. In a series of experiments they demonstrated that this GSH was largely parasitic in origin, rather than having been derived from the infected red cells, and that the parasites were oxidizing red cell NADPH to maintain parasitic glutathione in the reduced form (GSH). From these observations on erythrocytes in G6PD normal mice the authors then hypothesized that G6PD deficient erythrocytes would protect against fulminant malaria infection because utilization of NADPH by both the host erythrocyte and malaria parasite would overwhelm the limited ability of the G6PD deficient red cells to regenerate NADPH and the resultant decrement in GSH would lead to oxidant-induced hemolysis. They also suggested that the accumulation of oxidized glutathione (GSSG) in the parasitized G6PD deficient erythrocyte would inhibit parasite protein synthesis, an idea that had received some experimental support in an earlier study by Kosower and Kosower (1970).

Friedman (1979) cultured *P. falciparum* in normal and G6PD A (–) deficient erythrocytes in conditions which either enhanced or inhibited oxidant damage. Culture conditions under an atmosphere of 25% O₂ increased oxidant stress. This did not lead to a decrease in parasite multiplication in G6PD normal cells, but produced a 50% decrease in multiplication in G6PD deficient cells. Addition of the antioxidant compound vitamin E to the G6PD deficient culture mixture mitigated the oxidant stress somewhat so there was only a 30% decrease in parasite multiplication. Addition of DTT (dithiothreitol), a thiol reducing agent, to the G6PD deficient culture medium further mitigated the effect of the stress so that there was no decrease in *falciparum* multiplication and thus a total abolition of the protective effect of the G6PD deficient erythrocyte against *falciparum* parasitization. In order to approximate more closely in vivo conditions, the GSH that was a normal constituent of the culture medium was removed and under these circumstances *P. falciparum* multiplication was reduced by 62% in G6PD A (–) deficient erythrocytes at 17% O₂. In sum, this study demonstrated that *P. falciparum* multiplication was greater in G6PD normal than G6PD A (–) erythrocytes when an additional oxidant stress was applied to the system, and the author suggested that oxidants found in some foods could contribute to such an effect in vivo.

Roth et al. (1983)

A series of experiments by Roth et al. (1983) provided the first unequivocal evidence that *P. falciparum* multiplication is retarded in erythrocytes from G6PD deficient individuals under normal culture conditions. These studies were carried out on blood samples drawn from G6PD normal subjects, G6PD hemizygous deficient males, and G6PD heterozygous deficient females from Sardinia. None of the subjects had hemoglobin abnormalities and the hemoglobin F content of all subjects was less than 1%. Culture conditions were at 17% oxygen.

Five days after inoculation of *P. falciparum* into the culture the G6PD deficient hemizygous and heterozygous cultures both showed levels of parasitemia about one-third of that of the G6PD normal controls. There was a similar reduction in the parasitemias in the two cultures despite the fact that the cultures from the heterozygotes contained 30 to 40% histochemically G6PD normal erythrocytes compared to the hemizygous cultures which contained close to 100% G6PD deficient red cells.

The authors suggested that *falciparum* parasite growth inhibition was successful in their experiment under normal culture conditions because the G6PD defi-
cient erythrocytes were from Sardinian subjects who were more severely G6PD deficient, while the erythrocytes used by Friedman (1979) were from an individual with the less severely deficient G6PD A (−) variant and thus required the aid of an additional oxidant stress (25% O₂ culture conditions) to show a protective effect.

Luzzatto et al. (1983)

Luzzatto et al. (1983) inoculated synchronized cultures of *P. falciparum* in parallel into G6PD Mediterranean deficient and G6PD normal erythrocytes and found that the two types of cells were infected at about the same rate, but that there was approximately a 40% decreased rate of parasite growth in the G6PD (−) erythrocytes by the second schizogonic cycle. Similar findings were observed with G6PD Mahidol (−) and G6PD A (−). However, when they grew the *Plasmodium* parasites through several passages in the G6PD Mediterranean (−) erythrocytes and evaluated their ability to invade G6PD (+) and G6PD (−) erythrocytes they found that the parasitization rates were almost the same. It thus appeared that the surviving parasites had adapted to the G6PD (−) host cells and were capable of invading subsequent G6PD (−) erythrocytes almost as successfully as they were able to invade G6PD (+) erythrocytes.

Usanga and Luzzatto (1985)

Usanga and Luzzatto (1985) conducted a series of experiments designed to extend their previous findings. They reported that when *P. falciparum* parasites were grown through four successive cycles in G6PD A (−) red cells (adapted parasites) a "slight, not significant, difference was observed" in G6PD A (−) infection rates compared to G6PD A (+) infection rates, and that "in four separate experiments, multiplication of these adapted parasites, measured in a fifth cycle in G6PD-deficient (A−) RBC, was 79 ± 5% of the multiplication observed in transfer back to G6PD-normal RBC" (1985:794).

The investigators then evaluated the hypothesis that adaptation to the G6PD deficient erythrocytes was a consequence of induction of parasite G6PD enzyme activity in the G6PD deficient environment. When they examined G6PD activity in parasitized, compared to non-parasitized, G6PD (−) cells they found that G6PD activity was two to five times higher in the parasitized cells. When they examined G6PD extracts from parasitized G6PD A (−) erythrocytes they observed two bands of electrophoretically different G6PD activity, one of which was comparable to host cell enzyme while the other appeared to represent G6PD synthesized by *P. falciparum*. This process of induction did not occur when the parasites were grown through four cycles of G6PD A (+), an enzyme variant with normal activity, suggesting that this process of adaptation does not take place when the parasites invade erythrocytes with normal levels of G6PD activity.

Usanga and Luzzatto (1985:795) concluded that this observation explains why field studies have shown that "hemizygous G6PD-deficient males are not protected against infection and also explains the protection of heterozygous females." They argue that under in vivo conditions the *Plasmodium* parasites will adapt to the G6PD deficient erythrocytes in hemizygous and homozygous deficient individuals after a few cycles, thus overcoming the initial protection of the G6PD deficient condition. However, they maintain that in the heterozygote this process of adaptation will not take place as the parasites emerging from the G6PD normal erythrocytes will face an approximately 50% probability of entering a G6PD deficient erythrocyte to which they are not adapted and they imply that this should limit the parasitization. In another publication derived from this work Luzzatto et al. (1986) state that "However, in heterozygous females parasite adaptation will be persistently frustrated by the co-existence of G6PD normal and G6PD deficient red cells. . . . As a result, the susceptibility to malaria mortality of the Gd + /Gd-genotype is reduced...."

These are interesting findings which suggest that there is some degree of adaptation on the part of the *P. falciparum* parasite to the G6PD deficient host envi-
environment; however, such an observation in itself does not prove that "hemizygous G6PD-deficient males are not protected against infection" (Usanga and Luzzatto, 1985:795), which has been the conclusion of their own field studies (Bienzle et al., 1972; Luzzatto and Bienzle, 1979; Guggenmoos-Holzmann et al., 1981).

There are several objections to these findings. First, Usanga and Luzzatto (1985:794) report that parasites adapted through four cycles of G6PD (–) cells, when "measured in a fifth cycle in G6PD-deficient (A–) RBC, was 79 ± 5% of the multiplication observed on transfer back to G6PD-normal RBC." Although this decrease in parasitic growth rate is not as dramatic as occurs in P. falciparum that have not been adapted to G6PD (–) erythrocytes, it is still substantial and it can be argued that such a reduction is likely to slow the rate of development of the parasitemia to a biologically significant degree. Second, these studies should be carried out over a much larger number of parasite cycles in order to develop a better idea of the course of parasitization in G6PD deficient and G6PD normal erythrocytes. Third, it is difficult to see how the G6PD deficient heterozygote condition can be the most protective against falciparum multiplication. Approximately half of the erythrocytes in the heterozygote have normal G6PD activity and Luzzatto's own work has demonstrated that these erythrocytes have parasitization rates of two to 80 times those of the G6PD deficient erythrocytes in the same individuals under natural conditions (Luzzatto et al., 1969). Nothing is protecting these G6PD (+) red cells from parasitization, and if parasites that have adapted to the G6PD deficient condition then enter the G6PD normal red cells, there should be no impediment to their development.

In heterozygotes, parasites entering G6PD (–) cells after having cycled through G6PD (+) cells would have a growth rate of about 45% of the rate they would have had in G6PD (+) red cells (Usanga and Luzzatto 1985). However, this moderate diminution in growth in approximately 50% of the erythrocytes in heterozygous individuals should not necessarily convey greater antimalarial protection than a 20% diminution in parasite growth rate in all erythrocytes among parasites that have adapted to G6PD (–) red cells in hemizygous and homozygous individuals.

Further, if dietary oxidants potentiate the protective effect of G6PD (–) genotypes against falciparum parasitization (see below), then a far larger proportion of the erythrocytes will benefit from this phenomenon in G6PD deficient hemizygotes and homozygotes than in the heterozygotes. Therefore, the evidence provided by Luzzatto and co-workers in these in vitro studies to support the findings of their single field investigation (Bienzle et al., 1972) is not convincing. It does not support the view that there is only relative antimalarial protection in G6PD deficient heterozygotes and no protection against falciparum parasitization in hemizygous and homozygous G6PD deficient individuals.

Roth and Schulman (1988) further evaluated adaptation of P. falciparum parasites to G6PD deficient erythrocytes. They noted that it had been demonstrated that P. falciparum produced its own G6PD enzyme after several cycles of growth in G6PD (–) erythrocytes. In several experiments they investigated whether this parasite enzyme can enable the G6PD deficient host cell to resist oxidant stress as well as G6PD normal cells resist such stress. In these experiments they cultured P. falciparum in G6PD normal erythrocytes, G6PD deficient erythrocytes for one growth cycle only, and G6PD deficient erythrocytes for a minimum of five growth cycles. (G6PD deficient erythrocytes were G6PD Mediterranean with less than 1% of normal G6PD activity and G6PD A (–) with 12 to 15% activity.) All cultures were then challenged with acetylphenylhydrazine (APH), a powerful oxidant.

It was found that the unadapted malarial parasites added to the G6PD Mediterranean red cell medium showed very poor growth for 4 days (two cycles) after which growth approximated that of parasites in G6PD normal cells. However, falciparum parasites adapted through growth in G6PD Mediterranean red cells for five growth cycles and then added to a G6PD Mediterranean red cell medium
showed near normal growth. These findings replicated the work of Usanga and Luzzatto (1985) demonstrating the ability of \textit{P. falciparum} to adapt to G6PD deficient erythrocytes after several cycles.

The investigators then evaluated the sensitivity of the three different parasite-red cell systems to the inhibitory effects of the oxidant stress of APH over a dose range of 10 to 500 µg/ml. These experiments demonstrated that the unadapted parasites in G6PD Mediterranean erythrocytes were eight times more susceptible to the inhibitory effects of APH than parasites in G6PD normal systems. Compared to this baseline, adapted parasites in G6PD Mediterranean red cells were four times more sensitive to APH than parasites in G6PD normal erythrocytes. The parasite G6PD observed by Usanga and Luzzatto (1985) in \textit{P. falciparum} adapted to G6PD (−) cells increased the ability of the parasites to multiply at near-normal rates in G6PD Mediterranean red cells that were not under external oxidant stress. However, the adapted parasites in G6PD Mediterranean cell systems were still four times more vulnerable to additional oxidant stress than were parasites in G6PD normal cell systems.

The results with parasites grown in G6PD A (−) cells were somewhat different. Unadapted parasites began to grow without a lag period but growth was significantly reduced for the first two growth cycles, while parasites adapted to grow in G6PD A (−) red cells grew at a rate equivalent to those in G6PD normal erythrocytes. When APH was applied to parasite-G6PD A (−) cell systems the results were markedly different from those in the G6PD Mediterranean system. Both unadapted and adapted parasite-G6PD A (−) systems showed only slightly greater parasite sensitivity to the inhibitory effects of APH compared to parasite-G6PD normal cell systems.

It seems clear from these data that adaptation of \textit{P. falciparum} to G6PD Mediterranean red cells is minimal, as the parasites in these parasite-red cell systems were still four times more sensitive to oxidant stress than parasites in control parasite-G6PD (+) red cell systems. However, both unadapted and adapted parasites in G6PD A (−) erythrocytes were only about 30 to 40% more vulnerable to an oxidant stress than were parasites in G6PD normal cells. Although this greater sensitivity to oxidant stress is modest and was not statistically significant in this study, it is still probably biologically significant in protecting G6PD A (−) erythrocytes against \textit{falciparum} parasitization.

**IN VITRO STUDIES OF THE EFFECT OF OXIDANT STRESS FROM NATURALLY OCCURRING ANTIMALARIAL SUBSTANCES OF PLANT ORIGIN ON \textit{PLASMODIUM GROWTH IN HUMAN ERYTHROCYTES**

While parasitologists were carrying out in vitro investigations of the possible protective effects of G6PD deficient erythrocytes against \textit{P. falciparum} infection (see above), several nutritional biochemistry groups were exploring the nature of the hemolysis-producing compounds in fava beans (Beutler, 1970; Chevion et al., 1982, 1983; Jamalian, 1978; Jamalian et al., 1977; Higazi and Read, 1974). Since prophylactic administration of aminoquinoline antimalarials had been identified as the cause of hemolysis in G6PD A (−) subjects in early studies (Beutler et al., 1955; Beutler, 1957; Carson et al., 1956), the chemical nature of the favism-inducing compounds was of interest. This was not only from a public health point of view, but also in terms of whether these compounds might provide antimalarial protection. This would be particularly important for an understanding of how natural selection operates at the G6PD locus; if these compounds inhibit \textit{falciparum} development, it would be essential to ascertain whether they act differentially on the various G6PD genotypes to produce variation in vulnerability to \textit{falciparum} parasitization among the different G6PD genotypes in human populations.

A number of studies subsequently identified the source of the favism-producing compounds as the B-glycosides vicine and convicine, which constitute approxi-
mately 0.5% of the wet weight of the *Vicia faba* seed. These compounds are hydrolyzed in the gastrointestinal tract to form their unstable pyrimidine aglycones divicine and isouramil (Chevion et al., 1982; Chevion et al., 1983; Marquardt, 1989). The oxidized forms of these compounds have been shown to lower red cell reduced glutathione, and G6PD deficient erythrocytes, with their limited ability to regenerate reduced glutathione, hemolyze readily in the presence of these substances (Mager et al., 1965; Clark et al., 1989). These aglycones also generate hydrogen peroxide and free radical species (Chevion et al., 1982; Winterbourn et al., 1986).

It was thus clear from the chemical characterization of these substances that they place an oxidant stress on the red blood cell that is similar to that of the aminoquinoline antimalarials and that these compounds may have antimalarial effects (Etkin, 1979; Katz and Schall, 1979, 1986; Friedman, 1979).

Studies evaluating the antimalarial effects of divicine and isouramil

In vitro studies

Golenser et al. (1983). Golenser et al. (1983) carried out a series of studies on erythrocytes from a G6PD Mediterranean hemizygous male (<2% normal activity), two G6PD Mediterranean heterozygous females (40% and 50% normal activity), and two G6PD normal males. In each experiment there were two treatments of the erythrocytes from each subject: 1) they were bathed in a control solution; or 2) they were bathed in a solution of isouramil. All of the erythrocyte treatment conditions were then inoculated with the *P. falciparum* cultures.

The development of *P. falciparum* in the G6PD normal erythrocytes was not affected by the pretreatment of the red cells with isouramil. In both the control and the isouramil treated erythrocytes, 10% of the red cells were parasitized by the third day. Parasitemias reached similar levels in the G6PD deficient red cells that had been bathed in control solution, but in contrast, parasite growth was totally unsupported in the G6PD deficient erythrocytes that had been bathed in isouramil.

When evaluated as a function of the G6PD activity of the host erythrocytes, isouramil pretreatment totally suppressed parasite development in the G6PD hemizygous deficient (<2% activity) suspension, had an intermediate effect in the heterozygous suspensions (40 to 50% activity), and only had a very minor effect in the G6PD normal red cells. Young parasite stages (ring forms) were much less sensitive to the effect of isouramil than trophozoites and schizonts. The investigators concluded that G6PD normal individuals may be partially protected and G6PD deficient genotypes more fully protected against *falciparum* malaria. As a consequence of the ingestion of isouramil in fava beans.

Golenser et al. (1988). In this series of experiments Golenser et al. (1988) again examined *P. falciparum* parasite development in cultures of untreated G6PD normal and G6PD Mediterranean erythrocytes. They also studied parasite development in cultures of G6PD normal and G6PD Mediterranean parasitized erythrocytes that were exposed to crisis form factor (CFF; a normal blood constituent associated with the development of immunity to malaria), isouramil, and diamide.

In the first set of experiments with untreated G6PD Mediterranean erythrocytes they found that the total number of parasites in the deficient red cells was not lower than in normal erythrocytes. However, on closer examination they noted a profound retardation in the stage distribution of parasites in G6PD deficient red cells—with a predominance of less mature forms in the G6PD deficient red cells at comparable times in the parasitemia compared to the normal erythrocytes.

When parasites in the G6PD deficient and G6PD normal erythrocytes were exposed to CFF, the *P. falciparum* within the G6PD deficient erythrocytes were significantly more sensitive to the inhibitory activity of CFF, with a high percentage of ring forms failing to develop within these erythrocytes.
Exposure of parasitized G6PD deficient erythrocytes to the oxidant isouramil, derived from fava beans, and also to diamide, a thiol oxidizing agent, did not have a greater effect on parasite morphology than in parasitized G6PD normal erythrocytes. However, measures of parasite incorporation of [3H]-isoleucine and [H]-hypoxanthine showed that mature parasites in G6PD normal erythrocytes were more sensitive to the oxidant effect of isouramil than were the ring forms. Further, in G6PD deficient erythrocytes both ring forms and mature stages were more sensitive to oxidant stress than the corresponding stages in G6PD normal erythrocytes.

In summary, taken together these two studies (Golenser et al., 1983, 1988) indicate that G6PD Mediterranean erythrocytes are resistant to falciparum parasitization on their own and that this resistance is greatly potentiated by exposure to CFF and isouramil, a dietary oxidant derived from fava beans. In addition, isouramil also had a modest suppressant effect on mature parasite forms in G6PD normal erythrocytes.

In vivo studies

Clark et al. (1984). Clark et al. (1984) studied the effect of intravenous administration of divicine into mice that had been infected with Plasmodium vinckei, a mouse malarial parasite. Six days after infection with P. vinckei, after parasitemias were between 30 and 60%, the mice (n = 22) received an intravenous injection of divicine. By 1 hour after intravenous injection of the divicine microscopic examination indicated that 38% of the parasites had degenerated within the circulating erythrocytes. Further, the micrographs indicated that parasite membranes were damaged while the host erythrocyte membranes were intact. At 1 hour, the total parasitemia, counting intact and damaged parasites, had fallen by 25%, and 45% of the parasites observed by light microscope had shrunken with fragmented and pyknotic nuclei. Within 2 hours of injecting divicine the urine of the mice had changed to a dark black color and hemolysis was confirmed by hematocrit and plasma hemoglobin values, while unparasitized mice did not hemolyze under these conditions. Within 2 hours of the divicine injection parasite levels had fallen by approximately 65%, and the parasitemia in the mice was usually cleared by a single dose of divicine.

Intraperitoneal desferrioxamine (an iron chelator) blocked the antimalarial action of the divicine, while iron-saturated desferrioxamine did not limit the antimalarial effect. These data support the view that iron-catalyzed free radical production from divicine was responsible for the hemolysis and for the direct parasite killing effect.

These data provide strong evidence indicating that divicine has a powerful antimalarial effect in vivo. Although a mouse model with a P. vinckei parasitemia may be somewhat different than human falciparum malaria, the differences are likely to be minor. Also, human consumption of fava beans and other foods containing antimalarial substances is likely to result in a more gradual dose than that achieved with a massive intravenous injection. Therefore, these experimental findings probably differ somewhat from the manner in which these substances function in natural circumstances in human populations. Nevertheless, it is particularly noteworthy that this fava bean-derived substance produced an antimalarial effect in presumably G6PD normal mice, although, again, probably at doses greater than occur under natural circumstances in human populations. These data suggest that if divicine has antimalarial activity in vivo in G6PD normal erythrocytes, which are well-buffered against oxidant stress, then it is likely to do the same at much lower doses in G6PD deficient individuals, as the G6PD (−) erythrocytes are much more sensitive to the oxidant stress created by this compound.
As noted above, there is strong evidence that G6PD deficient erythrocytes achieve optimal antimalarial protection when they are placed under an additional oxidant stress due to the consumption of oxidant dietary substances such as divicine and isouramil in fava beans (Golenser et al., 1983, 1988; Clark et al., 1984, 1989), and probably from other oxidants in a variety of food crops (Etkin, 1979, 1986; Xiao, 1981). However, if this oxidant stress is excessive, G6PD deficient individuals, especially those with the severely deficient Class II enzyme variants like the Mediterranean variant, are at risk to experience an acute hemolytic episode which may be life-threatening (Beutler, 1983; Luzzatto and Mehta, 1989; Kattamis, 1986; Arese et al., 1986; De Flora et al., 1986; Belsey, 1973). Therefore, on theoretical grounds, human population adaptation would be enhanced if there were some mechanism whereby the dietary intake of these naturally occurring antimalarial substances was regulated so that dietary intake was adequate for antimalarial protection, but not so excessive as to produce hemolysis in G6PD deficient individuals. Adaptation would be further enhanced if dietary intake of these substances were maximized in G6PD normal individuals so as to provide optimal antimalarial benefits.

We have suggested that another genetic trait called "quinine" taste sensitivity (Fischer et al., 1961; Fischer, 1967; Smith and Davies, 1973) actually functions to regulate the intake of bitter-tasting naturally occurring antimalarial substances of plant origin, with sensitive tasters of quinine consuming more limited quantities of these compounds and individuals who are less sensitive tasters of quinine consuming greater amounts, or more concentrated doses, of these dietary substances (Greene et al., 1993). Further, although the genetic locus of quinine taste sensitivity is not known, we have postulated that it is on the X-chromosome and that the G6PD and "quinine" taste loci are closely linked, producing four possible haplotypes in the hemizygous individual: G6PD+/Q+; G6PD+/Q-; G6PD-/+Q; G6PD-/-Q. We argue that there has been co-evolution of the G6PD polymorphism and "quinine" taste sensitivity in populations living in areas where *falciparum* malaria has been endemic and where naturally occurring antimalarial substances of plant origin are consumed in the diet, in that there is a relative increase in the G6PD+/Q+ and G6PD-/-Q haplotypes.

We illustrate our argument by using the hemizygous deficient condition as an example and Q to denote acute taste sensitivity to quinine and Q- to denote an insensitivity to the taste of quinine. In our convention the Q does not represent a single allele, but a polygenically acting group of alleles on the X-chromosome. The alleles act additively, resulting in codominance. We suggest that the G6PD+/Q+ haplotype will have greater fitness than the G6PD+/Q- haplotype in areas where *falciparum* malaria is endemic and bitter-tasting oxidants are consumed in the diet, because of a propensity to avoid or limit the intake of these substances. The two haplotypes have equivalent antimalarial protection from their G6PD deficient condition, but the G6PD+/Q+ individuals should have a diminished hemolytic risk if they modulate their intake of these bitter-tasting, oxidant-containing foods. In a similar fashion, we suggest that the G6PD-/-Q haplotype would have greater fitness than the G6PD--/Q haplotype. Due to their taste insensitivity, there is a greater likelihood that individuals with this haplotype (who do not have the biologic protection against *falciparum* malaria of the G6PD deficient condition) would consume more bitter-tasting dietary oxidants and thus would have the continuing antimalarial benefits of this diet-mediated prophylaxis (see also Greene, 1974).

This hypothesis of a co-evolution of the G6PD polymorphism and "quinine" taste sensitivity was tested in a preliminary study of 17 G6PD deficient and 25 G6PD normal African-American subjects 15 to 40 years of age (Greene et al., 1993). The G6PD deficient subjects showed greater taste sensitivity to quinine sulfate, but not to sodium chloride (a control comparison) than did the G6PD normal subjects.
However, due to the small sample size these differences were not significant (ANOVA, P < .10). This study was limited by our inability to locate and recruit the proposed number of G6PD deficient subjects. Based on our data on G6PD activity in this population, a fair test of the hypothesis would require a sample of approximately 75 G6PD deficient and 75 G6PD normal subjects.

Although this hypothesis has not yet received empirical support, we believe that it is testable. If confirmed, it would significantly enhance the biocultural perspective presented above.

Fava bean consumption is widespread in the circum-Mediterranean region, the Near and Middle East, and southwest Asia, and malaria is common in all of these areas (Katz and Schall, 1986). Malaria is also common in the southern portion of China, the largest producer of fava beans in the world (Mikhael, 1986). However, the fava bean is only the best studied food crop with clear antimalarial activity. Etkin (1979, 1986) has provided evidence indicating that a number of plants used in traditional African medicine and diet have antimalarial activity. Xiao (1981) has provided similar evidence for several plants used in China. Therefore, it appears that in human populations there is widespread dietary use of plants with clear or suspected antimalarial activity.

SUMMARY AND CONCLUSIONS

This article has presented support for the view that all G6PD deficient genotypes are relatively protected against *falciparum* malaria, and that this protective effect is potentiated by the consumption of naturally occurring antimalarial substances of plant origin. Fava beans are the best known and best described source of these dietary oxidants, but not the only source. It also presents an hypothesis and a summary of preliminary data suggesting that what has been called “quinine” taste sensitivity functions to regulate the dietary intake of these substances and that this trait has co-evolved with the G6PD polymorphism in human populations. Although it appears clear that the G6PD deficient genotypes are relatively protected against *falciparum* and perhaps *vivax* malaria, it is also certain that there is strong selection against the hemizygous and homozygous G6PD deficient genotypes as a result of diet-, drug-, chemical-, and infection-related hemolysis and neonatal jaundice (Beutler, 1983; Belsey, 1973; Luzzatto and Mehta, 1989; Arese et al., 1986; Chevion et al., 1982; Calabrese, 1984). It is thus likely that the heterozygous G6PD deficient female is the most fit genotype in areas where malaria is endemic. Further, the G6PD/Q+ G6PD/Q- heterozygote should be the most fit genetic constitution in regions where malaria is endemic. These individuals would benefit from a moderate intake of dietary antimalarials due to their intermediate quinine taste sensitivity, and would have a limited risk of experiencing hemolysis.

Since G6PD occurs in all cells of the body (Luzzatto and Battistuzzi, 1985; Luzzatto and Mehta, 1989), G6PD deficiency may also be involved in cellular defense against other parasitic diseases and infections. This would be one possible explanation of why alleles for G6PD deficiency are at polymorphic frequencies in some populations in areas where malaria has apparently not been endemic (Livingstone, 1985). The plethora of G6PD deficient variants that have reached polymorphic frequencies also suggests that there may be unique relationships between each variant and particular dietary oxidants in regional cuisines, which would be an important area of future investigation.

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