Protective CD8+ T Cell Responses against the Pre-erythrocytic Stages of Malaria Parasites: an Overview

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CD8+ T cells have been implicated as critical effector cells in protection against the pre-erythrocytic stage of malaria in mice and humans following irradiated sporozoite immunization. Immunization experiments in animal models by several investigators have suggested different strategies for vaccination against malaria and many of the targets from liver stage malaria antigens have been shown to be immunogenic and to protect mice from the sporozoite challenge. Several prime/boost protocols with replicating vectors, such as vaccinia/influenza, with non-replicating vectors, such as recombinant particles derived from yeast transposon (Ty-particles) and modified vaccinia virus Ankara, and DNA, significantly enhanced CD8+ T cell immunogenicity and also the protective efficacy against the circumsporozoite protein of Plasmodium berghei and P. yeti. Based on these experimental results the development of a CD8+ T cell inducing vaccine has moved forward from epitope identification to planning stages of safety and immunogenicity trials of candidate vaccines.

Key words: malaria - cytotoxic T lymphocyte (CTL) responses - CTL and helper peptides - CD8+ and CD4+ epitopes - recombinant vectors - Ty-virus like particles (VLP)

Malaria remains one of the major causes of disease and death in the tropics. There are an estimated 300-500 million cases of malaria each year, resulting in over 1.5 to 2.7 million deaths, mainly of children under five years old in Africa (WHO 1997). The development of an effective vaccine represents one of the most promising alternatives to control the disease and over the past decade, there has been considerable progress in the identification of candidate vaccine antigens and their genes. This overview describes recent advances in the area of cell-mediated immunity with emphasis on cytotoxic T cells directed against sporozoite and liver stage antigens of malaria parasites and their role in protection.

It is well documented that immunization of mice (Nussenzweig et al. 1967), monkeys (Nussenzweig et al. 1970) and humans (Clyde et al. 1973) with radiation attenuated sporozoites of Plasmodium species protects against challenge with live sporozoites, but does not protect against challenge with infected erythrocytes. Therefore, the protective immunity induced by irradiated sporozoites is stage specific and must be against either extracellular sporozoites prior to their invading the hepatocytes or parasites developing within the hepatocytes. The sera from mice (Vandenberg et al. 1969) and humans (Clyde et al. 1973) immunized with irradiated sporozoite precipitate the surface coat of sporozoites (CS protein). Because of this, for many years antibodies against sporozoites were thought to be the major immune effectors responsible for the observed protection.

However, in 1977 it was reported that µ-suppressed mice immunized with irradiated sporozoites were protected against sporozoites challenge (Chen et al. 1977) and several years later it was shown that: (a) in vivo depletion of CD8+ T cells drastically reduced protective immunity against a challenge with live sporozoites (Schofield et al. 1987, Weiss et al. 1988); (b) passive transfer of cloned murine CD8+ T cells, specific for the circumsporozoite (CS) protein or the sporozoite surface protein 2 (SSP2), conferred protection to mice (Romero et al. 1989, Rodrigues et al. 1991, Khusmith et al. 1991) and (c) treatment of irradiated sporozoite immunized mice with monoclonal antibody against IFN-γ or with an inhibitor of inducible nitric oxide synthetase also eliminated protection (Schofield et al. 1987, Seguin et al. 1994).

The CD8+ T cells have been implicated as critical effectors cells in protection against the pre-erythrocytic stage of malaria. The target of these malaria specific CD8+ T cells is most likely to be...
the infected hepatocyte, which presents, on its surface, parasite-derived epitopes in association with class I MHC molecules. The CD8+ T cells subsequently secrete IFN-γ, which in turn induces the infected hepatocytes to produce nitric oxide that eliminates the infected hepatocytes or inactivates the intracellular parasite (Schofield et al. 1987, Nussler et al. 1993, Seguin et al. 1994, Klitz et al. 1995).

This hypothesis is supported by studies which indicated that in the rodent model, four antigens expressed in hepatocytes have been established as targets for the protective CD8+ T cell responses: the surface antigens of the sporozoites P. berghei (PbCSP) and P. yoelii (PyCSP and PySSP2) and the protein expressed on the surface of hepatocytes and erythrocytes – PyHEP17. The P. falciparum homologues of these antigens, PfCSP, PfSSP2/TRAP and PfExp-1, respectively, have been shown to be targets for CD8+ T cells after immunization with radiation-attenuated sporozoites and after natural exposure to P. falciparum (Rogers et al. 1992, Doolan et al. 1996a), as well as a fourth antigen, P. falciparum liver stage antigen 1-LSA1 (Guerin-Marchand et al. 1987).

While several antigens appear to be involved in protection, so far, a single protective epitope has been identified in the murine P. yoelii and P. berghei systems. These epitopes are located in the CS protein and their amino acid sequences are SYVPSAEQI and SYIPSAEKI respectively (Romero et al. 1989, Weiss et al. 1990). In the P. falciparum CS protein, a CD8+ epitope was identified using murine T cells obtained from B10.BR (H-2k) mice immunized with a vaccinia vector expressing the entire P. falciparum CS protein. CD8+ T cells from mice immunized with P. falciparum sporozoites also recognized this epitope. Furthermore, the same epitope sensitized target cells for lysis by CD8+ T cells derived from three out of four sporozoite-immunized volunteers (Mallik et al. 1991).

During the past decade, the major efforts on pre-erythrocytic malaria vaccine development have focused on inducing antibodies to the sporozoite surface and CD8+ T cells against infected hepatocytes; however, there is now considerable interest in protective CD4+ T cell responses against infected hepatocytes.

New data indicates that in several strains of mice, protection induced by irradiated sporozoites can be eliminated by treatment of mice with anti-CD4+ antibodies or anti-CD8+ antibodies. The CD4+ T cells specific for the CS protein produced IFN-γ, IL-2 and IL-6 (Tsuji et al. 1990, Renia et al. 1991, 1993). These authors have also demonstrated that CD4+ T cell clones against PyCSP eliminated infected hepatocytes from culture and conferred protection when adoptively transferred to naive animals (Renia et al. 1991). Immunization with synthetic peptides from two liver stage proteins induces consistent, genetically restricted, CD4+ T cell and IFN-γ dependent protection (Wang et al. 1996).

In view of these encouraging results, the challenge for the future is to develop a pre-erythrocytic vaccine capable of inducing not only protective antibodies against sporozoites but also CD4+ and CD8+ T cell responses against the hepatocytes. Remarkable progress in research on these forms in recent years has led to better understanding of the biology of these forms and attempts are currently being made to induce protective anti-plasmodia T cells in vivo, using live and synthetic immunogens.

**INDUCTION OF ANTI-MALARIA CD8+ T CELLS USING LIVE RECOMBINANT BACTERIAL AND VIRAL VECTORS**

Recombinant live bacteria and viruses expressing immunologically relevant antigens are attractive delivery systems, designed to elicit protective immunity against microbial pathogens. The most widely studied live virus vehicle is the recombinant vaccinia virus, which tolerates insertions of large segments of foreign DNA and replicates within the cytoplasm of the host cell. The CD8+ CTL epitope in P. falciparum CS protein was originally defined using cells obtained from mice immunized with a recombinant vaccinia virus expressing the CS protein (Kumar et al. 1988).

In subsequent studies, live viral vectors expressing the appropriate proteins and epitopes have been used as promising vaccine candidates (Li et al. 1992, Rodrigues et al. 1994, Murata et al. 1996). In these studies, conditions which influence the CD8+ T cell responses induced by immunization of mice with different recombinant viruses were also identified and analyzed. These live vectors were engineered to express either the entire sequence of the P. yoelii CS protein or only the H-2Kd restricted epitope, SYVPSAEQI. Using this system, a certain degree of protective immunity against this parasite could be induced by a single immunization with either virus but a second dose of the same recombinant virus failed to enhance this immunity (Li et al. 1993, Rodrigues et al. 1994, Murata et al. 1996). The immunization with recombinant vaccinia virus expressing only the CD8+ epitope was as efficient as with the version expressing the entire CS protein. In contrast, protective immunity induced with influenza virus could only be achieved when it expressed the CS B cell epitope (Rodrigues et al. 1994). The magnitude of these responses depended on the type of vector used for
immunization and appeared to be strictly dose dependent (Murata et al. 1996).

On the other hand, immunization with recombinant vaccinia virus after priming with influenza virus significantly increased the protective immunity (Li et al. 1993, Rodrigues et al. 1994, Murata et al. 1996). Furthermore, with this sequence of immunization, a strong inhibition (more than 90%) of parasite development in the liver was observed and approximately 50 to 70% of immunized mice failed to display parasitemia (Li et al. 1992, Rodrigues et al. 1994, Murata et al. 1996).

More recently, studies on evaluating the immunogenicity of a replication-defective recombinant adenovirus expressing the CS protein of P. yoelii showed that a single immunizing dose of 109 plaque forming units (pfus) of this recombinant virus induced a large number of CS-specific CD8+ and CD4+ T cells in the spleen of immunized mice and also induced high titers of anti-sporozoite antibodies, particularly when given by the subcutaneous or intramuscular route. Most importantly, a single dose of the recombinant virus resulted in a 93% inhibition of the liver stage development of the parasite, as measured by the level of parasite rRNA present in the liver of immunized mice, after sporozoite challenge. Furthermore, 40% of the immunized mice displayed complete resistance to infection. CD8+ T cells were the principal mediators of this protective effect, as shown by a greatly reduced level protective immunity following the depletion of this T cell subset (Rodrigues et al. 1997).

A highly attenuated strain of vaccinia virus (NYVAC) has been used to develop a multistage vaccine for malaria by including genes encoding seven P. falciparum antigens derived from sporozoite, liver, blood and ookinete stages. This vaccine designated NYVAC-P17 elicited high titers of specific antibodies in Rhesus monkey (Tine et al. 1996). In Phase I and II trials in humans, the vaccine proved to be safe and well tolerated but presented variable immunogenicity. Most of the vaccinated individuals presented a delayed time to onset of parasitemia but only 1 out of 35 failed to develop infection (Ockenhouse et al. 1998).

Bacterial vectors have also been used to induce CS specific CD8+ CTL in immunized mice. Initial studies using an attenuated strain of Salmonella typhimurium transformed with plasmid expressing the CS gene of P. berghei and P. yoelii demonstrated the capacity of this vector to induce CD8+ T cell dependent immune protection in 55%-70% of immunized BALB/c mice (Sadoff et al. 1988, Aggarwall et al. 1990, Flyn et al. 1990). This approach was recently evaluated in humans immunized orally with recombinant S. typhi expressing the CS protein of P. falciparum. In that study, only 3 out of 10 volunteers mounted an antibody response and 1 produced CSP specific CD8+ T cells (Gonzales et al. 1994).

The studies described above indicate that the use of live recombinant viruses is an attractive system for vaccine design based on the induction of CD8+ T cells against intracellular pathogens. However there are restrictions with regard to the induction of secondary CD8+ T cells or the in vivo expansion of antigen specific CD8+ T cells, since this can only be achieved under very specific conditions of immunization. In addition, such vectors are difficult to modify and also have the potential to cause diseases in immuno-compromised hosts, or even in healthy individuals if the attenuated vector reverts to a virulent phenotype.

**INDUCTION OF CD8+ T CELLS USING SYNTHETIC, NON-INFECTIVE IMMUNOGENS**

The use of simple proteins or synthetic peptides to induce CTL responses has also been explored. Free short peptides are poor immunogens for CTL and several approaches have been used to overcome this difficulty including the use of adjuvants, the chemical modification of peptides and the addition of helper epitopes.

A peptide containing a CD8+ T cell T epitope of the P. berghei CS protein, when modified by the addition of a palmitoyl-cys-ser lipid tail, induced murine CD8+ T cells specific for parasites (Deres et al. 1989). However, high cell densities of responding T cells by sensitization either in vivo or in vitro with a short peptide could not easily be achieved (Widmann et al. 1992, Valmori et al. 1994, Allsopp et al. 1996).

The induction of CD8+ T cells could be achieved by immunization with a synthetic peptide corresponding to the CTL epitope of the P. yoelii and P. berghei CS protein, however the number of antigen specific CD8+ T cells induced, was relatively modest. This response could be enhanced by the administration of this peptide together with peptides corresponding to CD4+ T cell epitopes (Romero et al. 1992, Widmann et al. 1992, Valmori et al. 1994).

Protection against malaria by a synthetic peptide vaccine designed to induce CD8+ CTL response against a single class I restricted epitope has been reported in few studies but the best result could only protect 40% of the challenged animals (Migliorini et al. 1993, Franke et al. 1997). More importantly, in peptide-primed animals, secondary CD8+ T cell responses could not be induced by a second dose of synthetic peptides or by the use of a recombinant vaccinia virus expressing the CS protein (Murata et al. 1996, Oliveira-Ferreira et al. 2000).
Recently, a new semi-synthetic system in which proteins self-assembled in particles, such as the particle-forming p1 protein encoded by the TYA gene of the yeast retrotransposon Ty, has been developed (Adams et al. 1987, Kingsman & Kingsman 1988). When this protein is expressed in yeast, high levels of virus like particles (Ty-VLP) are produced and proteins of interest can be fused to the C-terminus of the p1 protein, resulting in a hybrid Ty-VLP. In this system, defined protein sequences can be expressed without loss of their particulate structure, which has been reported to facilitate the generation of high levels of antibody and T cell responses to a number of antigens (Griffiths et al. 1991, Harris et al. 1993, Layton et al. 1993). Since protein sequences can be easily inserted and expressed by Ty-VLP, this system is very attractive for the eventual development of vaccines using non-replicating subunits.

Immunization with Ty-VLP carrying a CD8+ T cell epitope of the P. berghei CS protein (TyPb) resulted in an immune response (Allsopp et al. 1996). This study compared four adjuvants designed for the use with peptide, lipopeptides, recombinant salmonella, vaccinia virus and an attenuated vaccinia, plasmid DNA and Ty-VLPs. Only the lipopeptide and Ty-VLPs were able to stimulate CTL in all immunized mice. Subcutaneous immunization with Ty-VLPs without any adjuvant resulted in high levels of CTL and these CTL T cells were still present ten weeks after a single immunization.

Although Ty-VLP particles carrying a CD8+ T cell epitope of the CS protein of P. berghei and P. yoelii can induce CS specific CD8+ T cells, protection has only been achieved when mice were primed with the particles and boosted with vaccinia recombinant expressing the whole CS protein. This sequence of immunization induced high numbers of specific CD8+ T cells, inhibited the liver stage development (94%) and protected 62-95% of the mice from sporozoites infection (Plebanski et al. 1998, Oliveira-Ferreira et al. 2000).

These data suggest that particulate antigens in the form of hybrid Ty-VLP are potent immunogens and have the potential to constitute a multi-stage vaccine against malaria. Since protein sequences can be easily inserted and expressed by Ty-VLP, this system is very attractive for the eventual development of vaccines using non-replicating subunits.

**INDUCTION OF CD8+ T CELLS USING DNA PLASMID**

The demonstration that plasmid DNA can induce antigen encoded specific immune responses following intramuscular or intradermal injection or following administration of DNA in gold particles has suggested a different approach to vaccine development. The simplicity of the DNA approach implies that it is possible to combine many DNA sequences encoding different antigens and thereby broaden the immune response. By incorporating a complete antigen rather than few epitopes, DNA immunization may circumvent the problem of genetic restriction of the protective immune response to target epitopes.

Several research groups are working on the production of DNA vaccines against malaria and the results obtained so far, using animal models of malaria, are encouraging. It was first reported that BALB/c mice are protected against sporozoite challenge by intramuscular immunization with plasmid DNA encoding the PyCS gene; 56% of mice were protected against challenge with sporozoites and the levels of induced CTL were significantly higher than the levels induced by protective immunization with irradiated sporozoites (Sebedegah et al. 1994). However, it was demonstrated that this protective immunity was genetically restricted; 86% of BALB/c mice were protected while only 20% of the other four strains of mice studied were protected (Doolan et al. 1996a).

In contrast, immunization with plasmid DNA expressing the hepatocyte erythrocyte protein 17 (PyHEP17) gave more than 20% (up to 86%) of protection for three of five strains of inbred mice. Immunization with a mixture of the two plasmids circumvented some of the genetic restriction of protection, which, otherwise, was dependent on CD8+ T cells, IFN-γ and nitric oxide (Doolan et al. 1996b).

Recently sequential immunization with plasmid DNA encoding P. yoelii CS protein and with a recombinant vaccinia virus expressing the CS protein was found to generate a high CD8+ T cell response and to contribute to a substantial protection in mice (Schneider et al. 1998, Plebanski et al. 1998).

In the non-human primate model, vaccination using four plasmids encoding various malaria genes has been found to be immunogenic. In addition, administration of each plasmid alone was as effective at inducing CD8+ T cells as was the administration of a mixture of the four plasmids (Wang et al. 1998).

The first human vaccination trials with DNA plasmid encoding the CS protein of P. falciparum showed that malaria naive volunteers developed specific CD8+ T cells directed against ten peptides tested and these responses were genetically restricted by multiple HLA alleles (Wang et al. 1998). However, the magnitude of the CTL response seen
in some of the volunteers was considerably higher than the response seen in humans exposed to irradiated sporozoites or natural infection (Mallik et al. 1991, Sedegah et al. 1992, Doolan et al. 1993).

Although DNA vaccines are promising, the issue of long-term safety has not yet been assessed. In addition, vaccination in mice with plasmid DNA led to the generation of anti-DNA antibodies, although these antibodies did not initiate or accelerate the course of an autoimmune disease (Mor et al. 1997). Therefore, even if immunogenicity can be improved it will take several years before the mass vaccination of children with DNA can be achieved, owing to safety concerns.

CONCLUDING REMARKS

Studies in murine systems have shown that it is possible to induce the number and quality of CD8+ T cells, both of which appear to be necessary to stop the progression of malaria infection beyond the liver stages. These findings provide the rationale and the experimental basis for the development of immunogens capable of inducing both, humoral and T cell immune responses. This combination of immune effector mechanisms is likely to be much more efficient in protecting against infection than either alone. However, it is possible to induce the number and quality of CD8+ T cells, both of which appear to be necessary to stop the progression of malaria infection.

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