Identification of protective antibody responses to blood stage antigens of *Plasmodium falciparum*

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Abstract:

Introduction

Research into immunisations against *Plasmodium (P.) falciparum* has so far yielded vaccines providing partial protection against complications of malaria. Immunological correlates of clinical immunity against complications of malaria are not well understood. There is an urgent need for a pathway leading to the discovery of all antigens eliciting a protective antibody response to inform the development of effective vaccines. The aim of this review was to discuss the identification of protective antibody responses to blood stage antigens of *P. falciparum*.

Materials and methods

A narrative review of investigations into the role of antibodies in immunity to *P. falciparum* malaria was undertaken. Databases search included MEDLINE and PreMEDLINE. A review of methods for detection of antigens relevant in anti-plasmodial immunity and of types of antibodies found to be associated with protection against complications of malaria was undertaken.

Results

Specific IgG1 and IgG3 but not global antibody responses to merozoite antigens have been associated with immunity to complications of *P. falciparum* malaria. Current research has used biased investigation platforms.

Introduction

Immunity to clinical disease and therefore, reduction of morbidity and mortality in *Plasmodium (P.) falciparum* malaria is largely mediated by immunity to antigens of blood stages. People in hyperendemic areas often carry *P. falciparum* in their blood stream, but remain asymptomatic demonstrating that, there is a lack of development of natural immunity to infection, and pre-erythrocytic stages of the parasite including sporozoites and hepatic stages. Blood stages are merozoites, which are released from infected hepatocytes and red blood cells, and the intra-erythrocytic trophozoites and the schizonts. Each merozoite exiting the liver into the bloodstream can invade an erythrocyte and multiply up to 20-fold every two days in cycles of erythrocyte invasion, replication, erythrocyte rupture, and release of infectious merozoites. The erythrocyte rupture may release numerous *P. falciparum* blood stage antigens. Objectives of this critical review were:

- To establish the theoretical foundations for identification of the pattern of antibody responses associated with protection against clinical manifestations of severe *P. falciparum* malaria.
- To establish a pathway leading to the discovery of immuno-relevant blood stage antigens of *P. falciparum*, which could form the basis of future vaccines against blood stages of this parasite.

Materials and methods

In this narrative review, Pubmed (MEDLINE and PreMEDLINE) was searched for articles on protective antibody responses to *P. falciparum* blood stage antigens and methods to detect them. The methods found and results were critically analysed, and a pathway to discovery of immuno-relevant blood stage antigens designed, which uses the characteristics of protective antibody responses established in previous studies to identify those antigens.
Results
Specific antibody subclass responses and immunity to clinical malaria
The first experiments demonstrating the proof of principle of the role of antibodies in anti-plasmodial immunity in humans, were gamma-globulin transfer experiments from immune Gambian adults to children with asymptomatic *P. falciparum* parasitemia, resulting in a reduction to the parasite count to <1% of the initial value, and a progressive reduction of clinical symptoms. Later studies showed that, global immunoglobulin responses against *P. falciparum* were not associated with protection, but immunoglobulin subclasses IgG1, IgG2, and IgG3 in French Guinea, a cross-sectional study investigated the levels of antibodies comparing persons living in endemic areas without parasitemia and persons from endemic areas with acute malaria. Parasitemia was associated with lower IgG1 and IgG3 subclass responses. In terms of protective antibody responses to blood stage antigens previously investigated in immune-epidemiological studies, a recent systematic review with meta-analysis of population-based prospective studies and population-based treatment to reinfection studies found a limited number of studies and restricted to antigens/components of antigens comprising MSP-1α, MSP-1-EGF, MSP-1-BL1, MSP-1-BL2, MSP-2, MSP-2 ACD, MSP-3, GLURP, AMA-1, and EBA-175. The authors concluded that IgG responses to some, but not all, merozoite surface antigens were associated with protection against symptomatic *P. falciparum* infection in malaria endemic areas. The authors identified very few antigens that had been well studied, and a deficiency of studies done outside Africa. The authors concluded that more studies in different populations, examining multiple antigens at multiple time-points, are needed to better determine the role of anti-merozoite antibody subclasses in protection against malaria, with prospective cohort studies as the preferred study design to establish temporal causality.

Detection of immune-relevant antigens
Immunoglobulin subclasses IgG1 and/or IgG4 were associated with protection. This protection is provided by the ability of these subclasses to link antigens to monocytes stimulating them to produce tumour necrosis factor. This cytokine induces production of nitric oxide, which kills intracellular parasites as part of antibody dependent cellular inhibition (ADCI) and the generated nitric oxide is a vasodilator preventing cerebral vasospasm, which is significantly involved in the pathogenesis of cerebral malaria. IgG1 and IgG4 were therefore called cytophilic or opsonizing antibody subclasses. The initial investigations of antibody responses have mainly been carried out by ELISA using recombinant proteins or synthetic peptides usually representing subdomains of malarial proteins as test antigens. Such antigen preparations don’t reflect native parasite protein conformations. The first study using an unbiased approach to detection of immunoreactive antigens visualized antigens, which were precipitated by immunoglobulin of immune donors. This was achieved by labelling of the parasite with 35S-methionine and separating the immune-complexes by two-dimensional electrophoresis followed by autoradiographic detection of the position of the immune-complexes in relation to molecular weight standards. This method did not allow identification of the antigens, immunoglobulin subclasses or avidity. The method could also not exclude missing small antigens trapped in the immune-precipitants, which were not bound by immunoglobulins. This is particularly important for low abundance antigens.

The first study to enable a description of the antibody type as well as the molecular mass of antigens in people exposed to falciparum malaria used one-dimensional sodium dodecyl sulphate-polyacryl amide gel electrophoresis (SDS-PAGE) with 21 molecular weight markers followed by electrotransfer to a nitrocellulose paper. Antibody types and IgG subclasses were visualized and 38 detergent solubilized parasite antigens and 20 exoantigens were visualized without facility to identify these antigens or correlate them to clinical immunity. The immunoblotting patterns indicated that, immune adult sera of the investigated people from Burkina Faso contained antibodies of all IgG subclasses specific for the whole series of somatic antigens. IgG1 was predominant, and IgG4 was the least prevalent subclass.

Further progress in identification of immunoglobulin subclass responses to native *P. falciparum* blood stage antigens, correlating with clinical immunity was also achieved by one-dimensional SDS-PAGE and immunoblotting. People with different exposure to *P. falciparum* were investigated. There was a tendency for a broader IgG1 and IgG3 reactivity with increased exposure. Non-immune Danish travellers with a single malaria episode reacted against a few high-molecular weight antigens, whereas those from Liberian adults recognised a larger number of both high- and low-molecular weight antigens. The authors concluded that, the long time required to acquire clinical protection against *P. falciparum* malaria is not only related to isotype switching towards ADCI-effective antigens but also to a gradual development of IgG1 and IgG3 antibodies against some previously non-targeted antigens. This approach did not identify the antigens involved, but was a less biased approach compared to protein microarray methods using recombinant proteins or synthetic peptides being developed.

The most comprehensive approach to detection of antigens relevant in a protective immunoglobulin response was pioneered by Jungblut et al. and
Pitarch et al.\textsuperscript{,}\textsuperscript{1,2}, who subjected native proteins of \textit{Borrelliagarinii} and \textit{Candida albicans} respectively, to two-dimensional electrophoresis followed by Western blotting with serum of patients. This approach was first successfully applied to parasites with a more complex proteome for the nematode \textit{Trichosstrongylus colubriformis} in a later study by a different group\textsuperscript{10}.

An approach to identification of immunorelevant antigens of a protective immune response to \textit{P. falciparum} could be as follows (modified from Eisenhut M\textsuperscript{3}):

- Antigens against a wide spectrum of \textit{P. falciparum} antigens could be gained from purified parasitophorous vacuole membrane-enclosed merozoite structures (PEMS), which contain a highly homogeneous synchronous parasite population at the mature schizont stage, which is essentially free of contaminating host cell proteins\textsuperscript{7}.
- Antigen is prepared by saponin mediatedlysis of parasitized red blood cells followed by washing steps, and breaking up of parasites by trituration and ultrasonication.
- The antigen mixture is subjected to two-dimensional electrophoresis using isoelectric focusing in the first using a pH gradient of 2–12 in the IPG strip and PAGE in the second dimension. Four gels are generated for each participant.
- Antigens are blotted onto nitrocellulose where they get exposed to serum of a person with and separately without clinical immunity to \textit{P. falciparum}.
- For identification of high avidity antibodies, the blotted antigen-antibody complexes on two of the four nitrocellulose sheets per participant are subjected to a washing step, with a dissociation buffer including PBS-T-urea (8M) with vigorous shaking, followed by additional wash with PBS-T buffer\textsuperscript{11}.
- Bound immunoglobulin is visualized with anti-human immunoglobulin. Immunoglobulin subclasses are visualized by specific anti-subclass antibodies for the cytophilic antibody subclasses IgG\textsubscript{1} and IgG\textsubscript{2} conjugated with peroxidase. One set of two gels (one with and one without dissociation buffer treatment) is used for each antibody subclass.
- The amount of antibody of the participants serum bound to the antigen is quantified by transmission densitometry of the stains generated by the peroxidase reaction.
- Avidity is quantified by avidity index as ratio of the optical density of urea-treated spots to the optical density value of untreated spots multiplied by 100. An avidity index of less than 30\% is defined as low-avidity, between 30\% and 50\% as intermediate avidity, and greater than 50\% as high-avidity\textsuperscript{11}.
- The patterns emerging with this method are put into relationship to clinical immunity. Groups with clinical immunity are compared to patients hospitalized with acute malaria. Subgroup analysis is done for different age groups.
- Antigens found to be associated with a protective antibody response in prospective studies can be identified by mass spectrometry using sections of the gel where the antigen is situated. Amino acid sequences obtained from this process can be attributed to proteins using the fully deciphered \textit{P. falciparum} genome.

**Discussion**

The author has referenced some of his own studies in this review. None of these studies involved trials in humans or animals. Ethical approval or informed consent was not required.

The foundation of development of effective blood stage vaccines is comprehensive identification of protective antibody responses. These are characterized by cytophilic properties (i.e. IgG\textsubscript{1} and IgG\textsubscript{2}) and high avidity and directed against multiple antigens. Preliminary studies suggest that, in the context of host immune recognition of the \textit{P. falciparum} parasite, a large number of antigens are recognised, dispersed amongst a large fraction of the proteome\textsuperscript{12}.

A currently pursued approach to detection of antigens relevant for immunity to \textit{P. falciparum} is the detection of genetic signatures of balancing selection to identify targets of anti-parasite immunity. Balancing selection is selection that maintains different alleles within a population. Using this approach, a variety of given polymorphisms of genes is maintained rather than a reduction of numbers of gene variants by selection or persistence of a random neutral variety of polymorphisms. These gene sequences undergoing balancing selection can be identified by sequencing and comparison of the sequences obtained. Disadvantages include that over 5000 genes will have to be screened and epigenetic modification is not captured by this method. Another shortcoming difficult to overcome with this method is expansion of the population and heterogeneity of the population sampled\textsuperscript{13}.

The starting point for identification of effective antibody responses requires a detailed and comprehensive description of the “immunome” defined as the set of antigens or epitopes that interface with the host immune system\textsuperscript{12}.

The above detailed approach of two-dimensional PAGE followed by immunoblotting is unbiased compared to analysis of antibody responses to selected recombinant \textit{P. falciparum} antigens on high through put micro-and macroarrays. Recombinant proteins lack epigenetic modifications and glycosylation and may not have epitope forming tertiary and quaternary structures for example formed by disulphide bonds. Two-
dimensional electrophoresis as an approach also has limitations: Protein surface structure may be altered by detergent use. Hydrophobic proteins may not be released from their position in parasite or red blood cell membranes or fail to migrate in the gel. The resolution of the two-dimensional electrophoresis may not be high enough to enable detection of low abundance proteins with the wide range of the pH gradient used to capture a comprehensive set of blood stage antigens.

An additional agenda in research investigating protective antibody responses is looking for antigens inducing antibodies with adverse effects on the clinical manifestations of malaria:

IgG antibody responses to conserved regions of merozoite proteins and immune-complex concentrations were found to be elevated in patients with cerebral malaria compared to uncomplicated malaria. This may be partly due to down-regulation of Fc-receptors involved in the clearance of antibodies but could indicate a pathogenetic role of immune-complexes particularly of those containing IgE. Deposition of IgE containing immune-complexes could lead to the release of TNF, a cytokine that leads to increased cytoadherence of parasitized red blood cells by up-regulation of intercellular adhesion molecule-1 in endothelial cells.

*P. falciparum* malaria is commonly associated with features of autoimmune glomerulonephritis and future studies need to explore the specificity and antibody subclass of the IgG involved to avoid adverse effects of vaccine induced antibody responses.

**Conclusion**

Future research needs to employ the unbiased platform of two-dimensional electrophoresis combined with immunoblotting onto nitrocellulose and detection of high avidity and cytophilic antibody responses in sera of clinically immune compared to patients with severe malaria with identification of the immune-relevant antigens detected by mass spectrometry. This will enable development of a multi-epitope vaccine with a higher rate of protection.

**Reference**