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Review

Utilization of genomic sequence information to develop malaria vaccines

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Summary

Recent advances in the fields of genomics, proteomics and molecular immunology offer tremendous opportunities for the development of novel interventions against public health threats, including malaria. However, there is currently no algorithm that can effectively identify the targets of protective T cell or antibody responses from genomic data. Furthermore, the identification of antigens that will stimulate the most effective immunity against the target pathogen is problematic, particularly if the genome is large. Malaria is an attractive model for the development and validation of approaches to translate genomic information to vaccine development because of the critical need for effective anti-malarial interventions and because the *Plasmodium* parasite is a complex multi-stage pathogen targeted by multiple immune responses. Sterile protective immunity can be achieved by immunization with radiation-attenuated sporozoites, and anti-disease immunity can be induced in residents in malaria-endemic areas. However, the 23 Mb *Plasmodium falciparum* genome encodes more than 5300 proteins, each of which is a potential target of protective immune responses. The current generation of subunit vaccines is based on a single or few antigens and therefore might elicit too narrow a breadth of response. We are working towards the development of a new generation vaccine based on the presumption that duplicating the protection induced by the whole organism may require a vaccine nearly as complex as the organism itself. Here, we present our strategy to exploit the genomic sequence of *P. falciparum* for malaria vaccine development.

Key words: *Plasmodium*, *P. falciparum*, vaccine, genomics, proteomics, molecular immunology, immune screening, multi-epitope.

Challenges in malaria vaccine development

Malaria is the most important parasitic disease of man, exacting an estimated toll of 300–500 million new infections and 1.5–3 million deaths annually (World Health Organization, 2002). Moreover, recent analyses suggest that the medical impact of malaria has actually been significantly underestimated (Breman, 2001) and that the enormous economic impact of malaria has never been adequately considered (Gallup and Sachs, 2001). The profile of increasing resistance of the *Plasmodium* spp. parasite to chemoprophylactic and chemotherapeutic agents, the increasing resistance of the *Anopheles* spp. vector to insecticides, and the inability of the most affected countries to mobilize and sustain the resources required for malaria control, highlight the critical need for an effective vaccine against this disease.

The development of a malaria vaccine is, however, a formidable challenge. Despite a relatively intense and systematic research effort conducted since the 1960s and clinical trials of a large number of candidate vaccines with a range of delivery systems designed to induce protective antibody or cell-mediated immune responses against the sporozoite in circulation, the infected hepatocyte or the parasitized erythrocyte, few humans have been protected (reviewed in Richie and Saul, 2002). Even in animal models, vaccines have not been optimal. As compared to developing vaccines against viruses and bacteria, developing a vaccine

against malaria is complicated by the complexity of the parasite as well as the complexity of the host's response to the parasite. Challenges include the complex multi-stage parasite life cycle, a large 23 Mb genome encoding more than 5300 proteins, distinct stage-specific expression of the proteins, the requirement for distinct immune mechanisms targeting these different stages, the poor understanding of the protective immune mechanisms, allelic heterogeneity of parasite antigens between strains, antigenic variation within a single strain, sequence polymorphism of critical target epitopes, parasite evasion of host immune responses, and variant disease expression based on epidemiology, transmission dynamics and the genetic background and age of the host (Hoffman et al., in press).

Models demonstrating the feasibility of developing a malaria vaccine

The feasibility of developing a malaria vaccine is, nonetheless, suggested by two human models demonstrating that protective immunity can be induced by exposure to intact *Plasmodium falciparum* parasites.

Model 1. Sterile protective immunity

One model, immunization with radiation-attenuated sporozoites, is for the design of a vaccine to prevent all clinical manifestations of malaria (reviewed in Hoffman et al., 2002a). In 1967, it was demonstrated that mice immunized with *Plasmodium berghei* sporozoites, attenuated by exposure to x-ray or gamma-radiation such that they could invade the host hepatocyte and undergo limited development but could not mature into blood-stage parasites, were protected against challenge with infectious sporozoites (Nussenzweig et al., 1967). Since the parasite was unable to mature to the erythrocytic stage, clinical symptoms of disease and transmission of malaria did not occur. Immunization with heat-killed, formalin-inactivated or lysed sporozoites was not effective, but protective immunity could be induced in mice immunized with infectious sporozoites and treated with chloroquine to prevent erythrocyte infection. These data emphasize the requirement for live sporozoites targeting the liver. In 1973, it was demonstrated that human volunteers could be protected against *P. falciparum* sporozoite challenge by immunization with radiation-attenuated *P. falciparum* sporozoites (Clyde et al., 1973a,b). It is now well established that immunization of mice or humans with radiation-attenuated *Plasmodium* sporozoites confers sterile protective immunity against challenge with infectious, non-attenuated sporozoites in virtually all recipients (reviewed in Nussenzweig and Nussenzweig, 1989; Hoffman et al., 2002a). This protection is effective against challenge with massive doses of infectious sporozoites, is species-specific but not strain-specific, is efficacious in outbred and inbred mouse strains differing in genetic background, as well as major histocompatibility complex (MHC)-diverse humans, and persists for at least 9 months in humans. The importance of immune mechanisms that are active in the irradiated sporozoite model is highlighted by the fact that infection-blocking immunity in humans rarely, if ever, occurs under natural conditions.

The *Plasmodium* spp. parasite developing within the host hepatocyte is the major target of protective immune responses induced by immunization with irradiated sporozoites. $CD8^+$ T cells specific for peptide epitopes from proteins expressed by irradiated sporozoites in the hepatocyte are considered the primary immune effectors, and protection is mediated by interferon γ (IFN-γ) released by these $CD8^+$ T cells (as well as other cells) rather than by direct cytotoxic T cell-mediated lysis (Doolan and Hoffman, 1999, 2000; Good and Doolan, 1999; Plebanski and Hill, 2000). $CD4^+$ T cells that recognize parasite-derived peptide/class II MHC molecule complexes on the hepatocyte, as well as antibodies against sporozoite surface proteins that neutralize the infectivity of sporozoites for hepatocytes, may also play a role (reviewed in Sinnis and Nussenzweig, 1996; Good and Doolan, 1999). The targets of cellular immunity (both at the $CD8^+$ and $CD4^+$ T cell level) are largely unknown, however, and correlates of protection after sporozoite immunization are unclear. Also, it is not established whether the protective immune responses induced by immunization with irradiated sporozoites are narrowly focused on a few immunodominant antigens and epitopes or, alternatively, are broadly dispersed on a relatively large number of parasite antigens. Recently, we have demonstrated that 16 of 27 putative *P. falciparum* proteins identified by multidimensional protein identification technology (MudPIT) were recognized by volunteers immunized with irradiated sporozoites; nine proteins were highly antigenic, three were of intermediate reactivity, and four were weakly antigenic (Doolan et al., 2003). These data support our contention that protective immune responses induced by immunization with irradiated sporozoites are probably directed against multiple antigens, and against multiple epitopes on those antigens, with variable potency. 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 protection.

The induction of sterile immunity in humans by immunization with radiation-attenuated *P. falciparum* sporozoites provides proof-of-principle regarding the feasibility of a malaria vaccine that prevents blood-stage infection and clinical disease. This model also suggests a logical approach for making such a vaccine: identify the antigenic targets of irradiated sporozoite-induced immunity (those antigens expressed by irradiated sporozoites within hepatocytes that are recognized by sporozoite-induced T cell responses) and package these antigens or their critical minimal epitopes in a vaccine formulation that is immunogenic and suitable for manufacture and administration.

Model 2. Anti-disease immunity

The second model is that of naturally acquired immunity, for the design of a vaccine to prevent death and severe disease. In areas where malaria is transmitted, individuals that survive

past a certain age will become reinfected and will become clinically ill but will not develop severe disease or die. 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 *P. falciparum* infections, the prevalence and density of parasitemia, and the morbidity and mortality associated with *Plasmodium* spp. infection with natural exposure is consistent with acquisition of anti-malarial immunity in humans (Baird, 1995, 1998; Snow et al., 1998). Moreover, passive transfer of purified immunoglobulin derived from adults with naturally acquired immunity following lifelong exposure to endemic malaria results in a marked decrease in *P. falciparum* blood-stage parasitemia and resolution of symptoms in the recipients (Cohen et al., 1961; McGregor and Carrington, 1963; Sabchareon et al., 1991). A clinically important degree of erythrocytic stage immunity can be also induced by repeated experimental exposure to blood-stage infection (reviewed in Jeffery, 1966; Collins and Jeffery, 1999a,b).

In naturally acquired immunity, all arms of the immune system are probably activated against all stages of the parasite life cycle. However, most malariologists believe that the most important effectors in naturally acquired immunity are antibodies directed against parasite proteins expressed on the surface of erythrocytes that prevent sequestration in the microcirculation (Duffy et al., 2001), antibodies directed against parasite proteins expressed on the surface of merozoites that prevent invasion of erythrocytes (Sim et al., 2001), and antibodies expressed against either type of parasite protein that are capable of mediating antibody-dependent cellular inhibition (Bouharoun-Tayoun et al., 1995), whereby biologically active molecules, including cytokines, nitric oxide and free oxygen intermediates, are released from reticuloendothelial or other cells after activation through the Fc component of the bound antibody molecule. Furthermore, pathogenesis of the clinical disease (Miller et al., 1994; Marsh et al., 1996; Clark and Schofield, 2000) may be mediated by these same host-derived biologically active molecules or by putative toxins released from the infected erythrocytes (Playfair, 1996; Clark and Schofield, 2000; Schofield et al., 2002), and neutralization of these *via* antibodies may play a role in protection. Finally, antibody responses against sporozoites as well as T cell responses against parasite proteins expressed within infected hepatocytes probably also contribute to naturally acquired disease modulating immunity. By reducing the number of *Plasmodium* spp. parasites maturing within the host hepatocyte, these pre-erythrocytic stage immune responses would be expected to dramatically reduce the initial blood-stage parasite burden and consequently the magnitude of the subsequent asexual stage amplification. In both hospitalized populations and semi-immune populations, most investigators have demonstrated a direct correlation between *P. falciparum* parasite density and morbidity and mortality associated with *P. falciparum* infection (McElroy et al., 1994, 1997; Mbogo et al., 1995; Vounatsou et al., 2000). The critical antigens targeted by naturally acquired immune responses are yet to be defined. Given the complexity of the parasite, and the host, it is likely that tens, hundreds or even thousands of parasite proteins may be targeted. This breadth of response may be further expanded by exposure to many heterogeneous parasite strains.

The existence of naturally acquired immunity and the demonstrations that a clinically important degree of erythrocytic stage immunity can be induced by experimental (Jeffery, 1966; Collins and Jeffery, 1999a,b) or natural (Baird, 1995, 1998) exposure to repeated blood-stage infection provide a strong rationale for the identification of the antigenic targets of naturally acquired immunity (those antigens expressed on the surface of merozoites or infected erythrocytes or in apical organelles that are recognized by antibodies induced in the context of naturally acquired immunity) and the development of vaccines designed to induce high levels of antibody responses against these antigens.

Current approaches to malaria vaccine development

Based on the two human models for malaria vaccine development described above, we consider that there are two extremes of vaccine design: (1) induction of T cell-mediated immune responses, directed against parasite antigens expressed by irradiated sporozoites within hepatocytes (liver-stage antigens) and (2) induction of antibody responses directed against parasite proteins expressed on the surface of merozoites or infected erythrocytes or in apical organelles. Thus, a two-tiered approach to malaria vaccine development is likely to be the most effective. Induction of T cell responses against liver-stage antigens would destroy the majority of parasites during the five-day window of hepatic stage development. However, since each sporozoite can potentially develop into 10 000–30 000 merozoites, each of which may potentially undergo a series of asexual amplifications resulting in a 10–20-fold increase in the number of parasites in the bloodstream every 48 h, a hepatic-stage vaccine must be 100% effective. Induction of antibody responses against erythrocytic stage antigens would protect against severe disease and death in individuals who experience breakthrough blood-stage infections.

Our hypothesis is that by reducing the number of parasites emerging from the liver (*via* T cell immune responses directed against those antigens expressed by irradiated sporozoites in hepatocytes) and priming the immune system to erythrocytic stage antigens that will be boosted by infection from natural exposure (*via* antibody responses directed against parasite proteins expressed on the surface of merozoites or infected erythrocytes or apical organelles), one will reduce the severity and mortality of *P. falciparum* malaria.

Most current candidate malaria vaccines are designed to induce protective immune responses against pre-erythrocytic and/or erythrocytic stage antigens. Another type of vaccine being developed, a transmission-blocking vaccine, is designed

to protect the entire community rather than the immunized individual, by inducing protective antibodies against sexual stage antigens and thereby reducing the intensity of malaria transmission.

Two main approaches to malaria vaccine development are currently being pursued worldwide. The most work has been done, and progress achieved, on an approach focused on maximizing the magnitude and quality of immune responses to a single or a few key antigens, such as the *P. falciparum* circumsporozoite protein (CSP) or merozoite surface protein 1 (MSP1), by immunizing with synthetic peptides or recombinant proteins in an adjuvant (Mahanty et al., 2003). These vaccines are being designed to primarily induce antibody and $CD4^+$ T cell responses, but there is also interest in eliciting $CD8^+$ T cell responses. Researchers focusing on this approach consider that the subset of parasite antigens currently identified as potential vaccine targets are adequate, that any one given antigen is likely to be as good as another provided that it is expressed in an appropriate context and that the major obstacle to fielding an effective vaccine lies with optimizing the induction of the desired immune response by vaccination. Some success with this approach has been shown with the demonstration that a CSP-based vaccine formulated in a strong adjuvant can provide short-term protection of malaria-naïve volunteers against experimental challenge and of semi-immune adults against naturally transmitted malaria. However, the transient nature of this protection and the inability of the vaccine to induce the class I-restricted responses considered important for pre-erythrocytic stage protection (Lalvani et al., 1999) show that the vaccine is far from adequate as currently formulated. The questions of whether all antigens are the same and it is the vaccine delivery system that matters, whether the single 'key' antigen has already been identified or whether there is a single 'key' antigen that has not yet been identified remain.

The second approach to malaria vaccine development is to focus on all of the currently known, promising candidate antigens and to induce good immune responses against them; for example, by priming with plasmid DNA and then boosting with DNA, recombinant viruses or recombinant proteins. The goal is to elicit $CD8^+$ and $CD4^+$ T cell as well as antibody responses. Researchers focusing on this multi-valent, multi-immune response approach are skeptical about the ability of a vaccine based on a single antigen to protect against a parasite as complex as *Plasmodium* that, as evidenced by its ability to establish a chronic, recrudescing infection, has evolved mechanisms for resisting all arms of the host immune response. Furthermore, genetic restriction of the host immune response and parasite variation of target antigens and epitopes pose enormous obstacles for vaccine development. That a multivalent approach may be more successful than an approach based on a single or few antigens is supported by the experimental demonstration that immunization of mice with a mixture of DNA vaccines encoding two pre-erythrocytic stage antigens could circumvent the genetic restriction of protection seen with each vaccine alone and could confer additive protection in some genetically distinct mouse strains not protected or poorly protected by either of the individual vaccines (Doolan et al., 1996). Additional support is provided by recent data demonstrating enhanced protection with a tetravalent *Plasmodium knowlesi* vaccine as compared with vaccines based on only one or two of the four antigens (W. R. Weiss, unpublished results). Thus, researchers pursuing this second approach are focusing their efforts on the development of a multi-valent, multi-stage and multi-immune response vaccine (Doolan and Hoffman, 1997) structured around the two extremes of vaccine design. Efforts to date have focused primarily on the technology of DNA-based vaccines (Doolan and Hoffman, 2001; Moorthy and Hill, 2002). First-generation DNA vaccines have proved suboptimal, but a number of immune-enhancement strategies show promise, at least in animal models. Most impressive are heterologous prime/boost approaches with DNA, recombinant viruses or recombinant protein in adjuvant, which show great potential for the induction of high levels of T cell or antibody responses, and protection (reviewed in Schneider et al., 1999; McConkey et al., 2003; Dunachie and Hill, 2003).

A major shortcoming of this approach is that it is based on a limited panel of already well-characterized antigens that may or may not be the most optimal targets of protective immunity (if, in fact, all antigens are not created equal). Additional, and potentially more promising, antigens could be included once identified. Regardless, in order for the vaccine to be effective in genetically diverse host populations and against all antigenically distinct *P. falciparum* strains, sufficient diversity must be represented by the panel of antigens to ensure that multiple arms of the immune system are activated and to allow for overcoming or circumventing the genetic restriction of the host immune response and the polymorphism of critical target epitopes. Finally, this vaccine approach and the number of antigens that can be targeted will be limited by logistical considerations regarding the size of the insert that can be included in a given vaccine delivery system and the number of antigens that can be formulated or administered simultaneously in the absence of antigenic competition (Sedegah et al., in press), as well as by manufacturing considerations.

An alternative approach, designed to mimic whole-organism-induced protective immunity

Despite considerable efforts over the past decades to develop subunit malaria vaccines that provide protective immunity comparable to that conferred by the human models of irradiated sporozoite immunization and naturally acquired immunity, no vaccine candidate has proven sufficiently efficacious to warrant commercial development (reviewed in Richie and Saul, 2002). This may not be too surprising given that the current generation of subunit vaccines is based on a single or few antigens and therefore might elicit too narrow a breadth of response and provide neither optimal protection nor protection on genetically diverse backgrounds. Moreover, responses against characterized antigens, known to be

expressed in the hepatic stage of the *Plasmodium* life cycle and recognized by $CD4^+$ and $CD8^+$ T cells, that are induced by sporozoite immunization are not as robust as those induced by subunit vaccination and seem unable to account for the protection seen with whole-organism vaccination (Sedegah et al., 1994; D. L. Doolan and C. Dobano, unpublished results). This suggests that the protective immunity induced by irradiated sporozoite immunization is due to the summation of many immune responses of low magnitude against multiple targets, where the low magnitude of response is a result of the low density of epitope presentation inherent in the process. Similarly, the magnitude of antibody responses against candidate blood-stage vaccine antigens or whole parasites induced by natural exposure to malaria is modest, and in many instances lower than the levels induced by subunit vaccination (Holder, 1996; Berzins and Perlmann, 1996; Brown and Rogerson, 1996). Overall, these data support the contention that immunization with only one or a few parasite proteins will be unlikely to duplicate the broad and sustained immunity elicited by exposure to a parasite that has thousands of proteins.

We believe that the sterile immunity achieved after immunization with irradiated sporozoites is probably directed against a large number of proteins expressed by irradiated sporozoites in hepatocytes and against a number of epitopes on those proteins and that the naturally acquired immunity experienced by those living in endemic areas is probably directed against a large number of proteins expressed on the surface of merozoites or infected erythrocytes or in apical organelles. Small or modest immune response against tens, hundreds or thousands of parasite proteins may be additive or synergistic. We are working, therefore, towards the development of a new generation vaccine, based on the presumption that duplicating and sustaining the protective immunity induced by whole-organism vaccination may require a vaccine that mimics the complexity of the organism itself, incorporating antigens from multiple stages and accounting for the extraordinary diversity of natural parasite populations. This approach requires the identification of an unprecedented number of parasite-derived proteins, the minimal $CD8^+$ and $CD4^+$ T cell epitopes on those antigens, and development of a vaccine delivery system that reproduces the breadth and multiplicity of the whole-organism-induced protective immunity. Our strategy is to focus on the two human models of whole-organism-induced immunity – irradiated sporozoite immunization and naturally acquired immunity – and to systematically identify and prioritize the antigens in the *Plasmodium* parasite targeted by the different immune responses. The central assumption is that, of the ~5300 potential proteins in the *P. falciparum* genome (Gardner et al., 2002), there will be one subset that is the target of protective T cell responses directed against the proteins expressed by the liver-stage parasite (those antigens expressed by irradiated sporozoites in hepatocytes) and another subset that is the target of protective antibody responses (those antigens expressed on merozoites, on infected erythrocytes and in apical organelles). We also anticipate that results of genome-based studies will suggest a prioritization amongst these antigens, according to their relative magnitude of immune reactivity and presumed protective capacity.

The *P. falciparum* genome and proteome – the foundation for a 'Genomes-to-Vaccines' approach

In 1996, an international consortium of genome scientists and funding agencies was formed to sequence the genome of *P. falciparum*. It was anticipated that the data deriving from this and related projects would facilitate the development and fielding of effective interventions against malaria, including vaccines, drugs and diagnostics (Wirth, 2002). To provide access to the data and to the bioinformatics tools necessary for data mining, the consortium established a centralized web-based database, 'PlasmoDB' (http://www.plasmodb.org), for the collection, annotation and analysis of genomic sequence data, proteomic data, gene and protein expression data, single nucleotide polymorphism (SNP) and expressed sequence tag (EST) data sets, genetic data and other information relevant to genomics-based research (Bahl et al., 2003).

The genomic sequence of *P. falciparum* was completed and published in October 2002 (Gardner et al., 2002). The *P. falciparum* proteome represented by stage-specific sporozoites, merozoites, trophozoites and gametocytes was also elucidated using MudPIT, which combines in-line high-resolution liquid chromatography and tandem mass spectroscopy (Washburn et al., 2002), and was published simultaneously with the *P. falciparum* genome (Florens et al., 2002; Lasonder et al., 2002). Since then, additional erythrocyte-surface-expressed parasite proteins have been identified using high-throughput proteomics (L. Florens, X. Liu, Y. Wang, O. Yang, O. Schwartz, M. Peglar, D. J. Carucci, J. R. Yates, III, Jr and Y. Wu, manuscript submitted). More recently, the gene expression profile of the *P. falciparum* parasite during the different stages of the parasite life cycle has been completed (LeRoch et al., 2003). Putative hepatic-stage-specific proteins have also been identified through EST sequencing, additional proteomics studies and bioinformatic methods (J. Aguiar, unpublished; P. L. Blair, unpublished; L. Florens, unpublished). In total, these data provide a set of open reading frames (ORFs) corresponding to potential *P. falciparum* target antigens and evidence for expression of these genes in different stages of the parasite life cycle. This foundation can be exploited for the identification and prioritization of novel antigens and epitopes that may be targets of anti-malarial protective immunity.

The genomic sequences of a number of other human, monkey or rodent *Plasmodium* spp. used in vaccine or drug development research, including a *P. falciparum* clinical isolate, *P. vivax*, *P. knowlesi*, *P. reichanowi*, *P. yoelii* (Carlton et al., 2002), *P. chabaudi* and *P. berghei*, have been also determined or are in progress (Hoffman et al., 2002b; http://www.tigr.org). The complete genomic sequence of the human host (Venter et al., 2001; Lander et al., 2001) and the *Anopheles gambiae* mosquito vector (the most important vector of *P. falciparum*

in sub-Saharan Africa; Holt et al., 2002) are now also available. The hope is that these data sets will facilitate additional characterization of those *P. falciparum* antigens for which a monkey or mouse orthologue is identified and will allow for comparative genomics (Thompson et al., 2001; Waters, 2002) and other studies designed to characterize *Plasmodium* spp. parasite antigens and their biological function relative to the insect or vertebrate host or to identify potential avenues for the development of novel interventions (Wirth, 2002). However, the promise of these multiple genomic data sets cannot be fully realized without developing appropriate technologies for systematically converting genomic data into protective vaccines, drugs or diagnostics.

Platform technologies for *P. falciparum* Genomes-to-Vaccines

Despite the completion or impending completion of the genomic sequence of a large number of pathogens that threaten public health, there is currently no algorithm that can be used effectively to identify the target antigens or epitopes of protective T cell or antibody responses from genomic data. Furthermore, the identification of antigens that will stimulate the most effective immune responses against the target pathogen is problematic, particularly when the genome of the pathogen is large, as is the case for *P. falciparum*. Accordingly, we are developing, validating and implementing several platforms to identify from the *P. falciparum* proteome (1) the subset of antigens expressed by irradiated sporozoites within hepatocytes that are recognized by T cell responses, based on the irradiated sporozoite model and (2) the subset of parasite proteins expressed on the surface of merozoites or infected erythrocytes or in apical organelles that are recognized by antibody responses, based on the naturally acquired immunity model. These platforms include: (1) recombinatorial cloning (Gateway™); (2) transcriptionally active PCR (TAP); (3) protein arrays and (4) ImmunoSense epitope-based strategies.

Recombinatorial cloning (Gateway™)

Traditional methods of cloning are too inefficient, laborious and costly to be applied to *P. falciparum*. Moreover, the high adenine/thymine content of the *P. falciparum* genome results in a large proportion of clones containing internal deletions and rearrangements and large numbers of non-recombinant clones. There is also a need to clone selected ORFs into multiple vectors, depending on the intended application (DNA vaccination, protein expression, transfection studies, etc.). Accordingly, we have evaluated the potential utility of a highly efficient directional recombinatorial cloning system. The Gateway™ system (InVitrogen Inc., Carlsbad, CA, USA) is designed to rapidly and efficiently clone large numbers of genes into plasmid vectors by exploiting the well-characterized site-specific recombination between bacteriophage lambda and *Escherichia coli*. The recombination process is designed such that a 'suicide' gene in the entry vector (and expression vectors), which is not able to support bacterial growth on standard *E. coli* host cells, must be replaced by the gene of interest to permit survival, making the plasmid-to-plasmid cloning essentially 100% effective. Dr Joshua Labaer and colleagues at the Harvard Institute of Proteomics (Harvard Medical School, Boston, MA, USA) provided invaluable assistance in adapting the Gateway™ system for use with *P. falciparum*; these researchers have produced a complete set of more than 7000 Gateway™ clones from *Saccharomyces cerevisiae* and a set of clones from the human genome (Brizuela et al., 2001).

Initially, a set of Gateway™ entry clones and destination clones in a customized DNA vaccine plasmid (J. C. Aguiar, J. LaBaer, V. Y. Shamailova, M. Koundinya, J. A. Russrl, P. L. Blair, F. Huang, K. Strang, W. Mar, R. Anthony et al., manuscript in preparation) and in a recombinant protein expression vector are generated for a prioritized subset of *P. falciparum* ORFs. Then, antisera against each of the gene products are generated in mice and used to establish stage specificity and subcellular localization (see below; stage-specific expression) and perhaps inhibitory activity using functional assays such as the inhibition of sporozoite invasion, inhibition of liver-stage development or growth inhibition assays. Capacity of the putative proteins to be recognized by immune sera from individuals residing in malaria-endemic areas or T cells from irradiated-sporozoite-immunized volunteers can also be evaluated (see below; immune screening).

Proof-of-principle studies with a subset of 111 full-length single exon genes (J. C. Aguiar, J. LaBaer, V. Y. Shamailova, M. Koundinya, J. A. Russrl, P. L. Blair, F. Huang, K. Strang, W. Mar, R. Anthony et al., manuscript in preparation) demonstrated that more than 86% of ORFs were cloned into entry and DNA vaccine vectors, in the absence of sequence deletions or rearrangements, and that antibodies that recognized *P. falciparum* merozoites, sporozoites and gametocytes could be generated by *in vivo* immunization with the Gateway™-compatible DNA vaccines. These data establish the Gateway™ as a platform technology for exploiting *P. falciparum* genomic data for vaccine development.

In a parallel project, we are applying the Gateway™ system to the *Plasmodium yoelii* rodent model. The goal is to identify and characterize *P. yoelii* antigens that can protect against *P. yoelii* sporozoite challenge. The underlying assumption is that *P. falciparum* orthologues of such antigens would represent good candidate vaccine antigens. This project focuses on data from the recently completed genomic sequence of *P. yoelii yoelii* (17XNL; Carlton et al., 2002). Selected ORFs, predicted to be expressed in the sporozoite/liver stage, are cloned using approaches analogous to those established for *P. falciparum*. Inbred BALB/c mice are immunized with pools of plasmids and challenged with infectious *P. yoelii* sporozoites. The capacity of the gene to reduce liver-stage parasite burden is quantified using Taqman® RT-PCR (Witney et al., 2001). Plasmid pools associated with protection are then deconvoluted to identify the individual protective genes. The advantage to the pooled plasmid approach is that it offers a

higher probability of a positive outcome, given that DNA vaccines based on single antigens may not necessarily be capable of conferring a high degree of protection against parasite challenge and that the protection induced by the multi-antigenic *Plasmodium* sporozoite may reflect the summation of immune responses directed against a relatively large number of parasite antigens. Preliminary studies with 192 single exon ORFs selected from a *P. yoelii* sporozoite EST library (Kappe et al., 2001), with 19 of the 192 being evaluated *in vivo* to date, have established proof-of-principle for this approach (D. Haddad, E. Bilcikova and W. R. Weiss, manuscript in preparation).

The primary outcome of the *P. yoelii* studies is protection rather than immunogenicity (although we recognize the value in correlating results of *in vitro* immunological screening assays with *in vivo* protection). This is a major advantage in the context of vaccine development, since *in vivo* identification of protective antigens is not feasible in the *P. falciparum* system prior to clinical testing, and potentially protective *P. falciparum* antigens may be identified by extrapolation from the *P. yoelii* system. It should be recognized, however, that the demonstration that a *P. yoelii* antigen is capable of protecting against *P. yoelii* sporozoite challenge in a mouse model does not necessarily mean that its *P. falciparum* orthologue would protect against *P. falciparum* parasite challenge in humans. An additional confounder is that the *P. yoelii* studies are carried out in a single genetically homogeneous inbred mouse strain (BALB/c; H-2d) whereas the target population for a *P. falciparum* vaccine is a genetically heterogeneous outbred human population. Nonetheless, mouse models are generally considered as suitable animal models for malaria vaccine development (Renia et al., 2002; Sanni et al., 2002), and *P. falciparum* orthologues of all characterized and protective *P. yoelii* antigens are currently considered high priority vaccine candidates.

Transcriptionally active PCR (TAP)

With the Gateway™ system, transcriptionally active genes are created by cloning the gene of interest into a replication-competent expression vector, transforming and growing bacteria and then purifying the plasmid. Even given the efficient nature of the recombinatorial approach as compared with traditional cloning methods, this approach is time, labor and cost prohibitive if a large number of genes needs to be analyzed. Genome-wide screening is also limited by the ability to validate that the appropriate recall immune responses can be induced against identified targets.

One solution is to render PCR products transcriptionally active (Sykes and Johnston, 1999). In response to this need, Dr Philip Felgner and colleagues (Gene Therapy Systems Inc., San Diego, CA, USA) developed a technology called transcriptionally active PCR, which allows for rapid and efficient generation of hundreds or thousands of genes in a form that is transcriptionally active *in vitro* and *in vivo* and that therefore can be used for high-throughput functional screening on a genome-wide basis (Liang et al., 2002). The technology uses nested PCR, in which two or more DNA fragments can be joined in a desired orientation, to introduce functional promoter and terminator sequences onto the gene of interest. Additionally, the technology can be modified by adding either epitope-tags (influenza-HA) or mammalian [cytomegalovirus (CMV)] or prokaryotic (bacterial phage T7) promoters. Epitope-tagging facilitates detection and characterization of the protein by western blot, enzyme-linked immunosorbent assay (ELISA), immunocytochemistry and fluorescence-activated cell sorting (FACS). Adding a T7 promoter allows for the generation of gene-specific mRNAs in a cell-free system and the generation of proteins in a cell-free transcription/translation system. The TAP system therefore offers enormous potential for genome-wide screening. To assess immunological potency, for example, TAP fragments or TAP proteins can be screened in novel cellular or humoral assays to quantify the capacity of each antigen to induce recall cellular or humoral immune responses from malaria-immune individuals (see below; immune screening assays).

Using a panel of already well-characterized *P. falciparum* and *P. yoelii* antigens, as well as a limited panel of ORFs selected from the *P. falciparum* genomic sequence database, we have evaluated the potential of TAP in the context of *Plasmodium* (D. A. Regis, P. Quinones-Casas and D. L, Doolan, manuscript in preparation). We have identified conditions for the efficient amplification of all *P. falciparum* and *P. yoelii* genes from either genomic DNA or plasmid DNA template and have established that *Plasmodium*-derived TAP fragments can be expressed *in vitro* and i*n vivo* and are immunogenic in mice. We are currently developing and validating cellular and humoral immune screening assays based on TAP technology (see below; immune screening).

Protein arrays

Recognition of the less than perfect correlation between RNA and protein expression (Gygi et al., 1999) and the influence of post-translational modification of proteins on phenotypic and functional outcome, including recognition by the host immune system, highlights the importance of genome-wide screening based on the translated products of the gene sequences. Cell-free gene transcription and translation systems, which couple T7 RNA polymerase-driven transcription with translation, offer the potential for high-throughput protein production. Dr Philip Felgner and colleagues at the University of California, Irvine (Irvine, CA, USA) have adapted the cell-free transcription and translation system to a high-throughput protein expression platform using a robotics workstation, enabling 384 different purified proteins to be produced and purified in a single day. In collaboration with Dr Felgner, we are applying protein array technology for functional screening of the *P. falciparum* genome.

The *P. falciparum* proteome will be prepared in two forms. The first form will be on protein ‘chips’, and the chips will be used to quantify serum antibody titers from immune or semi-immune humans against each of the target ORFs. The arrays will be screened for capacity of the target antigens to be

recognized by sera collected from clinically distinct cohorts of individuals naturally exposed to malaria. For the second proteome format, each individual protein will be purified and stored in 96-well microtiter plates in a form that will enable them to be delivered to, and processed by, antigen-presenting cells (APCs). These APCs will then be used as targets for *in vitro* cellular assays to evaluate the capacity of each putative protein to be recognized by recall T cell responses from irradiated-sporozoite-immunized volunteers. It is anticipated that this quantitative array-based humoral and cellular immune response scan will provide a profile of immune responses against *P. falciparum* in humans.

ImmunoSense

The approaches detailed above are aimed at identifying the complement of *P. falciparum* antigens targeted by protective T cell and/or antibody responses. The ImmunoSense approach offers the opportunity to simultaneously identify the target antigens as well as the minimal epitopes on those antigens. It is an integrated approach (executed in collaboration with Dr Alessandro Sette and colleagues, La Jolla Institute of Allergy and Immunology, San Diego, CA, USA) that incorporates bioinformatic predictions, human leukocyte antigen (HLA) supertype considerations, high-throughput binding assays and cellular assays. In essence, it represents a process in which epitope predictions are utilized in 'reverse', as a tool to identify new antigens; epitope identification has thus far been seen only as a means to identify the epitopes contained within a protein considered as a target of cellular immunity.

A detailed description of the ImmunoSense approach, and its application in the malaria model, has been reported elsewhere (Doolan et al., 2003). In brief, amino acid sequences corresponding to the subset of *Plasmodium* sporozoite/liver-stage ORFs are scanned using allele-specific computerized algorithms for peptide epitopes predicted to bind with high affinity to Class I and Class II HLA supertypes. HLA supertypes are characterized by largely overlapping peptide repertoires and are expressed at high frequencies in all major ethnicities (Southwood et al., 1998; Sette and Sidney, 1998, 1999). Pools of peptides, each pool representing a putative antigen, are screened for immune reactivity by assays such as IFN-γ ELISPOT (enzyme-linked immunospot), using peripheral blood mononuclear cells (PBMCs) from individuals immunized with radiation-attenuated *P. falciparum* sporozoites or mock-immunized controls. Peptide pools that recall strong T cell responses are deconvoluted to identify the individual peptide epitopes that are recognized. These antigens and epitopes can be prioritized according to the magnitude of the recall response and their capacity to be preferentially recognized by protected *versus* non-protected volunteers, as well as by their ability to bind strongly to multiple members of the relevant superfamily.

We have validated this approach in the context of *P. falciparum* (Doolan et al., 2003). Starting with 27 ORFs thought to be expressed in the sporozoite proteome, we identified 16 novel proteins reproducibly recognized by irradiated-sporozoite-immunized volunteers. Nine antigens were highly antigenic (recognized by >50% of volunteers in >25% of assays), three antigens were of intermediate reactivity, and four were of low reactivity (Doolan et al., 2003). Significantly, a number of antigens identified using this strategy were more antigenic than well-characterized antigens currently considered the best vaccine candidate antigens. These experiments provide proof-of-concept for the Immunosense approach, thereby allowing for more comprehensive genome-wide analysis.

The anticipated outcome of these studies will be (1) the identification, from the complete *P. falciparum* proteome, of those proteins that correspond to immunodominant antigens recognized by volunteers immunized with radiation-attenuated *P. falciparum* sporozoites and their minimal target epitopes and (2) the prioritization of those antigens and epitopes on the basis of immune reactivity and according to their potential association with protection against *P. falciparum* sporozoite challenge. Additionally, it is expected that the studies will establish whether protective immune responses in humans immunized with irradiated *P. falciparum* sporozoites are narrowly focused on a few immunodominant antigens and epitopes or, alternatively, are broadly dispersed on a relatively large number of parasite antigens.

Selection and prioritization of *P. falciparum* ORFs for genome-wide screening by Genomes-to-Vaccines approaches

The complexity of the 23 Mb *P. falciparum* genome parasite, with an estimated 5268 predicted proteins (Gardner et al., 2002), dictates a rational approach to prioritize ORFs representing putative antigens for evaluation. Bioinformatics is recognized as an essential tool for mining genomic and proteomic databases. However, there is currently no algorithm that can be used to identify the targets of protective antibody or T cell responses from genomic sequence data. In the case of antibody responses, one approach has been to focus on predicted surface or secreted molecules presumably accessible to antibody, but there has been no systematic genome-wide validation of the accuracy of such predictions. In the case of T cell responses, the subcellular location and function of the target protein is less important than the presence of appropriate MHC-binding epitopes in the sequence. *Plasmodium* gene-predicting algorithms such as Glimmer M or Phat (http://www.tigr.org/softlab/glimmer) can be used to identify genes, exons and introns; programs such as Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) can identify structural and functional domains; TMHMM2 can predict transmembrane domains; SignalP can predict signal peptides; Prospero can predict repetitive regions; and BLAST can identify orthologous sequences amongst different *Plasmodium* species. A range of bioinformatics tools can be accessed *via* the PlasmoDB database (http://www.plasmodb.org) or *via* the web (e.g. http://www.epitope-informatics.com/References.htm#BcellEpitopes).

Other experimentally derived data can also be considered,

such as the relative level of expression of the gene or the corresponding protein or the degree of polymorphism as indicated by SNP analysis. For example, one outcome of the MudPIT analysis of *P. falciparum* sporozoites (Florens et al., 2002) is a database detailing the number of MudPIT runs in which corresponding peptides were identified, the number of peptide hits per protein, the percentage of the protein sequence covered by those peptides, and the unique locus identifier of the corresponding genomic sequence. ORFs within this data set can therefore be ranked according to their relative level of expression in the sporozoite proteome. Similarly, information regarding stage-specific gene expression is also available, as a result of AffyMatrix gene chip studies (LeRoch et al., 2003). Correlation between protein expression and gene expression data sets is currently in progress (J. R. Yates and E. Winzeler, personal communication).

Despite this wealth of information, and in the absence of genome-wide validation, it is not obvious which tools are the most appropriate and which criteria are the most valid for ORF selection in the context of vaccine development. We have elected to use a combination of bioinformatics and comparative genomics approaches based on a number of genomic, proteomic, gene expression and other large-scale *Plasmodium* functional genomic data sets to select and prioritize *P. falciparum* ORFs for genome-wide screening.

One approach (based on the irradiated sporozoite model) has been to combine transcription and proteome analyses, using sequences derived from a *P. yoelii* sporozoite EST database (Kappe et al., 2001), *P. falciparum* sporozoite EST database (J. C. Aguiar, J. LaBaer, V. Y. Shamailova, M. Koundinya, J. A. Russrl, P. L. Blair, F. Huang, K. Strang, W. Mar, R. Anthony et al., manuscript in preparation), complete *P. falciparum* genome expression profiling using gene chips (Le Roch et al., 2003) and proteome analyses (Florens et al., 2002; Lasonder et al., 2002). Potential *Plasmodium* liver-stage-specific expressed genes were down selected by comparing *P. falciparum* orthologues of a *P. yoelii* laser capture microdissected liver-stage library (Sacci et al., 2002) with annotated genes not present in current EST libraries and not possessing peptide matches in *P. falciparum* proteome analyses. Selected antigens were further examined and ranked using the PlasmoDB web-based database for predicted signal sequences and transmembrane domains (for sporozoite antigens only), Pfam and GO assignments, sequence similarities and exon/intron gene structure. These criteria led to a manageable set (~250 annotated genes) of putative vaccine targets for study (representing ~5% of the *P. falciparum* genome; P. L. Blair, unpublished results). Selected ORFs can then be subjected to an automated primer design algorithm, such as a Primer3 (White Head Institute, Cambridge, MA, USA), for predicting oligonucleotide primer pairs suitable for high-throughput cloning using the Gateway™ technology or other Genomes-to-Vaccines approaches.

Target credentialing

Target credentialing systematically assigns functional criteria to each protein and, depending on the application, allows for a reduction of the number of potential targets from ~5300 to a more realistic number for more comprehensive evaluation and/or to serve as the foundation for the development of new candidate vaccines, drugs or diagnostics.

Stage-specific expression and subcellular localization

Because the requirement for inducing different types of immune responses by vaccination depends on the stage during the parasite's life cycle at which the protein is expressed, identification of the stage-specific expression of putative antigens is critical. MudPIT analysis of different preparations of parasite material (e.g. sporozoites, merozoites, trophozoites, gametocytes; Florens et al., 2002; Lasonder et al., 2002) provides evidence for expression of putative proteins in the different stages of the parasite's life cycle but not relative expression between the different stages. Comprehensive gene expression data sets (Le Roch et al., 2003) provide information on expression of all ORFs within any given stage as well as between stages. *P. falciparum* DNA microarray studies (Hayward et al., 2003; Rathod et al., 2002) also provide information regarding stage-specific expression, but such data sets are currently limited to only a subset of the *P. falciparum* genome. Finally, proteomic analysis and protein structure prediction algorithms can suggest which proteins are likely to be membrane-associated surface proteins, but the accuracy of such predictions has yet to be validated on a genome-wide scale.

More specific information regarding the stage-specific expression of putative *P. falciparum* proteins can be obtained by screening antigen-specific sera against parasite preparations (Hoffman et al., 1998). These sera will be generated for those ORFs evaluated in the Gateway project. However, financial and logistical reasons preclude comprehensive *in vivo* immunization studies for the thousands of putative *P. falciparum* proteins in the proteome. Antisera are evaluated by immunofluoresence antibody tests (IFAT) against stage-specific slides prepared from *P. falciparum* sporozoites, cultured hepatoma cells infected with irradiated or non-irradiated *P. falciparum* sporozoites, erythrocytic-stage *P. falciparum* parasites from carefully synchronized cultures corresponding to ring trophozoites, mature trophozoites, and schizonts or gametocytes.

Information on the subcellular localization of putative proteins can also be obtained from the IFAT studies. Although IFAT does not have the resolution of immunoelectron microscopy, it is nonetheless possible to distinguish a number of subcellular localization patterns; for example, in sporozoites, predominant surface expression can be distinguished from internal expression associated with micronemes, a secretory organelle, and, in the erythrocytic-stage parasites, expression on the surface of infected erythrocytes, on the surface of parasites within the red cell, in the apical organelles (micronemes and rhoptries) and within the parasite cytoplasm can be reliably distinguished.

Immune screening

In our Genomes-to-Vaccines program, we propose to identify, from the complete *P. falciparum* proteome, (1) the subset of antigens expressed by irradiated sporozoites in hepatocytes that are recognized by protective T cell responses and (2) the subset of antigens expressed on the surface of merozoites or infected erythrocytes or apical organelles that are recognized by protective antibody responses. A critical component of our strategy is the evaluation of immune responses. Standard immunological assays are not suitable for high-throughput screening with plasmid DNA, PCR fragments or recombinant proteins. Therefore, we are developing and optimizing novel cellular and humoral immune assays that can be applied to genome-wide screening (P. Quinones-Cases and D. L. Doolan, unpublished results; J. Aguiar and G. T. Brice, unpublished results).

To identify targets of protective T cell responses, we are utilizing specimens from irradiated-sporozoite-immunized volunteers (both protected and not protected against sporozoite challenge), presuming that the entire repertoire of sporozoite-induced T cell specificities will be represented in those individuals. We are focusing on T cell-derived IFN-γ as the primary marker of cellular immunogenicity, since this is considered to be the most appropriate *in vitro* marker of pre-erythrocytic stage protection identified to date (Good and Doolan, 1999; Plebanski and Hill, 2000). However, where possible, we will apply information from other studies designed to identify robust and predictive *in vitro* marker(s) of anti-malarial protective immunity; e.g. immunologically relevant DNA microarray studies with specimens from irradiated-sporozoite-immunized volunteers (C. Dobano, P. Quinones-Casas and D. L. Doolan, unpublished results).

To identify the subset of *P. falciparum* proteins expressed on the surface of merozoites or infected erythrocytes or in apical organelles, we are assaying sera from adults with naturally acquired immunity, since it is presumed that the entire repertoire of antibody specificities is represented in those individuals. However, given the complexity of host–parasite interactions and the variant disease expression based on epidemiology, transmission dynamics and the genetic background and age of the host, we also propose to ultimately analyze the responses of well-characterized cohorts of naturally exposed individuals with distinct categories of clinical disease (e.g. severe malaria, mild malaria, asymptomatic and symptomatic), as well as individuals of different ages (neonates, infants, children and adults). Sera from volunteers immunized with radiation-attenuated *P. falciparum* sporozoites and collected at different time points during the immunization and challenge process will also be evaluated, to provide a kinetic profile of antibody responses to pre-erythrocytic stage antigens.

Biological function

One indication as to the value of a putative antigen for inclusion in a vaccine relates to its biological function, at least in the case of protective antibody responses where the target antigen or the critical linear or conformational B cell epitope(s) on the antigen must be accessible to the antibodies (e.g. surface or secreted molecules). The biological function of the target protein is less important in the case of T cell responses, where the presence of MHC-binding epitope(s) in the sequence and appropriate processing of the antigen and presentation of the T cell epitope in the context of the MHC Class I and/or Class II molecules for recognition by the host immune system is critical.

Unfortunately, there are no algorithms to predict how a given antigen will function *in vivo*, and there are not yet established methods to accurately assess biological function *in vivo*, particularly in a high-throughput manner. Some approaches, such as gene-knockout studies (Wickham et al., 2003), are potentially useful in this regard but are currently not adapted for genome-wide application. For those putative proteins where the desired immune effector mechanism is an inhibitory antibody, however, it is possible to assess biological activity *in vitro*. Specifically, the capacity of antigen-specific antisera to inhibit parasite development or invasion could be evaluated by the inhibition of sporozoite invasion assay, inhibition of liver-stage development assay, or growth inhibition assay.

Ultimately, the capacity of an individual or pool of erythrocytic-stage proteins to induce protective antibodies *in vivo* could be assessed by immunizing *Aotus* monkeys with plasmid DNA or recombinant proteins and challenging with *P. falciparum* blood-stage parasites (Collins, 2002). Demonstration of pre-erythrocytic protection against *P. falciparum* 3D7 strain is currently not feasible in animal models, since a reproducible *Aotus–P. falciparum* 3D7 sporozoite challenge model is not available, and mice cannot be infected with *P. falciparum*. However, reproducible infection of intact *Aotus lemurinus griseimembra* monkeys by intravenous inoculation with sporozoites from a monkey-adapted *P. falciparum* strain (Santa Lucia) and a wild-type *P. falciparum* strain (Cali-Colombia-4, FCC-4) has been recently reported (Zapata et al., 2002), allowing for the possibility of evaluating protection with heterologous *P. falciparum* sequences of a given antigen of interest. Also, a reproducible sporozoite challenge model is available for *P. knowlesi* in rhesus monkeys (Collins, 2002; Rogers et al., 2001) and for *P. yoelii* in mice (Sedegah et al., 1982), so protection could be evaluated for those *P. falciparum* antigens that have identified as *P. knowlesi* or *P. yoelii* orthologues.

The next step: multi-epitope vaccine development

How best to mimic the complexity of multi-antigenic whole organism vaccines by subunit vaccination is not obvious. Challenges include how to optimally combine multiple antigens or their epitopes, how to induce the appropriate responses against each antigen or epitope included in a vaccine and how to direct immune responses towards antibody production for some antigens or epitopes and T cell responses for others. A basic conundrum is that adding antigens to a

multi-antigen cocktail incrementally reduces the dose of each component and thus may reduce component immunogenicity to the point where protection is lost. Finally, for a vaccine to be optimally effective, it must elicit protective immune responses that are sustained over time, by boosting after natural exposure to the parasite or by specific vaccination strategies. Although progress has been made, no delivery system has yet been shown to be adequate. It is our contention that, amongst those vaccine technologies currently available, only multi-epitope-based approaches offer the potential for delivering a subunit vaccine that can duplicate the complexity of whole-organism-induced protection. Including only the protective epitopes from multiple antigens and discarding all other extraneous sequence provides an enormous efficiency of space, probably the only feasible way to package the antigenic complexity of the parasite as a subunit vaccine.

In other systems, induction of simultaneous responses against multiple epitopes derived from multiple antigens has already been demonstrated. The immunogenicity of multi-epitope constructs appears to be strongly influenced by a number of different variables, and the immunogenicity (or antigenicity) of the same epitope expressed in the context of different vaccine constructs can vary over several orders of magnitude. This situation underscores the necessity of a systematic study of different variables in order to establish clear criteria for the optimal design of multi-epitope vaccines (reviewed in Sette et al., 2001, 2002). To address this in the context of malaria, in collaboration with Jeff Alexander, Brian Livingston, Mark Newman and colleagues (Epimmune Inc., San Diego, CA, USA), we are designing and optimizing multi-epitope vaccines comprising a panel of $CD8^+$ and $CD4^+$ T cell epitopes derived from four *P. falciparum* pre-erythrocytic stage antigens. These epitopes were identified by Class I and Class II algorithm predictions and peptide binding/recognition strategies similar to those described above for the ImmunoSense approach and were recognized by recall immune responses from volunteers immunized with irradiated sporozoites or naturally exposed to malaria (Doolan et al., 1997, 2000). Studies are aimed at optimizing vaccine design by eliminating junctional epitopes, optimizing spacers between epitopes and considering the order of epitopes, effect of flanking regions, and cellular targeting to antigen processing and presentation pathways. Recognition of individual epitopes is demonstrated by immunogenicity assays utilizing HLA transgenic mice and/or antigenicity assays using human APCs transfected *in vitro* with the prototype vaccine. The simplest vaccine configuration capable of effective delivery of the selected sets of epitopes will also be determined. Subsequent studies will identify the optimal vaccine delivery strategy for simultaneous induction of immune responses against multiple epitopes, and the appropriate vaccine formulation studies. Overall, it is anticipated that these studies will define operational rules for the design and optimization of multi-epitope-based vaccines.

Finally, we propose to compare the efficacy of the multi-epitope vaccine with the whole-organism-irradiated sporozoite vaccine, as well as whole antigen subunit vaccines (DNA and viral vectored), *in vivo* in HLA transgenic mice and *in vitro* in antigenicity assays using humans APCs. It is anticipated that these studies will validate the multi-epitope approach beyond the level of antigen and epitope identification and will provide important information regarding the potential of multi-epitope-based approaches to mimic whole-organism-induced immunity.

Conclusion

The human models of irradiated sporozoite immunization and naturally acquired immunity suggest that the development of a malaria vaccine is feasible. We are working toward the development of a new generation malaria vaccine based on the presumption that duplicating the protection induced by the whole organism may require a vaccine approaching the complexity of the organism itself. We anticipate that the Genomes-to-Vaccines strategies described above – Gateway™ recombinatorial cloning, TAP, protein arrays and ImmunoSense epitope screening – will identify the complement of *P. falciparum* antigens recognized by individuals immunized with irradiated *P. falciparum* sporozoites (in the case of T cell responses) or naturally exposed to malaria (in the case of antibody responses). This will allow for the design, optimization and evaluation of new candidate malaria vaccines based either on a limited number of antigens and/or epitopes that are more immunogenic than those currently available or that include many more antigens and/or epitopes and therefore may be more effective. Finally, these data will allow for the development of a vaccine capable of reproducing the breadth and multiplicity of the whole-organism-induced protection, by incorporating an unprecedented number of parasite-derived proteins or their minimal epitopes that are targets of protective immune responses. It is our contention that the whole genome approach proposed here will probably be much more successful than other approaches in combating the complex *Plasmodium* parasite and that significant and long-term impact on public health may be achieved only by exploiting the enormous opportunities provided by sequencing the *Plasmodium* genome.

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