Hemoglobin reduces redox metals as well as reacts with O_2 to form both superoxide and H_2O_2. We have characterised both •OH and amino acid side chains oxidised to carbonyl groups with hemoglobin in the presence of O_2 and Cu(II). Chinese hamster ovary cells exposed to low levels of oxidative stress exhibit novel behaviour. They are structurally and metabolically intact, but are unable to undergo cell division. DNA is not oxidised. The site of oxidation appears to be localised on the membrane of the cell and involves redox metals. The mechanism of this signalling pathway for the regulation of the cell cycle is under study.

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About hemoglobins, G6PD and parasites in red cells

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Introduction

Kaspar Winterhalter is a towering figure in more ways than one. For those who are fortunate to know him in person, he is a congenial analyst of any topic germane to human endeavour and to the relationships between human beings and human societies. For the scientific community, he is a scientist who has made substantial contributions to the study of hemoglobins and to the understanding of the nature and function of the extracellular matrix. My own interactions with him are full of pleasant memories, and they are related to the fact that both of us have been caught throughout our working life on the '*Grenzgebiet*' between biochemistry, molecular genetics and hematology. I feel greatly privileged to have been invited by our mutual colleague Ernesto Di Iorio to pay tribute to Kaspar on the occasion of his 60th birthday. I thought on this occasion it may be appropriate to review some examples of how hematology and molecular biology have been mutually beneficial in understanding human disease.

Hemoglobin

Kaspar Winterhalter carried out some pioneering work on the subunit structure of hemoglobin (Hb), starting some 30 years ago[22], and he has contributed impressively to the study of the pathophysiology of hemolytic anemias associated with abnormal hemoglobins. Therefore I shall mention some recent developments which I find pertinent.

Unstable hemoglobin disease. This condition, which hematologists refer to also by the phrase of Congenital Heinz Body Anemia (CHBA), is a classical example of

a dominantly inherited condition in which dominance is explained through the deleterious effect of an abnormal protein, even though at least half of the Hb in heterozygotes is normal. We have recently investigated[16] a family in which six members are heterozygous for the unstable hemoglobin variant Hb Bushwick (β74E18 gly → val), and one young woman, from a first-cousin marriage, is homozygous for Hb Bushwick. Heterozygotes had a compensated hemolytic disorder. The homozygous patient had a chronic hemolytic anemia which became very severe concomitant with episodes of infection. Globin chain biosynthetic studies revealed that the abnormal β-chains of Hb Bushwick are intrinsically unstable. Thus, the homozygous state for an unstable hemoglobin is compatible with life, although it entails a severe hemolytic disorder which is also associated with ineffective erythropoiesis.

Sickle cell disease. In spite of the fact that the molecular basis of this condition was elucidated by V. M. Ingram in 1956[7], it has to be a humbling admission for every hematologist that its treatment is still unsatisfactory. One limiting factor has been the lack of an animal model. A few years ago I was fortunate to be involved in a collaborative project with Frank Grosveld's group which yielded the first such model. A crucial step was the discovery by Grosveld[5] of the DNA region, upstream of the β-globin gene cluster, which is now known as the locus control region (LCR). When an artificial construct consisting of the LCR, two human α-globin genes in tandem and one human β^S gene was injected into mouse blastocysts, some of the resulting transgenic embryos died in utero, some yielded apparently normal mice, but a few yielded mice whose red cells could be made to sickle in vitro. The different outcome turned out to be a function of the copy number of the transgene. One of the mice, with 6 copies of the transgene, had about 84% human Hb S in his red cells, the balance being mouse Hb[4]. Virtually all of the red cells sickled upon deoxygenation, and the presence of irreversibly sickled cells in the peripheral blood as well as electron microscopy of various organs proved that sickling also occurred in vivo. The phenotype of this mouse best mimicked that of a human genetic compound heterozygous for Hb S and for hereditary persistence of foetal Hb. Unfortunately the mouse was infertile, but several Hb S mouse lines have been since established in Grosveld's and in other labs, and they are proving useful in studying the pathophysiology of sickling and possible anti-sickling agents. In the meantime, we are still confronted with the plight of patients who suffer from sickle cell anemia. However, for the first time we have a powerful agent, hydroxyurea[3], that can increase in vivo the level of Hb F, a feature well known to be associated with relatively milder clinical manifestations of this condition. We are currently running a trial of hydroxyurea in severely affected patients. At the same time, the improved technology of bone marrow transplantation (BMT) has made a radical form of treatment available to patients with sickle cell disease who have an HLA-identical sib donor[19].

Glucose 6-phosphate dehydrogenase

This enzyme, G6PD in short, has been known in human red cells thanks to Warburg and Christian[21] since just about the time Kaspar was born. Whereas the globin genes are highly *cell lineage-specific*, the G6PD gene is ubiquitously expressed and it is a good example of a *housekeeping* gene. However, G6PD also became prominent in hematology some forty years ago, when Alving's group in Chicago[2] and Sansone and Segni in Genova[15] discovered that acute hemolytic anemias (AHA), previously referred to as *primaquine sensitivity* and *favism*, could be attributed to a genetically determined deficiency of G6PD in red cells. The explosive nature of the red cell destruction that takes place when G6PD deficient subjects are exposed to drugs, such as primaquine, to fava beans or to certain infections results from their inability to withstand a severe oxidative stress, and it is associated with massive denaturation of Hb. Thus, we have here an acutely acquired Heinz body anemia, in contrast to the chronic nature of CHBA mentioned in the section on hemoglobin of this article (cf. previous page). The close relationship between the two conditions has practical implications, and it seems topical to mention that the same agents that cause 'oxidative hemolysis' in G6PD deficient individuals also causes exacerbation of hemolysis in patients with unstable hemoglobins such as Hb Zürich: this has been a recurrent excuse for telephone conversations between Kaspar and myself!

G6PD deficiency displays a high degree of genetic heterogeneity. Biochemical analysis quickly identified in certain parts of the world several polymorphic variants which are generally asymptomatic but entail the risk of AHA; as well as rare sporadic variants associated with chronic non-spherocytic hemolytic anemia (CNSHA), and which can occur anywhere in the world. The main advance over the last few years has been that, following the cloning of the human G6PD gene[12,14], which maps to the chromosomal region Xq28[11], it has become possible to identify precisely the mutations underlying G6PD deficiency. From the analysis of some 80 different mutations a certain pattern is beginning to emerge[20]. First, the large majority of variants result from point mutations, i.e. single base changes producing single amino acid replacements. Second, the mutations are scattered throughout the coding region of G6PD. Third, as one might have expected, different mutations are always responsible for AHA versus CNSHA. Moreover, a significant proportion of the mutations associated with CNSHA are clustered in a region

corresponding to exon 10. Since most of the corresponding enzyme variants are highly unstable, this suggests that this region may be of distinct importance in the three-dimensional structure of the G6PD molecule (see ref. 14a). Another case of special interest is that of G6PD A-, the most common G6PD deficient variant in Africa and one of the most prevalent in the world. From a detailed analysis of enzyme kinetics it was suggested nearly 20 years ago that this variant might have two mutations[1], and this contention has been indeed validated[17]. Moreover, by in vitro mutagenesis we were able to show that either of the two mutations alone does not cause enzyme deficiency, and therefore this results from a unique interaction of the two mutations. One might surmise that, although one replacement is in amino acid position 68 and the other in amino acid position 126, they might be spatially adjacent.

Parasites in red cells

It is remarkable that, of the known widespread genetic polymorphisms in human populations, so many involve red cells. Certainly at least one reason is that which was first highlighted by J. B. S. Haldane[6], when he hypothesised that *malaria* had played a major role in shaping human evolution, because it had caused and is still causing so much mortality for so many generations. A body of evidence, which many of us regard as overwhelming, now supports the notion that the genes for Hb S, for thalassaemia and for G6PD deficiency have all been subject to malaria selection[9]. The parasite responsible, *Plasmodium falciparum*, is highly specific for the human species and its relationship to the red cell is most intimate, as is often the case with intracellular parasites. Indeed, the penetration into the erythrocyte, the intraerythrocytic development, the choice between asexual and sexual differentiation, and the erythrocyte lysis are all processes resulting from close interactions between the parasite and the host cell. Thus, it is not surprising that genetic changes in the red cells may affect the success of the parasite cycle. Interestingly, they seem to be able to do so in several different ways. With an abnormality of the 'band 3' membrane protein, as in the case of the ovalocytosis prevalent in Malaysia and in Oceania, there is hindrance to penetration[8] or – *failure of infection*. With G6PD deficiency there is marked inhibition of the intracellular development of the parasite – or *abortive infection*, – although the parasite is able to adapt to the abnormal red cell environment in subsequent cycles[18]. With Hb S, penetration and intracellular development are essentially normal, but the red cells containing mature parasites (trophozoites and schizonts) undergo selective sickling, and are consequently removed by macrophages: *suicidal infection*[10]. In order to understand better the mechanism of relative resistance against malaria afforded by G6PD deficiency, which seems to be a specific prerogative of heterozygous females, who are genetic mosaics as a result of X-chromosome inactivation, we have characterised the G6PD from *Plasmodium falciparum* and cloned the gene encoding it. This gene has a region of about 450 codons with an amino acid homology of about 39% when compared to human G6PD[13]. An unexpected finding was that the *Plasmodium falciparum* G6PD gene is much larger than any of those characterised thus far from other species. The N-terminal region has no homology to G6PD, but it contains a sequence reminiscent of the glutathione (GSH)-binding domain of several GSH transferases, and it may thus encode a different biochemical function which may be pertinent to GSH metabolism.

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