Brief Definitive Report

SYNTHETIC PEPTIDE VACCINE CONFERS PROTECTION AGAINST MURINE MALARIA

By FIDEL ZAVALA,* JAMES P. TAM,§ PHILIP J. BARR,‖ PEDRO J. ROMERO,* VICTORIA LEY,‡ RUTH S. NUSSENZWEIG,* AND VICTOR NUSSENZWEIG‡

*From the *Department of Medical and Molecular Parasitology and the ‡Department of Pathology, New York University School of Medicine, New York 10016; §The Rockefeller University, New York 10021; and the ‖Chiron Corporation, Emeryville, California 94608*

Sporozoites are the infective stage of malaria parasites present in the salivary glands of Anopheles mosquitoes. An effective vaccine against sporozoites should prevent infection and, together with the use of residual insecticides and chemoprophylactic agents, contribute to the control of the disease. Sporozoite vaccines against *Plasmodium falciparum*, containing part or the entire repeat domain of the circumsporozoite (CS) protein, have recently undergone their first clinical trials in human volunteers (1, 2). The choice of this antigen was based mainly on studies showing that in vitro incubation of sporozoites with mAb to the repeated subunit sequences of CS proteins abolished their infectivity. Moreover, passive transfer of the mAb to the mammalian hosts before the inoculation of sporozoites protected them from malaria infection (reviewed in reference 3).

Left unanswered are important questions regarding the effectiveness of vaccines containing CS protein repeats, and the role of antibody and T cell effector mechanisms in protection. These questions could only recently be approached experimentally, when the CS gene of *Plasmodium berghei*, a rodent malaria parasite, was cloned and the corresponding amino acid sequence was elucidated (4). This sequence contains a central region with tandemly repeated amino acid units, varying somewhat in composition, $\overset{A}{\text{DPP}}\text{PPNP}\overset{A}{\text{PN}}$. In competitive binding assays the $(DPPPPNPN)_2D$ peptide was 30–50 times better at inhibiting the interaction of an mAb (3D11) with *P. berghei* sporozoites than the alternative form, the $(DPAPPNAN)_2D$ peptide (4). Because the mAb 3D11 neutralizes the infectivity of sporozoites very effectively (5), we postulated that vaccination of mice with synthetic peptides containing the $(DPPPPNPN)_2D$ sequence might induce protective antibodies when coupled with an appropriate carrier. The present experiments address this question in a rodent model.

Materials and Methods

Synthesis of Peptides 17.1 and 17.2. A 17-mer peptide of the tandemly repeating domain of *P. berghei* $(DPPPPNPN)_2$, designated 17.1, and the alternative sequence

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$(DPAPPNAN)_2D$, designated 17.2, were synthesized as described (4). The peptides containing a *p*-hydroxybenzydrylamine handle were released from the resin by treatment with 10% methylamine in tetrahydrofuran: ETOH (1:1, vol/vol) for 14 h at 18°C.

Synthesis and Characterization of a Polymer of Peptide 17.1. The synthesis of the peptide 17.1 on a multiple antigen peptide system (MAPS) was initiated on a Boc-β-Ala-OCH_2-Pam-resin (6) on which successive Boc-Lys (BOZ) were coupled to give an MAPS with eight-reactive amino ends. The synthesis of 17.1 on this hexadecavalent MAPS was similar to the synthesis of the linear 17.1 peptide. The MAPS containing 17.1 was then liberated by the low trifluorometasulfonic acid method (7), and purified by dialysis in decreasing concentrations of urea. The MAPS 17.1, with a calculated molecular weight of 16,698, gave the correct molecular weight and a single band in SDS–gel electrophoresis. Analysis of the purified MAPS 17.1 in C_{18} reverse-phase HPLC gave a broad peak.

Expression in Yeast and Purification of the P. berghei CS Protein. Expression, and subsequent purification to homogeneity of the central repeat region and flanking sequences of the *P. berghei* CS protein were performed as in reference 8.

Coupling of Synthetic Peptides to Tetanus Toxoid (TT). The synthetic peptide 17.1 was crosslinked with TT using glutaraldehyde (GA) (9), or bisdiazobenzidine (BDB) (10).

Immunization and Indirect Immunofluorescence Assay (IFA). A/J mice were injected twice with 50 μg of the conjugates. The first dose was incorporated into CFA and 100 μl were given intraperitoneally. For the booster, the antigen was dissolved in PBS and 100 μl was administered intravenously 15 d later. Norway Brown rats were immunized with 100 μg of the conjugate in CFA and received two intravenous boosters 15 and 35 d after priming. An additional group of mice was immunized with X-irradiated sporozoites with two biweekly intravenous injections of 1.5×10^5 parasites. Immunofluorescence was performed using fixed sporozoites (11).

Detection of Exoerythrocytic Liver Stages (EEF) of P. berghei by DNA Hybridization. Rats were killed 44 h after challenge with sporozoites, and the liver DNA was purified. The amount of parasite DNA was estimated as described (12).

Immunoradiometric (IRMA) and Competitive Binding Assays. Flexible polyvinyl-chloride microtiter wells (No. 3911, Falcon Labware, Oxnard, CA) were coated with 20 μl of 1 μg/ml of the recombinant protein of *P. berghei*, or the polymer of the 17.1 peptide. The serum titrations were performed as described (9).

To compare the avidities of the antipeptide and antisporozoite antibodies for the synthetic peptide and the recombinant protein, pools of serum samples obtained after the last immunization were diluted in PBS-BSA to obtain similar concentrations of antibodies. The serum samples were incubated for 1 h with the different inhibitors and an aliquot of the mixtures deposited into antigen-coated wells. The results were expressed as percent of the binding obtained with the same sera in the absence of inhibitors.

Results and Discussion

First, we studied the immunogenicity of the two main repeat sequence units DPPPPNPN and DPAPPNAN when presented in the context of the native molecule, that is, in mice immunized with irradiated *P. berghei* sporozoites. We compared the ability of the peptides 17.1 $[(DPPPPNPN)_2D]$ and 17.2 $[(DPAPPNAN)_2D]$ to inhibit the binding of antibodies in the sera of these mice with immobilized recombinant protein (sporozoite extracts were not used as antigen because they contain mosquito-derived immunogens). Peptide 17.1 was a much better inhibitor: 40% inhibition was obtained with 25 μg of 17.1, while 400 μg/ml of 17.2 were necessary to achieve a comparable result (not shown).

Having selected 17.1 as the peptide to be included in the vaccine, we compared the immunogenicity of two conjugates of 17.1 with TT, prepared with either glutaraldehyde or bisdiazobenzidine as crosslinking agents. Groups of six A/J mice were injected with the two conjugates and controls were immunized with X-irradiated sporozoites. The sera obtained 10 d after the booster injection were

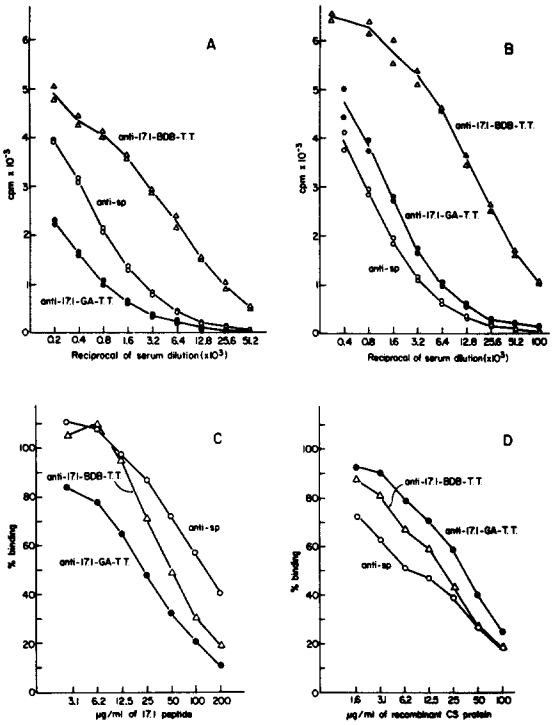

FIGURE 1. Titration of anti-17.1-GA-TT, anti-17.1-BDB-TT, and antisporozoite antibodies using as antigen immobilized *P. berghei* recombinant CS protein (*A*) or a synthetic polymer of the 17.1 peptide (*B*). Results of competitive binding assays comparing the binding of the different sera to the recombinant protein, in the presence of increasing concentrations of the synthetic peptide 17.1 or the recombinant protein, are shown in *C* and *D*, respectively. The results show that 17.1-BDB-TT is a better immunogen than 17.1-GA-TT. Higher levels of antibodies to the CS protein were found in the sera of mice injected with 17.1-BDB-TT (*top panels*), and their specificity resembled more closely that of antibodies to sporozoites (*bottom panels*).

pooled, and the levels of antibodies were determined by an IRMA using as the antigen the 17.1 polymer or the recombinant CS protein. The latter was chosen because it was more likely to mimic the configuration of the native protein and permit a better assessment of the quality of the antipeptide antibodies.

The highest levels of antibodies were found in the sera of mice immunized with 17.1-BDB-TT. The results of the titrations of the other two serum pools varied with the antigen used in the IRMA. With the CS protein as antigen, higher levels of antibodies were detected in the antisporozoite antisera than in anti-17.1-GA-TT antisera. The reverse was true when 17.1 was the antigen, indicating that the antipeptide and antisporozoite antibodies recognize best the homologous antigens (Fig. 1, *A* and *B*).

Next, we compared the binding avidities of the three pooled sera for the recombinant CS protein by performing competitive inhibition assays, the inhibitors being either the 17.1 peptide or the recombinant CS protein (Fig. 1, *C* and *D*, respectively). Both inhibitors were quite effective and there were no marked differences in the slopes of the titration curves. However, more recombinant CS protein was required to inhibit the antipeptide antibodies than the antisporozoite antibodies. When the inhibitor was 17.1, the reverse was observed, that is, more peptide was required to inhibit the antisporozoite antibodies. It appears, therefore, that the antipeptide antibodies have a higher binding avidity for the peptide, while the antisporozoite antibodies react better with the recombinant protein. Close inspection of the titration curves nevertheless shows that the patterns of inhibition of antibodies to 17.1-BDB-TT more closely resemble those of the antisporozoite antibodies. Because 17.1-BDB-TT induced higher titers of antibodies of higher avidity than 17.1-GA-TT, it was used as a vaccine.

TABLE I

Amounts of P. berghei DNA in the Liver of Immunized and Control Rats 44 h after Challenge with 10^5 Sporozoites

Immunogen	Rat Number	*P. berghei* DNA in liver	Mean ± SD
		ng	
Peptide 17.1 conjugated to tetanus toxoid with bisdiazobenzidine	1	0.0	
	2	21.0	
	3	13.3	6.2±8.8
	4	0.0	
	5	3.4	
	6	0.0	
Tetanus toxoid treated with bisdiazobenzidine	7	192.0	
	8	273.6	
	9	55.8	184.9±97.0
	10	316.6	
	11	122.3	
	12	149.2	

TABLE II

Protection from Malaria Infection by P. berghei Sporozoites after Immunization with a Synthetic Peptide Vaccine

Exp.	Immunogen	Range IFA Titers × 10^{-3}	Number protected/ challenged	Percent protection
1	17.1-BDB-tetanus toxoid	16–32	7/8	87
	BDB-tetanus toxoid	Neg	0/8	0
	X-irradiated sporozoites	4–16	6/7	85
	None	Neg	0/5	0
2	17.1-BDB-tetanus toxoid	16–64	6/8	75
	BDB-tetanus toxoid	Neg	0/8	0
	None	Neg	0/5	0

The effects of the 17.1-BDB-TT vaccine on sporozoite infectivity were evaluated using a specific DNA probe to measure the number of EEF developing in the livers of rats challenged with a large number (10^5) of *P. berghei* sporozoites (12). The mean titer of antibody in the serum of the rats of the experimental group was 10,660 ± 3,771. As shown in Table I, the livers contained 97% less parasite DNA than those of the controls vaccinated with TT. No parasite DNA was detected in the livers of three of six immunized rats.

To determine the degree of protection against malaria infection that this vaccine would provide, we immunized A/J mice. After the first dose, the serum titers of antibody against sporozoites were 1,635 ± 992; after the booster injection, they rose to 26,000 ± 7,745. 15 d later, the mice were challenged intravenously with 1,000 sporozoites. In two experiments, 87 and 75% of vaccinated mice did not develop parasitemia, a degree of protection comparable to that obtained by immunization with irradiated parasites (85%). All naive mice and all mice immunized with TT developed parasitemia 4 to 5 d after the challenge (Table II).

The T lymphocytes of the vaccinated mice did not proliferate after in vitro incubation with the synthetic peptide 17.1, although there was a strong response to both TT and 17.1-BDB-TT (data not shown). Furthermore, immunization of this strain of mice with the monomer or a polymer of the synthetic repeat

peptide, without the carrier protein, did not induce antibody production. These results strongly suggest that helper cells of A/J mice do not recognize the peptide, and that their protective immunity was mediated solely by antibodies.

In short, active immunization with a synthetic peptide representing the repeat domain of a CS protein can induce high levels of antibodies to the native protein, and effectively protect against sporozoite-induced malaria infection. Using the same experimental model, but with a different peptide vaccine formulation, others reported only marginal protection with 500 *P. berghei* sporozoites (13). The lower efficacy of that vaccine formulation might have been due to the use of a repeat peptide containing the alanine substitutions, instead of $(DPPPPNPN)_2D$. Furthermore, in those studies glutaraldehyde was used for coupling the peptide to the carrier protein. In our experiments, the levels and avidity of antibodies to the native protein were consistently lower in mice vaccinated with 17.1-GA-TT than in mice vaccinated with 17.1-BDB-TT. The reasons for this are not clear except that with glutaraldehyde the peptide is bound to the carrier through the NH_2-terminal and with BDB through the COOH-terminal handle. Striking effects of the orientation of a synthetic peptide on the immunogenicity and on the specificity of the induced antibodies have been reported (14).

We conclude that protective immunity to sporozoites can be obtained exclusively by antibodies to the repeats of the CS protein. A few volunteers had been vaccinated with X-irradiated sporozoites, and recently a synthetic and a recombinant vaccine against *P. falciparum* containing a portion of the repeat domain of the CS protein were tested in humans. In both instances, protection coincided with the presence of antisporozoite antibodies.

However, because sporozoites remain in circulation for a very short time before entering the liver cells, antibody levels would have to be maintained at sufficiently high levels to be fully protective. This may be difficult to achieve with peptide or recombinant vaccines containing only repeats. The identification of T cell epitopes in the CS polypeptide (or in other sporozoite-associated proteins) would permit their inclusion in vaccines. Sensitized T cells are a source of IFN-γ, which has a potent inhibitory effect on the liver stages of malaria parasites (15). Vaccines that contain sporozoite-specific T cell epitopes might be more advantageous because the antibody response would be boosted during infection, and T cell–dependent killing mechanisms would be triggered.

Summary

A synthetic peptide, $(DPPPPNPN)_2D$, representing a subunit of the repeat domain of the *Plasmodium berghei* circumsporozoite protein, was conjugated to tetanus toxoid using bisdiazobenzidine. Immunization of mice and rats with the conjugate induced high serum titers of antibodies to the parasite, and most of the animals were completely protected from malaria infection when challenged with sporozoites.

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References

1. 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:1287.
2. Herrington, D. A., D. F. Clyde, G. Losonsky, M. Cortesia, J. R. Murphy, J. Davis, S. Bagar, 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.
3. Nussenzweig, V., and R. S. Nussenzweig. 1986. Development of a sporozoite malaria vaccine. *Am. J. Trop. Med. Hyg.* 35:678.
4. Eichinger, D. J., D. E. Arnot, J. P. Tam, V. Nussenzweig, and V. Enea. 1986. Circumsporozoite protein of *P. berghei*: Gene cloning and identification of the immunodominant epitopes. *Mol. Cell. Biol.* 6:965.
5. 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.
6. Mitchell, A. R., B. W. Erickson, M. W. Ryabtsev, R. S. Hodges, and R. B. Merrifield. 1976. tert-Butoxycarbonylaminoacyl-4-(oxymethyl)-phenylacetamido-methyl-resin, a more acid-resistant support for solid-phase peptide synthesis. *J. Am. Chem. Soc.* 98:7357.
7. Tam, J. P., W. F. Heath, and R. B. Merrifield. 1986. Mechanism for the removal of benzyl protecting groups in synthetic peptides by trifluorometasulfonic acid-trifluoracetic acid-dimethylsulfide. *J. Am. Chem. Soc.* 108:5242.
8. Barr, P. J., H. L. Gibson, V. Enea, D. E. Arnot, M. R. Hollingdale, and V. Nussenzweig. 1987. Expression in yeast of a *P. vivax* antigen of potential use in human malaria vaccine. *J. Exp. Med.* 165:1160.
9. 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.
10. Johnson, J. 1985. Production and assay of murine anti-allotype antisera. *In* Immunological Methods I. I. Letkovits and B. Perris, editors. Academic Press Inc., Orlando. 202.
11. Nardin, E. H., R. S. Nussenzweig, I. A. McGregor, and J. H. Bryan. 1979. Antibodies to sporozoites and their frequent occurrence in individuals living in an area of hyperendemic malaria. *Science* (*Wash. DC*). 206:597.
12. Ferreira, A., V. Enea, T. Morimoto, and V. Nussenzweig. 1986. Malaria sporozoite infectivity measured with a DNA probe. *Mol. Biochem. Parasitol.* 19:103.
13. Egan, J. E., J. L. Wever, 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. Woollett, J. F. Young, and W. T. Hockmeyer. 1987. Efficacy of murine malaria sporozoite vaccines: implications for human vaccine development. *Science* (*Wash. DC*). 236:453.
14. Dyrberg, T., and M. B. A. Oldstone. 1986. Peptides as antigens. Importance of orientation. *J. Exp. Med.* 164:1344.
15. 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.