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

DENISE L. DOOLAN AND STEPHEN L. HOFFMAN*

Malaria Program, Naval Medical Research Institute, 12300 Washington Avenue, Rockville, MD 20852, USA
(hoffmans@nmripo.nmnc.navy.mil)

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 exo-erythrocytic 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

* Author for correspondence.

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-acquired 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.* 1991a) and *P. vivax* CSP (Charoenvit *et al.* 1991b) 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, 1990a,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.* 1987b) 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.* 1994a) and *P. cynomolgi* in monkeys (Hoffman *et al.* 1997). Recombinant tumour necrosis factor- α (TNF- α) protected mice (Nüssler *et al.* 1991b).

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.* 1987b). 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.* 1987a) but was not effective alone in primary cultures of hepatocytes infected with *P. berghei* (Mellouk *et al.* 1991) or *P. yoelii* (Nüssler *et al.* 1991a). 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.* 1991b). 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, cholera toxin, *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 HEPI7 DNA vaccines demonstrating that protective immunity is dependent on CD8⁺ T cells (Sedegah *et al.* 1994b; 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.

REFERENCES

- Aggarwal, A., Kumar, S., Jaffe, R., Hone, D., Gross, M. & Sadoff, J. 1991 Oral *Salmonella*: malaria circumsporozoite recombinants induce specific CD8⁺ cytotoxic T cells. *J. Exp. Med.* **172**, 1083–1090.
- Alonso, P. L., Lindsay, S. W., Armstrong, J. R. M., Conteh, M., Hill, A. G., David, P. H., Fegan, G., de Francisco, A., Hall, A. J., Shenton, F. C., Cham, K. & Greenwood, B. M. 1991 The effect of insecticide-treated bed nets on mortality of Gambian children. *Lancet* **337**, 1499–1502.
- Baird, J. K. 1995 Host age as a determinant of naturally acquired immunity to *Plasmodium falciparum*. *Parasitol. Today* **11**, 105–111.
- Beadle, C., McElroy, P. D., Oster, C. N., Beier, J. C., Oloo, A. J., Onyango, F. K., Chumo, D. K., Bales, J. D., Sherwood, J. A. & Hoffman, S. L. 1995 Impact of transmission intensity and age on *Plasmodium falciparum* density and associated fever: Implications for malaria vaccine design. *J. Infect. Dis.* **172**, 1047–1054.
- Bouharoun Tayoun, H., Attanath, P., Sabchareon, A., Chongsuphajaisiddhi, T. & Druilhe, P. 1990 Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion *in vitro*, but act in cooperation with monocytes. *J. Exp. Med.* **172**, 1633–1641.
- Bruce-Chwatt, L. J. (ed.) 1980 *Essential malariology*. London: Willaim Heinemann Medical Books. 354 pp.
- Charoenvit, Y., Mellouk, S., Cole, C., Bechara, R., Leef, M. F., Sedegah, M., Yuan, L. F., Robey, F. A., Beaudoin, R. L. & Hoffman, S. L. 1991a Monoclonal, but not polyclonal antibodies protect against *Plasmodium yoelii* sporozoites. *J. Immunol.* **146**, 1020–1025.
- Charoenvit, Y., Collins, W. E., Jones, T. R., Millet, P., Yuan, L., Campbell, G. H., Beaudoin, R. L., Brodersen, J. R. & Hoffman, S. L. 1991b Inability of malaria vaccine to induce antibodies to a protective epitope within its sequence. *Science* **251**, 668–671.
- Charoenvit, Y., Mellouk, S., Sedegah, M., Toyoshima, T., Leef, M. F., de la Vega, P., Beaudoin, R. L., Aikawa, M., Fallarme, V. & Hoffman, S. L. 1995 17 kDa *Plasmodium yoelii* hepatic and erythrocytic stage protein is the target of protective antibodies. *Exp. Parasitol.* **80**, 419–429.
- Chen, D. H., Tigelaar, R. E. & Weinbaum, F. I. 1977 Immunity to sporozoite-induced malaria infection in mice. I. The effect of immunization of T and B cell-deficient mice. *J. Immunol.* **118**, 1322–1327.
- Clyde, D. F., Most, H., McCarthy, V. C. & Vanderberg, J. P. 1973a Immunization of man against sporozoite-induced falciparum malaria. *Am. J. Med. Sci.* **266**, 169–177.
- Clyde, D. F., McCarthy V. C., Miller, R. M. & Hornick, R. B. 1973b Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am. J. Med. Sci.* **266**, 398–401.
- Clyde, D. F., McCarthy V. C., Miller, R. M. & Woodward, W. E. 1975 Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. *Am. J. Trop. Med. Hyg.* **24**, 397–401.
- Cochrane, A. H., Santoro, F., Nussenzweig, V., Gwadz, R. W. & Nussenzweig, R. S. 1982 Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesi*. *Proc. Natn. Acad. Sci. USA* **79**, 5651–5655.

- Cohen, S., McGregor, I. A. & Carrington, S. 1961 Gamma-globulin and acquired immunity to human malaria. *Nature* **192**, 733–737.
- Diggs, C. L., Welde, B. T., Anderson, J. S., Weber, R. M. & Rodriguez, E. 1972 The protective effect of African human immunoglobulin G in *Aotus trivirgatus* infected with Asian *Plasmodium falciparum*. *Proc. Helm. Soc. Wash.* **39**, 427–456.
- Doolan, D. L. & Hoffman, S. L. 1997 Multi-gene vaccination against malaria: a multi-stage, multi-immune response approach. *Parasitol. Today* **13**, 171–178.
- Doolan, D. L., Sedegah, M., Hedstrom, R. C., Hobart, P., Charoenvit, Y. & Hoffman, S. L. 1996 Circumventing genetic restriction of protection against malaria with multi-gene DNA immunization: CD8⁺ T cell, interferon- γ , and nitric oxide dependent immunity. *J. Exp. Med.* **183**, 1739–1746.
- Edelman, R., Hoffman, S. L., Davis, J. R., Beier, M., Sztein, M. B., Losonsky, G., Herrington, D. A., Eddy, H. A., Hollingdale, M. R., Gordon, D. M. & Clyde, D. F. 1993 Long-term persistence of sterile immunity in a volunteer immunized with X-irradiated *Plasmodium falciparum* sporozoites. *J. Infect. Dis.* **168**, 1066–1070.
- Egan, J. E., Hoffman, S. L., Haynes, J. D., Sadoff, J. C., Schneider, I., Grau, G. E., Hollingdale, M. R., Ballou, W. R. & Gordon, D. M. 1993 Humoral immune response in volunteers immunized with irradiated *Plasmodium falciparum* sporozoites. *Am. J. Trop. Med. Hyg.* **49**, 166–173.
- Egan, J. E., Weber, J. L., Ballou, W. R., Hollingdale, M. R., Majarian, W. R., Gordon, D. M., Maloy, W. L., Hoffman, S. L., Wirtz, R. A., Schneider, I., Woollett, G. R., Young, J. F. & Hockmeyer, W. T. 1987 Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. *Science* **236**, 453–456.
- Ferreira, A., Schofield, L., Enea, V., Schellekens, H., van der Meide, P., Collins, W. E., Nussenzweig, R. S. & Nussenzweig, V. 1986 Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. *Science* **232**, 881–884.
- Flynn, J. L., Weiss, W. R., Norris, K. A., Seifert, H. S., Kumar, S. & So, M. 1990 Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. *Molec. Microbiol.* **4**, 2111–2118.
- Gwadz, R. W., Cochrane, A. H., Nussenzweig, V. & Nussenzweig, R. S. 1979 Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterization of surface antigens of these parasites. *Bull. WHO* **57**, 165–173.
- Herrington, D. A., Nardin, E. H., Losonsky, G., Bathurst, I. C., Barr, P. J., Hollingdale, M. R., Edelman, R. & Levine, M. M. 1991 Safety and immunogenicity of a recombinant sporozoite malaria vaccine against *Plasmodium vivax*. *Am. J. Trop. Med. Hyg.* **45**, 695–701.
- Hoffman, S. L. & Sacci, J. B. Jr 1995 Rationale and approaches to constructing preerythrocytic malaria vaccines. In *Vaccine design: the subunit and adjuvant approach*. (ed. M. F. Powell & M. J. Newman), pp. 787–802. New York: Plenum Press.
- Hoffman, S. L., Franke, E. D., Hollingdale, N. R. & Druilhe, P. 1996 Attacking the infected hepatocyte. In *Malaria vaccine development* (ed. S. L. Hoffman), pp. 35–76. Washington, DC: ASM Press.
- Hoffman, S. L., Weiss, W., Mellouk, S. & Sedegah, M. 1990a Irradiated sporozoite vaccine induces cytotoxic T lymphocytes that recognise malaria antigens on the surface of infected hepatocytes. *Immunol. Lett.* **25**, 3–38.
- Hoffman, S. L., Isenbarger, D., Long, G. W., Sedegah, M., Szarfman, A., Mellouk, S. & Ballou, W. R. 1990b T lymphocytes from mice immunized with irradiated sporozoites eliminate malaria from hepatocytes. *Bull. WHO* **68**, 132–137.
- Hoffman, S. L., Isenbarger, D., Long, G. W., Sedegah, M., Szarfman, A., Waters, L., Hollingdale, M. R., van der Miede, P. H., Finbloom, D. S. & Ballou, W. R. 1989 Sporozoite vaccine induces genetically restricted T cell elimination of malaria from hepatocytes. *Science* **244**, 1078–1081.
- Hoffman, S. L., Crutcher, J. M., Puri, S. K., Ansari, A. A., Villinger, F., Franke, E. D., Singh, P. P., Finkelman, F., Gately, M. K., Dutta, G. P. & Sedegah, M. 1997 Sterile protection of monkeys against malaria after administration of interleukin-12. *Nature Med.* **3**, 80–83.
- Hollingdale, M. R., Nardin, E. H., Tharavani, S., Schwartz, A. L. & Nussenzweig, R. S. 1984 Inhibition of entry of *Plasmodium falciparum* and *P. vivax* sporozoites into cultured cells: an *in vitro* assay of protective antibodies. *J. Immunol.* **132**, 909–913.
- Huff, C. G., Marchbank, D. F. & Shiroishi, T. 1958 Changes in infectiousness of malarial gametocytes. II. Analysis of the possible causative factors. *Expl Parasitol.* **7**, 399–417.
- Khumsmith, S., Charoenvit, Y., Kumar, S., Sedegah, M., Beaudoin, R. L. & Hoffman, S. L. 1991 Protection against malaria by vaccination with sporozoite surface protein 2 plus CS protein. *Science* **252**, 715–718.
- Khumsmith, S., Sedegah, M. & Hoffman, S. L. 1994 Complete protection against *Plasmodium yoelii* by adoptive transfer of a CD8⁺ cytotoxic T cell clone recognizing sporozoite surface protein 2. *Infect. Immun.* **62**, 2979–2983.
- Li, S., Rodrigues, M., Rodriguez, D., Rodriguez, J. R., Esteban, M., Palese, P., Nussenzweig, R. S. & Zavala, F. 1993 Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8⁺ T-cell-mediated protective immunity against malaria. *Proc. Natn. Acad. Sci. USA* **90**, 5214–5218.
- Maheshwari, R. K., Czarniecki, C. W., Dutta, G. P., Puri, S. K., Dhawan, B. N. & Friedman, R. M. 1986 Recombinant human gamma-interferon inhibits simian malaria. *Infect. Immun.* **53**, 628–630.
- Mazier, D., Mellouk, S., Beaudoin, R. L., Texier, B., Druilhe, P., Hockmeyer, W., Trosper, J., Paul, C., Charoenvit, Y., Young, J., Miltgen, F., Chedid, L., Chigot, J. P., Galley, B., Brandicourt, O. & Gentilini, M. 1986 Effect of antibodies to recombinant and synthetic peptides on *P. falciparum* sporozoites *in vitro*. *Science* **231**, 156–159.
- McGregor, A. & Carrington, S. P. 1963 Treatment of East African *P. falciparum* malaria with West African human gamma-globulin. *Trans. R. Soc. Trop. Med. Hyg.* **57**, 170–175.
- Mellouk, S., Green, S. J., Nacy, C. A. & Hoffman, S. L. 1991 IFN-gamma inhibits development of *Plasmodium berghei* exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. *J. Immunol.* **146**, 3971–3976.
- Mellouk, S., Hoffman, S. L., Liu, Z. Z., De la Vega, P., Billiar, T. R. & Nüssler, A. K. 1994 Nitric oxide-mediated antiplasmodial activity in human and murine hepatocytes induced by gamma interferon and the parasite itself: enhancement by exogenous tetrahydrobiopterin. *Infect. Immun.* **62**, 4043–4046.
- Mellouk, S., Maheshwari, R. K., Rhodes-Feuillette, A., Beaudoin, R. L., Berbiguier, N., Matile, H., Miltgen, F., Landau, I., Pied, S., Chigot, J. P., Friedman, R. M. & Mazier, D. 1987 Inhibitory activity of interferons and interleukin 1 on the development of *Plasmodium falciparum* in human hepatocyte cultures. *J. Immunol.* **139**, 4192–4195.
- Migliorini, P., Betschart, B. & Corradin, G. 1993 Malaria vaccine: immunization of mice with a synthetic T cell helper epitope alone leads to protective immunity. *Eur. J. Immunol.* **23**, 582–585.
- Mitchell, G. H., Butcher, G. A. & Cohen, S. 1975 Merozoite vaccination against *Plasmodium knowlesi* malaria. *Immunology* **29**, 397–407.
- Nussenzweig, V. & Nussenzweig, R. S. 1989 Rationale for the development of an engineered sporozoite vaccine. *Adv. Immunol.* **45**, 283–334.
- Nussenzweig, R. S., Vanderberg, J., Most, H. & Orton, C. 1969 Specificity of protective immunity produced by X-irradiated *Plasmodium berghei* sporozoites. *Nature* **222**, 488–489.
- Nüssler, A., Drapier, J.-C., Renia, L., Pied, S., Miltgen, F., Gentilini, M. & Mazier, D. 1991a L-arginine-dependent destruction of intrahepatic malaria parasites in response to

- tumor necrosis factor and/or interleukin 6 stimulation. *Eur. J. Immunol.* **21**, 227–230.
- Nüssler, A., Pied, S., Goma, J., Renia, L., Miltgen, F., Grau, G. E. & Mazier, D. 1991b TNF inhibits malaria hepatic stages *in vitro* via synthesis of IL-6. *Int. J. Immunol.* **3**, 317–321.
- Pied, S., Civas, A., Berlot Picard, F., Renia, L., Miltgen, F., Gentilini, M., Doly, J. & Mazier, D. 1992 IL-6 induced by IL-1 inhibits malaria pre-erythrocytic stages but its secretion is down-regulated by the parasite. *J. Immunol.* **148**, 197–201.
- Potocnjak, P., Yoshida, N., Nussenzweig, R. S. & Nussenzweig, V. 1980 Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malaria infection. *J. Exp. Med.* **151**, 1504–1513.
- Renia, L., Grillot, D., Marussig, M., Corradin, G., Miltgen, F., Lambert, P. H., Mazier, D. & Del Giudice, G. 1993 Effector functions of circumsporozoite peptide-primed CD4⁺ T cell clones against *Plasmodium yoelii* liver stages. *J. Immunol.* **150**, 1471–1478.
- Renia, L., Marussig, M. S., Grillot, D., Pied, S., Corradin, G., Miltgen, F., Del Giudice, G. & Mazier, D. 1991 *In vitro* activity of CD4⁺ and CD8⁺ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc. Natn. Acad. Sci. USA* **88**, 7963–7967.
- Renia, L., Mattei, D., Goma, J., Pied, S., Dubois, P., Miltgen, F., Nüssler, A., Matile, H., Menegaux, F., Gentilini, M. & Mazier, D. 1990 A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells. *Eur. J. Immunol.* **20**, 1445–1449.
- Rieckmann, K. H., Beaudoin, R. L., Cassells, J. S. & Sell, D. W. 1979 Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull. WHO* **57**, 261–265.
- Rieckmann, K. H., Carson, P. E., Beaudoin, R. L., Cassells, J. S. & Sell, K. W. 1974 Sporozoite induced immunity in man against an Ethiopian strain of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **68**, 258–259.
- Rodrigues, M. M., Cordey, A.-S., Arreaza, G., Corradin, G., Romero, P., Maryanski, J. L., Nussenzweig, R. S. & Zavala, F. 1991 CD8⁺ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. *Int. Immunol.* **3**, 579–585.
- Rodrigues, M., Li, S., Murata, K., Rodriguez, D., Rodriguez, J. R., Bacik, I., Bennink, J. R., Yewdell, J. W., Garcia Sastre, A., Nussenzweig, R. S., Esteban, M., Palese, P. & Zavala, F. 1994 Influenza and vaccinia viruses expressing malaria CD8⁺ T and B cell epitopes. Comparison of their immunogenicity and capacity to induce protective immunity. *J. Immunol.* **153**, 4636–4648.
- Romero, P., Maryanski, J. L., Corradin, G., Nussenzweig, R. S., Nussenzweig, V. & Zavala, F. 1989 Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature* **341**, 323–325.
- Sabchareon, A., Burnouf, T., Ouattara, D., Attanah, P., Bouharoun-Tayoun, H., Chantavanich, P., Foucault, C., Chongsuphajaisiddhi, T. & Druihle, P. 1991 Parasitological and clinical human response to immunoglobulin administration in falciparum malaria. *Am. J. Trop. Med. Hyg.* **45**, 297–308.
- Sadoff, J. C., Ballou, W. R., Baron, L. S., Majarian, W. R., Brey, R. N., Hockmeyer, W. T., Young, J. F., Cryz, S. J., Ou, J., Lowell, G. H. & Chulay, J. D. 1988 Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science* **240**, 336–338.
- Satchidanandam, V., Zavala, F. & Moss, B. 1991 Studies using recombinant vaccinia virus expressing the circumsporozoite protein of *P. berghei*. *Molec. Biochem. Parasitol.* **48**, 89–100.
- Schofield, L., Ferreira, A., Nussenzweig, V. & Nussenzweig, R. S. 1987a Antimalarial activity of alpha tumor necrosis factor and gamma-interferon. *Fed. Proc. Fedn Am. Soc. Exp. Biol.* **46**, 760.
- Schofield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R. S. & Nussenzweig, V. 1987b Gamma-interferon, CD8⁺ T cells and antibodies required for immunity to malaria sporozoites. *Nature* **330**, 664–666.
- Sedegah, M., Finkelman, F. & Hoffman, S. L. 1994a Interleukin-12 induction of interferon-gamma-dependent protection against malaria. *Proc. Natn. Acad. Sci. USA* **91**, 10700–10702.
- Sedegah, M., Hedstrom, R., Hobart, P. & Hoffman, S. L. 1994b Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natn. Acad. Sci. USA* **91**, 9866–9870.
- Sedegah, M., Beaudoin, R. L., Majarian, W. R., Cochran, M. D., Chiang, C. H., Sadoff, J., Aggarwal, A., Charoenvit, Y. & Hoffman, S. L. 1990 Evaluation of vaccines designed to induce protective cellular immunity against the *Plasmodium yoelii* circumsporozoite protein: vaccinia, pseudorabies, and salmonella transformed with circumsporozoite gene. *Bull. WHO* **68**, 109–114.
- Sedegah, M., Chiang, C. H., Weiss, W. R., Mellouk, S., Cochran, M. D., Houghton, R. A., Beaudoin, R. L., Smith, D. & Hoffman, S. L. 1992 Recombinant pseudorabies virus carrying a plasmodium gene: herpes virus as a new live viral vector for inducing T- and B-cell immunity. *Vaccine* **10**, 578–584.
- Sedegah, M., Beaudoin, R. L., De la Vega, P., Leef, M. F., Ozcel, M. A., Jones, E., Charoenvit, Y., Yuan, L. F., Gross, M., Majarian, W. R., Robey, F. A., Weiss, W. & Hoffman, S. L. 1988 Use of a vaccinia construct expressing the circumsporozoite protein in the analysis of protective immunity to *Plasmodium yoelii*. In *Technological advances in vaccine development*. (ed. L. Lasky), pp. 295–309. New York: Liss.
- Stoute, J. A., Slaoui, M., Heppner, D. G., Monin, P., Kester, K., Desmons, P., Wellde, B. T., Garcon, N., Krzych, U., Marchand, M., Ballou, W. R. & Cohen, J. D. 1997 A preliminary evaluation of a recombinant circumsporozoite protein vaccine against *Plasmodium falciparum* malaria. *New Engl. J. Med.* **336**, 86–91.
- Tsuji, M., Romero, P., Nussenzweig, R. S. & Zavala, F. 1990 CD4⁺ cytolytic T cell clone confers protection against murine malaria. *J. Exp. Med.* **172**, 1353–1357.
- Verhave, J. P., Meuwissen, J. H. E. T. & Gokenser, J. 1978 Cell-mediated reactions and protection after immunization with sporozoites. *Israel J. Med. Sci.* **14**, 611–613.
- Wang, R., Charoenvit, Y., Corradin, G., De la Vega, P., Franke, E. D. & Hoffman, S. L. 1996 Protection against malaria by *Plasmodium yoelii* sporozoite surface protein 2 linear peptide induction of CD4⁺ T-cell and IFN- γ -dependent elimination of infected hepatocytes. *J. Immunol.* **157**, 4061–4067.
- Wang, R., Charoenvit, Y., Corradin, G., Porrozzzi, R., Hunter, R. L., Glenn, G., Alving, C. R., Church, P. & Hoffman, S. L. 1995 Induction of protective polyclonal antibodies by immunization with a *Plasmodium yoelii* circumsporozoite protein multiple antigen peptide vaccine. *J. Immunol.* **154**, 2784–2793.
- Weiss, W. R., Sedegah, M., Beaudoin, R. L., Miller, L. H. & Good, M. F. 1988 CD8⁺ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc. Natn. Acad. Sci. USA* **85**, 573–576.
- Weiss, W. R., Berzovsky, J. A., Houghton, R., Sedegah, M., Hollingdale, M. & Hoffman, S. L. 1992 A T cell clone directed at the circumsporozoite protein which protects mice against both *P. yoelii* and *P. berghei*. *J. Immunol.* **149**, 2103–2109.
- Weiss, W. R., Mellouk, S., Houghton, R. A., Sedegah, M., Kumar, S., Good, M. F., Berzovsky, J. A., Miller, L. H. & Hoffman, S. L. 1990 Cytotoxic T cells recognize a peptide from the circumsporozoite protein on malaria-infected hepatocytes. *J. Exp. Med.* **171**, 763–773.
- World Health Organization 1994 World malaria situation in 1992. *Wkly Epidemiol. Rec.* **69**, 309–314.
- Yoshida, N., Nussenzweig, R. S., Potocnjak, P., Nussenzweig, V. & Aikawa, M. 1980 Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science* **207**, 71–73.