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PRODUCTION OF ANTI-SPOROZOITE ANTIBODIES IN ABSENCE OF RESPONSE TO CARRIER BY COUPLING AN MDP DERIVATIVE TO A MALARIA PEPTIDE-TETANUS TOXOID CONJUGATE

Ellen R. Clough*[1], Michel Jolivet*, Françoise Audibert*,*
John W. Barnwell+, David H. Schlesinger‡, and Louis Chedid*

* C.N.R.S. UA-579, Immunothérapie Expérimentale, Institut Pasteur, 28, rue du Dr. Roux, 75015 Paris, France

+ Department of Parasitology, and ‡ Department of Medicine and Cell Biology, New York University Medical Center, New York, NY

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A synthetic peptide (pep) representing a portion of the Plasmodium knowlesi circumsporozoite protein attached to a tetanus toxoid (TT) carrier, has been shown to be immunogenic when delivered in saline with derivatives of the synthetic adjuvant, muramyl dipeptide (MDP).

The present study was designed to determine if the degree of substitution of pep and of MDP derivatives on the tetanus toxoid (TT) carrier, as well as the choice of MDP derivative used play a role in determining anti-pep and anti-TT antibody levels. One of the MDP derivatives used in the conjugates was ε-amino-caproic Murabutide, since Murabutide which is currently in clinical trials cannot be conjugated. The results show that low doses of this derivative coupled with pep on TT can be used to stimulate high levels of circulating anti-pep antibodies without augmenting the anti-carrier response. In addition, anti-pep antibodies elicited in response to one of the conjugates were biologically active since they produced shedding of the circumsporozoite coat of live parasites.

In numerous model systems, synthetic peptides are being used to elicit the production of antibodies which will cross react with the native intact protein (1-5) and in some cases protect against infectious challenge (1-3, 5). These compounds are generally administered to laboratory animals in complete Freund's adjuvant (CFA), an agent unacceptable for use in humans due to its unwanted side effects. Analogs of NAcMur-L-Ala-D-isoGln, muramyl dipeptide (MDP), the smallest adjuvant active component of CFA, but devoid of many of its side effects (6, 7) have been successfully used in several synthetic vaccine models to overcome the adjuvant problem (1, 8-10).

[1] Present address: International Minerals and Chemical Corp., 1810 Frontage Road, Northbrook, IL 60062

* To whom correspondence should be addressed

Abbreviations : Complete Freund's adjuvant (CFA); muramyl dipeptide (MDP); circumsporozoite protein (CSP); malarial peptide (pep); tetanus toxoid (TT); conjugate of malaria peptide to tetanus toxoid (pep-TT); enzyme-linked immunosorbent assay (ELISA); radioimmunoassay (RIA); sporozoite extract (SPZ); indirect fluorescence antibody test (IFA); circumsporozoite protein reaction (CSP).

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Murabutide (NAcMur-L-Ala-D-Gln-α-n-butyl-ester), a clinically acceptable MDP analog (11), when given in saline with a synthetic malarial peptide (representing the immunodominant epitope of the Plasmodium knowlesi circumsporozoite protein) stimulated high levels of circulating anti-peptide antibodies which elicited shedding of the circumsporozoite protein (CSP) (12).

Peptides are weakly immunogenic unless coupled to a carrier molecule. In the malaria model as well as in others, various approaches have been used to decrease the need for a carrier, including polymerization of peptides (5, 9), or copolymerization of peptide and MDP (13). In some cases, peptides have been conjugated to molecules which can act as carriers but do not themselves stimulate an antibody response (2, 14). An alternative approach has been tested in the present study : conjugation on the same carrier of both the synthetic malarial peptide and the adjuvant at different substitution ratios. In addition, two synthetic MDP derivatives differing in hydrophobicity were used because of the importance for an antigen of this property in eliciting antibody responses (14, 15). One of the derivative was ε-amino-caproic Murabutide since Murabutide which is currently in clinical trials cannot be conjugated. The effect of these variations on the anti-carrier and anti-peptide responses, as well as on the ability of the anti-peptide antibodies to mediate shedding of the CSP of live parasites was tested.

MATERIALS AND METHODS

Construction of immunogens

The identification and chemical synthesis of the immunodominant epitope of the P.knowlesi circumsporozoite protein, has been previously described (16) and in greater detail (17). In these studies an analogous peptide containing the 24 amino acids present in the natural sequence and two additional amino acids (a tyrosine at the N-terminal end and a cystine at the C-terminal end) was used. Varying amounts of the peptide and either MDP-lysine (18) or ε-amino-caproic Murabutide (19) were cross-linked to tetanus toxin (TT) using glutaraldehyde (20).

For conjugates A and D, 2.35 mg tetanus toxin, 3.75 mg malarial peptide and 2 mg MDP were used. Conjugates B and E contained 2.35 mg TT, 7.5 mg pep and 1 mg MDP. Conjugates C and F contained 11.7 mg TT, 18.75 mg pep and 2.5 mg MDP. Pep-TT was made by cross-linking 9.4 mg pep with 5.9 mg tetanus toxin. The toxin was used as a carrier since it has more reactive groups than the toxoid. Protein content in each conjugate was measured by Folin reaction and MDP content by a coloric method (21). The resultant conjugates are listed in Table 1. Complete detoxification by glutaraldehyde treatment of each product was controlled in mice before use.

Study of immunogenicity

The immunogenicity of the compounds was tested using groups of six adult female BALB/c mice (Institut Pasteur, Rennemoulin, France) according to protocols described in the Results section.

Sera from animals in each group were pooled and tested for total anti-peptide and anti-tetanus toxoid antibodies by ELISA as previously described (2). Recognition of the native protein by anti-peptide antibodies was assessed by radioimmunoassay (RIA) using sporozoite extract (SPZ) fixed to plastic plates (SPZ) (22), and an indirect fluorescence antibody test (IFA) (23, 24). The circumsporozoite protein reaction (CSP) (22, 25) was used to test the biologic activity of anti-pep antibodies.

Table 1. List of conjugates

	Conjugates containing MDP-Lys			ε-amino-caproic Murabutide		
	A	B	C	D	E	F
NH_2 eq. (TT : pep : adj)	1:1:2	1:2:1	1:1:0.5	1:1:2	1:2:1	1:1:0.5
Adjuvant used (μg/mouse)	1	0.6	0.3	1	0.6	0.3

In all experiments 50 μg of each conjugate were administered subcutaneously.

Antibody production in vitro

Spleen cells were asceptically removed, washed and suspended at a concentration of 10^7 cells/ml in RPMI 1640 (Seromed) containing 2 g/l $NaHCO_3$, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 % fetal calf serum, and 2 x 10^{-5}M 2-mercaptoethanol. 100 μl aliquots were placed in each well of 96 well tissue culture plates (Costar). 20 μl of TT or pep-TT at 0.1 μg/ml was added to each well and the final volume was adjusted to 0.2 ml. Control wells received no antigen. Plates were incubated in 10 % CO_2, 90 % O_2 at 37°C. At four days, the plates were washed to remove the antigen. Six days later the supernatants were collected and tested for antibody by ELISA.

RESULTS

In vivo antibody responses to conjugates with different substitution ratios of pep, TT and MDP-lysine

Animals received on days 1 and 30, 50 μg of either conjugate A, B, or C. Two control groups received 50 μg of pep-TT alone or mixed with 100 μg of MDP-Lys on day 1. Both groups were boosted by pep-TT alone. Animals were bled on days 28 and 35. The results (Table 2) show that mice immunized with the conjugate containing the most peptide had the highest anti-pep response. Increasing the amount of adjuvant from 0.3 μg to 1 μg did not result in a higher anti-pep

Table 2. Effect on anti-peptide and anti-carrier responses of different substitution ratios of malaria peptide, and MDP-lysine on tetanus toxoid

Conjugate	Group	NH_2 equivalents TT:pep:MDP-lysine	D 28		D 35	
			anti-pep	anti-TT	anti-pep	anti-TT
	1	pep-TT + saline	3.17	1.5	4.32	1.19
	2	pep-TT + MDP-lysine	5.3	1.3	8.6	.92
A	3	1 : 1 : 2	14	13	12.3	6
B	4	1 : 2 : 1	38	3.8	22	3.11
C	5	1 : 1 : 0.5	10.8	4.48	9	1.42

Titers are expressed as inverse of the dilution of serum giving an O.D. in ELISA of 2x background x 10^4.

response but favored a strong anti-TT response. All groups treated with conjugates containing MDP-lysine had higher anti-pep and anti-TT titers than mice which received a pep-TT (1:1) conjugate either mixed with 100 µg MDP-lysine or given without adjuvant.

In vivo antibody responses to conjugates containing ε-amino-caproic Murabutide

Groups of 6 mice received 50 µg of conjugates on day 1 and day 25. Control groups received the conjugate pep-TT (50 µg) in saline alone or mixed with 100 µg of Murabutide on day 1. Both groups received the antigen alone on day 25. Animals were bled on days 14, 32 and 123.

Representative results from one out of two experiments are shown in Table 3. All groups receiving adjuvant, either mixed with or conjugated to pep-TT, had high anti-peptide primary and secondary responses although conjugates D-F contained only 1 µg, 0.6 µg, and 0.3 µg of adjuvant per 50 µg of conjugate respectively. These levels of anti-peptide antibodies were comparable to those obtained by using 100 µg of the adjuvant mixed to 50 µg of the pep-TT conjugate. A sharp contrast between groups was seen in the anti-carrier response. Animals receiving the ε-amino-caproic Murabutide-pep-TT conjugate had remarkably low anti-TT titers (< 400 to 1400 for the primary and 10,000 to 50,000 for the secondary) while those receiving pep-TT mixed with the adjuvant had 8-40 times higher anti-carrier titers. The difference in anti-carrier responses between groups 1-2 and 3-5 was even more pronounced after the boost, and administration of pep-TT in saline, dramatically favored anti-TT antibody production. This phenomenon could be observed as late as d123.

In vitro antibody production by spleen cells from mice primed with pep-TT-ε-amino-caproic Murabutide conjugate

Due to the strinkingly low levels of circulating anti-TT antibodies in mice treated with conjugates containing Murabutide, the ability of spleen cells from mice of group 5 to produce

Table 3. Effect on anti-peptide and anti-carrier responses of different substitution ratios of malaria peptide and ε-amino-caproic Murabutide on tetanus toxoid

Conjugate	Group	NH_2 equivalents TT:pep:adjuvant	D 14		D 32		D 123	
			pep	TT	pep	TT	pep	TT
	1	pep-TT in saline	0.28	1.22	1.06	24.6	3.9	4.60
	2	pep-TT + Murabutide	0.80	1.66	29.8	39.7	10.7	7.40
D	3	1 : 1 : 2	0.83	<0.04	19.6	1.15	9.30	0.160
E	4	1 : 2 : 1	1.15	<0.04	14.5	2.55	10.5	0.340
F	5	1 : 1 : 0.5	1.00	0.14	8.85	5.70	7.46	0.80

Titers are expressed as inverse of the dilution of serum giving an O.D. in ELISA of 2x background x 10^4.

Table 4. Production of biologically active antibodies by ε-amino-caproic Murabutide mixed or conjugated to malaria peptide tetanus toxoid conjugate

Group	NH_2 equivalents TT:peptide:adjuvant	ELISA anti-pep	ELISA anti-TT	RIA SPZ	IFA	CSP
1	pep-TT in saline	10,600	246,000	320	2,500	< 5
2	pep-TT + Murabutide	298,000	397,000	20,000	20,000	10
3	1 : 1 : 2	196,000	11,500	2,500	41,000	40
4	1 : 2 : 1	145,000	25,500	1,280	10,000	< 5
5	1 : 1 : 0.5	88,500	57,000	2,500	5,000	< 5

anti-TT antibodies in vitro following stimulation with pep-TT or free TT was tested as compared with mice of groups 1 and 2. ELISA on undiluted supernatants from these cultures show that spleen cells from group 5 stimulated by the conjugate made only anti-pep antibodies (OD > 2), whereas no anti-TT antibodies were detected if these cells were stimulated either by the conjugate or by TT. In contrast, cells from mice of group 2 made an anti-peptide response (OD 1.35) but also strong anti-TT response (OD > 2). Cells of mice of group 1 which were immunized with pep-TT in absence of adjuvant made no detectable anti-peptide antibodies although they could respond to TT (OD > 2). These results further demonstrate that coupling low doses of ε-amino-caproic MDP to pep-TT results in less effective priming to the TT carrier than does mixing the adjuvant with antigen.

Biological activity of anti-pep antibodies

Day 32 sera from all groups of the experiment described (Table 3) were tested for reactivity with the natural circumsporozoite protein by RIA and by IFA. In view of evaluating whether antibodies which recognize the parasite coat-protein can also elicit shedding of the circumsporozoite protein of live parasites their biologic activity, as measured by CSP, was also tested. The results are shown in Table 4. Although titers of sera from all groups were elevated over saline controls, sera from mice treated with pep-TT mixed with adjuvant exhibited by far the best reactivity with a sporozoite extract as measured by RIA. This group also had high IFA and CSP titers. However, for both IFA and CSP, sera from animals treated with conjugate D had the highest titers.

DISCUSSION

These experiments demonstrate that the degree of substitution of adjuvant on carrier as well as properties of the adjuvant itself can greatly influence the antibody response to both the

peptide and the carrier. These factors can also affect the degree to which anti-pep antibodies recognize the native protein. Thus, when hydrophilic MDP-lysine was used as adjuvant, both circulating anti-pep and anti-TT levels were high even when low doses of adjuvant were coupled to pep-TT. Within the conjugate-treated groups, the anti-pep response was highest in animals receiving the conjugate containing the most peptide. Increasing the quantity of MDP-Lys with respect to peptide resulted in elevated anti-carrier responses. When ε-amino-caproic Murabutide was used sera from mice treated with the conjugate containing 1 μg of adjuvant had the highest titers of antibodies recognizing the peptide and the native protein. They also elicited the best CSP reaction, a reaction which has been shown to correlate well with degree of protection in mice and monkeys (25). Thus, low doses (1 μg/50 μg conjugate) of ε-amino-caproic Murabutide, when coupled directly to pep-TT, seem able to produce more protective antibodies than pep-TT mixed with 100 μg of the same adjuvant or of MDP-lysine (unpublished observations).

Sera from mice treated with the conjugates containing the Murabutide had markedly lower titers of anti-TT antibodies than those from animals receiving pep-TT. The reduced anti-carrier secondary response in vivo and in vitro of animals treated with ε-amino-caproic pep-TT conjugate may be a result of changes in antigenicity of the TT-carrier due to conformational modifications or to the creation of new epitopes occurring during the cross-linking of peptide and adjuvant on carrier. A second possibility is that use of the amphiphilic adjuvant may reduce the immunogenicity of TT by making some determinants inaccessible to the immune system during the in vivo priming process.

In conclusion, by varying the degree of substitution of pep and adjuvant on TT as well as the chemical structure of the adjuvant conjugates containing low doses of adjuvant have been obtained which can elicit high titers of anti-peptide antibodies and low anti-carrier titers. The results indicate that one of the conjugates containing -amino-caproic Murabutide may be used effectively to stimulate production of protective anti-sporozoite antibodies. Since this conjugate was administered in saline and contained a derivative of Murabutide it may represent a suitable clinical model for a Plasmodium falciparum peptide.

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