INFECTION AND IMMUNITY, June 1985, p. 839–842
0019-9567/85/060839-04$02.00/0

Vol. 48, No. 3

Biologically Active Antibodies Elicited by a Synthetic Circumsporozoite Peptide of *Plasmodium knowlesi* Administered in Saline with a Muramyl Dipeptide Derivative

ELLEN R. CLOUGH,[1] FRANÇOISE M. AUDIBERT,[1] JOHN W. BARNWELL,[2] DAVID H. SCHLESINGER,[3] RUTH ARNON,[4] AND LOUIS A. CHEDID[1]*

G.R.31, Centre National de la Recherche Scientifique, Immunothérapie Expérimentale, Institut Pasteur, 75015 Paris, France[1]; Departments of Parasitology[2] and Medicine and Cell Biology,[3] New York University Medical Center, New York, New York 10016; and The Weizmann Institute of Science, Rehovot 76100, Israel[4]

Received 26 December 1984/Accepted 18 February 1985

A synthetic peptide whose sequence was derived from the circumsporozoite protein of *Plasmodium knowlesi* coupled to bovine gamma globulin has been shown to be immunogenic when administered with Freund complete adjuvant. The present experiments were designed to test the immunogenicity of the peptide when attached to a tetanus toxoid carrier and administered with alum or murabutide, both acceptable clinical adjuvants. In both cases, the use of an adjuvant increased the levels of circulating anti-peptide antibodies over those observed when no adjuvant was used. However, when the antisera were tested for reactivity with the native protein, animals of the group receiving the conjugate associated with murabutide always had titers greatly exceeding those observed in animals that received the conjugate with alum. Moreover, the sera of the murabutide-treated group were shown to be more active in eliciting shedding of the circumsporozoite protein than were sera of animals of the Freund complete adjuvant-treated group. The use of tetanus toxoid in secondary immunizations could be eliminated when the mice primed with peptide-tetanus toxoid and murabutide were boosted with a polymer of the peptide. The results indicate that the synthetic malarial peptide-tetanus toxoid conjugate is capable of stimulating high levels of biologically active antibodies only when administered with murabutide.

The sporozoite stage of *Plasmodium knowlesi* is highly immunogenic, and repeated injections of X-irradiated sporozoites have been shown to protect against malarial infection in mice, monkeys, and humans (4). The identification and synthesis of a peptide representing a 24-amino-acid tandemly repeating epitope from the circumsporozoite protein of *P. knowlesi* has recently been reported (5). Antibodies against this synthetic peptide, produced in rabbits, also cross-link the circumsporozoite protein on the membrane of the sporozoite and neutralize sporozoite infectivity (7a). Other synthetic peptides representing epitopes from bacterial (1, 9, 10) and viral (2; L. Chedid, C. Carelli, and F. Audibert, Proc. Symp. Advances in Carriers and Adjuvants for Veterinary Biologics, Ames, Iowa, 7 and 8 May, 1984) pathogens have been shown to produce antibodies that recognize the natural structure when administered in saline with muramyl dipeptide or analogs. In the case of diphtheria toxin and of streptococci these antibodies were shown to be protective (1, 9). It was therefore interesting to determine whether the malarial peptide could generate high titers of antipeptide antibodies recognizing the native circumsporozoite protein when delivered under conditions suitable for humans. To this end, Freund complete adjuvant, unacceptable for human use due to its unwanted side effects, was replaced by a synthetic muramyl dipeptide, murabutide, now undergoing clinical trials with another vaccine (3; F. Oberling, C. Bernard, L. Chedid, J. Choay, C. Giron, and J. M. Lang, Int. Symp. on Immunomodulation by Microbial Products and Related Synthetic Compounds, Osaka, 1981). In most experiments, the peptide was coupled to a tetanus toxoid (TT) carrier (pep-TT), mixed with murabutide, and administered in saline to mice. The anti-peptide responses and the reactivity of these anti-peptide antibodies for the native protein were measured.

MATERIALS AND METHODS

A synthetic 24-amino-acid peptide representing the immunodominant epitope of the sequence of the circumsporozoite protein of *P. knowlesi* was synthesized as previously described (5). A second peptide with two additional amino acids, tyrosine at the N terminus and cysteine at the C terminus, was also synthesized to facilitate labeling and coupling of the peptide to carrier. The peptide was conjugated to a TT carrier (pep-TT) by using glutaraldehyde (1) or *N*-ethyl-*N'*-(3-dimethylamino)propyl-carbodiimide (6). However, in one case no carrier was used; to increase the size of the molecule, the 26-amino-acid sequence was polymerized with glutaraldehyde.

Adult female BALB/c mice (Institut Pasteur, Rennemoulin) were immunized subcutaneously with 10, 50, or 100 μg of the pep-TT, with or without adjuvant. Alum was used at a dose of 200 μg per mouse; murabutide was used at 100 μg per mouse. Mice were boosted 4 weeks later with the same amount of antigen in the absence of adjuvant. In experiments with the polymerized peptide, either animals received 100 μg of the pep-TT conjugate subcutaneously two times 4 weeks apart, or they received a primary immunization with 100 μg of pep-TT followed 4 weeks later by 100 μg of the polymer or peptide alone. All animals were bled 2 weeks after primary immunization and 2, 3, and 4 weeks after the boost. Pooled sera from each group were tested for reactivity with the peptide in an enzyme-linked immunosorbent assay (ELISA) and a radioimmunoassay (RIA) as previously described (1, 15). Recognition of the natural protein by

* Corresponding author.

TABLE 1. Secondary anti-peptide and anti-SPZ response after immunization with various doses of pep-TT in saline with alum or murabutide[a]

Treatment (μg)	Antibody (ELISA) titer		Sporozoite RIA titer (day 52)
	Day 45	Day 52	
pep-TT (10)	7,800	8,300	
pep-TT (50)	27,000	13,000	
pep-TT (100)	42,000	41,000	300
pep-TT (10) plus alum (200)	41,300	21,000	
pep-TT (50) plus alum (200)	49,800	28,900	
pep-TT (100) plus alum (200)	96,000	110,000	2,500
pep-TT (10) plus murabutide (100)	54,000	53,000	
pep-TT (50) plus murabutide (100)	130,000	47,000	
pep-TT (100) plus murabutide (100)	120,000	150,000	25,000

[a] BALB/c mice (six per group) received subcutaneously the conjugate in saline either alone or with adjuvant. At 4 weeks they were boosted with the same amount of conjugate without adjuvant. Anti-peptide titers were measured by ELISA 2 and 3 weeks after the boost, and anti-sporozoite titers were measured by RIA.

antipeptide antibodies was assessed by RIA with a sporozoite extract fixed to plastic plates (15), the circumsporozoite reaction (14), and an indirect fluorescence antibody test (11).

RESULTS

Anti-peptide response elicited by various dosages of pep-TT. The immunogenicity of the peptide alone or pep-TT and given with or without adjuvant was tested in experiments to determine the minimum dose of pep-TT needed under different adjuvant conditions (Table 1). At all doses both alum and murabutide increased levels of circulating anti-peptide antibodies over saline controls; the highest titers were observed with the highest dose of pep-TT associated with murabutide. Third-week sera of mice receiving 100 μg of pep-TT were tested for binding to the sporozoite. Sera from mice immunized with pep-TT in saline contained only a very low level of anti-peptide antibodies that recognized the sporozoite (titer, 300). Although the alum-treated group had high titers of anti-peptide antibodies by the ELISA procedure (110,000), minimal binding to the sporozoite could be detected (titer, 2,500). In contrast, sera from the murabutide group, which had comparable anti-peptide antibody titers by ELISA (150,000), had markedly higher anti-sporozoite antibody titers (25,000).

Boosting effect of pep-TT, free peptide, and polymerized peptide. Preliminary experiments showed that two administrations of the free peptide alone or peptide with murabutide could not elicit an anti-peptide response. In the following experiment mice (six per group) were primed with pep-TT associated with murabutide and received a boost of either the conjugate, the free peptide, or the polymerized peptide without adjuvant. By far the highest anti-peptide antibody titers both 1 week (day 35) and 2 weeks (day 42) after the boost were observed in the group treated twice with the conjugate, although on day 35 the polymerized peptide elicited a better secondary response than did the free peptide (Table 2).

Anti-peptide and anti-native protein responses after pep-TT administration. In previous experiments, only the anti-peptide antibody titers, as measured by ELISA, and sporozoite recognition, as evaluated by RIA, were presented. In this experiment, immunofluorescence and the circumsporozoite reaction were used to further test the reactivity of anti-peptide antibodies with the native protein. Mice (six per group) received 100 μg of pep-TT alone, with alum or with murabutide. A positive control group received 100 μg of the conjugate in Freund complete adjuvant. Four weeks later, all animals received a boost of 100 μg of pep-TT without adjuvant.

RIA with peptide-coated plates confirmed previous ELISA results, showing that anti-peptide antibodies were clearly enhanced when the conjugate was administered with any of the adjuvants (Table 3). Moreover, as previously observed, the anti-sporozoite antibodies as measured by RIA were increased. The use of either murabutide in saline or Freund complete adjuvant resulted in higher anti-sporozoite titers than did alum treatment. However, when these sera were tested for reactivity with the native protein in the circumsporozoite assay, titers for the murabutide-treated group were higher. It must be stated, however, that in mice titers obtained after immunization with *P. knowlesi* peptides are lower than those obtained after immunization by the natural pathogen of the mouse, *Plasmodium berghei* sporozoite (14). Although the levels of anti-peptide antibodies in the alum treated group were high, sera from these mice did not elicit the circumsporozoite reaction. It is likely, based on earlier results obtained by immunization with attenuated intact sporozoites in rhesus monkeys, that anti-circumsporozoite titers of the magnitude observed in the murabutide-treated group can be correlated with immunity against parasite challenge (7).

DISCUSSION

Siddiqui et al. (13) have reported successful immunization of owl monkeys with liposomes containing a natural malarial merozoite antigen and a fatty acid derivative of muramyl dipeptide. In the present paper we demonstrate that a synthetic 26-amino-acid sequence representing a portion of the circumsporozoite protein of *P. knowlesi* (5), when given in saline with a muramyl dipeptide derivative, murabutide, can elicit high titers of anti-peptide antibodies that recognize the native protein. Murabutide has also been shown to be adjuvant active with a TT vaccine in monkeys (A. Morin, personal communication).

The use of either Freund complete adjuvant or murabutide in primary immunization with the pep-TT resulted in potent anti-peptide responses after boosting with the conjugate in the absence of adjuvant. Although both adjuvant treatments resulted in equivalent titers of antibody capable of binding to the peptide, an important difference was observed in the

TABLE 2. Secondary response elicited by pep-TT, free peptide, or polymerized peptide after a primary immunization with the conjugate[a]

Immunization		ELISA titer	
Primary	Secondary	Day 35	Day 42
pep-TT	pep-TT	21,300,000	499,000
pep-TT	Free peptide	5,000	2,900
pep-TT	Polymerized peptide	38,000	1,400

[a] The antigens were given at a dose of 100 μg for both primary and secondary immunizations. For the primary immunization murabutide (100 μg) was associated.

TABLE 3. Antibody response of mice to pep-TT with different adjuvants

Treatment (μg)	Immunization		Titer			
			RIA		IFA	Circumsporozoite
	No.	Day	Peptide[a]	Sporozoite		
pep-TT (100)	1	21	Negative	Negative		
	2	35	40	320	160	<5
	3	105	5,120	10,240	640	<5
pep-TT (100) plus murabutide (100)	1	21	160	320		
	2	35	5,120	20,480	2,560	20
	3	105	40,960	81,920	10,240	40
pep-TT (100) plus Freund complete adjuvant (100)	1	21	80	160		
	2	35	5,120	20,480	2,560	5
	3	105	40,960	81,920	10,240	20
pep-TT (100) plus alum (200)	1	21	40	40		
	2	35	1,280	2,560	320	<5
	3	105	163,840	40,960	2,560	<5

[a] Peptide of 24 amino acids.

ability of these antibodies to elicit the circumsporozoite reaction. This in vitro test has been shown to have a positive correlation with the degree of protection in mice and monkeys (4). The use of alum as the adjuvant resulted in high anti-peptide antibody titers only after a third immunization with pep-TT. Even then these antibodies recognized the native protein less well than the murabutide group as judged by RIA, immunofluorescence, and the circumsporozoite reaction.

An attempt was made to decrease the need for repeated injections of TT and thus the total amount of carrier given in the primary and secondary immunization, since TT itself is a potent immunogen. Immunization with two doses of the peptide itself in adjuvant, in the absence of carrier protein, was unsuccessful, possibly due to the small size of the peptide. To increase the length of the peptide in the hopes that it could then act as both hapten and carrier, the peptide was polymerized. Utilization of the polymerized peptide after priming with pep-TT resulted in an elevated anti-peptide response. Similar attempts to reduce the amount of carrier protein used have been successful in the streptococcal M protein model system (9).

It is also possible to copolymerize the adjuvant with the peptide to make a longer synthetic immunogen that has inherent adjuvanticity. In this way, the need for the larger carrier molecule can often be eliminated (9). Experiments are underway to test the efficacy of immunization with a muramyl dipeptide-lysine-malaria peptide copolymer, thus avoiding the use of TT even in the primary immunization.

Preliminary experiments in rhesus monkeys suggest an important role for the carrier molecule in eliciting a good anti-peptide response. Two monkeys immunized with pep-TT and murabutide produced good anti-peptide RIA titers (5,000 to 10,000), whereas two monkeys immunized with peptide conjugated to bovine gamma globulin and murabutide produced only low anti-peptide titers (10 to 80) as measured by RIA. Thus, TT appears to be a suitable carrier, whereas bovine gamma globulin is not (J. Barnwell, unpublished results). However, administered in saline, even when conjugated to TT, the peptide did not elicit biologically active antibodies unless the conjugate had been associated with murabutide. Other factors that may be important in eliciting the anti-peptide response while stimulating a minimal anti-carrier response are being explored, including the degree of substitution of peptide and adjuvant on the carrier.

ACKNOWLEDGMENTS

Ellen Clough was supported by a fellowship from the French/American Exchange Program of the Centre National de la Recherche Scientifique and the National Science Foundation and by a grant from the Rockefeller Foundation. This work has been partly supported by Centre National de la Recherche Scientifique Action de Soutien Programmée 956.11.27.

We are grateful to Michel Jolivet and Maurice Hattab for preparing the pep-TT and polymerized peptide. We thank P. Lefrancier (Laboratoire Choay) for providing the murabutide and C. de Champs for typing the manuscript. We thank Ruth and Victor Nussenzweig for fruitful discussions while performing this work and preparing the manuscript.

LITERATURE CITED

1. **Audibert, F., M. Jolivet, L. Chedid, R. Arnon, and M. Sela.** 1982. Successful immunization with a totally synthetic diphtheria vaccine. Proc. Natl. Acad. Sci. U.S.A. **79**:5042–5046.
2. **Audibert, F. M., G. Przewlocki, C. D. Leclerc, M. E. Jolivet, H. S. Gras-Masse, A. L. Tartar, and L. A. Chedid.** 1984. Enhancement by murabutide of the immune response to natural and synthetic hepatitis B surface antigens. Infect. Immun. **45**:261–266.
3. **Chedid, L. A., M. A. Parant, F. M. Audibert, G. J. Riveau, F. J. Parant, E. Lederer, J. P. Choay, and P. Lefrancier.** 1982. Biological activity of a new synthetic muramyl peptide adjuvant devoid of pyrogenicity. Infect. Immun. **35**:417–424.
4. **Cochrane, A. H., R. S. Nussenzweig, and E. H. Nardin.** 1980. Immunization against sporozoites, p. 163–175. *In* J. P. Kreier (ed.), Malaria in man and experimental animals. Academic Press, Inc., New York.
5. **Godson, G. N., J. Ellis, P. Svec, D. H. Schlessinger, and V. Nussenzweig.** 1983. Identification and chemical synthesis of a tandemly repeated immunogenic region of *Plasmodium knowlesi* circumsporozoite protein. Nature (London) **305**:29–33.
6. **Goodfriend, T. L., L. Levine, and G. D. Fasman.** 1964. Antibodies to braddykinin and angiotensin. A use of carbodimides in immunology. Science **144**:1344–1346.
7. **Gwadz, R. W., A. H. Cochrane, V. Nussenzweig, and R. S. Nussenzweig.** 1979. Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterization of surface antigens of these parasites. Bull. W.H.O. **57**(Suppl. I):165–173.

7a. **Gysin, J., J. Barnwell, D. H. Schlesinger, V. Nussenzweig, and R. S. Nussenzweig.** 1984. Neutralization of the infectivity of sporozoites of *Plasmodium knowlesi* by antibodies to a synthetic peptide. J. Exp. Med. **160**:935–940.

8. **Hollingdale, M. R., F. Zavala, R. S. Nussenzweig, and V. Nussenzweig.** 1982. Antibodies to the protective antigen of *Plasmodium berghei* sporozoites prevent entry into cultured cells. J. Immunol. **128**:1929–1930.

9. **Jolivet, M., F. Audibert, E. H. Beachey, A. Tartar, H. Gras-Masse, and L. Chedid.** 1983. Epitope specific immunity elicited by a synthetic streptococcal antigen without carrier or adjuvant. Biochem. Biophys. Res. Commun. **117**:359–366.

10. **Leclerc, C., A. Morin, and L. Chedid.** 1983. Potential use of synthetic muramyl peptides as immunomodulating molecules. Recent Adv. Clin. Immunol. **3**:187–204.

11. **Nardin, E. H., R. S. Nussenzweig, and R. Gwadz.** 1979. Characterization of sporozoite surface antigens by indirect immunofluorescence: application of this technique to dectect stage and species specific anti-malarial antibodies. Bull. W.H.O. **57**(Suppl.):211–217.

12. **Santero, F., A. H. Cochrane, V. Nussenzweig, E. H. Nordin, R. S. Nussenzweig, R. W. Gwadz, and A. Ferreira.** 1983. Structural similarities between the protective antigens of sporozoites from different species of malaria. J. Biol. Chem. **258**:3341–3345.

13. **Siddiqui, W. A., D. W. Taylor, S. C. Kan, K. Kramer, S. M. Richmond-Crum, S. Kotani, T. Shiba, and S. Kusumoto.** 1978. Vaccination of experimental monkeys against *Plasmodium falciparum*: a possible safe adjuvant. Science **201**:1237–1239.

14. **Vanderberg, J., R. S. Nussenzweig, and H. Most.** 1969. Protective immunity produced by injections of X-irradiated sporozoites of *Plasmodium berghei*. V. *In vitro* effects of immune serum on sporozoites. Mil. Med. **134**(Suppl.):1183–1190.

15. **Zavala, F., A. H. Cochrane, E. H. Nordin, R. S. Nussenzweig, and V. Nussenzweig.** 1983. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J. Exp. Med. **157**:1947–1957.