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Review

Malaria vaccines: Genomic search for profiling naturally acquired immunity

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ABSTRACT

Malaria remains a major public health problem worldwide. Despite many efforts to control or to eliminate the disease many malaria cases and deaths are still reported. The current measures to control malaria include quick diagnosis and treatment of malaria cases, vector control strategies, active research for malaria vaccine discovery and new drugs. Resistance of *Plasmodium falciparum* to the common and affordable antimalarial drugs as well as the resistance of the malaria vector to usual insecticides has been reported. An effective and affordable malaria vaccine would be the most important tool to control malaria. To date there is no licensed malaria vaccine. Traditional approaches toward malaria vaccine discovery have shown their limitations because of the limited number of proteins that were evaluated. The completion of the genome sequence of *Plasmodium falciparum* as well as the availability of the high throughput protein microarray immunoassay and other bioinformatic approaches offer the possibility to accelerate and to widen research on malaria vaccine candidates. The harnessing of the well-established model of the naturally acquired immunity (NAI) developed in people living in malaria endemic areas is an important way for the discovery of novel malaria vaccine candidates. Protein microarray is a high throughput technology, which allows the profiling of the humoral immune response to many proteins expressed at different stages of the *Plasmodium* life cycle in a single experiment. However, despite the hope generated by the sequencing of *P. falciparum* malaria genome no antigen identified by the protein microarray technology has yet been developed as a potential vaccine. On the other hand identification of structurally stable domains with limited or no polymorphism has led to the development of one candidate tested in phase 1a and 1b clinical trials.

KEYWORDS: *Plasmodium*, genomic research, protein microarrays, naturally acquired immunity, malaria vaccine.

INTRODUCTION

Malaria remains a major public health problem, especially in developing tropical and subtropical countries. Despite many efforts to control the disease 212 millions of malaria cases and 429 000 deaths occurred in 2015 [1]. Key interventions to control malaria include prompt and effective treatment with common antimalarial drugs, vector control strategies and active malaria vaccine research. An emerging resistance of the most virulent malaria parasite, *Plasmodium falciparum,* to the most effective and affordable antimalarial drugs, artemisinin, has been

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reported [2] and, as expected, the resistance of the vector to common insecticides is increasing [3]. An effective and affordable vaccine would be the best way of controlling malaria burden. To date there is no licensed vaccine against malaria. Traditional approaches toward malaria vaccine discovery have shown their limitations because of the limited number of proteins that were evaluated. The completion of the genome of the malaria parasite *P. falciparum* [4] and the description of its proteome [5] offer a great opportunity to identify genes and their products as potential vaccine candidates. Moreover, the availability of high-throughput protein microarray and peptides synthesis technologies allows a functional study of the *Plasmodium* proteomes and an opportunity to enhance research of antimalarial strategies including vaccine candidate discovery. The naturally acquired immunity (NAI) developed in humans living in malaria endemic areas has already been reported as one of the models of immunity that attest the feasibility of malaria vaccine. Indeed, in individuals living in malaria endemic areas exposed to malarial infection naturally acquired immunity increases progressively during the first two decade of life and results in decreased clinical severity of the disease and mortality [6]. Moreover, experimental studies carried out using sera from naturally exposed donors have given a strong evidence that triggering a protective immune response is possible [7-9]. Sero-epidemiological studies carried out in malaria endemic areas allow the harnessing of this model of immunity for the discovery of potential malaria vaccine candidates. Nowadays, sero-epidemiological studies conducted in malaria endemic areas take advantage of functional genomics, protein microarray and protein structure prediction for the evaluation of a wide number of potential malaria vaccine candidates.

In this review we highlight the main findings obtained from sero-epidemiological studies conducted in endemic areas using whole genome bioinformatics and high-throughput protein/peptide production to profile naturally acquired humoral immune responses.

Naturally acquired immunity to malaria

Malaria immunity may be defined as the state of resistance to the infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication [10]. Naturally acquired immunity developed in humans living in endemic area plays a key role in the control of the infection. The inadequate understanding of naturally acquired immunity may contribute to the failure in developing an efficacious malaria vaccine. Acquire immunity may be either passive or active. Passive acquired immunity refers to the prenatal or postnatal transfer of protective substances from mother to child or by the injection of such substances (reviewed by [10]). Active acquired immunity is an enhancement of the defense mechanism of the host as a result of a previous encounter with the pathogen (or parts thereof). Various types of acquired immunity have been defined in humans: (i) the anti-disease immunity, conferring protection against clinical disease, which affects the extent and risk of morbidity associated with a given parasite density; (ii) the anti-parasite immunity, conferring protection against parasitemia, which affects the density of parasites and (iii) premunition, providing protection against new infections by maintaining low-grade and generally asymptomatic parasitemia (reviewed by [10]). In sub-Saharan Africa where the disease is holo-endemic, people are almost continuously infected by *P. falciparum* and the majority of infected adults rarely experience overt disease. Naturally acquired immunity is compromised in pregnant women, especially primigravidae, and adults leaving temporarily endemic areas lose naturally acquired immunity. Thus, exploration of naturally acquired immunity is a key element in the design and rational development of vaccines and other malaria-control tools.

Models of acquired immunity to malaria

The feasibility of developing malaria vaccine is suggested by two human models of immunity demonstrating that protective immunity can be induced by exposure to intact *P. falciparum* parasite (reviewed by [11]).

Sterile protective immunity

The first model is based on immunization with radiation-attenuated sporozoites which aims to prevent infection and all of the clinical manifestations of the disease. In 1973, it was demonstrated that human volunteers immunized with radiation-

attenuated *P. falciparum* sporozoites were protected [12, 13]. It is now well established that immunization in mice or humans with radiation-attenuated *Plasmodium* sporozoites confers sterile protective immunity against challenge with infectious, non-attenuated sporozoites in virtually all naïve recipients [14]. On the other hand, when a similar experiment was performed in volunteers living in endemic areas sterile protection was limited in time [15]. The *Plasmodium* parasites developing within the host hepatocyte is the major target of protective immune responses induced by immunization against irradiated sporozoites. $CD8^+$ T cells specific for peptides derived from proteins expressed by irradiated sporozoites in the hepatocyte are considered the primary immune effectors and protection is mediated by interferon gamma release by these $CD8^+$ T cells (as well as other cells) rather than by direct cytotoxic T cells [16-18]. $CD4^+$ T cells that recognize parasite-derived peptides/ class II MHC molecule complexes on the hepatocyte, as well as antibodies against sporozoites surface proteins that neutralize the infectivity of sporozoites for hepatocytes may also play a role [19]. The protective immune responses induced by immunization with irradiated sporozoites are probably directed against multiple antigens and epitopes thereof [10]. Thus, a multi-antigen vaccine that induces $CD8^+$ as well as $CD4^+$ T cell responses against liver-stage antigens may be required to mimic the breadth and complexity of the irradiated sporozoite-induced sterile protection in naïve volunteers but seems not adequate to confer protection in people living in endemic countries [15]. Various mechanisms and hypotheses have been put forward to explain this discrepancy [20, 21]. The induction of sterile immunity in humans by immunization with radiation-attenuated *P. falciparum* sporozoites under experimental conditions provides proof-of-principle regarding the feasibility of a malaria vaccine that prevents blood-stage infection and clinical disease in naïve populations (travelers, seasonal workers, military personnel etc.) while it seems that an anti-disease vaccine is needed in malaria endemic areas.

Anti-disease immunity

This second model consists in the exploitation of naturally acquired immunity for the design of a vaccine to prevent disease and death. In malaria endemic areas, individuals who survive infections develop anti-disease immunity, which protects them from severe malaria. In areas with annual, stable transmission there is little to no severe disease or malaria-associated deaths after the age of 7-10 years. In areas with very intense transmission this transition may occur as early as the second or third year of life. The decrease in the incidence of *Plasmodium* infections, the prevalence and density of the parasitemia and the morbidity and mortality associated with *Plasmodium spp*. infection with natural exposure is consistent with acquisition of anti-malarial immunity in humans [22]. Moreover, passive transfer of purified immunoglobulin derived from adults with naturally acquired immunity following lifelong exposure to endemic area results in a marked decrease in *P. falciparum* blood-stage parasitemia and resolution of symptoms in the recipients [7, 9]. In naturally acquired immunity all the arms of the immune system are probably activated against all stages of the parasite life cycle. However, given the protective efficacy of antibody passive transfer, naturally acquired immunity is most likely based on antibodies directed against parasite proteins expressed on the surface of the infected erythrocytes that prevent sequestration in the microcirculation [23], antibodies directed against parasite proteins expressed on the surface of merozoites that prevent invasion of erythrocytes [24] and antibodies expressed against proteins expressed in different blood stages capable of mediating antibody-dependent cellular inhibition and/or opsonization [25, 26].

Several critical antigens targeted by naturally acquired immune responses have been identified and, among them, RH5 has been recently emerged as a possible effective candidate [27]. Other candidates associated with RH5 may be proven of additional protective benefit. On the other hand, given the complexity of the parasite and the host, it is very likely that many other parasite proteins may be targeted. The existence of naturally acquired immunity and the demonstration that a clinically important degree of erythrocytic stage immunity can be induced by experimental or natural exposure to repeated blood-stage infection provides a strong rationale for the identification of protective naturally acquired immunity targets and the development of the relevant vaccines [28, 29]; [6, 22].

Protein microarray technology in malaria research

Until recently only a few studies took advantage of the genomic data, and malaria vaccine development efforts were focused almost exclusively on a handful of well-characterized *P. falciparum* antigens [30]. The completion of *Plasmodium* genomes, in principle, allows a more rational approach to vaccine development, based on selecting the optimal antigens for inclusion in a vaccine. Selection of the optimal antigens can be based on the established models of protective immunity.

Available protein microarray technology represents a formidable tool to investigate the humoral response against the entire proteomes of complex pathogens [31]. Protein microarrays can be divided into two conceptually-distinct groups named abundance-based microarrays and function-based microarrays [32].

Analytical microarrays, functional microarrays and reverse phase microarrays are the three types of protein microarrays used to study the biochemical activities of proteins. Functional protein microarrays are, in principle, composed by full-length proteins or protein domains. These protein chips are used to study the biochemical activities of an entire proteome in a single experiment. This assay format incorporates key features, such as true parallelism, miniaturization and high throughput that could overcome most of the current enzyme-linked immunosorbent assay (ELISA) limitations [33]. Several studies have shown that protein microarray could provide a practical means to quantify thousands of different protein species in clinical or research applications [34-37]. Protein microarrays allow the profiling of the humoral immune responses with small amount of samples. Compared to conventional ELISA they appear to be more sensitive and specific. One of the most challenges in *P. falciparum* protein microarrays construction concerns the expression and the purification of the proteins because of the high A-T composition of its genome. Indeed, *P. falciparum* genome is the most A-T sequenced genome to date [4]. High throughput platforms have been set up to overcome these limitations. The microarray immunoassay requires high technology and expertise, which may not be available in routine laboratory located in developing countries. A technology transfer to set this platform in research laboratories in these countries requires a capacity strengthening through training of human resources and acquisition of adequate instruments.

Antibody response profile and malaria transmission level

Protein microarrays have been used to characterize antibody responses in populations from malaria endemic regions in Africa, South-East Asia, and South America. Several published malaria proteome studies have been aimed at understanding the phenomenon of naturally acquired immunity to malaria. A sero-epidemiological study conducted in Mali showed that naturally exposed individuals produce antibodies against hundreds of *P. falciparum* antigens. Both children and adults have more reactivity after the high transmission season and adults show more reactivity than children [38]. When adolescent children who survived the malaria season without experiencing symptoms were compared with age-matched children who had malaria episodes, 49 antigens were identified to be associated with protection against clinical malaria [38]. Similar observation from a low transmission setting of the Peruvian Amazon showed a limited set of *P. falciparum* protein antigens associated with the development of naturally acquired clinical immunity [39].

In another study conducted in Mali, protein microarray containing 1204 proteins was used at understanding gametocyte-specific antibody responses in exposed populations. From the results of that study, 91 proteins were classified as gametocyte-specific and 69 as proteins not expressed by gametocytes. The overall breadth and magnitude of gametocyte-specific IgG responses increased during the malaria season [40].

In a sero-epidemiological study conducted in Kenya, protein microarray containing 854 proteins was used to profile the antibody responses in two endemic areas in the western Kenyan highlands with differing malaria transmission intensities. This allowed the identification of 107 proteins as serum antibody targets. Highly immunogenic antigens that produce stable antibody responses from early age as well as less immunogenic proteins that require repeated exposure for stable responses to develop and produce different sero-conversion rates

between sites have been observed [41]. Results of that study showed that the season of sample collection affected the intensity of the antibody response in residents from the hilltop but not from the valley bottom. Sera collected at the valley bottom site where *P. falciparum* transmission levels are higher showed no significant difference in the intensity of antibody responses between the dry and wet seasons [41].

Antibody response profiles and age

In the first protein microarray-based study of variant antigens in malaria, an array was produced containing 123 var domains derived from *P. falciparum* field isolates from Papua New Guinea [42]. The data showed that the anti-PfEMP1-DBLα antibody responses increased in diversity, magnitude and prevalence with age. In Mali, a protein microarray containing 21 fragments of pfEMP1 was used to study the sero-reactivity of malaria-exposed children and adults. The results showed that sero-reactivity to pfEMP1 fragments was higher in adults than in children; intracellular conserved fragments were more widely recognized than extracellular hypervariable fragments [43]. These results show that protein microarray is a powerful tool to identify sero-reactive domains of polymorphic malaria antigens. This approach could be used to identify extracellular pfEMP1 domains critical for the development of protective immunity to malaria. In another study conducted in Kenya, protein microarray containing 824 unique *P. falciparum* proteins was used to study the naturally acquired immunity in children and adults. The results of that study showed that antibody from immune adults reacted with a total of 163 *P. falciparum* proteins. The children gradually acquired antibodies to the full repertoire of antigens recognized by adults. Antibody levels to 106 antigens were significantly higher in children who were protected from symptomatic malaria than in those who were not [44].

Antibody response profiles and protection to clinical malaria

Protein microarray was used in Gambia to study the reactivity profiles in serum of children with different levels of clinical immunity to *P. falciparum* malaria. The results of that study showed that serum reactivity to individual antigens did not correlate with the immune status. By contrast combined recognition of some antigens was significantly associated with protection against clinical malaria [45]. A protein microarray has also been used to compare the reactivity profile of exposed patients with clinical malaria to unexposed-subjects. Samples from exposed patients were collected in Yunnan, an area of China to detect antibody responses *to Plasmodium falciparum* merozoites proteins. Sera from unexposed-subjects were collected from malaria non endemic area. A total of 30 highly immunoreactive merozoite antigens were identified including well characterized blood-stage vaccine candidates as well as novel proteins [46]. Another study conducted in Papua Novel Guinea used protein array containing 4441 recombinant proteins expressed during the blood stage of *P. falciparum* and *P. vivax* to characterize the antibody response to the blood stage malaria in symptomatic and asymptomatic children. The sera from children recognized hundreds of the arrayed recombinant *P. falciparum* and *P. vivax* proteins. In general responses in asymptomatic children were highest in those with parasitemia, suggesting that antibody levels are associated with parasite load. Symptomatic children carried fewer antibodies than asymptomatic children with infection detectable by microscopy [47].

In Uganda, protein microarray was used during a sero-epidemiological study to profile humoral immune response and look for novel malaria vaccine candidates. The array contained 1827 recombinant proteins. The results of that study showed 128 proteins that are significantly associated with protection from symptomatic malaria. From the 128 proteins, 53 were down-selected as the most plausible targets of host protective immune response [48].

In Kenya, protein microarray was used to study the influence of HIV infection on the acquisition of immunity to malaria. In that study individuals from areas of high and low endemicity had different antibody profiles. HIV-infected patients with normal $CD4^+$ counts had the same reactivity against malaria antigens as HIV-negative individuals leading to the conclusion that early stage HIV-infected patients are at no risk of losing naturally acquired immunity [49].

To elucidate the profile of antibodies that develop after natural or experimental infection or after vaccination with attenuated organisms aiming to identify immunoreactive antigens of interest for vaccine development, a protein microarray containing 250 proteins was used to screen sera from Kenyan volunteers. The sera from naturally exposed individuals recognized 33 antigens from which 16 were characterized and 17 were novel ones [50].

A protein microarray containing 515 *P. vivax* and 500 *P. falciparum* proteins was used in India to profile the immune responses in residents at three sites. The samples showed significant sero-reactivity to 265 *P. vivax* and 373 *P. falciparum* antigens. The most immunogenic antigens of both *Plasmodium* species were associated with asymptomatic malaria [51].

Antibody response profiles and susceptibility to malaria

The array technology has also been applied to compare antibody profiles between the Fulani and Dogon ethnic groups in Mali, which differ in susceptibility against malaria [52, 53]. A protein microarray containing 1087 *P. falciparum* antigens was used in this study to probe the plasma samples of study participants. The results showed that the breadth and magnitude of *P. falciparum*-specific IgM and IgG were significantly higher in the malaria-resistant Fulani versus the malaria-susceptible Dogon. The *P. falciparum*-specific IgM responses more strongly distinguished the two ethnic groups [54] with higher titers in less susceptible Fulani compared to Dogon ethnic group.

Antibody response profiles and pregnancy

In Mali, a protein microarray containing five overlapping fragments of the 3D7 VAR2CSA extracellular region was used to profile the humoral immune response in relation with pregnancy. The results showed that Malian women with a history of at least one pregnancy had antibody recognition of four of these fragments and had stronger reactivity against the two distal fragments than did nulliparous women, children and men [55].

Antibody response profiles and Hemoglobin type

Protein microarray was also used to study the effect of sickle-cell trait on the *P. falciparum* specific antibody response in a cohort of naturally exposed individuals from Mali [56]. Heterozygous states of hemoglobin (Hb) A and HbS (HbAS; sickle cell trait) or HbC (HbAC) protect against *P. falciparum* malaria but the mode of protection is unclear. One hypothesis is that HbAS and HbAC accelerate the acquisition of immunity to malaria, possibly by enhancing *Plasmodium falciparum*-specific antibody responses. However, in that study, protein microarray data showed that there were no significant differences in antibody profiles between normal and sickle-cell trait [56].

Protein structure-based vaccine discovery

Limitations of the protein microarray approach are the lack of information on the structure of the antigens expressed, the fine specificity/epitopes recognized by the immune sera and their degree of polymorphism. An approach that would address these considerations was to target specific protein 3-dimemsional domains with limited or no polymorphism. Hence the approach taken by the groups of Corradin and Kayava was to focus on specific structurally defined stable domains present in the *P. falciparum* proteome, and, in particular, α-helical coiled coil and intrinsically unstructured domains with limited or no polymorphism, which are easily identified by a bioinformatics search of the *P. falciparum* genome [57]. The advantage of focusing to these two structurally domains are: 1) α-helical coiled coil fragments are around 30-40 amino acid residues long, self-assembling as helices of 2-6 strands, amenable to high throughput peptide synthesis and 2) there is no need to worry about the 3-D structure of unstructured regions. In addition, both structures can be easily determined by circular dichroism measurements. About 180 α-helical coiled coils and 1000 50-100 aa long unstructured antigens were identified in the erythrocytic cycle of *P. plasmodium* [58]. All of the 180 α-helical coiled coils with a decreasing bioinformatics score that form stable structures were synthesized and tested for antigenicity with immune sera. Subsequently, affinity-purified antibodies specific for selected antigens were tested for functional activities and their polymorphism determined ([57]; Kulangara *et al.*, 2009; unpublished data). After this initial work, the sequence of the most promising antigens was scanned for the presence of interesting protein

domains as the unstructured one. One of these, P27A derived from the blood-stage protein PFF165c, seems to be particularly interesting for its length of 104 aa and the presence of little polymorphism (aa position 293, E/G, 60/40 %) [59]. Alternatively strings of interesting α-helical coiled coil antigens derived from different proteins can be produced and their immunological properties and vaccine potential can be determined [60]. One of these potential vaccine antigens derived from the protein PFF165c, P27A, has been tested in a phase 1a and 1b clinical trials with promising safety and immunological results (manuscript submitted). Similar approach was recently taken to identify α-helical coiled coil domains in *P. vivax* blood stage proteins. A number of α-helical coiled coil segments have been identified [61] some of which are associated with protective immune responses [62].

CONCLUSION

All the reviewed studies show that profiling the naturally acquired humoral immune response to malaria parasite through protein microarray and/or structure-based vaccine discovery approaches allow the rapid identification of novel antigenic proteins which must be further validated for their immunogenicity, low polymorphism and efficacy to control malaria infection. Given the complexity of the malaria parasite multivalent vaccine design targeting one or more stages of the parasite seems at this stage a priority for the development of an efficacious malaria vaccine.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

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