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DNA Vaccine Against Malaria: A Long Way To Go

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ABSTRACT: Vaccination is the attempt to mimic certain aspects of an infection for the purpose of causing an immune response that will protect the individual from that infection. Malaria, a disease responsible for immense human suffering, is caused by infection with *Plasmodium* spp. parasites, which have a very complex life cycle — antigenically unique stages infect different tissues of the body. It is a parasitic disease for which no successful vaccine has been developed so far, despite considerable efforts to develop a subunit vaccine that offers protective immunity. Due to the spread of drug-resistant malaria, efforts to develop an effective vaccine have become increasingly critical. DNA vaccination provides a stable and long-lived source of protein vaccine capable of inducing both antibody- and cell-mediated immune responses to a wide variety of antigens. Injected DNA enters the cells of the host and makes the protein, which triggers the immune response. According to present needs, the flexibility of DNA vaccine technology permits the combination of multiple antigens from both the preerythrocytic and erythrocytic stages of malaria parasite. DNA vaccines with genes coding for different antigenic parts of malaria proteins have been created and presently some of these are undergoing field trials. The results from these trials will help to determine the likelihood of success of this technology in humans. This review presents an update of the studies carried out in malaria using DNA vaccine approach, the challenges, and the future prospects.

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ABBREVIATIONS: **Ab**, antibody; **AMA1**, apical membrane antigen1; **CSP**, circumsporozoite protein; **CTL**, cytotoxic T lymphocytes; **EBA-175**, 175-kDa erythrocyte binding antigen; **EIA**, enzyme immunoassay; **EXP-1**, exported protein-1; **GM-CSF**, granulocyte macrophage colony stimulating factor; **GST**, glutathione-*S*-transferase; **HEP17**, hepatocyte erythrocyte protein17; **Hsp60**, heat shock protein60; **IFAT**, indirect fluorescence antibody test; **IFN**, interferon; **IgG**, immunoglobulin; **LSA**, liver stage antigen; **MSP**, merozoite surface protein; **Pfg27**, *Plasmodium falciparum* gametocyte antigen27; **Pfs25**, *Plasmodium falciparum* surface antigen25; **RESA**, ring-infected erythrocyte surface antigen; **SERA**, serine repeat antigen; **SSP2**, sporozoite surface protein 2; **TRAP**, thrombospondin-related anonymous protein.

I. INTRODUCTION

Historically, malaria is one of the greatest causes of human misery and death. Despite continued efforts to control the disease, it remains a major health problem in many regions of the world. Malaria remains one of the world's worst health problems, with 1.5 to 2.7 million deaths annually; these deaths are primarily among children under 5 years of age and pregnant women in sub-Saharan Africa (Phillips, 2001). It is the world's major parasitic disease, for which effective control measures are urgently needed. Parasitic diseases present particular problems for vaccine development. One of the difficulties encountered in successful vaccine design against *Plasmodium* is an inadequate knowledge of antigens eliciting protective immunity, the precise type of immune responses for which to aim and the ways in which these can be induced. However, over the last decade there has been considerable progress in the understanding of immune mechanisms involved in conferring protection to malaria and in the identification of vaccine candidate antigens and their genes. Despite considerable efforts to develop subunit vaccines that offer protective immunity, no successful vaccine has been developed so far (Anders and Saul, 2000). Malaria vaccines are being developed to achieve both protection of the vaccinated individual and the reduction of malaria transmission through the community (Taylor-Robinson, 2000).

II. LIFE CYCLE OF MALARIA PARASITE

There are four stages in the life cycle of the malaria parasite. The cycle in human for the parasite *Plasmodium falciparum* begins when an infected anopheles mosquito bites and leaves the saliva containing thousands of threadlike sporozoites into the bloodstream. Within no time, the sporozoites home in on the liver and infect liver cells. The sporozoites begin multiplying vigorously in liver cells. Each sporozoite forms a schizont, which contains about 30,000 round, compact merozoites. In about a week's time these schizonts rupture, killing the liver cells in the process and releasing millions of merozoites into the bloodstream.

The merozoites quickly infect red blood cells, where they can hide safely from both antibodies and killer T cells. In these cells the merozoites have two choices, either they can take part in a repeated process of amplification and each merozoite forms another schizont with 20 new merozoites in it, resulting in 20-fold amplification of the parasite burden in the blood cells every 2 days, or the merozoites that do not form schizonts in red blood cells can develop into a sexual stage known as gametocytes and reinfect mosquitoes. These are taken up into the mosquito's gut where after developing they come to the salivary glands of mosquito and are ready to reinfect humans. These stages have evolved in part to avoid the two primary defenses of the human immune sys-

tem: antibodies, which seek out and destroy invaders in body fluids and cytotoxic T cells that attack infected cells. The sporozoites do not stay in the bloodstream long enough to be hunted down by antibodies, and the merozoites also do not stay in the liver long enough for cytotoxic T cells to mobilize and attack the infected cells. At the same time, the parasite multiplies vigorously at every step of its cycle, so that even if an immune response is 99% efficient at clearing out the parasite at any one stage, there are enough parasites left to multiply and cause the disease.

The characteristic fever and chills of malaria arise when the schizonts rupture the red blood cells and during the process release not only merozoites but a malaria toxin also. As the parasites amplify in the blood they can eventually infect two out of every three red blood cells. This is the reason of severe anemia in patients having malaria.

Plasmodium is a complex organism with a multistage life cycle spent partly in the guts and salivary glands of mosquitoes and partly in the liver and blood of human beings. At each stage of its life cycle it expresses different proteins, allowing it to play hide and seek with the human immune system. The malaria parasite has 14 chromosomes, about 7000 genes and a four-stage life cycle as it passes from humans to mosquitoes and back again (Taubes, 2000). It is very efficient at evading the human immune response. For the malaria parasite the stages that cause the disease are different from the stages that transmit the parasites from the mosquito vector to the human host and vice versa. Therefore, to achieve the effects at different stages the vaccines are being developed against the different stages of the parasite. Liver-stage vaccines will help to reduce the chances of a person becoming sick. The vaccine against the blood stage will reduce the severity of the disease and risk of death during infection. Finally, the vaccine against stages that infect mosquitoes will directly prevent the spread of malaria through the community.

III. MALARIA VACCINE

Sterile protective immunity against challenge with *Plasmodium* spp. sporozoites can be induced in multiple model systems and humans by immunization with radiation-attenuated *Plasmodium* spp. sporozoites. The infected hepatocyte has been established as the primary target of this protection, but the underlying mechanisms have not been completely determined. Taking the complex life cycle of malaria into account, few researchers believe that any vaccine made from a single malaria antigen will be sufficient to control malaria. Immunization with irradiated sporozoites protects animals and humans against malaria, and the circumsporozoite protein is a target of this protective immunity. Vaccines for malaria have been developed all over the world using 30 or more likely looking proteins, but so far none alone has evoked a strong enough immune response to form the basis of a malaria prevention program. At present, about 40 antigens are under study from the different stages of the parasites life cycle. The researchers hope to elicit both antibodies and cytotoxic T cells so as to attack the parasite wherever it makes itself visible. Some researchers are aiming at producing a vaccine that will stimulate cytotoxic T cells to destroy all the infected cells in the liver before the parasite has a chance to multiply. Another point of attack is when the parasite leaves the liver, but before it invades the red blood cells, where it becomes unreachable. This could be approached by targeting the surface proteins of the merozoite. If successful, this antibody attack against these proteins might prevent the merozoite from

penetrating the red blood cells. This intervention in turn should prevent the cyclic amplification that leads to the clinical disease.

The molecular events controlling sporozoite invasion and exo-erythrocytic (EE) development within hepatocytes are largely not understood, and EE parasites are probably better defined immunologically than biologically. The observation that the *P. falciparum* sporozoite antigen TRAP (thrombospondin-related anonymous protein) contains multiple adhesive domains that recognize endothelial and hepatocyte receptors indicates that, like leukocyte passage across capillaries, sporozoite invasion probably involves a coordinated interaction between sporozoite and hepatic molecules (Hollingdale et al., 1998). The parallel with leukocyte extravasation is strengthened by the finding that TRAP contains a functional, integrin-like I domain. EE parasites are an important target of immunity elicited by irradiated sporozoites, and much current effort is focused on developing malaria vaccines targeting EE parasites. Only one EE-specific antigen, liver-stage antigen 1 (LSA-1), is known to be expressed during EE development and may contribute to protective immunity elicited by irradiated *P. falciparum* sporozoites. In a study in Papua New Guinea, resistance to *P. falciparum* infection correlated with $CD8^+$ T-cell interferon-γ responses to an LSA-1 epitope that contains an HLA A11-restricted sequence. As A11 is >40% frequent in this population, it is reasonable to suggest that, as with B53 responses to LSA-1 in The Gambia, *P. falciparum* has driven genetic selection of certain HLA haplotypes, as proposed by Haldane nearly 50 years ago. Thus, LSA-1 is an important vaccine candidate and is being expressed in bacterial and phage vectors (Hollingdale et al., 1998).

Although several candidate vaccine antigens have been developed for malaria, there is as yet no effective single vaccine available. The ultimate malaria vaccine perhaps will be multivalent, requiring the identification of a suitable cocktail of antigens. The right mixture of antigens such as some liver-stage antigen, some blood-stage antigen, a transmission blocking component, something specific against *P. vivax,* and something against *P. falciparum* would be an ideal combination for an ideal malaria vaccine.

IV. DNA VACCINE

Vaccination has eradicated smallpox and greatly decreased mortality and morbidity associated with a variety of viral and bacterial infectious diseases. However, conventional methodologies have failed to provide vaccines against many widespread deadly human diseases, among them HIV, malaria, and cancer. Recombinant DNA vaccines have shown great promise in animal models in inducing protective immunity. In recent studies the DNA vaccines rather than the more conventional protein or peptide vaccines are becoming more popular. Vaccines made of DNA are being developed as a form of gene therapy that uses the patient's own cellular machinery to make foreign proteins that stimulate an immune response. DNA-based vaccines are being considered due to their ease of production, low cost, long shelf-life, lack of requirement for a cold chain, and ability to induce both humoral and cellular immune responses (Doolan et al., 1998; Tuteja, 1999; Apostolopoulos and Plebanski, 2000; Carucci, 2001). These are stretches of 'naked DNA' containing genes for viral proteins that are expressed when the DNA is taken up by muscle and other cells in the body. The transgene product is, however, rarely found in the circulation, and little is known about how

the antigen presentation occurs (Davis et al., 1993). DNA vaccines are relatively easy to make compared to a synthetic or recombinant protein. It is also easier to make cocktails of genes by using different antigenic parts of different malaria proteins. Numerous studies have already shown that the immunization of experimental animals with plasmid DNA encoding antigens from a wide spectrum of bacteria, viruses, protozoa, and cancer leads to protective humoral- and cell-mediated immunity (Tuteja, 1999; Tuteja et al., 2000).

Direct intramuscular injection of plasmid DNA has been used ubiquitously to induce immune responses. In few cases it has been reported that co-injection of toxic agents intended to cause muscle necrosis and repair either prior to or concurrently with injection of DNA can increase gene transfer and expression. These include local anesthetics such as bupivicaine and myotoxins such as cardiotoxin (Davis et al., 1993; Wang et al., 1993; Vitadello et al., 1994). The introduction of DNA can be accomplished by simple intramuscular or intradermal injections using needles, as well as by propelling DNA-coated gold particles to various tissues, preferentially the dermis (Fynan et al., 1993). Although the greatest experience is with injection into skeletal muscle, other tissues have also been shown to express gene products after DNA injection, including cardiac muscle, liver, and dermis (Raz et al., 1994).

The introduction of DNA vaccine technology has facilitated an unprecedented multiantigen approach to developing an effective vaccine against complex pathogens such as the *Plasmodium* spp. parasites. However, the basic science of DNA vaccines has yet to be clearly defined and ultimately will determine the success or failure of this technology to find a place in the immunological arsenal against disease. The main disadvantage of DNA vaccines is that nobody knows whether DNA vaccines can induce a sufficient enough immune response to protect a human against any disease.

A number of studies have been carried out in last few years using different genes and different model systems (Hoffman et al., 1997). DNA vaccines on their own are not optimal, and it has been demonstrated that heterologous prime/boost immunization strategies involving priming with DNA and boosting with poxvirus or recombinant protein in adjuvant works better (Doolan and Hoffman, 2001).

A. Studies Involving Circumsporozoite Gene

The circumsporozoite protein (CSP) from the surface of sporozoite stage *Plasmodium* spp., a malaria parasite, is among the most important of the malaria vaccine candidates. It is a protein that constitutes the bulk of the parasite's surface coating. A number of studies have been carried out using the CSP gene from different species of *Plasmodium*. These are described in the following sections.

It has been shown that intramuscular injection of BALB/c mice with *PyCSP* plasmid DNA induced higher levels of antibodies and cytotoxic T lymphocytes against the *P. yoelii* CSP than did immunization with irradiated sporozoites. This vaccine protected 9 of the first 16 mice immunized (Table 1) (Hoffman et al., 1994). It has been reported that intramuscular injection of mice with plasmid DNA encoding the *P. yoelii* CSP protein induced higher levels of antibodies and cytotoxic T lymphocytes against the *P. yoelii* CSP protein than did immunization with irradiated sporozoites (Sedegah et al., 1994). Mice immunized with this vaccine had an 86% reduction in liver-stage parasite burden after challenge with infec-

TABLE 1
Antigens Used as DNA Vaccine

DNA VACCINE CANDIDATE		SPECIES	MODEL	OBSERVATIONS	REMARK	REFERENCE
GENE	**ENCODED PROTEIN**					
Circumsporozoite gene (*CSP*)	CSP	*P. yoelii*	Mice	Antibody and CTL responses present	68% protection (CD8+T cells dependent)	Hoffman et al, 1994
	CSP	*P. yoelii*	Mice	Antibody and CTL responses present	28% protection with CSP and 58% along with GM-CSF	Weiss et al, 1998
	CSP	*P. yoelii*	Aotus monkeys	Antibody response present	Response was equivalent to MAP/adjuvant based vaccine	Gramzinski et al, 1997
	CSP	*P. yoelii*	Mice	Antibody and CTL responses present	44% protection with DNA and 69% after boosting with vaccina PyCSP	Sedegah et al, 1998

	CSP	*P. yoelii*	Mice	Antibody and CTL responses present	60% protection on priming with PyCSP and GM-CSF and boosting with poxvirus PyCSP	Sedegah et al, 2000
	CSP	*P. berghei*	Mice	Antibody and CTL responses present	Ab increased on increasing the interval between immunizations	Leitner et al, 1997
	CSP	*P. berghei*	Mice	Antibody responses present	71% and 33% protection by gene bombardment into liver & skin respectively	Yoshida et al, 2000
	CSP	*P. berghei*	Mice	Antibody responses present	Intradermal/ Gene-gun injection gives Th1/Th2 type response respectively	Weiss et al, 2000
	CSP	*P. berghei*	Mice	Antibody responses present	CSP+A and CSP-A induced different immune responses	Scheiblhofer et al, 2001

TABLE 1 (continued)

Liver stage Antigen-3 gene (LSA-3)	LSA-3	*P. falciparum*	Mice	Antibody and CTL responses present	Protection against challenge by *P. yoelii* sporozoites	Sauzet et al, 2001
Merozoite surface protein-1 gene (MSP-1)	MSP-1	*P. yoelii*	Mice	Antibody response present	C-terminus of MSP-1 fused with GST and used as antigen	Daly and Long 1995; Kang et al, 1998
	MSP-1	*P. yoelii*	Mice	Antibody response present	Protective Immunity against *P. yoelii* sporozoite challenge	Becker et al, 1998
	MSP-1	*P. chabaudi*	Mice	Antibody response present	Protection of mice with plasmid coding for C terminus of MSP-1 and Hep. B surface fusion protein	Wunderlich et al, 2000
	MSP-1	*P. vivax*	Mice	Antibody response present	Entire N/C-terminus fused to Hep. B surface antigen were antigenic	De Oliviera et al, 2001

Serine repeat antigen gene (SERA)	SERA	*P. falciparum*	Mice	Antibody response present	-Entire protein is more immun-ogenic than N-terminal domain only. -Co-immuni-zation with hep.B surface antigen plasmid increases the antibody titer	Belperron et al, 1999
	SERA	*P. falciparum*	Mice	Antibody response present	Co-inoculation of cytokine expression plasmid enhan-ces the humoral response	Sakai et al, 1999
Merozoite surface protein 4 gene (MSP-4)	MSP-4	*P. falciparum*	Mice	Antibody response present	Antibodies induced by gene inoculation were lower than by protein inoculation	Wang et al, 2000

tious doses of sporozoites. Eighteen (68%) of 28 mice that received two or three doses of vaccine were protected against challenge with sporozoites, and the protection was dependent on $CD8^+$ T cells (Sedegah et al., 1994). A later study examined the nature and localization of the antibody and cytokine secreting cells activated by immunization with this plasmid construct (Mor et al., 1995). The initial humoral response was localized to the draining lymph nodes and was characterized by the production of IgG1 anti-PyCSP antibodies and the Th2 cytokine IL-4. However, the secondary response was dominated by IFN-γ (a Th1 cytokine) production and the secretion of IgG2a anti-PyCSP antibodies in the spleen. *PyCSP* DNA and mRNA were detected only in the quadriceps muscles (sites of DNA injection), but these sites lacked either cytokine- or antibody-secreting cells. These findings indicate that circulating lymphocytes encounter plasmid-encoded antigen in the muscles, initiate a humoral response in the lymph nodes, and then seed distal lymphoid organs (Mor et al., 1995).

Despite efforts to develop vaccines that protect against malaria by inducing $CD8^+$T cells that kill infected hepatocytes, no subunit vaccine has been shown to circumvent the genetic restriction inherent in this approach, and little is known about the interaction of subunit vaccine-induced immune effectors and infected hepatocytes. It has been reported that immunization with plasmid DNA encoding the *P. yoelii* CSP protected one of five strains of mice against malaria (H–2d, 75%), a *PyHEP17* (hepatocyte erythrocyte protein) DNA vaccine protected three of the five strains (H-2a, 71%; H-2k, 54%; H-2d, 26%). The combination of these two DNAs protected 82% of H-2a, 90% of H-2k, and 88% of H-2d mice (Doolan et al., 1996). Protection was absolutely dependent on $CD8^+$T cells, INF-γ, or nitric oxide. These data introduce a new target of protective preerythrocytic immune responses, *PyHEP17* and its *P. falciparum* homologue, and provide a realistic perspective on the opportunities and challenges inherent in developing malaria vaccines that target the infected hepatocyte (Doolan et al., 1996).

The immunogenicity and efficacy of two DNA plasmids expressing different amounts of *P. berghei* CSP were evaluated by immunizing mice intramuscularly or epidermally and by varying the number of immunizations (one to three doses) and the interval between immunizations (Leitner et al., 1997). Expanding the interval between immunizations gave the strongest effect, increasing the efficacy and boosting antibody (Table 1). The strongest homoral immune response and the greatest level of protection were induced by vaccinating epidermally with high expresser plasmid using a gene gun to administer three doses at 6-week intervals (Leitner et al., 1997). *P. yoelii* CSP DNA vaccine has been used for a study in Aotus monkeys. It was shown that an intradermal route of immunization with this plasmid DNA generates antibody responses equivalent to a multiple antigen peptide/adjuvant based vaccine (Table 1) (Gramzinski et al., 1997).

Using *P. yoelii* as a model for malaria vaccine development, it has been shown that a DNA plasmid encoding the *P. yoelii* CSP can protect mice against sporozoite infection (Weiss et al., 1998). Co-administration of a new plasmid PyCSP1012 with a plasmid encoding murine granulocyte-macrophage colony stimulating factor (GM-CSF) was shown to increase protection against malaria (Table 1). While PyCSP1012 alone protected 28% of mice, the protection increased to 58% when a GM-CSF encoding plasmid was added. GM-CSF plasmid increased antibodies to PyCSP of IgG1, IgG2a, and IgG2b isotypes and also IFN-gamma responses of $CD8^+$ T cells to the PyCSP 280 to 288 amino acid epitope in-

creased. The increases in antigen specific IL-2 production and $CD4^+$ T cell proliferation were also dramatic. It has been proposed that GM-CSF may act on dendritic cells to enhance the presentation of the PyCSP antigen, with enhanced IL-2 production and $CD4^+$ T cell activation driving the increases in antibodies and $CD8^+$ T cell function (Weiss et al., 1998).

The prime-boost immunization strategy with a combination of various recombinant vaccinia virus strain and plasmid DNA has been investigated (Schneider et al., 1998). Using plasmid DNA encoding *PbCSP* and *PbTRAP* as priming agents and boosting with recombinant modified virus Ankara (MVA) complete protection against sporozoite challenge was observed in both BALB/c and C57BL/6 mice. The specific order of immunization, DNA priming followed by MVA boosting was essential for protection (Schneider et al., 1998). The sequential immunization with *P. yoelii* CSP DNA vaccine, recombinant vaccinia, and synthetic peptide *P. yoelii* CSP vaccine was also tested in another study (Sedegah et al., 1998). Mice were immunized with *PyCSP* DNA, PyCSP multiple antigenic peptide, or recombinant vaccinia expressing PyCSP were boosted 9 weeks later with the same immunogen or one of the others and were challenged. Only mice immunized with DNA and boosted with vaccinia PyCSP (D-V) (69%) or DNA (D-D) (44%) had greater protection than controls. D-V mice had significantly higher individual levels of antibodies and class I-restricted cytotoxic T lymphocyte activity than did D-D mice; interferon γ production was also higher in D-V than in D-D mice. The results indicated that priming with *PyCSP* DNA and boosting with recombinant vaccinia expressing PyCSP were associated with greater immunogenicity and protective immunity than priming and boosting with *PyCSP* DNA alone (Table 1) (Sedegah et al., 1998).

To evaluate the safety of a plasmid DNA vaccine, tissue distribution studies in mice and safety studies in mice and rabbits were conducted with a plasmid DNA encoding *P. falciparum* CSP (Parker et al., 1999). After intramuscular injection plasmid DNA was detected initially in all of the vascularized tissues, but at a later time point it was found primarily in the muscle at the site of injection. After intravenous administration plasmid DNA is initially distributed at a relatively low frequency to all the tissues except gonads and brain. Afterward the DNA is rapidly cleared and by 4 weeks postadministration it could be detected only in the lung of some of the animals. In a safety study in mice and rabbits repeated intramuscular injections of the plasmid DNA at different doses had no adverse effects on clinical chemistry or hematology and did not result in any organ pathology or systemic toxicity. No evidence of autoimmune-mediated pathology, antinuclear antibodies or antibodies to double-stranded DNA were observed in the animals tested (Parker et al., 1999).

It is well known now that intramuscular immunization with a naked DNA plasmid expressing the *P. yoelii* CSP (pPyCSP) protects mice against challenge with *P. yoelii* sporozoites. It has also been shown that this protection can be improved either by coadministration of a plasmid-expressing murine GM-CSF (pGMCSF) or by boosting with recombinant poxvirus expressing the PyCSP. It has been reported in a recent study that combining these two strategies, by first mixing the priming dose of pPyCSP with pGMCSF and then boosting with recombinant virus, can substantially increase vaccine effectiveness (Table 1) (Sedegah et al., 2000). This strategy not only improved the immune responses and protection, but the pPyCSP dose could be lowered from 100 μg to 1 μg with little loss of immunogenicity after boost with recombinant pox-

virus. Comparing mice primed by the 1-μg doses of pPyCSP plus 1 μg pGMCSF with mice primed by 1-μg doses of pPyCSP alone, the former were better protected (60% vs. 0) and had higher concentrations of antibodies. These mice also had more *ex vivo* CTL activity (25% vs. 7% specific lysis), and more IFN-γ-secreting cells by enzyme-linked immunospot assay (1460 vs. 280 IFN-γ-spot-forming cells/10^6 cells). Priming with plasmid vaccine plus pGMCSF and boosting with recombinant poxviruses strongly improves the immunogenicity and protective efficacy of DNA vaccination and allows for significant reduction of dose (Sedegah et al., 2000).

In a recent study a *PbCSP* gene plus the mouse IL-12 gene was bombarded directly by a gene gun into the mouse liver once or into the skin twice (Yoshida et al., 2000). A marked protective effect was induced by gene bombardment into the liver (more than 71%) compared with that into the skin (less than 33%). A Th-1 type immune response was observed in the hepatic lymphocytes from mice bombarded into the liver, resulting in more effective protection compared with those bombarded into the skin (Table 1) (Yoshida et al., 2000).

Gene gun injection of genetic vaccines encoding *P. berghei* CSP induces a significant protective effect against sporozoite challenge; however, intramuscular injection does not (Table 1) (Weiss et al., 2000). In a recent study the immune responses and protective effects induced by *P. berghei* CSP genetic vaccines delivered intradermally with a needle or epidermally with a gene gun were compared. Mice were immunized three times at 4-week intervals and challenged by a single infectious mosquito bite. Although 50 times more DNA was administered by needle than by a gene gun, the latter method induced significantly greater protection against infection. Intradermal injection of the CSP genetic vaccine induced a strong Th1-type immune response characterized by a dominant CSP-specific IgG2a humoral response and high levels of interferon γ produced by splenic T cells. Gene gun injection induced a predominantly Th2-type immune response characterized by a high IgG1/IgG2a ratio and significant IgE production. Neither method generated measurable cytotoxic T lymphocyte activity. The results indicate that a gene gun-mediated CS-specific Th2-type response may be best for protecting against malarial sporozoite infection when the route of parasite entry is via mosquito bite (Weiss et al., 2000).

The C terminus of the CSP is anchored to the parasite cell membrane by a glycosylphosphatidylinositol (GPI) glycolipid. This GPI signal sequence functions poorly in heterologous eukaryotic cells, causing CSP retention within internal cell organelles during genetic immunization. The immunogenicity and protective efficacy of DNA vaccines encoding the *P. berghi* CSP has been studied recently. It has been shown that cellular location of antigen has quantitative and qualitative effects on immune responses induced by genetic immunization (Table 1) (Scheiblhofer et al., 2001). Removal of the GPI signal sequence had a profound effect on induction and efficacy of CSP-specific immune response after genetic immunization of BALB/c mice with a gene gun. The CSP produced from the plasmid lacking the GPI anchor signal sequence (CSP-A) was secreted and soluble, but that produced by the plasmid containing the GPI anchor signal sequence (CSP+A) was not. The CSP-A plasmid induced a highly polarized Th2-type response, in which the CSP-specific IgG antibody titer was three- to fourfold higher, and the protective effect was significantly greater than that induced by the CSP+A plasmid. Thus, these two physical forms of CSP induced quantitatively and qualitatively different immune responses that also differed in protective efficacy (Scheiblhofer et al., 2001).

In a recent study the needle-free jet device Biojector with syringe/needle as a method to administer a DNA vaccine encoding PfCSP in albino rabbits was compared (Aguiar et al., 2001). A group of three rabbits was injected by the intramuscular (IM) route using a syringe/needle combination, a second group IM with the Biojector device and a third group both IM and intradermal (ID) using the Biojector. When animals were immunized with the Biojector IM or IM/ID when compared with the syringe/needle IM, 10- and 50-fold greater antibody titers were observed as measured by enzyme immunoassay (EIA) and indirect fluorescence antibody test (IFAT), respectively. It was also observed that the Biojector conferred a greater ability to prime the immune system when compared with the needle. The subsequent boosting of all animals with a recombinant canary pox virus (ALVAC) expressing PfCSP induced significantly higher titers in both Biojector groups of rabbits when compared with the needle and naive animals. These results provided the foundation for a clinical trial using the same regime (Aguiar et al., 2001).

B. Studies Involving Liver Stage Antigens

Recently, a liver-stage antigen 3 (LSA-3) has been identified; this is expressed both in the mosquito- and liver-stage parasites. This *P. falciparum* protein is highly conserved and shows promising antigenic and immunogenic properties. In chimpanzees, the primates most closely related to humans and that share a similar susceptibility to *P. falciparum* liver-stage infection, immunization with LSA-3 protein-induced protection against successive heterologous challenges with large numbers of *P. falciparum* sporozoites (Daubersies et al., 2000). Therefore, LSA-3 of *P. falciparum* is a suitable candidate for effective vaccination against preerythrocytic stages.

In continuation of this study, DNA-based immunization of mice by *P. falciparum* LSA-3 (PfLSA3) was evaluated (Table 1) (Sauzet et al., 2001). The animals developed a dominant Th1 immune response (high γ interferon T-cell responses and predominance of immunoglobulin G2a) to each of three recombinant proteins spanning the molecule. The immunological cross-reactivity of PfLSA3 with its putative homologue on sporozoites of the rodent parasite *P. yoelii* has been shown, and it has also been shown for the first time that responses induced by PfLSA3 in mice significantly protect against a heterologous challenge by *P. yoelii* sporozoites. These results support a significant effect of DNA-induced immune responses on preerythrocytic stages of the parasite (Sauzet et al., 2001).

C. Studies Involving Blood-Stage Antigen Genes

Merozoite surface protein 1 (MSP-1) has been the focus of intense efforts to develop malaria blood-stage vaccines. A large body of evidence from *in vitro* studies with *P. falciparum* and from challenge studies in murine and primate models indicate that MSP-1 can be a target of protective immune responses, suggesting that a vaccine against MSP-1 could protect humans against malaria.

It has been shown that when the C terminus of *P. yoelii* MSP-1 was expressed with glutathione-*S*-transferase (GST) as a fusion protein (GST-PyC2), it elicited antibody-mediated protective immune responses in BALB/c mice (Table 1) (Daly and Long, 1995). In continuation of this study, the humoral immune responses to a DNA vac-

cine (V3) encoding GST-PyC2 have been examined (Kang et al., 1998). When BALB/c mice were immunized with V3 plasmid, anti-PyC2 antibodies were successfully induced. In contrast to protein immunization, there was no significant increase in the avidity of antibodies during the course of DNA immunization. These data suggest that there may be little or no affinity maturation of specific antibody during DNA immunization in this system (Table 1) (Kang et al., 1998). Immunization of mice with DNA vaccines encoding the full-length form and C and N termini of *P. yoelii* MSP-1 provided partial protection against sporozoite challenge and resulted in boosting of antibody titers after challenge (Table 1) (Becker et al., 1998). Mice immunized with full-length PyMSP-1 DNA vaccine exhibited statistically significant reductions in parasitemia on days 15 through 21. It has been suggested that this protection may have been antibody mediated (Becker et al., 1998).

It has been shown that the genetic immunization of rodents with a plasmid coding for a *P. chabaudi* MSP1 (C terminus)-hepatitis B virus surface fusion protein (pPcMSP1(19)-HBs) provided protection of mice against subsequent lethal challenge with *P. chabaudi chabaudi* PC1-infected red blood cells (Table 1) (Wunderlich et al., 2000). The percentage of survivor mice was higher in DNA-immunized mice than in animals immunized with a recombinant rPcMSP1(19)- glutathione-*S*-transferase fusion protein administered in freund adjuvant. In all mice immunized with the pPcMSP1(19)-HBs, a Th1-specific response, including the production of anti-MSP1(19)-specific immunoglobulins predominantly of the IgG2a subtype and reacting almost exclusively against discontinuous epitopes, was elicited. The co-injection of Th1-type cytokine-expressing plasmids (γ interferon, interleukin-2, and granulocyte-macrophage colony-stimulating factor) mostly abolished protection and boosting of MSP1(19)-specific antibodies. The inclusion of a lymph node-targeting signal did not significantly increase protection. Further, these results support that MSP1(19)-HBs DNA constructs might be useful as components of a genetic vaccine against the asexual blood stages of *Plasmodium* (Wunderlich et al., 2000).

In a recent study the antigenic properties of the of *P. vivax* MSP-1 (PvMSP-1), were studied (Table 1) (de Oliveira et al., 2001). Thus, seven recombinant bacterial plasmids coding different regions of the PvMSP-1 protein were constructed and used to immunize BALB/c mice. The results demonstrated that a plasmid encoding the entire N-terminus comprising 682 amino acids and a plasmid encoding the C-terminus, including the two juxtaposed epidermal growth factor (EGF)-like domains fused to the Hepatitis B surface antigen, were antigenic. Moreover, the elicited immune responses were similar to those reported for these same PvMSP-1 regions in natural human infections (de Oliveira et al., 2001).

The liver- and blood-stage-expressed serine repeat antigen (SERA) of *P. falciparum* is a candidate protein for the malaria vaccine. The immune responses induced in mice immunized with SERA-expressing plasmid DNA vaccines delivered by intramuscular injection or delivered intradermally by gene gun immunization were compared (Table 1) (Belperron et al., 1999). Mice were immunized with a plasmid encoding the entire 47-kDa domain of SERA, or the N-terminal domain (amino acids 17 to 110) of SERA. The antibody responses were minimal following DNA vaccination with N-terminal domain of SERA suggesting that N-terminal domain alone is not highly immunogenic by this route of vaccine delivery. Immunization of mice by gene gun delivery of the 47-kDa domain of SERA elicited a significantly higher serum antibody titer to the antigen than immunization

of mice by intramuscular injection of the same plasmid. The predominant isotype subclass of the antibodies elicited to the SERA protein following intramuscular and gene gun immunization with SERA plasmid DNA was immunoglobulin G1. Co-immunization of mice with SERA plasmid DNA and a plasmid expressing the hepatitis B surface antigen by the intramuscular route resulted in higher anti-SERA titres than those generated in mice immunized with the SERA DNA plasmid alone (Belperron et al., 1999).

In another study mice were immunized with plasmid expressing the 47-kDa amino-terminal domain of the *P. falciparum* SERA using a gene gun and humoral immune response to SERA antigen were investigated (Table 1) (Sakai et al., 1999). Significant SERA-specific antibodies were observed in BALB/c mice after immunization three times with SERA expression plasmid. Furthermore, these levels were increased by the co-inoculation of cytokine (IFN-γ, IL-4, GM-CSF, or IL-12) expression plasmid. With respect to the SERA-specific Ig subclasses, co-inoculation of IFN-γ, GM-CSF, or IL-12 expression plasmid increased the levels of SERA-specific IgG2a, and these were much higher than that in mice immunized with SERA expression plasmid alone. In contrast to the SERA-specific IgG2a, co-inoculation of any cytokine expression plasmid did not change the levels of SERA-specific IgG1. These results indicate that cytokine expression plasmid enhances and regulates humoral immune response elicited by SERA DNA immunization (Sakai et al., 1999).

The humoral immune responses elicited by priming with a DNA plasmid and boosting with either the plasmid or the corresponding recombinant protein in alum adjuvant were compared (Haddad et al., 1999). The plasmid DNA encoded a sequence (M3) derived from the *P. falciparum* blood-stage antigen Pfl55/RESA (ring-infected erythrocyte surface antigen), and the recombinant protein consisted of the identical malarial sequence fused to an albumin-binding region of streptococcal protein G. Mice of different genetic backgrounds (CBA, BALB/c, and C57BL/6) were primed with plasmid DNA and boosted with either plasmid or recombinant protein. In all strains of mice, boosting with protein elicited higher anti-M3 antibody levels than obtained by boosting with plasmid, yet the kinetics and longevity of the secondary responses were comparable. Antiserum obtained after protein boosting displayed an immunoglobulin G (IgG) subclass profile skewed to the IgG1 isotype, regardless of the mouse strain (Haddad et al., 1999). In contrast, mice receiving a second injection with plasmid responded with a more mixed IgG subclass profile. Inclusion of a *P. falciparum* CSP protein-derived T-helper epitope (CS.T3) in the immunization plasmid as well as in the fusion protein did not significantly change the humoral responses to M3. These results show the potential of DNA vaccination for the purpose of priming an antibody response against the Pfl55/RESA. This DNA priming, when combined with a protein boost, results in high titred and long-lasting anamnestic responses (Haddad et al., 1999).

P. falciparum merozoite surface protein 4 (MSP4) is being developed as a component of a subunit vaccine against asexual stages of malaria (Wang et al., 2000). Three DNA constructs were produced that induced expression of MSP4 either in the cytoplasm of transfected cells or secreted from cells under the control of the human tissue plasminogen activator (TPA) signal or the native *P. falciparum* MSP4 signal. Only the construct containing the TPA signal induced detectable antibodies in mice, although gene expression was demonstrated in all constructs, and MSP4 was shown to be secreted using either signal by *in vitro* transient transfection of COS cells. Two recombinant MSP4 proteins that encoded the same se-

quence as the plasmid DNA were produced in *E. coli* (EcMSP4-His) and *Saccharomyces cerevisiae* (yMSP4-His) and used to raise antibodies in mice. Comparison of the antibodies elicited by these various antigen formulations showed differences in titer, isotype, and epitope recognition. The titer of antibodies induced by DNA vaccination was lower than that induced by yMSP4-His, which in turn was lower than that induced by EcMSP4-His (Table 1). The isotype profiles of the antibodies were also different. The plasmid DNA induced predominantly IgG2a responses, whereas the two proteins induced predominantly IgG1 responses. The antibodies induced by DNA and yMSP4-His recognized predominantly the C-terminal epidermal growth factor (EGF)-like domain of the protein, whereas EcMSP4-His induced antibodies recognizing all domains of the protein equally. The antibodies induced by DNA vaccination were directed almost extensively to conformational epitopes so that reactivity with native MSP4 was abolished after disulfide bonds in the protein were disrupted. The antibodies induced by recombinant proteins recognized linear epitopes as well, and reactivity to native MSP4 was preserved after reduction and alkylation of parasite proteins (Wang et al., 2000).

D. Studies Involving a Variety of Antigens

CD8(+) T cells have been implicated as critical effector cells in protective immunity against malaria parasites developing within hepatocytes. A vaccine that protects against malaria by inducing CD8(+) T cells will probably have to include multiple epitopes on the same protein or different proteins, because of parasite polymorphism and genetic restriction of T-cell responses. In developing a multiantigen, multiplasmid malaria vaccine for humans, it was important to know if plasmids encoding *P. falciparum* malaria genes were immunogenic in nonhuman primates and if mixing plasmids affected the response to individual component antigens. To determine if CD8(+) T-cell responses against multiple *P. falciparum* proteins can be induced in primates by immunization with plasmid DNA, rhesus monkeys were immunized intramuscularly with a mixture of DNA plasmids encoding four *P. falciparum* proteins or with individual plasmids. All six monkeys immunized with *PfCSP* DNA, seven of nine immunized with *PfSSP2* DNA, and five of six immunized with *PfExp-1* or *PfLSA-1* DNA had detectable antigen-specific cytotoxic T lymphocytes (CTL) after *in vitro* restimulation of peripheral blood mononuclear cells. CTL activity was genetically restricted and dependent on CD8(+) T cells. By providing the first evidence for primates that immunization with a mixture of DNA plasmids induces CD8(+) T-cell responses against all the components of the mixture, these studies provide the foundation for multigene immunization of humans (Wang et al., 1998a).

The potential of building multicytotoxic T lymphocyte (CTL) epitope antigens in combination with the nucleic acid immunization technology is explored for development of malaria vaccines (Hanke et al., 1998). A novel minimal vector pTH for direct gene transfer was constructed for efficient expression of vaccine antigens and used as a vehicle for *P. falciparum*-derived poly-epitope genes. Two murine epitopes were included into these constructs to allow for testing of vaccine immunogenicity in small animals. The results showed that a single DNA injection generated CTL responses in all 15 vaccinated mice. The elicited CTL precursor frequencies were estimated in an interferon-γ-based ELISPOT

assay and found to be an average of 300 (range 4 to 1346) peptide-responding cells per 10^6 splenocytes (Hanke et al., 1998).

The immunogenicity of *P. falciparum* preerythrocytic stage DNA vaccines, *PfCSP*, sporozoite surface protein 2 (PfSSP2), carboxyl terminal of liver stage antigen 1 (PfLSA-1 c-term), and exported protein 1 (PfExp-1) has been tested in mice (Table 2) (Hedstrom et al., 1998). It was observed that mice injected with these plasmids produced antigen-specific antibody and cytotoxic T lymphocyte responses (Hedstrom et al., 1998).

The *PvCSP, PvSSP2,* Pv apical membrane antigen 1 (*PvAMA1*), and *PvMSP1* genes were used to study the immune response in mice (Table 2) (Rogers et al., 1999). These constructs induced high levels of specific antibody in mice regardless of whether the antigen was expressed in native form or fused to a human tissue plasminogen activator leader peptide. High titer antibodies induced against PvCSP did not react with the protective AGDR epitope within the sequence of this antigen (Rogers et al., 1999).

In a study, two antigens present on the sexual stages of *P. falciparum,* Pfs25 (Pf surface antigen25) and Pfg27 (Pf gametocyte antigen27), have been used to induce biologically important antibodies that can block the development of the parasite in the mosquito and thus transmission of the disease. It has been shown that DNA encoding *Pfs25* when administered intramuscularly, either alone or with DNA encoding *Pfg27,* had the most potent transmission-blocking effects, resulting in up to a 97% decrease in oocyst numbers in mosquito midguts and a 75% decrease in rate of infection (Lobo et al., 1999). Immunization with DNA encoding a Pfg27-Pfs25 fusion protein was less effective and DNA encoding *Pfg27* elicited antibodies in sera that had only modest effects on the infectivity of the parasite. These results show that DNA vaccination can result in potent transmission-blocking antibodies in mice (Table 2) (Lobo et al., 1999).

In a recent study, expression library immunization was used as a tool for the discovery of sequences protective against malaria infection (Smooker et al., 2000). A genomic *P. chabaudi* expression library was constructed comprising 10 separate pools of 3000 plasmids. Using biolistic delivery of pools composed of 616 to 30,000 plasmids up to 63% protection of mice from a challenge with *P. chabaudi* has been reported. Overall expression library immunization protected 36% of vaccinated mice against virulent challenge compared with only 3.2% survival of control mice (Smooker et al., 2000).

The gene encoding the 60-kDa heat shock protein of *P. yoelii, PyHsp60,* was cloned into the VR1012 and VR1020 mammalian expression vectors (Sanchez et al., 2001). Mice were immunized intramuscularly at 0, 3, and 9 weeks with 100 μg of *PyHsp60* DNA vaccine alone or in combination with 30 μg of pmurGMCSF (plasmid-encoding murine granulocyte macrophage colony stimulating factor). The sera from immunized mice but not from vector control groups recognized *P. yoelii* sporozoites, liver stages, and infected erythrocytes in an indirect fluorescent antibody test. Two weeks after the last immunization, mice were challenged with 50 *P. yoelii* sporozoites. In one experiment the vaccine pPyHsp60-VR1012 used in combination with pmurGMCSF gave 40% protection. In a second experiment this vaccine did not protect any of the immunized mice but induced a delay in the onset of parasitemia. In neither experiment was there any evidence of a protective effect against the asexual erythrocytic stage of the life cycle. In a third experiment mice were primed with *PyHsp60* DNA, were boosted 2 weeks later with 2×10^3 irradiated *P. yoelii* sporozoites and were challenged several weeks later. The pres-

TABLE 2
Mixture of Antigens Used as DNA Vaccine

DNA VACCINE CANDIDATE		SPECIES	MODEL	OBSERVATIONS	REMARK	REFERENCE
MIXTURE OF ANTIGENS	**COMPO-NENTS**					
	CSP SSP2 LSA-1 Exp-1	*P. falciparum*	Mice	Antibody and CTL responses present	Immune responses against all the components	Hedstrom et al, 1998
	CSP SSP2 AMA1 MSP1	*P. vivax*	Mice	Antibody response present	Immune responses against all the components	Rogers et al, 1999
	Pfs25 Pfs27	*P. falciparum*	Mice	Antibody response present	Potent transmission blocking antibodies	Lobo et al, 1999
	CSP SSP2 AMA1 MSP1	*P. knowlesi*	Mice monkeys	Antibody response present	Partial protection observed in monkeys on sporozoite challenge	Rogers et al, 2001

ence of *PyHsp60* in the immunization regimen did not lead to reduced blood-stage infection or development of parasites in hepatocytes. *PyHsp60* DNA vaccines were immunogenic in BALB/c mice but did not consistently, completely protect against sporozoite challenge (Sanchez et al., 2001).

In a recent study the antigenic properties of EBA-175 (175-kDa erythrocyte binding antigen) protein have been evaluated in monkeys (Jones et al., 2001). Aotus monkeys received four doses of *P. falciparum* EBA-175 region II vaccine as plasmid DNA (Dv-Dv) or recombinant protein in adjuvant (Pv-Pv) or as three doses of DNA and one dose of protein (Dv-Pv) (Jones et al., 2001). After three doses, antibody titers were approximately 10^4 in DNA-immunized monkeys and 10^6 in protein-immunized monkeys. A fourth dose did not significantly boost antibody responses in the Dv-Dv only or Pv-Pv only groups, but titers were boosted to approximately 10^6 in monkeys in the Dv-Pv group. Four weeks after the last immunization, the animals were challenged with 10^4 *P. falciparum*-parasitized erythrocytes. Peak levels of parasitemia were lower in the 16 monkeys that received region II-containing plasmids or proteins than in the 16 controls. Three of 4 monkeys in the Dv-Pv group did not require treatment. These results demonstrate that immunization with EBA-175 region II induces a significant antiparasite effect *in vivo* (Jones et al., 2001).

P. falciparum merozoites bind to and invade human erythrocytes via specific erythrocyte receptors. This establishes the erythrocytic stage of the parasite life cycle that causes clinical disease. The hypothesis that *P. falciparum* ligand, EBA-175 region II (RII), which binds its erythrocyte receptor glycophorin A during invasion, can be used as an immunogen to induce antibodies that block the binding of RII to erythrocytes and thereby inhibit parasite invasion of erythrocytes was tested in a recent study (Sim et al., 2001). Accordingly, mice, rabbits, and monkeys were immunized with DNA plasmids that encoded the 616 amino acid RII. DNA vaccine plasmids that targeted the secretion of recombinant RII protein with and without the universal T-cell helper epitopes P2P30 were used to immunize mice, rabbits, and Aotus monkeys. *In vitro*, RII specific antibodies were assessed by IFA, ELISA, blocking of native EBA-175 binding to human erythrocytes and growth inhibition assays. The RII DNA plasmids were highly immunogenic as measured by ELISA and IFA. The anti-RII antibodies blocked the binding of native EBA-175 to erythrocytes and rosetting of erythrocytes on COS-7 cells expressing RII. Most important, murine and rabbit anti-RII antibodies inhibited the invasion of merozoites into erythrocytes. Non-human primates were also immunized, and it was shown that the RII-DNA plasmids were immunogenic and well tolerated in these monkeys. Monkeys were challenged with parasitized erythrocytes; one of three monkeys that received RII DNA plasmid was protected from fulminant disease. After challenge with live parasites, anti-RII antibody titers were boosted in the immunized monkeys. By proving the hypothesis that anti-RII antibodies can block merozoite invasion of erythrocytes, these studies pave the way for the clinical evaluation of EBA-175 as a receptor-blockade vaccine (Sim et al., 2001).

A series of preclinical studies were conducted in mice and rabbits to evaluate the safety of MuStDO 5 in a recent study (Parker et al., 2001). MuStDO 5 is a multivalent plasmid DNA vaccine for malaria comprised of five plasmid DNAs encoding five proteins from *P. falciparum* and one plasmid DNA encoding human GM-CSF. In pharmacology studies in mice, GM-CSF could not be detected in the serum following either intramuscular or a combined intramuscular/intradermal administration of the vac-

cine but was readily detected in the muscle following intramuscular administration. In a tissue distribution study in mice, MuStDO 5 plasmid DNA was detected by PCR initially in highly vascularized tissues, while at later time points the plasmid DNA was detected primarily at the site(s) of injection. In safety studies in mice and rabbits, repeated intramuscular/intradermal administration of the MuStDO 5 vaccine was found to be safe and well tolerated without any evidence of autoimmune pathology (Parker et al., 2001).

A non-human primate model for malaria vaccine development allowing reliable, stringent sporozoite challenge, and the evaluation of both cellular and antibody responses is needed. A multicomponent, multistage DNA vaccine for the simian malaria species *P. knowlesi* has been used in a recent study (Rogers et al., 2001). This vaccine includes the CSP (PkCSP), sporozoite protein 2 (PkSSP2), and two blood-stage antigens, apical merozoite antigen 1 (PkAMA1) and merozoite surface protein 1 (PkMSP1p42), as well as recombinant canarypox viruses encoding the four antigens (ALVAC-4). It was observed that the DNA vaccine plasmids expressed the corresponding antigens *in vitro* and induced antiparasite antibodies in mice. The various groups of four monkeys received three doses of a mixture of the four DNA vaccine plasmids and a plasmid encoding rhesus granulocyte-monocyte colony-stimulating factor. All the groups were boosted with a single dose of ALVAC-4. Animals immunized by any route (intramuscular, intradermal, or biojector) developed antibody responses against sporozoites and infected erythrocytes and against a recombinant PkCSP protein, as well as gamma interferon-secreting T-cell responses against peptides from PkCSP. It has also been shown that following challenge with 100 *P. knowlesi* sporozoites, one of 12 experimental monkeys was completely protected and the mean parasitemia in the remaining monkeys was significantly lower than that in four control monkeys (Table 2) (Rogers et al., 2001).

Development of nucleic acid-based vaccines against parasitic diseases shows great promise, although certain concerns about safety aspects of conventional DNA-vaccines have been raised. A recent study presents a comparison of antibody responses induced in mice by DNA and RNA-based immunization with vectors encoding a part of the *P. falciparum* antigen Pf332 (Andersson et al., 2001). Two types of plasmids were used, one conventional DNA plasmid containing a cytomegalovirus promoter and one suicidal DNA plasmid encoding the Semliki Forest virus (SFV) replicase. RNA, encoding the SFV replicase and the relevant antigen, was delivered either as naked RNA or packaged in SFV suicide particles. In general, the antibody responses induced by the DNA plasmids were low and peaking after three injections, the conventional plasmid giving the highest responses. Also, the RNA delivered in SFV particles consistently induced antibody responses, although comparatively low. Analyses of the ratio of immunoglobulin IgG1/IgG2a subclasses in the responses indicated that all plasmids resulted in a bias for a Th2-type of response, while the SFV particles elicited a Th1 type of response. Importantly, all these immunogens induced an immunological memory, which could be efficiently activated by a booster injection with the corresponding protein, with unchanged patterns of IgG subclasses (Andersson et al., 2001).

E. Studies Involving Human Subjects

In a study on the use of malaria DNA vaccine in humans it has been shown that volunteers developed antigen-specific, ge-

netically restricted, CD8(+) T-cell-dependent CTL responses (Wang et al., 1998b). The responses were directed against all 10 peptides tested and were restricted by six human lymphocyte antigen (HLA) class I alleles (Wang et al., 1998b).

The safety and tolerability of DNA vaccine encoding *PfCSP* was studied. Twenty healthy adult volunteers were enrolled in a Phase I clinical study (Le et al., 2000). Volunteers were given three intramuscular injections of one of four different dosages (20, 100, 500, and 2500 μg) of the *PfCSP* plasmid DNA at monthly intervals and were followed for up to 12 months. There were few and mild local reactogenicity and systemic symptoms. There were no serious adverse events, clinically significant biochemical or hematological changes, or detectable anti-dsDNA antibodies. Despite induction of excellent CTL responses, intramuscular DNA vaccination via needle injection failed to induce detectable antigen-specific antibodies in any of the individual (Le et al., 2000).

In a study, it was investigated whether naturally induced immunity to TRAP contributes to protection against malaria in humans (Dolo et al., 1999). The study was carried out in children living in an endemic region of West Africa to reveal associations between PfTRAP seroprevalence and the risk of cerebral malaria. Children with uncomplicated malaria had a significantly higher PfTRAP seroprevalence when compared with children with cerebral malaria. The risk of developing cerebral malaria appeared to depend on the reciprocal relationship between sporozoite inoculation rates and humoral immunity against PfTRAP. Experimentally induced immunity against TRAP in different rodent models has consistently proven to elicit a high degree of protection against malaria. The results suggest that naturally induced humoral immunity against PfTRAP contribute to the development of protection against severe malaria (Dolo et al., 1999).

The immunogenicity of a malaria DNA vaccine was assessed by administering it by needle intramuscularly or needle less jet injection (i.m. or i.m./intradermally [i.d.]) in 14 volunteers (Wang et al., 2001). Antigen-specific IFN-γ responses were detected by enzyme-linked immunospot (ELISPOT) assays in all subjects to multiple 9 to 23 amino acid peptides containing class I- and/or class II-restricted epitopes, and were dependent on both CD8(+) and CD4(+) T cells. Overall, frequency of response was significantly greater after i.m. jet injection. CD8(+)T-dependent cytotoxic T lymphocytes (CTL) were detected in 8/14 volunteers. Demonstration in humans of elicitation of the class I-restricted IFN-γ responses necessary for protection against the liver stage of malaria parasites brings closer to an effective malaria vaccine (Wang et al., 2001).

V. CONCLUSIONS AND FUTURE PROSPECTS

When the parasites first arrive in the liver and enter its cells they are relatively few in number and the person infected has to show symptoms of illness. Once they have taken up residence in the cells, they divide rapidly before bursting out into the bloodstream in large numbers. The aim is to produce a vaccine that will stimulate cytotoxic T cells to destroy all the infected cells in the liver before the parasite has a chance to multiply and take over the entire immune system.

Since the demonstration of the technology a few years ago, DNA vaccines have emerged as a promising method of vaccination. Typically, multiple injections of plasmid DNA via the intramuscular or intrader-

mal route yield both antibody and cellular responses with long-lasting immunity. DNA vaccines have been shown not only to induce potent immune responses but also to offer many advantages in terms of ease of construction, testing, and production. The ease with which DNA may be manipulated means that vaccines can be custom designed to meet many needs. DNA immunization circumvents the often difficult and time-consuming requirement to produce peptides, recombinant proteins, and recombinant live vectors as immunogens and the need for adjuvants to enhance immune responses. Vaccines made of DNA are being developed as a form of gene therapy that uses the patient's own cellular machinery to make foreign proteins that stimulate an immune response.

DNA vaccination can induce both types of effector response. Unlike conventional protein vaccines, plasmid DNA vaccination leads to antigen processing and loading onto both MHC class I and class II molecules. Improved gene expression combined with improved immunostimulatory (ISS) sequences inserted into the plasmid DNA backbone or use of ISS-containing oligonucleotides as adjuvant are some of the measures proven to enhance antibody responses. The priming with plasmid DNA and boosting with recombinant vaccinia expressing the same protein is also associated with greater immunogenicity and protective immunity than priming and boosting with plasmid DNA alone. These findings support assessing sequential immunization with plasmid DNA and recombinant vaccinia in further studies. It is well established now that immunization with a mixture of plasmid DNA induces immune responses against all the components of the mixture. These findings provide the foundation for multigene immunization of humans as it is the need of the hour for protection against malaria.

Some of the plasmid-based genetic vaccines have already entered clinical trials to test their safety and efficacy in healthy human volunteers. It has already been established that the level of integration it if occurs at all is not considered to pose a significant safety concern. Although several DNA vaccines have already entered phase I/II human clinical trials, there are several hurdles that need to be overcome on the road to the safe use of DNA vaccines widely.

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