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T CELL RESPONSES TO REPEAT AND NON-REPEAT REGIONS OF THE CIRCUMSPOROZOITE PROTEIN DETECTED IN VOLUNTEERS IMMUNIZED WITH *PLASMODIUM FALCIPARUM* SPOROZOITES

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The design of a malarial vaccine based on the circumsporozoite (CS) protein, a major surface antigen of the sporozoite stage of the malaria parasite, requires the identification of T and B cell epitopes for inclusion in recombinant or synthetic vaccine candidates. We have investigated the specificity and function of a series of T cell clones, derived from volunteers immunized with Plasmodium falciparum *sporozoites, in an effort to identify relevant epitopes in the immune response to the pre-erythrocytic stages of the parasite. CD4+ T cell clones were obtained which specifically recognized a repetitive epitope located in the 5' repeat region of the CS protein. This epitope, when conjugated to the 3' repeat region in a synthetic MAPs construct, induced high titers of antisporozoite antibodies in C57B1 mice. A second T cell epitope, which mapped to aa 326-345 of the carboxy terminal, was recognized by lytic, as well as non-lytic, CD4+ T cells derived from the sporozoite-immunized volunteers. The demonstration of CD4+ CTL in the human volunteers, and the recent studies in the rodent model (Renia et al., 1991; Tsuji et al., 1990), suggest that CS-specific CD4+ T cells, in addition to their indirect role as helper cells in the induction of antibody and CD8+ effector cells, may also play a direct role in protection against sporozoite challenge by targeting EEF within the liver.*

Key words: malaria sporozoites – CS protein – human CD4+ T cells – epitopes – vaccines

The demonstration that sporozoite-immunized human volunteers, as well as rodents and monkeys, could be protected against malaria has provided the impetus for research on sporozoite-induced immune mechanisms and vaccine development (Nussenzweig & Nussenzweig, 1989). The unique aspects of the *Plasmodium* life cycle render the sporozoite susceptible to both humoral and cellular effector mechanisms. The identification T cell epitopes recognized by helper and cytotoxic T cells has, therefore, become a priority for the design of effective malaria vaccines.

In an effort to characterize the cell-mediated immune responses to malaria sporozoites, we have derived T cell lines and clones from several volunteers immunized by multiple exposures to the bites of irradiated *P. falciparum* infected mosquitoes (Herrington et al., 1991). A T cell line was derived from one volunteer, by stimulating his PBL *in vitro* with a recombinant *P. falciparum* circumsporozoite (rPfCS) protein (Nardin et al., 1989). These CD4+ T cells proliferated and secreted gamma interferon when challenged with the rPfCS protein. The yeast-derived recombinant protein contains approximately 70% of the total CS sequence, including the entire repeat region consisting of 5' repeats of alternating NANP and NVDP sequences and a 3' repeat containing only the NANP tetramer (Nardin et al., 1990). The CD4+

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T cell clones also recognized the native CS protein contained in extracts of *P. falciparum* sporozoites but not extracts of other malarial species.

Mapping of the T cell epitope recognized by the sporozoite specific CD4+ T cell clones was carried out using truncated recombinant CS proteins produced in *Escherichia coli*. The ability of the recombinants to stimulate the T cell clones depended on the presence of the 5' repeat sequence consisting of alternating NANP and NVDP tetramers. Deletion of the 5' repeats abrogated the T cell response.

The identification of the T cell epitope within the 5' repeat region was confirmed using a 12-mer synthetic peptide that contained the NANPNVDPNANP 5' repeat sequence which stimulated the T cell clone to the same extent as the rPfCS protein. A peptide containing the 3' repeat (NANP)3 sequence, which contains the immundominant B cell epitope of the *P. falciparum* CS protein (Zavala et al., 1985), did not induce proliferation or gamma interferon production by the T cell clones.

The 3' and the 5' repeat sequences of the CS protein are conserved in all isolates of *P. falciparum* sequenced to date (Dame et al., 1984; del Portillo et al., 1987; Lockyer & Schwarz, 1987; Caspers et al., 1989). Therefore, vaccines based on the repeats would be expected to generate an immune response effective in different endemic areas. In order to investigate the role of the T cell epitope in the anti-parasite immunity, we examined the ability of a synthetic vaccine containing the 5' repeat T cell epitope when combined with the 3' repeats, to induce an antisporozoite antibody response in mice (Munesinghe et al., 1991).

The mice were immunized with a synthetic peptide polymer called the Multiple Antigen Peptide system (MAPs), which consists of a poly-lysine core matrix on which peptides are synthesized to form dendritic arms or branches (Tam, 1988). MAPs containing T and B cell epitopes of the CS protein of *P. berghei* rodent malaria have been shown to induce high levels of antisporozoite antibodies and protect immunized mice against viable sporozoite challenge (Tam et al., 1990).

The *P. falciparum* MAPs used to immunize the mice contains equimolar ratios of the *P. falciparum* T and B cell repeat epitopes in each one of its four branches or arms (Fig. 1). The antigenicity of the T cell epitope was not altered by incorporation into MAPs. The human T cell clones, derived from the sporozoite-immunized volunteer, proliferated and produced gamma interferon when challenged with all the MAPs constructs containing the 5' repeats regardless of configuration (Munesinghe et al., 1991).

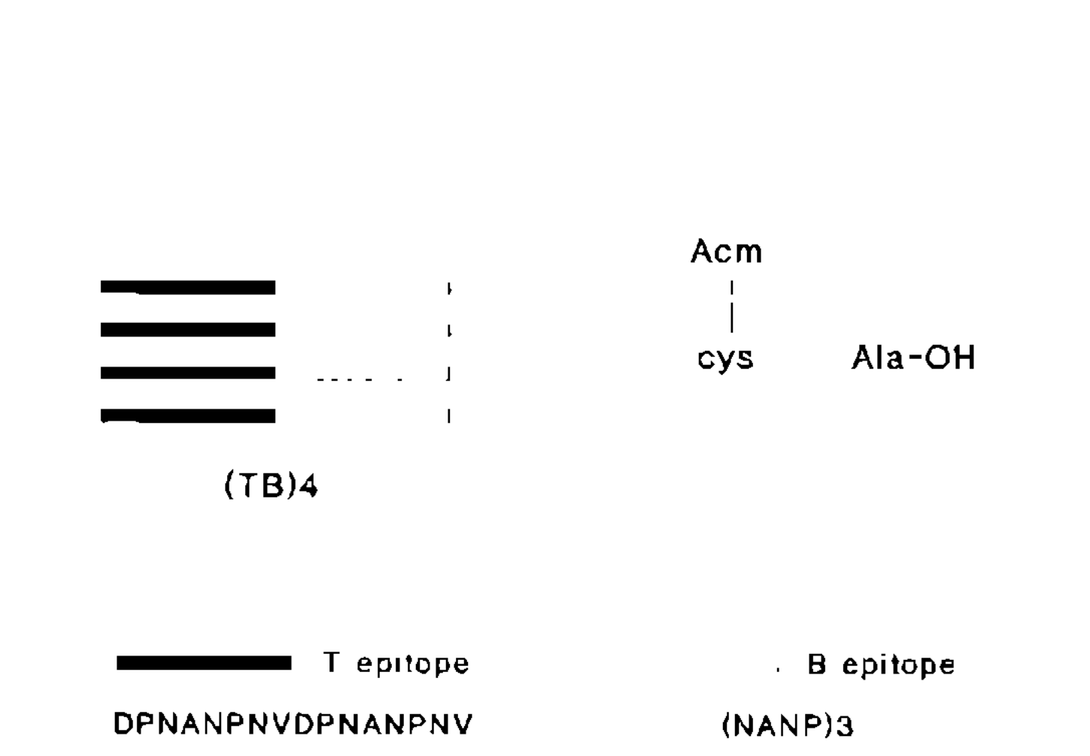

Fig. 1: a schematic representation of the (TB)4 MAP construct which contains equimolar ratios of T and B cell epitopes in each one of the four branches linked to the $(lys)_3$-cys-ala core. The DPNANPNVDPNANPNV sequence represents the T cell epitope located in the 5' repeat region and $(NANP)_3$ represents the B cell epitope located in the 3' repeats of the *Plasmodium falciparum* CS protein.

Based on a series of MAPs constructs having different orientations and molar ratios of T and B cell epitopes, the (TB)4 construct was found to be optimally immunogenic (Fig. 2). Following immunization with MAPs, mice of four different inbred strains behaved either as high, intermediate or non-responders. Balb/c mice belonged to this last category, since they failed to produce antibody when immunized with any of the MAPS constructs containing the 3' and 5' repeats of the *P. falciparum* CS protein.

After the first immunizing dose of (TB)4 in Freund's Adjuvant, mice of the three responder strains, C57B1 (H-2b), A/J (H-2a) and C3H (H-2k), developed anti-repeat antibodies and this response increased after the subsequent boosters on day 21 and 42. The anti-repeat antibody response in the intermediate responder stains suggests that mice of the H-2k and H-2a haplotypes recognize a T helper epitope within

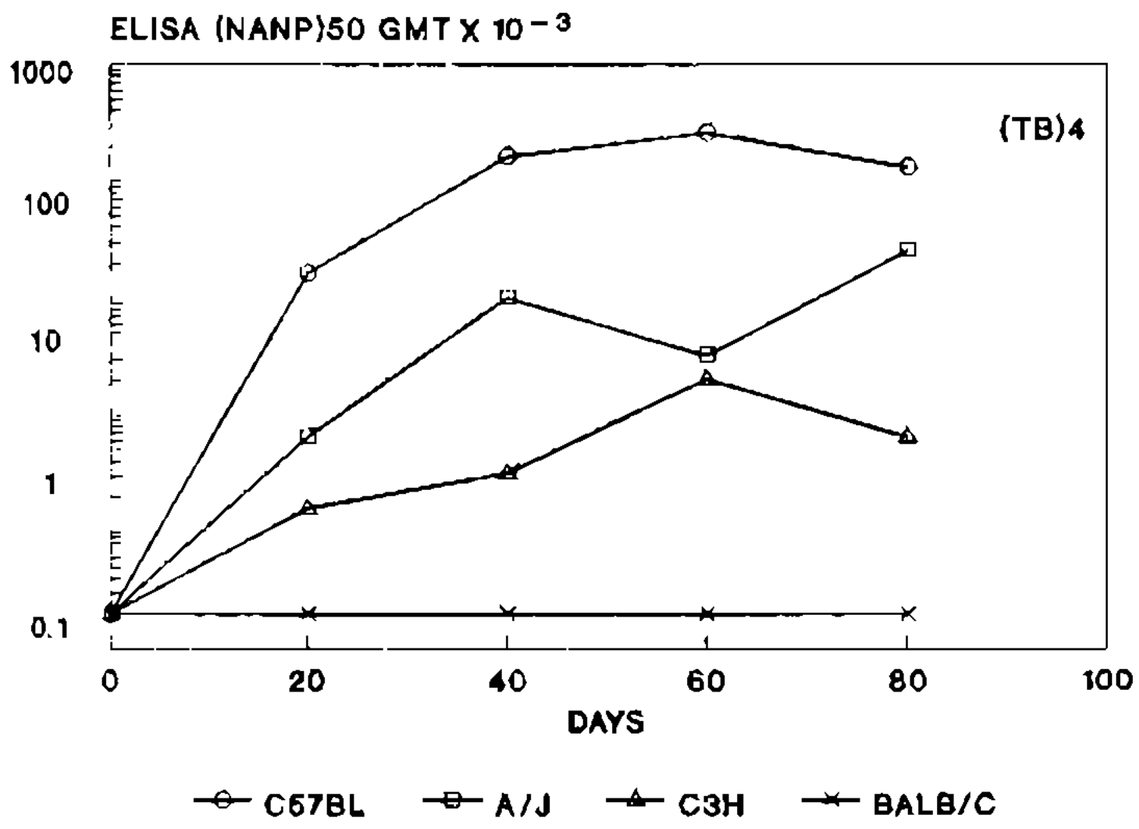

Fig. 2: the ELISA geometric mean titers (GMT) x 10^{-3} of sera obtained from four inbred strains of mice immunized with 50µg of (TB)4 MAPs emulsified in Freund's Adjuvant, administered on day 0, 21 and 42. Sera from four mice in each group, obtained 20 days following each immunizing dose, was assayed in the ELISA using (NANP)50 as antigen. (from Munseinghe et al., 1991).

the 5' repeat region, since only the H-2b haplotype can recognize a T cell epitope in the 3' repeats (Del Giudice et al., 1986; Good et al., 1986).

The anti-peptide antibodies generated by MAPs immunization were highly cross-reactive with the native CS protein on the sporozoite surface as shown by the indirect immunofluorescent (IFA) titers. In the high responder strain, a geometric mean IFA titer of 826,000 was obtained in the sera of C57B1 mice, with peak IFA titers reaching 1 million in sera of some individual mice.

Therefore, the studies with the MAPs constructs have shown that the 5' repeat region of the CS protein is recognized by T helper cells that function in the production of antisporozoite antibodies. However, in addition to CD4+ T helper cell epitopes, the CS protein of *P. falciparum* is also recognized by CD8+ T cells obtained from sporozoite-immunized human volunteers (Malik et al., 1991). Cytotoxic cells specific for the CS protein of several rodent malarias can passively protective naive recipients against sporozoite challenge (Romero et al., 1989, Rodrigues et al., 1991).

In an effort to detect cytotoxic T cells, we screened PBL obtained from the sporozoite-immunized volunteers for cytotoxic activity. PBL of one volunteer, obtained after he had successfully resisted challenge with infective *P. falciparum* sporozoites, lysed 34% of the target cells pulsed with a pool of synthetic peptides that covered the entire *P. falciparum* CS protein sequence (Moreno et al., 1991).

Six of the ten sublines derived from this line displayed high levels of lytic activity and the response of a representative clone is shown (Fig. 3). When T cells were incubated with ^{51}Cr-labelled target cells pulsed with a peptide pool covering aa 1 - 405 of the CS protein, 82% of the target cells were lysed.

In order to identify the epitope recognized by the CTL, target cells were pulsed with pools of 10-20 peptides representing either the amino terminal, the repeats, or the carboxy terminal sequences of the *P. falciparum* protein. High levels of lytic activity were obtained only when target cells were pulsed with a pool of peptides representing the carboxy terminal sequence aa 281-345. When the target cells were pulsed with each one of the eight peptides contained in the aa 281-345 pool, only the target cells pulsed with the 20-mer peptide containing the amino acid sequence EYLNKIQNSLSTEWSPCSVT, aa 326-345, were lysed. The degree of lysis was similar to that observed using target cells pulsed with the entire CS peptide pool (aa 1 - 405).

The phenotype of the lytic T cells was found to be predominantly CD4+ and consistent with this phenotype, the lytic, as well as the proliferative, responses of the lines and clones were found to be class II restricted. Peptide pulsed cells that were either matched or mismatched with the HLA DR 1,7 determinants of the T cell donor were tested for ability to stimulate the T cell clones. Only the peptide cells that expressed the class II DR 7 allele were lysed by the CTL. The CTL also showed high levels of proliferation when the 326-345 peptide was presented by autologous APC cells or by cells expressing DR 5,7, but not the DR 1,4 alleles.

Therefore, the class II restricted CD4+ CTL recognize an epitope in the C terminal region of the *P. falciparum* CS protein that is distinct from the epitope recognized by the class I restricted CD8+ CTL (KPKDELDYENDIEKKICKMEKCS) recently identified using cells obtained from sporozoite-immunized rodents and human volunteers (Kumar et al., 1988; Malik et al., 1991).

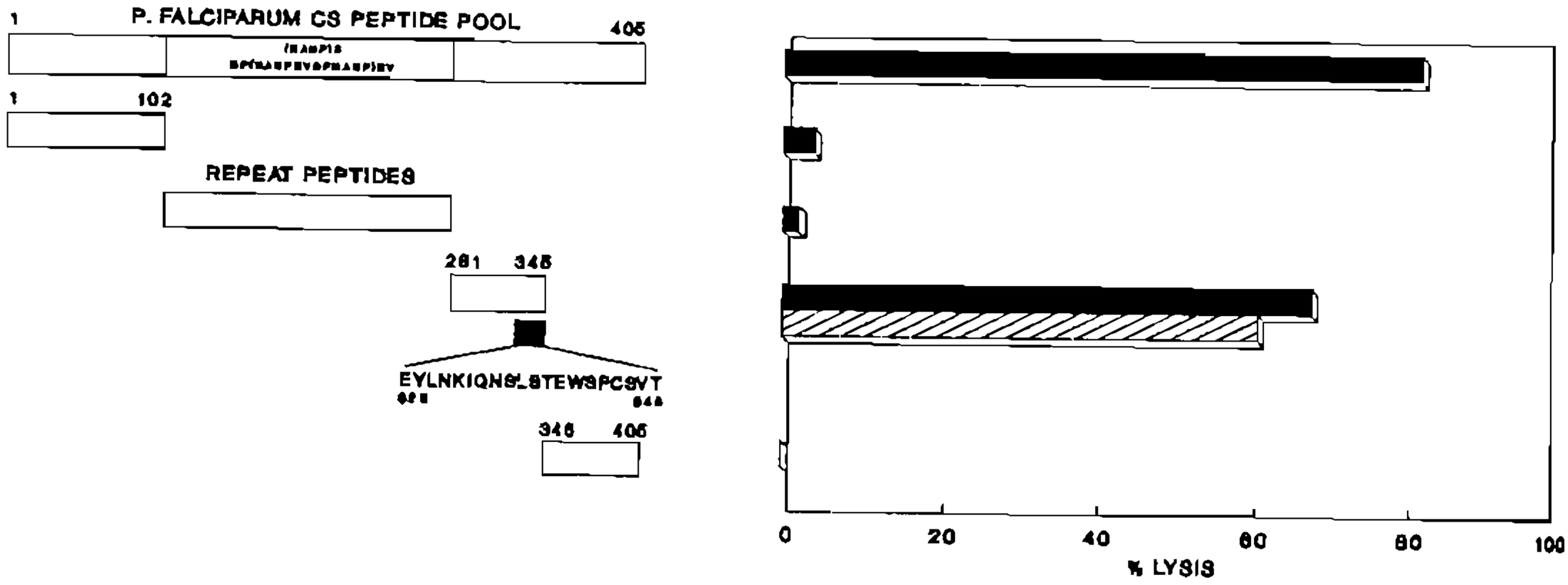

Fig. 3: peptide specificity of a representative cytolytic CD4+ T cell clone. The results are shown as percent lysis, at an E:T ratio of 50:1, of ^{51}Cr-labelled autologous B cells targets pulsed with peptides representing different regions of the *Plasmodium falciparum* CS protein. Numbering of amino acids is based on the CS protein sequence of the NF 54 strain of *P. falciparum* (Caspers et al., 1989). The dark bars represent lysis obtained using target cells pulsed with a pool of peptides representing the entire CS sequence (aa 1-405) or with smaller peptide pools representing the N-terminal (aa 1-102), repeat regions (5' and 3' repeat sequences) or the C terminal sequences (aa 281-345 and aa 346-405). The hatched bar indicates the lysis obtained using target cells pulsed with peptide aa 326-345. The amino acid sequence is shown in single letter code (from Moreno et al., 1991).

The lytic activity of the human CD4+ T cell clones, along with the recent demonstration that mice could be protected against sporozoite challenge following passive transfer of cytotoxic CD4+ T cells specific for the *P. yoelli* CS protein (Del Giudice et al., 1990; Renia et al., 1991), or a non-CS antigen that is expressed in both sporozoites and blood stages of *P. berghei* (Tsuji et al., 1990), would suggest that CD4+ T cells may play a direct role in protection against sporozoite challenge, in addition to their indirect role as helper cells in the induction of antibody and CD8+ effector cell mechanisms of immunity.

REFERENCES

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

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

DEL GIUDICE, G.; COOPER, J.; MERINO, J.; VERDINI, A.; PESSI, A.; TOGNA, A.; ENGERS, H.; CORRADIN, G. & LAMBERT, P., 1986. The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is I-A^b restricted: Possible implications for malaria vaccines. *J. Immunol., 137:* 2952-2955.

DEL GIUDICE, G.; GRILLOT, D.; RENIA, L.; MULLER, I.; CORRADIN, G.; LOUIS, J.; MAZIER, D. & LAMBERT, P-H, 1990. Peptide-primed CD4+ cells and malaria sporozoites. *Immunol. Lett., 25:* 59-63.

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

GOOD, M.; BERZOFSKY, J.; MALOY, W.; HAYASHI, Y.; FUJII, N.; HOCKMEYER, W. & MILLER, L., 1986. Genetic 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.

HERRINGTON, D.; DAVIS, J.; NARDIN, E.; BEIER, M.; CORTESE, J.; EDDY, H.; LOSONSKY, G.; HOLLINGDALE, M.; SZTEIN, M.; LEVINE, M.; NUSSENZWEIG, R.; CLYDE, D. & EDELMAN, R., 1991. Successful immunization of humans with irradiated sporozoites: Humoral and cellular responses of the protected vaccinees. *Am. J. trop. Med. Hyg., 45:* 539-547.

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

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

MALIK, A.; EGAN, J.; HOUGHTEN, R.; SADOFF, J. & HOFFMAN, S., 1991. Human cytotoxic T lymphocytes against *Plasmodium falciparum* circumsporozoite protein. *Proc. Natl. Acad. Sci. U.S.A., 88:* 3300-3305.

MORENO, A.; CLAVIJO, P.; DAVIS, J.; EDELMAN, R.; SZTEIN, M.; HERRINGTON, D. & NARDIN, E., 1991. Cytotoxic CD4+ T cells from a sporozoite-

immunized volunteer recognize the *P. falciparum* CS protein. *Int. Immunol., 3:* 997-1003.

MUNESINGHE, D.; CLAVIJO, P.; CALLE, M.; NUSSENZWEIG, R. & NARDIN, E., 1991. Immunogenicity of multiple antigen peptides (MAPs) containing T and B cell epitopes located within the repeat region of the *P. falciparum* circumsporozoite protein. *Eur. J. Immunol., 21:* 3015-3020.

NARDIN, E.; HERRINGTON, D.; DAVIS, J.; LEVINE, M.; STUBER, D.; CASPERS, P.; BARR, P.; ALTSZULER, R.; CLAVIJO, P. & NUSSENZWEIG, R. S., 1989. Conserved repetitive epitope recognized by CD4+ clones from a malaria-immunized volunteer. *Science, 246:* 1603-1606.

NARDIN, E.; NUSSENZWIEG, R.; ALTSZULER, R.; HERRINGTON, D.; LEVINE, M.; MURPHY, J.; DAVIS, J.; BATHURST, I.; BARR, P.; ROMERO, P. & ZAVALA, F., 1990. Cellular and humoral immune responses to a recombinant *Plasmodium falciparum* CS protein in sporozoite-immunized rodents and human volunteers. *Bull. WHO Suppl., 68:* 85-87.

NUSSENZWEIG, V. & NUSSENZWEIG, R.S., 1989. Rationale for the development of an engineered sporozoite malaria vaccine. *Adv. Immunol., 45:* 283-334.

RENIA, L.; MARUSSIG, M.; GRILLOT, D.; PIED, S.; CORRADIN, G.; MILTGEN, F.; DEL GIUDICE, G. & MAZIER, D., 1991. *In vitro* activity of CD4+ and CD8+ T lymphocytes from mice immunized with a synthetic malaria peptide. *Proc. Natl Acad. Sci. U.S.A., 88:* 7963-7967.

RODRIGUES, M; CORDEY, A-S.; ARREAZA, G.; CORRADIN, G.; ROMERO, P.; MARYANSKY, J.; NUSSENZWEIG, R. & ZAVALA, F., 1991. CD8+ cytolytic T cell clones derived against the *Plasmodium yoelii* circumsporozoite protein protect against malaria. *Int. Immunol., 3:* 579-585.

ROMERO, P.; MARYANSKY, J.; CORRADIN, G.; NUSSENZWEIG, R.; NUSSENZWEIG, V. & ZAVALA, F., 1989. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature, 341:* 323-326.

TAM, J., 1988. Synthetic peptide vaccine design: Synthesis and properties of a high-density multiple antigenic peptide system. *Proc. Natl Acad. Sci. U.S.A., 85:* 5409-5413.

TAM, J.; CLAVIJO, P.; LU, Y-A; NUSSENZWEIG, V.; NUSSENZWEIG, R. & ZAVALA, F., 1990. Incorporation of T and B epitopes of the circumnsporozoite protein in a chemically defined synthetic vaccine against malaria. *J. Exp. Med., 171:* 299-306.

TSUJI, M.; ROMERO, P.; NUSSENZWEIG, R. ZAVALA, F., 1990. CD4+ cytolytic T cells clone confers protection against murine malaria. *J. Exp. Med., 172:* 1353-1357.

ZAVALA, F.; TAM, J.; HOLLINGDALE, M. ; COCHRANE, A.; QUAKYI, I.; NUSSENZWEIG, R. & NUSSENZWEIG, V., 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science, 228:* 1436-1440.