Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *87*, Suppl. III, 241-247, 1992

PLASMODIUM FALCIPARUM CS PROTEIN – PRIME MALARIA VACCINE CANDIDATE: DEFINITION OF THE HUMAN CTL DOMAIN AND ANALYSIS OF ITS VARIATION

DENISE L. DOOLAN & MICHAEL F. GOOD

The Tropical Health Program, Queensland Institute of Medical Research Bramston Terrace, Herston 4006, Brisbane, Queensland, Australia 4006

Studies in mice have shown that immunity to malaria sporozoites is mediated primarily by cytotoxic T lymphocytes (CTL) specific for epitopes within the circumsporozoite (CS) protein. Humans, however, had never been shown to generate CTL against any malaria or other parasite protein. The design of a sub-unit vaccine for humans relies on the epitopes recognized by CTL being identified and polymorphisms therein being defined. We have developed a novel technique using an entire series of overlapping synthetic peptides to define the epitopes of the Plasmodium falciparum *CS protein recognized by human CTL and have analyzed the sequence variation of the protein with respect to the identified CTL epitopic domain. We have demonstrated that some humans can indeed generate CTL against the* P. falciparum *CS protein. Furthermore, the extent of variation observed for the CTL recognition domain is finite and the combination of peptides necessary for inclusion in a polyvalent vaccine may be small. If ways can be found to increase immune responsiveness, then a vaccine designed to stimulate CS protein-specific CTL activity may prevent malaria.*

Key words: malaria vaccine – sporozoite immunity – cytotoxic T lymphocytes – sequence variation

The sporozoite coat protein, the circumsporozoite (CS) protein is a prime candidate vaccine antigen for the sporozoite stage in the life cycle of the malaria parasite. Vaccination with irradiated sporozoites can provide protective immunity in animals (mice, monkeys and birds) and humans (Mulligan et al., 1941; Nussenzweig et al., 1979; Clyde et al., 1973, 1975; Rieckmann et al., 1979) (reviewed by Nussenzweig & Nussenzweig, 1984). Sporozoite immunity appears, however, to wane over a period of months in mouse models (Nussenzweig & Nussenzweig, 1984). The protective immunity induced by irradiated sporozoites appears to be mediated in part by neutralizing antibodies that are directed mainly against the repeat domain of the CS protein (Potocnjak et al., 1980; Hollingdale et al., 1982). High concentrations of antibodies are, however, required to protect against sporozoite challenge (Potocnjak et al., 1980; Egan et al., 1987) and even then, unlike irradiated sporozoites, antibodies protect only against moderate challenge. Furthermore, human immunity is not correlated with the circulating level of naturally acquired antibody (Hoffman et al., 1987). Murine studies have suggested that CTL can protect more efficiently. The demonstration that athymic mice could not be immunized with irradiated sporozoites was indicative of a role for T-cell effector mechanisms in mediating protection (Chen et al., 1977; Spitalny et al., 1977). It was subsequently shown that T cells from sporozoite-immunized mice could transfer protection (Verhave et al., 1978; Egan et al., 1987) and that treatment of sporozoite-immunized mice with monoclonal anti-CD^{8+} antibody, but not anti-CD^{4+} antibody, completely abrogated immunity (Schofield et al., 1987; Weiss et al., 1988). Protection could also be induced by oral vaccination of mice with a recombinant salmonella expressing the *Plasmodium berghei* CS protein which conferred protection in the absence of antibody indicating that the CS protein may be the target of protective T cells (Sadoff et al., 1988). This protection correlated with cellular immunity (Sadoff et al., 1988) and was shown to be $CD8^+$ dependent (Aggarwal et al., 1990). CTL specific for the CS protein were shown to be present after immunization with irradiated sporozoites (Kumar et al., 1988; Romero et al., 1989; Weiss et al., 1990) and recombinant vaccines (Aggarwal et al., 1990; Flynn et al., 1990). CS-specific CTL

epitopes were identified in *P. falciparum* and, more recently, *P. berghei* and *P. yoelli* mouse models and all were restricted to single peptide epitopes in the carboxy terminal regions of the respective CS protein (Kumar et al., 1988, Romero et al., 1989; Weiss et al., 1990). The CTL epitopes of both species of murine malaria appeared to share a high degree of homology (Weiss et al., 1990). It was subsequently shown that $CD8^+$ T lymphocytes from mice immunized with irradiated sporozoites recognized malarial antigens, including the CS protein, on the surface of infected hepatocytes and could eliminate malaria-infected hepatocytes from *in vitro* culture in an antigen-specific and Major Histocompatibility Complex (MHC) – restricted manner (Hoffman et al. 1989; Weiss et al., 1990). The protective capacity of CTL was demonstrated by Romero et al. (1989) when adoptive transfer of a *P. berghei* CS-specific CTL line was able to confer a high degree of protection against *P. berghei* sporozoite challenge. This protective effect was species – and stage – specific. This provided the first direct evidence that $CD8^+$ T cells that are specific for a defined epitope could confer protection against a parasitic infection.

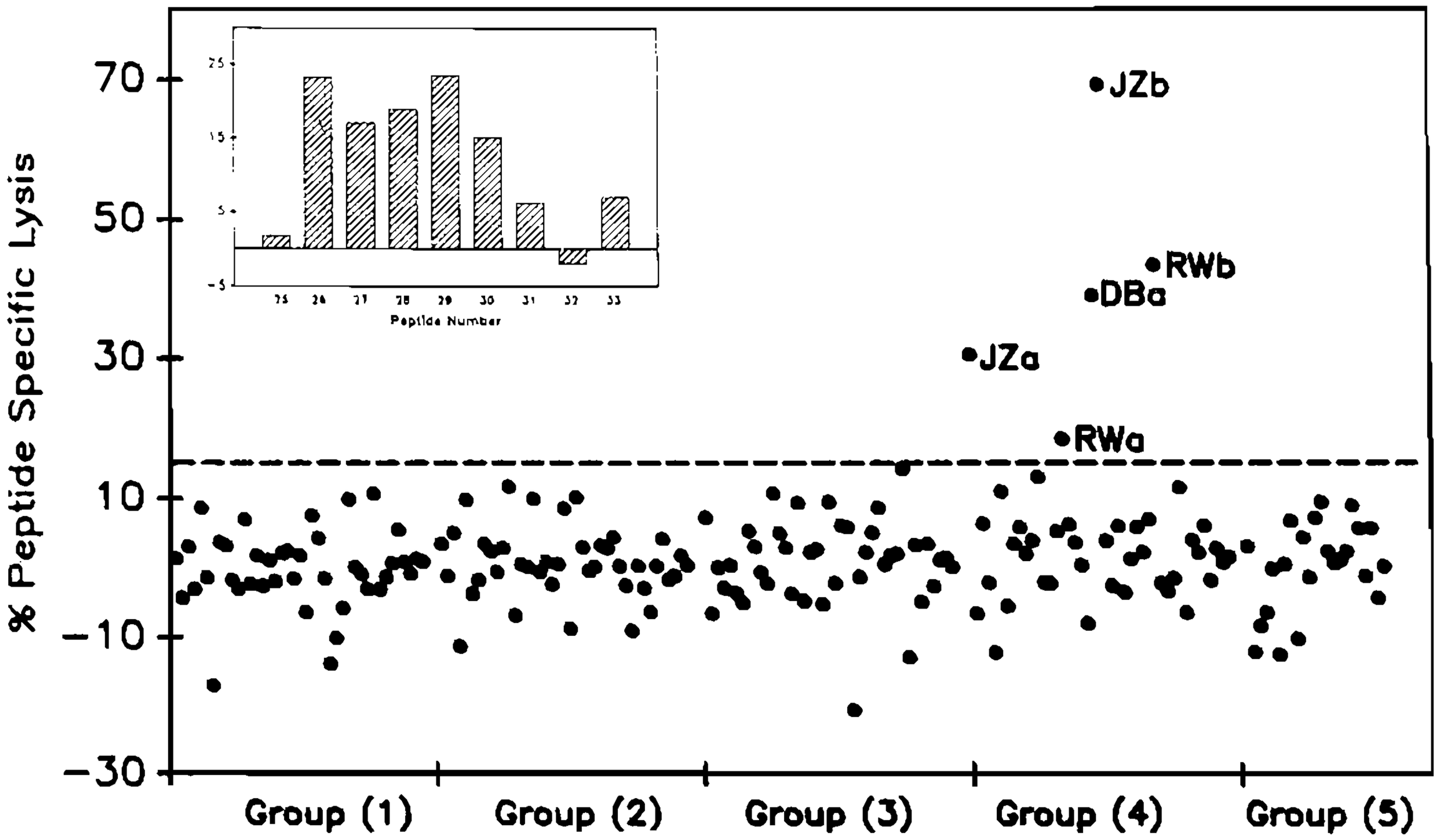
DEFINITION OF HUMAN CS-SPECIFIC CTL

Murine studies have thus shown that CTL specific for epitopes within the CS protein can prevent malaria. This has provided a model for the development of a sporozoite vaccine. Until recently, however, it had not been shown whether humans could mount a CTL response to the CS protein nor what determinants on the protein could be considered as target epitopes and thus warrant inclusion in a sporozoite vaccine.

We have developed a novel technique (Doolan et al., 1991a) using synthetic overlapping peptides representing four different CS protein isolates: 7G8 (Brazil); WEL (Nigeria), LE5 (Liberia), T9-98 (Thailand) to define the epitopes of the CS protein recognized by human peripheral blood CTL (Doolan et al., 1991b). We were able to reproducibly find high CTL activity in three of 42 individuals naturally exposed to malaria in different endemic countries (Fig.). Epitopes within the carboxy terminal region of the protein (residues 351-395, 7G8 strain) were recognized. Data indicated that two separate CTL epitopes occurred in this region. There was evidence, however, that the prevalence of such CTL is low and that recent malaria exposure may be a prerequisite for finding such CTL. The location of the CTL recognition region mapped to one of the two domains known to contain the bulk of the sequence variation and also the region recognized by murine CTL (Kumar et al., 1988). The only other reported CTL epitope shared between mouse and human was recently described for HIV 1 reverse transcriptase (Hosmalin et al., 1990).

Malik et al. (1991) were independently able to generate CTL from the peripheral blood of three from four volunteers experimentally infected with the bites of hundreds of infected mosquitoes. Upon subsequent sporozoite challenge, two of these three individuals were protected. Again, there was evidence that the frequency of CS-specific CTL precursors may be low. Activity was found to be $CD8^+$ T cell dependent and genetically restricted and mapped to the same location as the epitopes defined by us (above).

Thus, two reports have now been published revealing that sporozoite-exposed humans can generate CTL which recognize CS protein epitope(s) in the same polymorphic carboxy-terminal region of the CS protein. The fact that not all individuals could mount a CTL response may reflect genetic non-responsiveness to the protein of certain MHC genes (Good et al., 1990). This has been well documented in the mouse model (Good et al., 1986, 1988b; Weiss et al., 1989). That not all individuals were protected against challenge with a sporozoite clone does not imply that CTL can not protect against sporozoite challenge. Factors such as difference in CTL activity between peripheral blood and the hepatocyte in terms of the number of effector cells, antigen presentation etc, the apparent low frequency of CS-specific CTL precursors as shown in *P. falciparum* exposed mice, and that the CTL response is probably to a small number or even a single epitope within the protein of a parasite that is inoculated infrequently and probably in small numbers, must be considered. Nevertheless, this *in vitro* data suggests that most people with heavy recent sporozoite exposure have CS-specific CTL and that such CTL memory cells quickly decline following exposure.



Peripheral blood mononuclear cells (PBM) from 42 malaria-exposed donors were stimulated with groups of synthetic CS peptides and activity measured against PHA blast target cells incubated with the peptides. The order of the dots indicates the sequence in which various individuals were tested. For group 4 peptides, JZ and RW were retested against the entire group. The dotted line represents 15.75% specific lysis, a figure representing the 95% confidence interval for all values determined after the initial screening of all individuals. In this figure, the value of lysis of PHA blasts in the absence of added peptide (control lysis) has been subtracted from the value of lysis of targets in the presense of peptides. This control lysis was usually between -5 and +5% (mean 0.3%). *Inset:* A CTL line was established from DB by repetitive weekly stimulation with the group of peptides and tested against each of the individuals peptides in group 4. From Doolan et al., 1991b, with permission.

SEQUENCE VARIATION OF THE *P. FALCIPARUM* CS PROTEIN

Although the CS gene is largely invariant, several nucleotide substitutions, all of which result in amino acid changes, have been found in different *P. falciparum* isolates from different geographical regions (Dame et al., 1984; Lockyer & Schwartz, 1987; de la Cruz et al., 1987; Del Portillo et al., 1987; Caspers et al., 1989; Lockyer et al., 1989; Yoshida et al., 1990). It is a matter of concern that this sequence variation has been noted to occur predominantly in the T cell regions, in both the Th2R and Th3R helper T cell domains (Good et al., 1988a; Dontfraid et al., 1988; de la Cruz et al., 1988; De Groot et al., 1989; Zevering et al., 1990) as well as the human and murine CTL domains (Kumar et al., 1988; Doolan et al., 1991; Malik et al., 1991). The extent of polymorphism within these T cell epitopes suggests problems with the sub-unit approach to vaccine development.

Here we also report the analysis of the sequence variation of a 354 bp fragment (nucleotides 886-1239) (residues 296-412) encompassing the defined CTL domain, as well as previously defined $CD4^+$ T cell domains, of 41 geographically diverse isolates (Doolan et al., submitted). Deduced amino acid sequence comparisons revealed polymorphisms which were always non-synonymous, supporting the concept that pressure at the protein level, possibly CTL, was selecting the variation. There appeared, however, to be marked constraints in the ability of the parasite to change. Variation preferentially occurred at certain positions and some residue substitutions were much more common than others. Of 41 isolates analyzed, five separate sequences occurred in the Th2R domain and six in the Th3R and CTL domains. In several instances, the CS genes of isolates which encoded the same Th2R sequence differed in their corresponding Th3R sequences, and vice versa, giving a larger repertoire of Th2R/Th3R variant combinations. Eight vari-

ant sequences were found when considering the region encompassing both the Th2R and Th3R domains, of which three were previously undescribed.

A striking feature revealed by our analysis was that the sequences of all 22 Papua New Guinea (PNG) isolates were identical. This contrasts markedly with the extensive polymorphism of *P. falciparum* isolates from other countries. Furthermore, when 25 caucasian individuals with a history of extensive sporozoite exposure were tested for CTL recognition of the specific PNG sequence, no cytotoxic activity was detected. Given that the Th3R domain (361-380) represents the only region of variation within the human (351-395) (Doolan et al., 1991b; Malik et al., 1991) and murine (368-390) (Kumar et al., 1988) CTL epitopes, activity against the variant sequences would be predicted if CTL are indeed selecting the sequence variation. Although more individuals need to be tested, one possibility is that this sequence *per se* does not represent a CTL epitope, arising perhaps as the end result of significant selective pressures by CTL. This needs to be confirmed by studying the native Papua New Guinean response to the CS protein.

A similar situation has been reported in Brazil. Data presented by Yoshida et al. (1990) revealed that of 24 field isolates of *P. falciparum,* 19 displayed a sequence identical to that of the Brazilian sequences, 7G8. A second sequence was represented by four of the isolates and differed at five nucleotide positions in the Th2R domain. A third Brazilian sequence was noted for one clone (ItG2) analyzed in this study and was the same as that reported by Lockyer et al. (1989) for a similar clone (ItG1 G2). Furthermore, the sequence of the PNG isolates was not the same as the Brazilian sequences. The Th3R and CTL domains were invariant amongst all PNG isolates and amongst the Brazilian isolates reported by Yoshida et al. (1990). The third Brazilian sequence identified in this study and that of Lockyer et al. (1989) was identical in the Th3R and CTL domains to that of the PNG isolates.

In both studies (Doolan et al., 1991b; Yoshida et al., 1990) comparison of *P. falciparum* isolates from widely separated geographical regions revealed more extensive heterogeneity. Lockyer et al. (1989), in the only other comprehensive analysis had reported extensive T cell site polymorphism in a single small endemic area (5 mile radius) of the Gambia. They concluded that the degree of sequence variation in the Th2R and Th3R domains of parasites present at the same time and place was so extensive as to preclude the use of all variants in a polyvalent subunit vaccine. This conclusion is not, however, supported by our data nor that of Yoshida et al. (1990), which indicates that the polymorphism of CS protein T cell sites is very limited among *P. falciparum* isolates prevalent in certain geographical regions, notably Brazil and in particular PNG.

Furthermore, although heterogeneity of the *P. falciparum* CS protein has well documented, the variation does appear to be limited. Assimilating observed variation with that reported in the literature reveals that a total of 21 different sequences are representative of the entire spectrum of T cell variation in the *P. falciparum* CS protein observed to date (Doolan et al., 1992). Twelve separate sequences of 119 are noted in the immunodominant Th2R domain and nine separate sequences of 98 in the Th3R domain and in the human and murine CTL domain. In any given sequence, substitutions occur at no moire than five positions in Th2R and two positions in Th3R (relative to the 7G8 sequence) and there are no more than three different residues at each of the varying positions.

There has been much discussion in the literature about whether CTL have selected the variation that occurs in the CS protein. It is suggested that CTL are mediating the pressure through selection on the CS gene and given a measure of biological advantage to select parasites (de la Cruz et al., 1987; Good et al., 1988a, b; Arnot, 1989; McCutchan & Waters, 1990). Parasite antigens recognized by $CD8^+$ T cells must be presented to the host during immunization with irradiated sporozoites. Any parasite antigen on the hepatocyte surface recognized by CTL would thus be expected to come under selective pressure. The fact that there is an overlap of the CTL recognition region with one of the polymorphic domains and the immunodominant $CD4^+$ epitopes with both polymorphic domains and that all mutations within the CS gene are coding change mutations, indicative of selective pressure at the protein level, supports this hypothesis. This is, however, a matter of controversy (Nussenzweig & Nussenzweig, 1990; Yoshida et al., 1990) and will not be discussed further here. It suf-

fices to say that data of the authors support this concept.

IMPLICATIONS

Clearly more epidemiological studies are necessary to determine whether the CS antigen polymorphism represents a major obstacle for the development of an anti-sporozoite vaccine. The extend of variation observed for the CS protein CTL recognition domain suggests that any vaccine designed to stimulate this form of immunity will need to be polyvalent. Multiplicity of parasite CTL epitopes would, however, circumvent the lack of presentation by certain MHC alleles and parasite evasion of T cell epitopes (Good et al., 1987). The fact that immunization with *P. berghei* irradiated sporozoites protects all strains of mice indicates that many such antigens may exist (Hoffman et al., 1989). The ideal vaccine may thus contain CS-specific CTL epitopes combined with $CD4^+$ T helper epitopes from the same protein to allow natural boosting following exposure to the parasite, as well as other sporozoite/liver stage antigens (e.g. SSP2) (Khusmith et al., 1991). The feasibility of such an approach will, however, depend upon the degree of polymorphism in these epitopes exhibited by parasite populations as it seems likely that many of the defined sequences within the T cells domains will not cross-react. Nonetheless, the extent of variation observed for the CS protein CTL recognition region, as well as the $CD4^+$ T cell domains, appears to be finite and the combination of peptides necessary for inclusion in a polyvalent vaccine thus may be small. These antigens between them may be immunogenic for most people and may elicit cross-reactive responses to those parasite strains not represented. If ways can be found to increase immune responsiveness, then a vaccine designed to stimulate CS protein-specific CTL activity may prevent malaria.

REFERENCES

AGGARWAL, A.; KUMAR, S.; JAFFE, R.; HONE, D.; GROSS, M. & SADOFF, J., 1990. Oral salmonella: malaria circumsporozoite recombinants induce specific $CD8^+$ cytotoxic T cells. *J. Exp. Med., 172:* 1083-1090.

ARNOT, D., 1989. Malaria and the Major Histocompatability Complex. *Parasitol. Today, 5:* 138-142.

CASPERS, P.; GENTZ, R.; MATILE, H.; PINK, J.R. & SINIGAGLIA, F., 1989. The circumsporozoite protein gene from NF54, a *Plasmodium falciparum* isolate used in malaria vaccine trials. *Mol. Biochem. Parasitol., 35:* 185-190.

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.; McCARTHY, V.C.; MILLER, R.M. & HORNICK, R.B., 1973. Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am. J. Med. Sci., 266:* 398-403.

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.

DAME, J.B.; WILLIAMS, J.L.; McCUTCHAN, T.F.; WEBER, J.L.; WITZ, R.A.; HOCKMEYER, W.T.; MALOY, W.L.; HAYNES, J.D.; SCHNEIDER, I.; ROBERTS, D.; SANDERS, G.S.; REDDY, E.P.; DIGGS, C.L. & MILLER, L.H., 1984. Strucutre of the gene encoding the immunodominant surface antigen of the sporozoite of the human malaria parasite *Plasmodium falciparum. Science, 225:* 593-599.

de la CRUZ, V.F.; LAL, A.A. & McCUTCHAN, T.F., 1987. Sequence variation in putative functional domains of the circumsporozoite protein of *Plasmodium falciparum:* implications for vaccine development. *J. Biol. Chem., 262:* 11935-11939.

de la CRUZ, V.F.; MALOY, W.L.; LAL, A.A.; GOOD, M.F. & McCUTCHAN, T.F., 1988. Lack of cross-reactivity between variant T cell determinants from malaria circumsporozoite protein. *J. Immunol., 141:* 2456-2460.

DE GROOT, A.S.; JOHNSON, A.H.; MALOY, W.L.; QUAKYI, I.A.; RILEY, E.M.; MENON, A.; BANKS, S.M.; BERZOFSKY, J.A. & GOOD, M.F., 1989. Human T cell recognition of polymorphic epitopes from malaria circumsporozoite protein. *J. Immunol., 142:* 4000-4005.

DEL PORTILLO, H.A.; NUSSENZWEIG, R.S. & ENEA, V., 1987. Circumsporozoite gene of a *Plasmodium falciparum* strain from Thailand. *Mol. Biochem. Parasitol., 24:* 289-294.

DONTFRAID, F.; COCHRAN, M.A.; POMBO, D.; KNELL, J.D.; QUAKYI, I.A.; KUMAR, S.; HOUGHTEN, R.A.; BERZOFSKY, J.A.; MILLER, L.H.& GOOD, M.F., 1988. Human and murine CD4 T cell epitopes map to the same region of the malaria circumsporozoite protein: Limited immunogenicity of sporozoites and circumsporozoite protein. *Mol. Biol. Med., 5:* 185-196.

DOOLAN, D.L.; HOUGHTEN, R.A. & GOOD, M.F., 1991a. Assessment of human cytotoxic T cell activity using synthetic peptides: potential for field application. *Peptide Res., 4:* 125-131.

DOOLAN, D.L.; HOUGHTEN, R.A. & GOOD, M.F., 1991b. Location of human cytotoxic T cell epitopes within a polymorphic domain of the *Plasmodium falciparum* circumsporozoite protein. *Int. Immunol., 3:* 511-516.

DOOLAN, D.L.; SAUL, A.A. & GOOD, M.F., 1992. Geographically restricted heterogeneity of the *Plasmodium falciparum* circumsporozoite protein; relevance for vaccine development. *Infect. Immun., 60:* 675-682.

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.; SCHNEIER, 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.

FLYNN, J.L.; WEISS, W.R.; NORRIS, K.A.; SEIFERT, H.S. & KUMAR, S., 1990. Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen delivery system. *Mol. Microbiol., 4:* 2111-2118.

GOOD, M.F.; BERZOFSKY, J.A.; MALOY, W.L.; HAYASHI, Y.; FUJII, N.; HOCKMEYER, W.T. & MILLER, L.H., 1986. Geneti control of the immune response in mice to a *Plasmodium falciparum* sporozoite vaccine. Widespread nonresponsiveness to single malaria T epitope in highly repetitive vaccine. *J. Exp. Med., 164:* 655-660.

GOOD, M.F.; MALOY, W.L.; LUNDE, M.N.; MARGALIT, H.; CORNETTE, J.L.; SMITH, G.L.; MOSS, B.; MILLER, L.H. & BERZOFSKY, J.A., 1987. Construction of synthetic immunogen: use of new T helper epitope on malaria circumsporozoite protein. *Science, 235:* 1059-1062.

GOOD, M.F.; POMBO, D.; QUAKI, I.A.; RILEY, E.M.; HOUGHTEN, R.A.; MENON, R.; ALLING, D.W.; BERZOFSKY, J.A. & MILLER, L.H., 1988a. Human T cell recognition of the circumsporozoite protein of *Plasmodium falciparum.* Immunodominant T cell domains map to the polymorphic regions of the molecule. *Proc. Acad. Sci. USA, 85:* 1199-1203.

GOOD, M.F.; MILLER, L.H.; KUMAR, S.; QUAKYI, I.A.; KEISTER, D.; ADAMS, J.H.; MOSS, B.; BERZOFSKY, J.A. & CARTER, R., 1988b. Limited immunological recognition of critical malaria vaccine candidate antigens. *Science, 242:* 574-577.

GOOD, M.F.; KUMAR, S.; DE GROOT, A.S.; WEISS, W.R.; QUAKYI, I.A.; DONTFRAID, F.; SMITH, G.E.; COCHRAN, M. & BERZOFSKY, J.A., 1990. Evidence implicating MHC genes in the immunological nonresponsiveness to the *Plasmodium falciparum* CS protein. *Bull. WHO, 68 (Suppl):* 80-84.

HOFFMAN, S.L.; OSTER, C.N.; PLOWE, C.V.; WOOLLETT, G.R.; BEIER, J.C.; CHULAY, J.D.; WIRTZ, R.A.; HOLLINDGALE, M.R. & MUGAMBI, M., 1987. Naturally acquired antibodies to sporozoites do not prevent malaria: vaccine development implications. *Science, 237:* 639-642.

HOFFMAN, S.L.; ISENBARGER, D.; LONG, C.W.; SEDEGAH, M.; SZARFMAN, A.; WATERS, L; HOLLINGDALE, M.R.; VAN DER MEIDE, 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.

HOLLINGDALE, M.R.; ZAVALA, F.; NUSSENZWEIG, R.S. & NUSSENZWEIG, V., 1982. Antibodies to the protective antigens of *Plasmodium berghei* sporozoites prevent entry into cultured cells. *J. Immunol., 128:* 1929-1930.

HOSMALIN, A.; CLERICI, M.; HOUGHTEN, R.A.; PENDLETON, C.D.; FLEXNER, C.; LUCEY, D.R.; MOSS, B.; GERMAIN, R.N.; SHEARER, G.M. & BERZOFSKY, J.A., 1990. An epitope in human immunodeficiency virus reverse transcriptase recognized by both mouse and human cytotoxic T lymphocytes. *Proc. Nat Acad. USA, 87:* 2344-2348.

KHUSMITH, 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.

KUMAR, S.; MILLER, L.H.; QUAKYI, I.A.; KEISTER, D.B.; HOUGHTEN, R.A.; MALOY, W.L.; MOSS, B.; BERZOFSKY, J.A. & GOOD, M.F., 1988. Cytotoxic T cells specific for the circumsporozoite protein of *Plasmodium falciparum. Nature* (London), *334:* 258-260.

LOCKYER, M.J.; MARSH, K. & NEWBOLD, C.I., 1989. Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T-cell epitopes of the circumsporozoite protein. *Mol. Biochem. Parasitol., 37:* 275-280.

LOCKYER, M.J. & SCHWARTZ, R.T., 1987. Strain variation in the circumsporozoite protein gene of *Plasmodium falciparum. Mol. Biochem. Parasitol., 22:* 101-108.

MALIK, A.; EGAN, J.E.; HOUGHTEN, R.A.; SADOFF, J.C. & HOFFMAN, S.L., 1991. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. *Proc. Natl Acad. Sci. USA, 88:* 3300-3304.

McCUTCHAN, T.F. & WATERS, A.P., 1990. Mutations with multiple independent origins in surface antigens mark the targets of biological selective pressure. *Immunol. Lett., 25:* 23-26.

MULLIGAN, H.W.; RUSSEL, P.F. & MOHAN, B.N., 1941. Active immunization of fowls against *Plasmodium gallinaceum* by injections of killed homologous sporozoites. *J. Mal. Inst. India, 4:* 25-34.

NUSSENZWEIG, R.S. & NUSSENZWEIG, V., 1984. Development of sporozoite vaccines. *Phil. Trans. R. Soc. Lond., 307:* 117-128.

NUSSENZWEIG, V. & NUSSENZWEIG, R.S., 1990. Sporozoite malaria vaccine: where do we stand? *Ann. Parasit. Hum. Compr., 65 (Suppl):* 49-52.

NUSSENZWEIG, R.S.; VANDERBERG, J.P.; MOST, H. & ORTON, C., 1969. Protective immunity produced by the injection of X-irradiated sporozoites of *Plasmodium berghei.* III. Specificity of the protection. *Nature* (London), *222:* 488-489.

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.

RIECKMANN, K.H.; BEAUDOIN, R.L.; CASSELS, J.S. & SELL, K.W., 1979. Use of attenuated sporozoites in the immunization of human volunteers against falciparum malaria. *Bull. WHO (Suppl), 57:* 261-265.

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:* 323-326.

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 *Salmonela typhimurium* vaccine expressing circumsporozoite protein protects against malaria. *Science, 240:* 336-338.

SCHOFIELD, L.; VILLAQUIRAN, J.; FERREIRA, A.; SCHELLEKENS, H.; NUSSENZWEIG, R. & NUSSENZWEIG, V., 1987. γ interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. *Nature, 330:* 664-666.

SPITALNY, G.L.; VERHAVE, J.P.; MEUWISSEN, T.H. & NUSSENZWEIG, R.S., 1977. *Plasmodium berghei:* T cell dependence of sporozoite-induced immunity in rodents. *Exp. Parasitol., 42:* 73-81.

VERHAVE, J.P.; STRICKLAND, G.T.; JAFFE, H.A. & AHMED, A., 1978. Studies of the transfer of protective immunity with lymphoid cells from mice immunne to malaria sporozoites. *J. Immunol., 121:* 1031.

WEISS, W.R.; GOOD, M.F.; HOLLINGDALE, M.R.; MILLER, L.H. & BERZOFSKY, J.A., 1989. Genetic control of immunity to *Plasmodium yoelii* sporozoites. *J. Immunol., 143:* 4263-4266.

WEISS, W.R.; MELLOUK, S.; HOUGHTEN, R.A.; SEDEGAH, M.; KUMAR, S.; GOOD, M.F.; BERSOFSKY, J.A.; MILLER, L.H. & HOFFMAN, S.L., 1990. Cytotoxic T cells recognize a peptide from the circumporozoite protein on malaria-infected hepatocytes. *J. Exp. Med., 171:* 763-773.

WEISS, W.R.; SEDEGAH, M.; BEAUDOIN, R.L.; MILLER, L.H. & GOOD, M.F., 1988. $CD8^+$ T cells (cytotoxic/suppressor) are required for protection in mice immunized with malaria sporozoites. *Proc. Natl Acad. Sci. USA, 85:* 573-576.

YOSHIDA, N.; DI SANTI, S.M.; DUTRA, A.P.; NUSSENZWEIG, R.S.; NUSSENZWEIG, V. & ENEA, V., 1990. *Plasmodium falciparum:* restricted polymorphism of T cells epitopes of the circumsporozoite protein in Brazil. *Exp. Parasitol., 71:* 386-392.

ZEVERING, Y.; HOUGHTEN, R.A; FRAZER, I.H. & GOOD, M.F., 1990. Major population differences in T cell response to malaria vaccine candidate. *Int. Immunol., 2:* 945-955.