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Pre-erythrocytic-stage immune effector mechanisms in *Plasmodium* spp. infections

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SUMMARY

The potent protective immunity against malaria induced by immunization of mice and humans with radiation-attenuated *Plasmodium* spp. sporozoites is thought to be mediated primarily by T-cell responses directed against infected hepatocytes. This has led to considerable efforts to develop subunit vaccines that duplicate this protective immunity, but a universally effective vaccine is still not available and *in vitro* correlates of protective immunity have not been established. Contributing to this delay has been a lack of understanding of the mechanisms responsible for the protection. There are now data indicating that CD8+ T cells, CD4+ T cells, cytokines, and nitric oxide can all mediate the elimination of infected hepatocytes *in vitro* and *in vivo*. By dissecting the protection induced by immunization with irradiated sporozoite, DNA and synthetic peptide–adjuvant vaccines, we have demonstrated that different T-cell-dependent immune responses mediate protective immunity in the same inbred strain of mouse, depending on the method of immunization. Furthermore, the mechanism of protection induced by a single method of immunization may vary among different strains of mice. These data have important implications for the development of pre-erythrocytic-stage vaccines designed to protect a heterogeneous human population, and of assays that predict protective immunity.

1. INTRODUCTION

Malaria ranks with acute respiratory infections and diarrhoeal diseases as one of the major causes of mortality worldwide. Approximately 40% of the world's population is currently at risk. It is estimated that there are 2.7 million people infected with the malaria parasite annually, with 300–500 million new *Plasmodium* infections and 2–5 million deaths annually due to malaria (World Health Organization 1994). Furthermore, such estimates may be low. Infection is not synonymous with disease and deaths are not confined to the first infection. In areas of high endemicity, children aged less than 5 years are predominantly affected. The acquisition of partial clinical immunity with age is reflected in the decreased frequency of disease and mortality in older individuals, despite that such individuals continue to harbour malaria parasites. In areas of low endemicity, all age groups are targeted and clinical immunity is rarely achieved.

Malaria parasites were first described in human blood in 1880 by Laveran, and nearly two decades later Manson and Ross identified the mosquito as the vector of malaria. The liver stage in the life cycle was first documented in 1949 (summarized from Bruce-Chwatt 1980). However, despite intense research and many Phase I and Phase II clinical trials of potential vaccines, including recent promising results (Stoute *et al*. 1997), an efficacious malaria vaccine is not yet available for general use. Contributing to this failure is a lack of understanding of the mechanisms responsible for protective immunity and the inability to define correlates of protection *in vitro*.

2. LIFE CYCLE OF *PLASMODIUM* SPP.

Malaria is a disease caused by parasites of the genus *Plasmodium*. It is initiated when an infectious female *Anopheles* mosquito inoculates sporozoites into the circulation of the host during a blood meal. Sporozoites migrate to the liver, where they invade hepatocytes within 30–60 min of inoculation and develop as exoerythrocytic forms during a period of 5–10 d, depending on the species. As many as 30 000 merozoites are produced. During this hepatic stage, there is no clinical disease. When the liver schizont eventually ruptures, the merozoites enter the circulation, invade erythrocytes and undergo another asexual amplification of the parasite population, producing as many as 36 merozoites per mature schizont. This erythrocytic stage is responsible for the symptoms and pathology of malaria. Many red blood cells are destroyed in the process, contributing to the anaemia of malaria, and infected cells often sequester and adhere to vascular endothelium with occlusion of vessels. Some parasitized erythrocytes do not undergo asexual amplification but instead develop into the sexual forms (male and female gametocytes). These are taken up by mosquitoes during

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a blood meal and emerge from within the red blood cells as gametes, which then fertilize within the mosquito midgut. New sporozoites migrate to the salivary gland, and the life cycle recommences.

3. FEASIBILITY OF A MALARIA VACCINE

Cellular and molecular strategies allow the parasite to evade the host's immune response. Such strategies include, but are not restricted to, (i) the unique antigenicities of the different stages of the life cycle; (ii) genetic control of the immune responses; (iii) low immunological responsiveness; (iv) immunological tolerance; (v) antigenic variation; and (vi) allelic variation or antigenic diversity (reviewed by Doolan & Hoffman (1997)).

Regardless of these strategies, the feasibility of designing a malaria vaccine is supported by an abundance of experimental data. Sterile protective immunity against *Plasmodium berghei*, *P. knowlesi*, *P. falciparum* or *P. vivax* sporozoite challenge has been induced by immunization with radiation-attenuated sporozoites in every model system tested, including mice (Nussenzweig *et al*. 1969), monkeys (Gwadz *et al*. 1979) and humans (Clyde *et al*. 1973*a*,*b*, 1975; Rieckmann *et al*. 1974, 1979; Herrington *et al*. 1991; Edelman *et al*. 1993; Egan *et al*. 1993). With reference to the blood stage, in chickens and turkeys, humoral-mediated transmission blocking immunity has developed after immunization with killed asexual-stage parasites (Huff *et al*. 1958); in monkeys, persistent blood-stage infection has been cleared by immunization with *P. knowlesi* asexual-stage parasites in adjuvant (Mitchell *et al*. 1975); and in monkeys (Diggs *et al*. 1972) and humans (Cohen *et al*. 1961; McGregor & Carrington 1963; Bouharoun Tayoun *et al*. 1990; Sabchareon *et al*. 1991), passive transfer of purified immunoglobulin from individuals with lifelong malaria exposure to naive recipients has resulted in a marked decrease in *P. falciparum* blood-stage parasitaemia. Furthermore, because the prevalence, incidence, morbidity and mortality associated with malaria decrease markedly with age, there appears to be some degree of natural age-aquired immunity against malaria (reviewed by Baird (1995)).

4. PRE-ERYTHROCYTIC STAGE IMMUNITY

Vaccine strategies are generally targeted at only a single stage of the parasite's life cycle, although it is probable that a multistage vaccine may ultimately be required to combat a parasite as complex as *Plasmodium* (Doolan & Hoffman 1997). Both asexual erythrocytic-stage and sexual-stage vaccines would prevent or reduce morbidity and mortality, the former by eliminating or reducing the parasite load in the host's circulation, the latter by affecting the transmission of malaria in the community. Indeed, transmission-blocking vaccines would confer no immunity on the vaccine's recipient. In contrast, if completely effective, a vaccine designed to target the pre-erythrocytic stage (the sporozoite in circulation and/or the infected hepatocyte) would preclude both clinical disease and death and the transmission of malaria, by halting the parasite's development before the asexual erythrocytic stage where the clinical symptoms of malaria first manifest and the sexual stage where fertilization occurs. If only partly effective, such a vaccine may nevertheless reduce morbidity and mortality: the use of insecticide-impregnated bednets does not markedly reduce the incidence of parasitaemia in Gambian villages but has a dramatic effect on mortality (Alonso *et al*. 1991), and there is an association between intensity of malaria transmission and parasite density in children (Beadle *et al*. 1995). For these reasons, the present research has focused on the induction of protective immunity against the pre-erythrocytic stage.

For many years, the infected hepatocyte was considered an immune-privileged site, and the parasites developing within the hepatocytes were thought to be sequestered from immune attack. Pre-erythrocytic-stage immunity was therefore thought to be directed against the sporozoite in circulation. However, protection comparable to that achieved by immunizing with irradiated sporozoites was not obtained after immunization with asexual blood-stage antigens, heat-killed, formalin-inactivated or lysed sporozoites, or sporozoite antigens. Protective immunity was induced in mice immunized with non-irradiated sporozoites and treated with chloroquine to prevent erythrocyte infection (reviewed in Nussenzweig & Nussenzweig 1989). These data emphasized the requirement for live sporozoites targeting the liver and implicated the infected hepatocyte as the primary target of protective immunity induced by irradiated sporozoites. Furthermore, because the extracellular sporozoite stage lasts for 30 min or less in the mammalian host, immune responses targeted at the sporozoite in circulation must be active and complete within minutes of infection. In contrast, the liver stage of the malaria parasite's life cycle lasts for 24–48 h in the rodent malarias and for 5–14 d in the human malarias, depending on the species. It is now well established that CD8+ and CD4+ T cells can recognize parasite-derived peptides presented by class I or class II molecules, respectively, on the surface of infected hepatocytes (Hoffman *et al*. 1989, 1990*a*,*b*; Weiss *et al*. 1990, 1996; Renia *et al*. 1993). Indeed, because hepatocytes are the only host cells encountered by the parasite during the course of its life cycle that express major histocompatibility complex (MHC) molecules, the infected hepatocyte is the primary target of cell-mediated immune responses.

5. POTENTIAL MECHANISMS OPERATIVE DURING THE PRE-ERYTHROCYTIC STAGE OF THE *PLASMODIUM* LIFE CYCLE

Distinct immune mechanisms are required to combat the different stages of the parasite's life cycle; these are described in detail elsewhere (Doolan & Hoffman 1997). Those that may be active against the pre-erythrocytic stage are summarized in table 1. Moreover, the protective immunity induced by immunization with irradiated sporozoites is stage-specific but not strain-specific, and is efficacious in MHC-diverse humans and outbred as well as inbred

Table 1. *Potential mechanisms active against the pre-erythrocytic stage of* Plasmodium *spp. parasites*

(From Doolan & Hoffman (1997).)

life-cycle stage	immune response
sporozoite	antibodies that block sporozoite invasion of hepatocytes
	antibodies that kill the sporozoite, via opsonization
infected hepatocyte	CD8+ or CD4+ CTL that directly kill the infected hepatocyte
	CD4+ T cells that provide help for the activation and differentiation of CTL precursors
	CD8+ or CD4+ T cells that indirectly kill or inactivate the intracellular parasite, via cytokines or other factors
	cytokines released by activated CD8+ or CD4+ T cells or non-T cells that directly kill the infected hepatocyte or indirectly induce the infected hepatocyte to kill or inactivate the intrahepatic parasite
	antibodies that kill the infected hepatocyte or kill or inactivate the intrahepatic parasite, either directly, with complement, or via antibody-dependent cellular cytotoxicity

mouse strains differing in genetic background. These data indicate that multiple *Plasmodium* spp. proteins are the targets of protective immune responses. Both humoral and cellular (CD4+ and CD8+ T-cell) mechanisms have been implicated. Indeed, it has been established that a number of individual immune mechanisms can completely protect against sporozoite-induced malaria in the absence of other parasite-specific immune responses.

Monoclonal antibodies against the central repeat of the *P. berghei* circumsporozoite protein (CSP) (Potocnjak *et al*. 1980; Yoshida *et al*. 1980; Egan *et al*. 1987), *P. yoelii* CSP (Charoenvit *et al*. 1991*a*) and *P. vivax* CSP (Charoenvit *et al*. 1991*b*) passively protected mice and monkeys or neutralized *P. knowlesi* sporozoite infectivity for monkeys (Cochrane *et al*. 1982). Polyclonal antibodies against the central repeat of the *P. berghei* CSP (Egan *et al*. 1987) and *P. yoelii* CSP (Wang *et al*. 1995) passively protected mice. *In vitro*, a monoclonal antibody against the *P. falciparum* CSP repeats prevented invasion and development of *P. falciparum* in human hepatocytes (Hollingdale *et al*. 1984; Mazier *et al*. 1986) and a monoclonal antibody against a single epitope in the carboxyl terminus of the *P. yoelii* hepatocyte erythrocyte protein 17 (PyHEP17) eliminated infected hepatocytes from culture (Charoenvit *et al*. 1995).

The role of T cells was first demonstrated when irradiated sporozoite immunized μ-suppressed mice (which cannot make antibodies) were protected against challenge (Chen *et al*. 1977). Subsequently, *in vivo*, adoptive transfer of spleen cells from sporozoite-immunized mice (Verhave *et al*. 1978) or immune T cells (Egan *et al*. 1987) into naive mice protected against malaria in the absence of antibodies. *In vitro*, spleen cells from immune mice eliminated infected hepatocytes from *in vitro* culture in an MHC-restricted and species-specific manner (Hoffman *et al*. 1989, 1990*a*,*b*); this activity was not reversed by anti-interferon-γ antibody and not duplicated by culture supernatants.

The protective immunity induced by irradiated sporozoite immunization of A/J mice with *P. berghei* (Schofield *et al*. 1987*b*) and of BALB/c mice with *P. yoelii* (Weiss *et al*. 1988) was abrogated by *in vivo* depletion of CD8+ T cells but not CD4+ T cells, implicating CD8+ T cells as critical effector cells in protection.

CD8+ cytotoxic T lymphocytes (CTL) against a single epitope in the carboxyl terminus of the *P. berghei* CSP (Romero *et al*. 1989) or *P. yoelii* CSP (Rodrigues *et al*. 1991; Weiss *et al*. 1992) conferred protection in mice, even if transferred 3 h after sporozoite inoculation when the sporozoites had left the circulation and invaded hepatocytes (Rodrigues *et al*. 1991). The protective immunity transferred by one clone was abrogated by *in vivo* treatment with anti-interferon-γ (Weiss *et al*. 1992). A CD8+ CTL clone against the *P. yoelii* sporozoite surface protein 2 (PySSP2) also protected mice (Khusmith *et al*. 1994). CD4+ CTL from mice immunized with irradiated *P. berghei* sporozoites that recognized an undefined antigen common to sporozoites and blood stages also adoptively conferred protection (Tsuji *et al*. 1990). CD4+ T cells of the Th1 phenotype against a single epitope in the amino terminus of the *P. yoelii* CSP (Renia *et al*. 1993) conferred protection in the absence of any detectable cytotoxicity. Immunization of mice with a synthetic peptide representing the major repeat of PySSp2 conferred protection in a CD4+ T cell, interferon-γ dependent manner (Wang *et al*. 1996). Immunization of mice with a synthetic peptide representing a single CD4+ T cell epitope in the amino terminus of the *P. berghei* CSP (Migliorini *et al*. 1993) also protected; cytotoxicity was not assessed.

In vitro, CD8+ CTL against a single epitope in the carboxyl terminus of the *P. yoelii* CSP eliminated infected hepatocytes from culture in an antigen-specific, MHC-restricted manner (Weiss *et al*. 1990). CD4+ T cells directed against a single epitope in the amino terminus of the *P. yoelii* CSP eliminated infected hepatocytes from culture in an interferon-γ-independent manner (Renia *et al*. 1991, 1993). A heat-shock-like protein on the hepatocyte surface was a target of *in vitro* antibody-dependent cell-mediated cytotoxic mechanisms by liver non-parenchymal cells (Renia *et al*. 1990).

A role for cytokines has also been established. Systemic administration of interferon-γ partly protected against sporozoite challenge with *P. berghei* in mice (Ferreira *et al*. 1986) and *P. cynomolgi* in monkeys (Maheshwari *et al*. 1986). Administration of recombinant interleukin-12 (IL-12) completely protected against sporozoite challenge with *P. yoelii* in mice (Sedegah *et al*. 1994*a*) and *P. cynomolgi* in monkeys (Hoffman *et al*. 1997). Recombinant tumour necrosis factor-α (TNF-α) protected mice (Nüssler *et al*. 1991*b*).

The protective immunity induced by irradiated *P. berghei* sporozoites in A/J mice was abrogated by *in vivo* depletion of interferon-γ (Schofield *et al*. 1987*b*). In

contrast, the immunity in BALB/c mice immunized with irradiated *P. berghei* (Hoffman *et al*. 1989) or *P. yoelii* (Rodrigues *et al*. 1991) sporozoites was not affected by interferon-γ depletion. *In vitro,* treatment of hepatocytes infected with *Plasmodium* spp. with interferon-γ eliminated *P. berghei* (Ferreira *et al*. 1986; Mellouk *et al*. 1991) and *P. falciparum* (Mellouk *et al*. 1987) parasites from culture; this activity was dependent on nitric oxide (Mellouk *et al*. 1991, 1994). TNF-α inhibited development of *P. berghei* in a hepatoma cell line (Schofield *et al*. 1987*a*) but was not effective alone in primary cultures of hepatocytes infected with *P. berghei* (Mellouk *et al*. 1991) or *P. yoelii* (Nüssler *et al*. 1991*a*). However, in co-cultures of hepatocytes and non-parenchymal cells, TNF-α induced parasite inhibition via IL-6 secretion from the non-parenchymal cells (Nüssler *et al*. 1991*b*).The inhibitory effect of IL-1 on intrahepatic development of *P. yoelii* (Pied *et al*. 1992) and *P. falciparum* (Mellouk *et al*. 1987) has also been reported.

6. RATIONALE BEHIND ELUCIDATION OF IMMUNE MECHANISM

How vaccines will be designed to produce protective broad-ranging immune responses against malaria has not been obvious. Although more than 15 vaccines designed to induce protective antibody- or cell-mediated immune responses against the infected hepatocyte have been evaluated in humans, only small numbers of humans have been protected. Vaccines have been constructed to include B-cell epitopes, helper T-cell epitopes and/or CTL epitopes, generally in the form of synthetic peptides or purified recombinant proteins. Strategies employed to optimize the interaction between the antigenic epitope, antigen-presenting cells, and effector cells have been recently reviewed (Hoffman & Sacci 1995). Carrier proteins have included tetanus toxoid, meningococcal outer membrane protein, choleragenoid, *Pseudomonas aeruginosa* toxin A, the nonstructural protein of influenza A, the flanking regions or carboxyl terminus of the CSP, and surface antigen particles of hepatitis B. Adjuvants have included aluminium hydroxide, monophosphoryl lipid A, cell-wall skeleton of mycobacteria, QS-21, and oil–water emulsions. Delivery systems have included self-assembling lipid spheres such as liposomes, hepatitis B surface antigen particles, multiple-antigen peptide constructs, live vectors such as vaccinia virus, *Salmonella*, pseudorabies, *Mycobacterium bovis* BCG, and DNA plasmids.

Even in rodent models, the subunit vaccines found to be protective have not been optimal. They do not induce protection that can withstand massive sporozoite challenge, do not induce high and consistent protective immunity in a number of genetically diverse mouse strains or in outbred mice, and do not induce lifelong protection against malaria. An added complication has been that some vaccine delivery systems have induced the desired immune response, namely antigen-specific CD8+ CTL, but in many cases this response has not correlated with protection. In the *P. berghei* system, immunization of mice with attenuated *Salmonella typhimurium* expressing the *P. berghei* CSP induced *P. berghei* CSP-specific CTL and protected 50–75% of mice against challenge with *P. berghei* sporozoites (Sadoff *et al*. 1988) in a CD8+ T-cell-dependent and antibody-independent manner (Aggarwal *et al*. 1991). However, immunization with a recombinant vaccinia virus expressing the *P. berghei* CSP induced *P. berghei* CSP-specific CD8+ CTL but provided no protection (Satchidanandam *et al*. 1991). Likewise, in the *P. yoelii* system, mice immunized with recombinant vaccinia (Sedegah *et al*. 1988), *Salmonella typhimurium* (Sedegah *et al*. 1990) or pseudorabies (Flynn *et al*. 1990; Sedegah *et al*. 1992) expressing the *P. yoelii* CSP were not protected against *P. yoelii* sporozoite challenge, despite high levels of genetically restricted, *P. yoelii* CSP-specific CD8+ CTL (Flynn *et al*. 1990; Sedegah *et al*. 1992). CD8+ T-cell-dependent protection was, however, achieved after immunization with recombinant P815 mastocytoma cells expressing the *P. yoelii* CSP (67%), PySSP2 (50%), or a combination of PyCSP and PySSP2 (100%) (Khusmith *et al*. 1991), and with a recombinant influenza virus–vaccinia virus combination (Li *et al*. 1993; Rodrigues *et al*. 1994). The CTL generated in mice immunized with an attenuated pseudorabies virus construct containing the *P. yoelii* CSP could eliminate infected hepatocytes from *in vitro* culture, even though there was no protection *in vivo* (Sedegah *et al*. 1990). These data suggest that CD8+ T cells rather than CD8+ CTL *per se* may be the effector cells responsible for pre-erythrocytic stage immunity. This has been supported by recent data generated with *P. yoelii* CSP and HEP17 DNA vaccines demonstrating that protective immunity is dependent on CD8+ T cells (Sedegah *et al*. 1994*b*; Doolan *et al*. 1996), interferon-γ and nitric oxide (Doolan *et al*. 1996). A rational approach to the design of an effective malaria vaccine dictates that the underlying mechanisms responsible for protection be elucidated, and specific markers of protective immunity be identified.

7. PRELIMINARY DATA ON DIFFERENT PROTECTIVE MECHANISMS IN DIFFERENT STRAINS

We have investigated the mechanisms of protection against pre-erythrocytic-stage malaria induced by immunization with four distinct vaccines: irradiated sporozoites, DNA, multiple-antigen peptide (MAP), and linear peptide. By using genetically distinct inbred mice, outbred mice, gene-knockout mice, and *in vivo* depletion methods, it has been established that there is a diversity of protective immune responses (D. L. Doolan and S. L. Hoffman, unpublished data).

Protective immunization with irradiated sporozoites, the gold standard of pre-erythrocytic-stage protection, appears to be completely dependent on CD8+ T cells in all strains studied. In some strains, however, protection is not absolute and here CD4+ T cells are also crucial. In almost all cases, protection appears to be mediated via a cytokine cascade involving interferon-γ and nitric oxide. In most but not all cases, both IL-12 and natural killer (NK) cells play a role (D. L. Doolan and S. L. Hoffman, unpublished data). It is proposed that the CD8+ T cell activated by interacting with the MHC-peptide complex on the surface of the infected

hepatocyte secretes interferon-γ, which then induces the infected hepatocyte to produce nitric oxide that renders the parasite non-infectious. Furthermore, there may be a feedback loop whereby CD8+ T-cell-derived interferon-γ induces production of IL-12 by macrophages, dendritic cells and perhaps other cells, and the IL-12 then stimulates the production of additional interferon-γ from NK cells and T cells. However, in at least one mouse strain, depletion of cytokines *in vivo* has no effect on protection; this result indicates that in some cases protection may be mediated via classical CD8+ T-cell cytotoxicity (D. L. Doolan and S. L. Hoffman, unpublished data).

As with immunization with irradiated sporozoites, in a number of genetically distinct mouse strains the protection induced by immunization with plasmid DNA encoding either PyCSP or PyHEP17 is dependent on CD8+ (but not CD4+) T cells, interferon-γ, IL-12 and nitric oxide. In contrast, the mechanism induced by immunization with a MAP construct containing the PyCSP CTL epitope appears to be classical CD8+ T-cell cytotoxicity (D. L. Doolan and S. L. Hoffman, unpublished data). Interestingly, in one strain of mouse, immunization with a linear peptide of 18 amino acids from PySSP2 induces a high degree of protection, which is dependent on CD4+ (but not CD8+) T cells and interferon-γ (Wang *et al*. 1996) but not on IL-12 or nitric oxide.

The diversity of protective immune responses in the mouse model is exemplified in H-2^{d} mice, in which immunization with irradiated sporozoites or with plasmid DNA is completely dependent on CD8+ T cells, interferon-γ, IL-12 and nitric oxide, and partly dependent on NK cells. In contrast, protection in the same strain induced by immunization with a MAP construct is dependent on CD8+ T cells, but not on IL-12, and protection of H-$2^{d/k}$ mice induced by linear peptide immunization is dependent on CD4+ T cells and interferon-γ, but not on IL-12 or nitric oxide.

8. CONCLUSION

Malaria remains a major threat to public health, despite the considerable resources dedicated to vaccine development. Contributing to this is the fact that specific markers of protection have not been identified and the mechanisms of protective immunity required to be induced by vaccination have not been elucidated. The information presented here underscore the complexity of the murine host response to a parasitic infection and suggest that an outbred human population may behave similarly. Data nevertheless suggest that a vaccine that induces the requisite immune responses should be feasible. We believe that it is only by understanding the mechanisms of protective immunity that the goal of inducing consistent, long-lasting, sustainable protective immunity in humans may be achieved.

The experiments reported here were conducted according to the principles set forth in the *Guide for the care and use of laboratory animals*, Institute of Laboratory Animal Resources, National Research Council (National Academy Press). The opinions and assertions herein are those of the authors and are not to be construed as official or as reflecting the views of the US Navy or naval services at large. This work was supported by the Naval Medical Research and Development Command work units 62787A00101EVX, 61102A000101BFX, and 62787A00101EFX. This work was performed in part while D.L.D. held a National Research Council–Naval Medical Research Institute Research Associateship.

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