INCORPORATION OF T AND B EPITOPES OF THE CIRCUMSPOROZOITE PROTEIN IN A CHEMICALLY DEFINED SYNTHETIC VACCINE AGAINST MALARIA

By JAMES P. TAM,* PEDRO CLAVIJO,‡ YI-AN LU,* VICTOR NUSSENZWEIG,§ RUTH NUSSENZWEIG,‡ AND FIDEL ZAVALA‡

*From *The Rockefeller University, New York, New York 10021; and New York University School of Medicine, ‡Department of Medical and Molecular Parasitology and §Department of Pathology, New York, New York 10010*

Multiple antigen peptide systems (MAPs)[1] for peptide-based vaccines were designed by one of us to overcome the ambiguity and requirement of conjugation to a protein carrier by synthetic peptides (1, 2). The MAP system consists of an oligomeric branching lysine core, usually comprised of seven lysines, and eight dendritic arms of peptides containing antigenic epitopes. Since each arm of peptide may consist of 10 to more than 20 amino acids, the overall appearance of the MAP system is of a macromolecule with a high density of surface peptide antigen and a molecular weight exceeding 10,000, surrounding a lysine scaffolding core of molecular weight of <800.

The immunogenicity of monoepitope MAPs has been tested in animals (1, 2). All model MAPs elicited high-titered antisera, most of which recognized the cognate protein from which the epitopes were derived. However, some issues concerning the possible use of the MAP system for production of vaccines were not resolved. One important remaining question is whether the MAPs can be engineered to contain not only B cell epitopes but also functional T cell epitopes, selected from protein molecules that are candidates for development of subunit vaccines. In addition, the effect of the stoichiometry, orientation, and arrangement of the B and T epitopes on the immunogenicity of the MAPs has not been determined.

To study these problems we prepared 10 MAP models containing T and B epitopes of the circumsporozoite (CS) protein of the rodent malaria parasite, *Plasmodium berghei*. The immunodominant B cell epitope of the *P. berghei* CS protein is contained within its repeat domain and can be represented by a 16-residue peptide $(PPPNPND)_2$ (3, 4). Monoclonal and polyclonal antibodies against the repeat domains of the CS protein neutralize in vitro and in vivo the infectivity of malaria

This work was supported by the Agency for International Development (DPE 0453-A-00-5012-00), National Institutes of Health grant AI-28701, the MacArthur Foundation, and Bachem, USA. F. Zavala is a recipient of an Irma T. Hirschl Career Development Award.

Address correspondence to Dr. James P. Tam, The Rockefeller University, 1230 York Avenue, Box 294, New York, NY 10021.

[1] *Abbreviations used in this paper:* CS, circumsporozoite; IFA, immunofluorescence assay; IRMA, immunoradiometric assay; MAPs, multiple antigen peptide systems.

sporozoites (reviewed in reference 5). Recently, several T helper epitopes of the *P. berghei* CS protein have been identified, thus providing the opportunity not only to develop di-epitope MAPs, but also to test their efficacy as vaccines in a well-characterized rodent malaria model. One of these T helper epitopes, between residues 265 and 276 (KQIRDSITEEWS), was selected for inclusion in the di-epitope MAPs because it was recognized by several inbred strains of mice and particularly because it displayed helper activity in vivo (6).

Materials and Methods

Synthesis and Characterization of MAPs. The monomeric BT peptide was synthesized as described (6) by a stepwise solid-phase peptide synthesis method of Merrifield (7). MAPs were synthesized as described, on a tertbutoxycarbonyl (Boc)-Ala-Pam resin with a low loading (0.1 mmol/g of resin) (1). In the MAP models containing four copies of the peptide antigens [T-(4), B-(4), BT-(4), and TB-(4)], the core was synthesized with only three lysines prepared from branching with two levels of Boc-Lys(Boc), while in the MAP models containing eight copies of the antigens [T-(8), B-(8), BT-(8), and TB-(8)], the core was synthesized with seven lysines prepared from branching with three levels of Boc-Lys(Boc). In the MAP models containing eight copies of B or T antigens and one copy of T or B antigen [B-(8)-T, T-(8)-B], the B or T antigen was first synthesized linearly on the Boc-Ala-Pam resin and then branched with three levels of lysine to give the subsequent eight copies of T or B antigens. All peptides and MAPs were cleaved from the resin supports by the low/high HF procedure (8) to minimize side reactions, and the peptides were extracted from the resin with, and extensively dialyzed in, 8 M urea in 0.1 M Tris/HCl buffer, pH 8.0. All MAP products were characterized by high-performance gel chromatography. Amino acid analysis gave satisfactory results that agreed with the expected composition.

Immune Responses of MAPs. Groups of five mice of the H-2^a haplotype (A/J strain) were injected with 50 μg i.p. of a given MAP, or a mixture of MAPs [T-(8) + B-(8)], emulsified in CFA on day 0, and boosted with 50 μg of the same antigen in IFA on day 21. Sera were collected 21 d later. Sera were pooled and antibody titers were determined by an immunoradiometric assay using a recombinant CS protein as antigen or by indirect immunofluorescence (IFA) using glutaraldehyde-fixed sporozoites as antigen. The titers were expressed as the reciprocal of the highest positive serum dilution.

Results and Discussion

The 10 MAP models (Fig. 1) included tandemly connecting B and T epitopes, with four or eight peptide antigen arms [models BT-(4), and BT-(8)], and similar models in which the orientation of the B and T epitopes was reversed [models TB-(4), TB-(8)]. To test the importance of the stoichiometry of the B and T cell epitopes, model B-(8)-T contained eight copies of the B epitope and only one copy of the T epitope, while model T-(8)-B contained eight copies of the T epitope and only one copy of the B epitope. As controls, we prepared four different models of MAPs containing either B or T cell epitopes alone. Two of the control models [T-(4), B-(4)] contained four copies of either B or T epitopes, and the other two [T-(8), B-(8)] contained eight copies of either epitope. For comparison, a monomeric peptide [BT] containing only one copy each of the B and T epitopes was prepared.

To assay for the presence of the B epitope in the MAPs, we used the 3D11 monoclonal antibody (9) raised against *P. berghei* sporozoites and which reacts with the 16 amino acid B epitope (10). All MAPs containing the B epitopes were strong inhibitors of the binding of radiolabeled 3D11 to sporozoites immobilized in wells of microtiter plates. 50% inhibition of binding was observed at MAPs concentrations

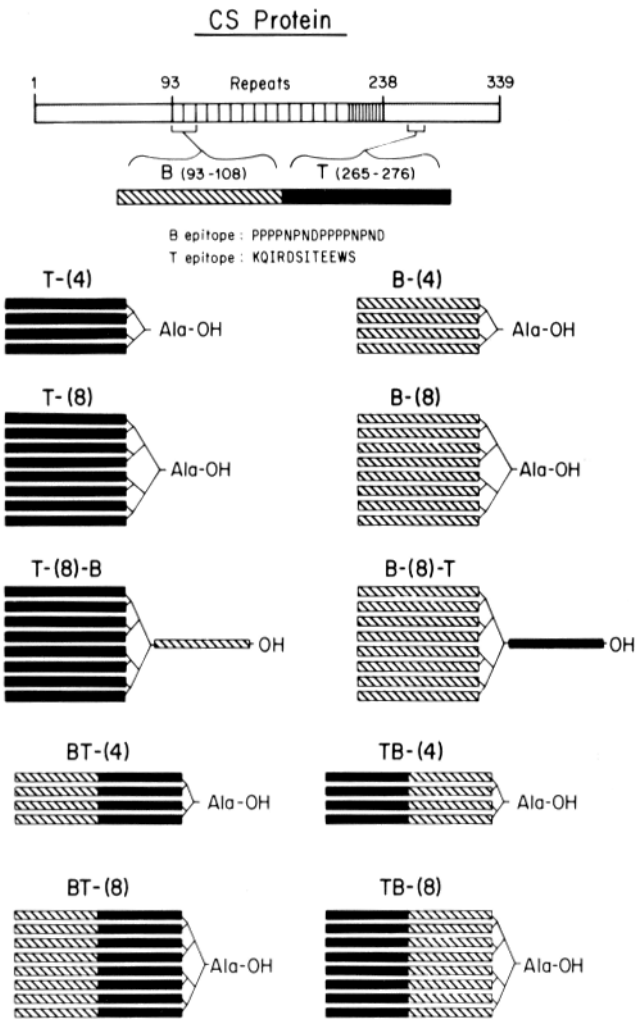

FIGURE 1. Schematic representation of the structure of the CS protein of *P. berghei* of the monomeric form of a peptide containing tandem B and T cell epitopes and of 10 MAP models (see text for explanation).

of 10^{-10} to 10^{-13} M, while the same degree of inhibition required $\sim 10^{-8}$ M of the monomeric peptide BT (data not shown). The MAPs containing only the T epitope were noninhibitory. These results indicated that the B epitopes in the MAPs functioned as antigens.

We had earlier shown that immunization with the *P. berghei* B epitope alone, either as a monomer or as an octameric MAP, did not elicit antibody responses in A/J and several other inbred strains of mice (6). To test for the capacity of the newly designed MAP antigens to elicit the production of antibodies to this B epitope, groups of five A/J mice were immunized with each of the 10 models of MAPs. Additional control groups were immunized with a BT monomer, or with a mixture of equal amounts of B-(8) and T-(8). The pooled sera from each group of animals were assayed for their reactivities with the recombinant CS protein by immunoradiometric

assay (IRMA) and with glutaraldehyde-fixed sporozoites by indirect immunofluorescence assay (IFA).

The primary antibody responses, measured 3 wk after the first antigen dose, showed that the MAPs that contained equimolar T and B epitopes, linked in tandem, were highly immunogenic. The best immunogen, BT-(4), produced serum antibody levels that were detectable at dilutions greater than 10^5. The secondary antibody responses observed 21 d (Fig. 2) and 34 d after administration of a second dose of the MAPs were significantly higher, but the ranking order of immunogenicity was similar to that observed in the primary responses. The serum titers of mice injected with BT-(4), were greater than 4×10^5, while the other three di-epitope MAPs containing equimolar T and B peptides, elicited titers between 10^5 and 4×10^5. The animals immunized with the mono-epitope B-(4) and B-(8) MAPs did not respond. There was a poor antibody response to T-(8)-B, B-(8)-T, T-(4), and T-(8). TB monomers or a mixture of equimolar amounts of B-(8) and T-(8) failed to elicit an antibody response. Thus, a covalent high molecular weight structure containing multiple copies of tandemly arranged B and T epitopes was required for good immunogenicity.

The IFA titers of the sera 34 d after the booster injection are shown in Table I. The BT(4)-injected mice had IFA titers of 1.28×10^5 against sporozoites. These titers are at least 10 times higher than those usually found in the serum of mice hyperimmunized with irradiated sporozoites. Immunization of A/J mice with similar doses of recombinant *P. berghei* CS protein incorporated in CFA, encompassing amino acids 81–277, resulted in much lower IFA titers (2×10^3) (6). It should be pointed out that although the present results were obtained with the *P. berghei* MAPs incorporated in CFA, high antibody titers were elicited with other MAPs when the adjuvant was alum, or even in the absence of adjuvant (in preparation). A plausible explanation for the greater immunogenicity of the MAPs may be that they contain a high

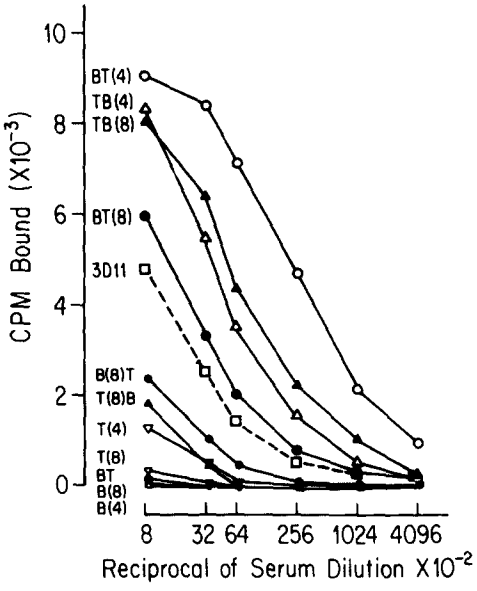

FIGURE 2. Secondary antibody responses of groups of mice immunized with different MAP models.

TABLE I
Protective Efficacy of Different MAP Models in Mice Challenged with 2,000 P. berghei Sporozoites

Immunogen	Antisporozoite (IFA titer × 10^{-3})*	Number‡ protected/challenged	Protection
			%
BT-(4)	128	4/5	80
TB-(4)	32	3/5	60
TB-(8)	32	3/5	60
BT-(8)	8	2/4	50
T-(4)	<0.2	0/4	0
B-(4)	<0.2	0/5	0
B-(8)	<0.2	0/5	0
BT monomer	<0.2	0/5	0
No immunogen	-	0/5	0

* Titer determined by IFA using glutaraldehyde-fixed sporozoites, 34 d after the booster injection of antigen.
‡ Mice were challenged by intravenous inoculations of 2,000 sporozoites 35 d after the booster injection. Peripheral blood smears were examined daily for parasitized erythrocytes. Protection is defined as absence of parasites from day 3 to 12 after challenge.

density and multiple copies of peptide antigens. In the recombinant CS protein, there are single copies of the T epitope and multiple B epitopes. Such an arrangement was simulated in the design of the B-(8)-T model that was a poor immunogen. In addition, as reported for other antigens, the CS protein may contain epitopes that suppress antibody responses (11).

Because the tandemly connected B and T cell epitopes in the MAP are relatively long, it is conceivable that the steric interactions between branches, and the epitope orientations of MAPs, may influence their immunogenicity. For example, the less bulky model BT-(4) produced ~50-fold higher antibody titers than BT-(8). However, there was little difference in immune response between the reversely oriented models TB-(4) and TB-(8). Although the present results indicate that there is no advantage to increasing the number of MAP branches from 4 to 8, the effects on the immune response of the number of the B and T epitopes in the MAP models may be sequence dependent, and may require optimization in a given experimental system. It is noteworthy that the B cell epitope tested in this study is exceptionally rich in proline (50%), while the T cell epitope has a strong propensity for amphipathic helix formation.

The efficacy of the MAPs as vaccines was evaluated by intravenous challenge of the immunized mice with 2,000 *P. berghei* sporozoites 35 d after the booster injection. 80% of the mice immunized with BT-(4) were protected, and in the groups of mice immunized with the other three di-epitopes MAPs, there was 50–60% protection (Table I). The levels of antibodies to sporozoites (IFA) in the vaccinated mice correlated well with the degree of protection. No protection was observed in mice immunized with the mono-epitope MAPs or the monomer BT.

Although the IFA titers of mice hyperimmunized with irradiated *P. berghei* sporozoites are much lower than those after immunization with the di-epitope MAPs, the mice

resist challenge with much higher doses of sporozoites, underscoring the importance of T cell effector mechanisms in protection (12). Early experiments had shown that μ-suppressed mice, which do not make antibodies, can be effectively vaccinated with irradiated sporozoites (13). In this model of immunization, $CD8^+$ cytotoxic T cells are required for protection (14, 15), and their target is most likely the liver stage of the parasite (16, 17). In fact, passive transfer to naive mice of $CD8^+$ cloned T cells recognizing an epitope of the CS protein of *P. berghei* can confer a high degree of protection against challenge with sporozoites (18).

While considerable progress has been made in understanding the mechanisms of protection in murine malaria models, the relative importance of serum antibodies and effector T cells in mediating protection against human malaria sporozoites has not been established (5, 19). Two *P. falciparum* malaria vaccines have undergone human trials. One was a recombinant fusion protein containing multiple copies of NANP (20), the B epitope of the *P. falciparum* CS protein (21), and the other was a synthetic vaccine consisting of $(NANP)_3$ coupled to a tetanus toxoid carrier (22). In both trials, one out of six and one out of three volunteers were protected, although the serum levels of antisporozoite antibodies were low. The frequency and magnitude of the antibody response in the volunteers receiving the synthetic vaccine increased with the dose of the conjugate. Unfortunately, however, the peptide vaccine dose could not be raised due to the toxicity of the tetanus toxoid carrier. It is also conceivable that the immune response to the peptide was suppressed because the volunteers had been previously vaccinated with tetanus toxoid (23). These two problems, carrier toxicity and epitopic suppression, may severely limit the effectiveness of this synthetic malaria vaccine and of other similarly designed vaccines. Both obstacles can be overcome by di-epitope MAPs. In addition, as shown here, these chemically unambiguous structures can be highly immunogenic and engineered to contain at least two and perhaps several different functional B and T epitopes. MAPs may therefore serve as a basis for developing subunit vaccines for diseases in which circulating antibodies play a role in protection.

Summary

We show here an effective and novel approach to engineer peptide-based vaccines using a chemically defined system, known as multiple peptide antigen systems (MAPs), to protect an inbred mouse strain from infection against rodent malaria. 10 mono- and di-epitope MAP models containing different arrangements and stoichiometry of functional B and/or T helper cell epitopes from the circumsporozoite protein of *Plasmodium berghei* were used to immunize A/J mice. While these mice did not respond to the mono-epitope MAP bearing only the B or T epitope, very high titers of antibody and protective immunity against sporozoite challenge were elicited by di-epitope MAPs, particularly those with the B and T epitopes in tandem and present in equimolar amounts. These results, obtained in a well-defined rodent malaria model, indicate that MAPs may overcome some of the difficulties in the development of synthetic vaccines, not only for malaria but also for other infectious diseases.

We thank the joint UNDP/World Bank/WHO Special Programme for Research and Training

in Tropical Diseases and Rockefeller Foundation for their continued support, and Christina Sang for the preparation of the manuscript.

Received for publication 16 August 1989.

References

1. Tam, J. P. 1988. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigen system. *Proc. Natl. Acad. Sci. USA.* 85:5409.
2. Posnett, D. N., H. McGrath, and J. P. Tam. 1988. A novel method for producing anti-peptide antibodies using a peptide derived from the T cell antigen receptor β-chain constant region. *J. Biol. Chem.* 263:1719.
3. Eichinger, D. J., D. E. Arnot, J. P. Tam, V. Nussenzweig, and V. Enea. 1986. Circumsporozoite protein of *Plasmodium berghei*: gene cloning and identification of the immunodominant epitopes. *Mol. Cell. Biol.* 6:3965.
4. Weber, J. L., J. E. Egan, J. A. Lyon, R. A. Wirtz, Y. Charoenvit, W. L. Maloy, and W. T. Hockmeyer. 1987. *Plasmodium berghei*: cloning of the circumsporozoite protein gene. *Exp. Parasitol.* 63:295.
5. Nussenzweig, V., and R. S. Nussenzweig. 1989. Rationale for the development of an engineered sporozoites malaria vaccine. *Adv. Immunol.* 45:283.
6. Romero, P. J., J. P. Tam, D. Schlesinger, P. Clavijo, P. J. Barr, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala. 1988. Multiple T-helper cell epitopes of the circumsporozoite protein of *Plasmodium berghei*. *Eur. J. Immunol.* 18:1951.
7. Merrifield, R. B. 1986. Solid phase synthesis. *Science (Wash. DC).* 232:341.
8. Tam, J. P., W. F. Heath, and R. B. Merrifield. 1983. S_N2 deprotection of synthetic peptides with a low concentration of HF in dimethylsulfide: evidence and application in peptide analysis. *J. Am. Chem. Soc.* 105:6442.
9. Yoshida, N., R. S. Nussenzweig, P. Potocnjak, V. Nussenzweig, and M. Aikawa. 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science (Wash. DC).* 207:71.
10. Zavala, F., J. P. Tam, P. J. Barr, P. J. Romero, V. Ley, R. S. Nussenzweig, and V. Nussenzweig. 1987. Synthetic peptide vaccine confers protection against murine malaria. *J. Exp. Med.* 166:1591.
11. Adorini, L., M. A. Harvey, A. Miller, and E. E. Sercarz. 1979. Fine specificity of regulatory T cells. II. Suppressor and helper T cells are induced by different regions of hen egg white lysozome in a genetically non-responder mouse strain. *J. Exp. Med.* 150:293.
12. Spitalny, G. L., and R. S. Nussenzweig. 1973. *Plasmodium berghei*: relationship between protective immunity and anti-sporozoite (CSP) antibody in mice. *Exp. Parasitol.* 33:168.
13. Chen, D. H., R. E. Tigelaar, and F. I. Weinbaum. 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.
14. Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig. 1987. Gamma interferon, $CD8^+$ T cells and antibodies required for immunity to malaria sporozoites. *Nature (Lond.).* 330:664.
15. Weiss, W. R., M. Sedegah, R. L. Beaudoin, L. H. Miller, and M. F. Good. 1988. $CD8^+$ T cells (cytotoxic/suppressors) are required for protection in mice immunized with malaria sporozoites. *Proc. Natl. Acad. Sci. USA.* 85:573.
16. Ferreira, A., L. Schofield, V. Enea, H. Schellekens, P. van der Meide, W. E. Collins, R. S. Nussenzweig, and V. Nussenzweig. 1986. Inhibition of development of exoerythrocytic forms of malaria parasites by gamma-interferon. *Science (Wash. DC).* 232:881.

17. Maheshwari, R. K., C. W. Czarniecki, G. P. Dutta, S. K. Puri, B. N. Dhawan, and R. M. Friedman. 1986. Recombinant human gamma interferon inhibits simian malaria. *Infect. Immun.* 53:628.
18. Romero, P., J. L. Maryanski, G. Corradin, R. S. Nussenzweig, V. Nussenzweig, and F. Zavala. 1989. Cloned cytotoxic T-cells recognize an epitope in the circumsporozoite (CS) protein and protect against malaria. *Nature (Lond.).* In press.
19. Egan, J. E., J. L. Weber, W. R. Ballou, M. R. Hollingdale, W. R. Majarian, D. M. Gordon, W. L. Maloy, S. L. Hoffman, R. A. Wirtz, I. Schneider, G. R. Woolett, J. F. Young, and W. T. Hockmeyer. 1987. Efficacy of murine malaria sporozoite vaccines: Implications for human vaccine development. *Science (Wash. DC).* 236:453.
20. Zavala, F., J. P. Tam, M. R. Hollingdale, A. H. Cochrane, I. Quakyi, R. S. Nussenzweig, and V. Nussenzweig. 1985. Rationale for the development of a synthetic vaccine against *P. falciparum* malaria. *Science (Wash. DC).* 228:1436.
21. Ballou, W. R., S. L. Hoffman, J. A. Sherwood, M. R. Hollingdale, F. A. Neva, W. T. Hockmeyer, D. M. Gordon, I. Schneider, R. A. Wirtz, J. F. Young, G. F. Wasserman, P. Reeve, C. L. Diggs, and J. D. Chulay. 1987. Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet.* i:1277.
22. Herrington, D. A., D. F. Clyde, G. Losonsky, M. Cortesia, J. R. Murphy, J. Davis, S. Baqar, A. M. Felix, E. P. Heimer, D. Gillessen, E. Nardin, R. S. Nussenzweig, V. Nussenzweig, M. R. Hollingdale, and M. M. Levine. 1987. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature (Lond.).* 328:257.
23. Herzenberg, L. A., and T. Tokuhisa. 1983. Epitope-specific regulation. I. Carrier-specific induction of suppression for IgG anti-hapten antibody responses. *J. Exp. Med.* 155:1730.