Sickle cell protection from malaria

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Abstract

A linkage between presence of Sickle Haemoglobin (HbS) and protection from malaria infection and clinical manifestations in certain areas was suspected from early observations and progressively elucidated by more recent studies. Research has confirmed the abovementioned connection, but also clarified how such protection may be abolished by coexistence of sickle cell trait (HbS trait) and alpha thalassemia, which may explain the relatively low incidence of HbS trait in the Mediterranean. The mechanisms of such protective effect are now being investigated: factors of genetic, molecular and immunological nature are prominent. As for genetic factors attention is given to the role of the red blood cell (RBC) membrane complement regulatory proteins as polymorphisms of these components seem to be associated with resistance to severe malaria; genetic ligands like the Duffy group blood antigen, necessary for erythrocytic invasion, and human protein CD36, a major receptor for P. falciparum-infected RBC's, are also under scrutiny: attention is focused also on plasmodium erythrocyte-binding antigens, which bind to RBC surface components. Genome-wide linkage and association studies are now carried out too, in order to identify genes associated with malaria resistance. Only a minor role is attributed to intravascular sickling, phagocytosis and haemolysis, while specific molecular mechanisms are the object of intensive research: among these a decisive role is played by a biochemical sequence, involving activation of haeme oxygenase (HMO-1), whose effect appears mediated by carbon monoxide (CO). A central role in protection from malaria is also played by immunological factors, which may stimulate antibody production to plasmodium antigens in the early years of life; the role of agents like pathogenic CD8 T-cells has been suggested while the effects of molecular actions on the immunity mechanism are presently investigated. It thus appears that protection from malaria can be explained by interaction of different factors: the elucidation of such mechanisms may prove valuable for the prevention and treatment strategy of a disease which still affects large parts of the world.

Introduction

Early suggestions of a possible relationship between extension and prevalence of haemoglobinopathies in some Mediterranean areas and malaria infection date back to the late 40's.[1,2] It was thereafter widely agreed that red cell genetic disorders, like Thalassemia, Sickle cell Disease and Glucose-6-phosphate dehydrogenase deficiency (G6PD) may confer resistance to Plasmodium infection.[3-5] A special role in preventing complications of severe malaria has been postulated for HbC.[6] Ovalocytosis, caused by a deletion in the band-3 gene, has also shown to exert a strong protection from malaria in southeast Asia.[7] Beside G6PD, another red cell disorder from enzyme deficiency, like pyruvate kinase deficiency, has also recently shown to interfere, as it was demonstrated that the most common mutation in the PKLR gene, protects red cells of the homozygote against the parasite *in vitro*.[8]

While a geographical correspondence between the distribution of thalassaemia and malaria was confirmed in the Mediterranean region as well as in other locations,[9] a similar relationship between HbS and malaria was discovered in Africa.[10,11] As far as SCD is concerned, it was suggested that, while sickle-cell homozygous individuals usually die before adulthood, the gene responsible for SCD could reach high frequencies because of resistance conferred against malaria by the heterozygous carrier state, resulting in a balanced polymorphism;[12] it has been actually observed that it is rather difficult for P. Falciparum to develop in HbS-contaning red cells and is also rare to find an HbS carrier struck by cerebral malaria, a common cause of death in this disease.[13]

Population Studies

It has been actually shown by studies using family-pedigree based genetic variance analysis that, as a whole, genetic factors account for a substantial proportion of the variability that is seen between children with regard to their risk of developing clinical P. falciparum malaria.[14] For instance, protection from malaria may be abolished by coexistence of sickle trait and alpha-thalassemia by a complex mechanism, which explains the relative rarity of the sickle cell trait in the Mediterranean: in contrast with theories attributing such rarity to its recent import from Africa into thalassemia-carrying populations,[15] it has been proposed that in populations with alpha-Thal and therefore low levels of normal β^+, βS was progressively excluded even if it did arrive early in the area, so that it is almost negligible at present; the coinheritance of both traits removes in this way their highly protective action from malaria.[16] This observation can be considered an example of *negative epistasis* between conditions like alpha-Thal and HbS trait,[17] confirming the old assumption that malaria protection is related to HbS level and HbS concentration in sickle cells.[18]

Among other genic interaction between Hbpathic patients it is of interest to examine West African populations, in which the HbS carrier state was found to be negatively associated with all major forms of severe P. falciparum malaria, while the negative associations of the carrier states of HbC and alpha-thalassemia appeared to be limited to cerebral malaria and severe anemia, respectively; as for HbC, protection from malaria is enjoyed by heterozygotes but to a lesser degree than βS heterozygots; a slight protection offered to homozygotes may however explain the persistence and even a minimal increase of HbC in these regions.[13]

Only very recently, however, extensive geostatistical studies provided the first quantitative evidence for a geographical link between the global distribution of HbS and malaria endemicity: a strong link between the highest HbS allele frequencies and high malaria endemicity was observed at the global scale, but this observation is based primarily on data found in Africa. The gradual increase in HbS allele frequencies from epidemic areas to endemic areas in Africa is consistent with the hypothesis that malaria protection by HbS involves the enhancement of both innate and acquired immunity to P. Falciparum; interactions with haemoglobin C might explain the lower HbS allele frequencies in West Africa.[19]

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An interesting aspect is the relationship between blood flow in peripheral vessels and deoxygenation of red cells in SCD: it is generally assumed that at least 80% of AS cells (from SCD heterozygotes) become reoxygenated in the circulation before they can become sickled.[20] However, if the AS cells are trapped in the venous part of the circulation, the great majority become sickled: this is thought to occur during the late stages of P. falciparum multiplication. The quantity and orientation of aligned sickle hemoglobin polymers (AHP) in sickle cells probably play an important role in the flow of sickled RBC through the circulation: actually, it has been shown that erythrocytes with the greatest degree of deformation (sickle cells) contain a large amount of aligned Hb polymers.[21]

Mechanism of protection

It is always a fascinating question to determine the extent by which living subjects (whether animals or plants) can develop mechanisms of resistance to parasites, or even display tolerance, a kind of damage limitation: as far as malaria is concerned some experiments in mice have shown an interesting genetic variation in tolerance, with a negative correlation between tolerance and resistance, suggesting that animals may evolve two distinct mechanisms of defense against pathogenic agents.[22] What are then the mechanisms responsible for sickle red cells protection fom malaria? It is in the first place important to realize that invasion of red cells by parasitic merozoites is a multi-step process, involving interactions between genetic, biochemical and immunological determinants; we may therefore take into account the following factors.

Genetic factors

Many factors of genetic character may be involved in the mechanism of protection. A number of studies have been focused on different components of the red cell membrane and special attention was given to the role of complement regulatory proteins, particularly complement receptor 1(CR1).[23] It has been actually shown that, in common with other malaria protective genes, the frequencies of many of the CR1 polymorphisms are high in a number of malaria endemic areas: for example, Cockburn *et al.*[24] showed that, polymorphisms resulting in low CR1 expression are associated with alpha thalassemia in malaria-endemic regions of Papua NewGuinea, affording protection from severe falciparum malaria itself. Complement receptor-1 polymorphism associated with resistance to severe malaria was also demonstrated in Kenya.[25]

Studies on other genetic factors have also produced important results: for instance, genetic ligands like the Duffy group blood antigen have been pointed out as necessary for erythrocytic invasion by some Plasmodium Vivax forms and have been recently identified; receptor-binding domains of erythrocyte-binding proteins (EBPs) have been mapped to conserved cysteine-rich domains referred to as Duffy-binding-like (DBL) domains.[26] As for P. falciparum infection, a high frequency of the Gerbich-negative blood group (a mutated gene encoding glycophorin C, or GYPC-Dex3) was earlier found to be associated with malaria resistance in populations of Papua New Guinea, where malaria is endemic;[27] later on, the GYPC-Dex3 gene was found to confer resistance against some P. Falciparum merozoites possessing the peculiar erytrocye-binding 140 antigen.[28] Recent research has confirmed that antigens in the Gerbich blood group system are expressed on glycophorin C (GPC) and glycophorin D (GPD), which are both encoded by a single gene, GYPC. It has also ben confirmed that Gerbich antigens act as receptors for the malarial parasite Plasmodium falciparum, while reduced levels of GPC and GPD are associated with hereditary elliptocytosis.[29]

Another line of investigation focused on the human protein CD36, which is a major receptor for P. falciparum-infected red blood cells[30] (RBC's) and may contribute to the disease by sequestering infected RBC's and inhibiting the immune response to the parasite so that the acquisition of immune responses that kill parasites is delayed through modulation of the function of antigen-presenting cells:[31] it was actually found by Aitman *et al.*[32] that African populations contain an exceptionally high frequency of mutations in CD36 and that these mutations, which cause CD36 deficiency, are associated with susceptibility to severe malaria; this may suggest that CD36 was a determinant of the risk of developing CM as compared to other severe malaria complications. However the role of the CD36 gene in malaria is still debated and no confirmation was obtained more recently.[33]

Studies have also been directed for many years to erythrocyte-binding antigens, found in the supernatant fluids of cultured human malaria parasite (Plasmodium falciparum): members of the erythrocyte binding-like (ebl) family representing proteins involved in the merozoite's invasion of the erythrocyte have received special interest as they bind with high affinity to glycoproteins on the surface of the erythrocyte.[34] One of them, the erythrocyte binding antigen-175 (EBA-175) binds to glycophorin A and mediates an invasion pathway for merozoite entry into erythrocytes.[35] The receptor for some of these proteins, which bind to the RBC membrane (like the EBA 175), has been identified and its participation in the invasion process has been determined;[36] for other antigens the receptor still has to be identified.

As the involvement of host genetic factors is receiving increased attention, there is now a trend to set up genome-wide studies to identify malaria resistance genes that determine the structure or function of red blood cells. In this context it is interesting to consider a genome-wide linkage and association study for P. falciparum infection intensity and mild malaria attack among a Senegalese population of children and young adults from 2 to 18 years old, belonging to a unique ethnic group: the subjects were closely followed-up during 3 years: this study identified three candidate regions, two of them containing genes that seem to be implicated in a response to malaria infection. Moreover, one gene associated with malaria infection was found in the 5q31q33 region.[37]

Another recent study, carried out on cerebral malaria (CM) genetics in the Angolan population, showed that transforming growth factor beta 2 (TGFB2) gene variants contribute to the risk of developing the CM syndrome when compared to other forms of severe malaria in Angolan children. The results also suggest that haeme-oxidase-1 (HMOX1) gene variants are associated to CM risk and control the HMOX1 mRNA expression in peripheral blood cells of children with CM.[38] Such results are regionally limited and require confirmation in larger population samples; it becomes anyway apparent that there might be a special role for genetic factors in controlling the clinical progression of the disease.

Role of sickling

It is of interest that malaria might act as a trigger for intravascular sickling: parasitized cells from A/S heterozygotes tend indeed to sickle more readily than non-parasitized cells, a phenomenon which facilitates their trapping by a normal reticuloendothelial system.[39] As for the modality of the red cell transformation to a sickle cell, it was known long ago that the red cell shape depends on the rate of deoxygenation: upon deoxygenation, HbS forms paracrystalline needles, transforming both SS and SA cells into their characteristic sickle shape and altering the intracellular viscosity. Slow deoxygenation results in a single fiber domain that initially grows in one general direction, with the classic sickled shape resulting from oblique alignment of adjacent fibers through interactions of their helical surfaces;[40] in contrast, rapid deoxygenation produces cells that do not appear sickled at all, having a nearly normal biconcave disc shape, even though the cells are filled with multiple domains of polymerized hemoglobin.[41,42]

A small reduction in the intracellular sickle

hemoglobin concentration will markedly increase the delay time of hemoglobin S polymerization and transit of red cells though the microcirculation and explains why stimulation of F-cell production from hydroxyurean and other therapeutic agents, by which sickle hemoglobin is diluted by replacement with fetal hemoglobin, has proven to be effective.[43]

Specific molecular mechanisms

A number of biochemical interactions, which have important impact on resistance to malaria, may be outlined as follows: i) considering the higher rate of free heme release by HbS than normal Hb it has been suggested that such faster heme loss from HbO2-S is due to accelerated autoxidation, as shown from spectrophotometric maesurements; this would be in agreement with previous observations about an excessive generation of superoxide by sickle erythrocytes and the abnormal deposition of heme and heme proteins on membranes of sickle red cells.[44] The effect of free heme, actually one of the factors involved in the pathogenesis of cerebral malaria, can however be opposed, as before mentioned, by haeme-oxygenase (HO-I), which is a stress-responsive enzyme converting the protoporphyrin IX ring of heme into biliverdin, releasing iron (Fe) and producing carbon monoxide (CO);[45] ii) it has been shown that HO-I expression is remarkably stimulated by a variety of agents, including of course heme but also heavy metals, hyperthermia, UV irradiation, and inflammatory cytokines; HO-1 expression appears mediated by the transcription factor NrF2.[46] Moreover, the heme/HO-1 system controls the pathogenesis of experimental cerebral malaria in mice, a neuroinflammatory syndrome that resembles cerebral malaria in children infected by P. falciparum.[47]

It was later realized that most, if not all, HO-1 inducers stimulate the production of reactive oxygen species or deplete glutathione levels or both; moreover the fact that heme is a potent pro-oxidant has led to postulate that the HO-1 gene (HMOX-1) is frequently activated under a variety of cellular stress conditions and that HO-1 activity is a component of the cellular defense mechanism against oxidant stress; HMOX-1 is also unique as it appears directly regulated by four stress-responsive transcription factors, namely heat-shock factor, nuclear factor-κB, nuclear factor-erythroid 2, and activator protein-1 families.[48]

A protective effect against sickle cell anemia and malaria by HO-1 seems thus fairly established and appears mediated by the same end-product of heme catabolism, namely CO.[49] This gaseous signaling molecule, or *gasotransmitter*, inhibits Hb oxidation and subsequently heme release from Hb, thus preventing free heme from participating in the pathogenesis of ECM; moreover, CO might have additional protective effects that contribute to prevent the lethal outcome of Plasmodium infection.[50]

Other end-products of heme catabolism by HO-1, such as labile iron, might also contribute to the protective effect conferred by sickle Hb against malaria. While cytotoxic per se, labile iron induces the expression of ferritin H chain (FtH), which confers cytoprotection against free heme *in vitro*.[51]

Phagocytosis

Following the observation that enhanced phagocytosis of rings, the early intraerythrocytic form of the parasite, was an important factor of protection in G6PD deficiency, it was suggested that enhanced phagocytosis of ring-parasitized mutant RBCs may represent the common mechanism for malaria protection in non immune individuals affected by sickle trait and beta-thalassemia trait;[52] it has been actually shown that HbS is substantially more unstable than HbA and has been suggested that, by clustering red cell membrane proteins, it may accelerate their removal by phagocytic cells.[53]

Haemolysis

It has been indeed shown that certain RBC mutations, like for HbC, alpha or beta-thalassemia as well as membrane protein defects[7,23,53] confer protection from malaria and that many of these mutations are capable of causing haemolysis, together with accumulation of free heme; it has therefore been suggested that chronic hemolysis might be protective per se against severe forms of malaria, following the observation of improved survival observed in human carriers of several RBC mutations; in this context heme administration to naïve mice, is sufficient *per se* to elicit a protective response, presumably through the induction of the Nrf2/HO-1 system.[49] The subject is still open to investigation as the same Nrf2/HO-1 system seems to offer protection against the pathological effect of the same RBC mutations.[54]

Immune mechanisms

Immune mechanisms are certainly operational: malaria protection increases in the first 10 years of life, an event considered as an accelerated acquisition of immunity.[14] Clonally variant surface antigens (VSA) of Plasmodium falciparum, inserted into the membranes of infected erythrocytes, have been recognized long ago and it was suggested that they constitute targets of antibody responses, enhanced with age and associated with protection from malaria:[55] a highly significant independent association was actually found between presence of HbAS and IgG anti-VSA responses, confirming that enhanced levels of cross-reactive anti-VSA responses in children with HbAS may be strongly associated with protection against malaria.[56]

Other immunological factors may be relevant in CM pathogenesis, like pathogenic CD8 T cells. The existence of a *multiple hit* system, by which free heme synergizes with other cytotoxic agonists, e.g. pathogenic CD8$^+$ T cells, to trigger disease has been postulated.[57] However, both the accumulation of pathogenic free heme and the activation and/or expansion of pathogenic CD8$^+$ T cells seem to be repressed by HbS: the inhibition of the formation of free heme after infection, via the HO-1/CO system, seems , in turn, to oppose the activation and/or expansion of pathogenic CD8$^+$ T cells. There is therefore a special form of immune regulation, which may explain the protective effect of sickle cell trait against severe malaria.[58]

As before mentioned, another important line of defence against infection is tolerance, which is also mediated by the immune system. A traditional definition of tolerance (in microbiological terms) is the capacity to limit the obnoxious action of a parasite or microbe burden:[59] titre, density and biomass of the pathogen determine the extent of such burden. It thus appears that both tolerance and resistance are opposite factors which may control the effect of a pathogenic agent.

Development of tolerance is also based on immunologic factors, although the mechanisms of tolerance are still not well understood, particularly in animal hosts; however a recent report describes a mechanism of host tolerance during infection with Plasmodium parasites, showing that the heme detoxification activity of HO-1 is a critical component of host tolerance to Plasmodium infection-associated liver failure.[60] It has been however stressed that tolerance, unlike resistance, does not impair the performance of the parasite.[59]

Future directions

A promising area of investigation, recently developed, is the possible relevance of interplay between different pathogenic factors; it has been actually stressed that multiple factors are involved in the variability of host's response to P. falciparum infection, which depends on a variety of factors, like the intensity and seasonality of malaria transmission, the virulence of parasite and host characteristics like age or genetic constitution; it has also been noted that related individuals often share a common environment (like a house) and that environmental factors play a major role in the risk of infectious diseases: it is thus difficult to disentangle environmental and genetic effects.[61]

Recent investigations also try to elucidate

the role of molecular and immunological factors: it has been already mentioned that the protective effect of HO-1 against sickle cell and against malaria is mediated by the same end-product of heme catabolism, namely CO; moreover, CO might have additional protective effects that contribute to prevent the lethal outcome of Plasmodium infection.[50] More efforts have been recently devoted to investigate the modality of sickle Hb protection against Plasmodium infection in mice, as well as induction of tolerance to such infection. Following previous reports using different mouse and Plasmodium strains,[62] it was found that survival advantage occurs irrespectively of parasite load and is apparently not associated with parasite sequestration in different organs, confirming induction of tolerance by sickle human Hb to Plasmodium infection.[22,63]

A very interesting research, carried out in a Mali village during the season of highest exposure to Plasmodium infection (September to November), confirmed in the first place that the survival advantage conferred by HbS against malaria in human populations can occur without a significant decrease of parasite load but also demonstrated a significant delayed time to first malaria episode, about 34 days, in children with sickle cell trait (HbAS): this may be due to one of two possibilities, (or both), namely either some prolongation of the time to achieve symptomatic parasite density or an increase of asymptomatic infections abortion.[64] More research is expected in this direction, including Hb typing in all investigations on time delay before the first episode of malaria.

Research is also in progress about dual aspects of HbS action, which not only prevents accumulation of pathogenic free heme, but also activation and expansion of pathogenic $CD8^+$ T cells: the former activity takes place, via the HO-1/CO system, while the latter action, on $CD8^+$ T cells, is exerted by a still unclear modality, which however appears to be independent from HO-1 or Nrf2.[65]

Attention is also devoted to the modality of cellular immunity developing in regions of high endemicity: the role of gamma-interferon, mainly produced by gamma-delta T-cells, cells is particularly examined, together with the involvement of natural killer (NK) cells and some cytokines, like Il-12 and Il-18 for IFN production.[66] Very recent investigations by Sharma *et al.*, performed on global gene expression profiling, found also that type I interferons, typically associated with antiviral immunity, were enriched in cells isolated from febrile patients with malaria. As type I interferons can cause immune pathology, the role of the STING-TBK1-type I interferon pathway was examined in a mouse model of cerebral malaria, and genetic ablation of type-1 interferon pathway resulted in protection from diseas.[67] The possibility that this pathway may be important to induce cerebral malaria in humans should therefore require further investigation.

The latest example of interactions between different pathways comes from a very recent study by Schuldt *et al.*,[68] carried out on 1200 infected children in Ghana: they found that a mutation in the FAS gene promoter encoding the protein C- 95 induces high levels of the protein, resulting in a reduction of severe malaria incidence. As C-95 promotes apoptosis, this effect may be ascribed to a C-95 – aided killing of immune cells, thus preventing an excessive immune response, another case of molecular-immunological interplay still waiting to be clarified.

It is finally worthwhile to emphasize the importance of the study of the natural mechanisms of defense as the basis for numerous attempts to develop vaccines, which may either prevent the development of malaria at all or reduce its major complications: *induction of immunity* must take into account the different stages of P. Falciparum in humans, namely the pre-erythrocytic stage, which initiates the infection; the asexual blood stage, which causes disease; and the gametocyte stage, which infects mosquitoes that transmit the parasite.[69] Several multistage vaccine candidates are currently under evaluation in clinical trials: a major example is the first trial in which two existing vaccination strategies are combined to produce a vaccine capable of inducing immune responses to both the pre-erythrocytic and blood stages of the P. falciparum life cycle.[70] More efforts are presently addressed in a number of directions: given the scale of the malaria burden on present large populations, such endeavours are not only a scientific, but a humanitarian task as well.

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