Studies on Peptides. CLXVII.[1,2] Solid-Phase Syntheses and Immunological Properties of Fragment Peptides Related to Human Malaria Circumsporozoite Protein

Kenichi AKAJI,[a] Yoshio HAYASHI,[a] Nobutaka FUJII,[a] Teh-yung LIU,[b] Ira BERKOWER,[b] and Haruaki YAJIMA*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Sakyo-ku, Kyoto 606, Japan and Office of Biologics Research and Review, Food and Drug Administration,[b] Bethesda, MD 20892, U.S.A. *Received November 14, 1988*

A glycine-linked tetramer of Asn–Ala–Asn–Pro, a tandem repeated sequence of malaria circumsporozoite (CS) protein, was synthesized by the Boc-based solid phase method, followed by deprotection with 1 M trimethylsilyl trifluoromethanesulfonate–thioanisole in trifluoroacetic acid. In addition, three tetramer-related peptides were similarly synthesized, *i.e.*, a 34-residue peptide [linked with TH, a proposed T-cell epitope of CS, at the C-terminus of the tetramer], a 46-residue peptide and a 59-residue peptide [linked with HA or HA′, two proposed T-cell epitopes of influenza hemagglutinin protein, at the N-terminus of the above 34-residue peptide]. Their immunological properties were examined by enzyme-linked immunosorbent assay, for which three different congenic strains of mouse were used to raise the specific antibodies. Despite conjugation of T-cell epitopes to the tetramer, the mice of low-responder strains to the tetramer failed to produce any antibody specific to the tetramer. However, with the aid of recombinant interleukin 2 as an adjuvant, the low-responder mice produced antibody with relatively high titers.

Keywords synthetic malaria vaccine; malaria circumsporozoite protein; solid-phase peptide synthesis; trimethylsilyl trifluoromethanesulfonate deprotection; malaria B-cell epitope; malaria T-cell epitope; adjuvant interleukin 2; congenic mice

The human malaria parasite, *Plasmodium falciparum*, has three stages of multiplication in the life cycle, named sporozoite, merozoite, and gametocyte.[3] The malaria parasite is carried by female mosquitoes that inoculate sporozoites into the bloodstream of human while they ingest the blood. The inoculated sporozoites soon pass into the liver and in a hepatic cell, one sporozoite multiplies to approximately 20000 merozoites. Vaccination with attenuated sporozoites prepared by X-ray irradiation is known to be effective to protect monkeys and humans against malaria infection.[4] However, the malaria parasite can only be cultured in red cells in limited amounts and the vaccines obtained from such a culture still have safety problems because of contamination with many infective agents. To overcome these problems, synthetic malaria vaccines produced by chemical or recombinant deoxyribonucleic acid (DNA) technology seem to be of particular importance.[5]

In 1984, Dame *et al.*[6] determined the nucleotide sequence of the circumsporozoite (CS) protein of *Plasmodium falciparum* which covers the surface membrane of the sporozoite. The gene, encoding 412 amino acids, contains 37 tandem repeats of a tetrapeptide, Asn–Ala–Asn–Pro (NANP[2]), at its central third region. Ballou *et al.*[7a] and Zavala *et al.*[7b] reported that polyclonal antibodies raised against this repeated tetrapeptide, $(NANP)_3$, reacted with the surface of the sporozoite and neutralized its infectivity. However, in our previous experiment,[8] a repeat peptide, $(NANP)_4$, could not produce specific antibodies able to recognize this repeat sequence, except in H-2^b strain mice, and this finding has been confirmed by others.[9] The results indicated that only the H-2^b mouse has the ability to induce a T-cell response, then to produce antibody against the malaria-encoded repeat sequence, but other mice do not. To overcome such T-cell controlled low immune response to peptide antigens, two possible procedures have been proposed: i) use of a synthetic peptide antigen, a B-cell epitope, covalently linked with a peptide fragment which is expected to work as a helper T-cell epitope[10]; ii) use of interleukin 2 (IL-2) as an adjuvant.[11]

In order to examine the usefulness of the above two procedures for possible preparation of synthetic malaria sporozoite vaccine, we have synthesized four peptides containing the tetramer, NANP, *i.e.*, (1) a 17-residue peptide [$(NANP)_4$–Gly] as a positive standard, (2) a 34-residue peptide [$(NANP)_4$–T-cell epitope (TH)], (3) a 46-residue peptide [T-cell epitope (HA)–$(NANP)_4$–TH], and (4) a 59-residue peptide [T-cell epitope (HA′)–$(NANP)_4$–TH] (Table I). From the previous data,[7] the tetramer of NANP can be judged to be one of the B-cell epitopes of CS protein. As T-cell epitopes, three peptides were selected. One is an octadecapeptide, SDKHIEQYLKKIKNSIST,[2] predicted as a helper T-cell site (TH) of CS protein[12] and found to be a nonrepetitive T-cell site on H-2^k strain mice.[13] This sequence is located at about 40 residues from the C-terminal site of the tandem repeated region of the CS protein. The other two were selected from the well characterized sequence of influenza hemagglutinin protein. SSFERFEIFPK (HA) is known to be a T-cell epitope on H-2^d mice[14] and SPKYVKQNTLKLATGMRNVPEKQT (HA′) is a T-cell epitope recognized by human T-cell clone.[15] For these syntheses, Gly was used as a spacer for the peptide 1, and aminohexanoic acid for the peptide 2, 3, and 4. In

TABLE I. Synthetic Peptides Related to the CS Protein of *P. falciparum*

No.	Peptide	Sequence
1	$(NANP)_4$–G	$(NANP)_4$–G
2	$(NANP)_4$–TH	$(NANP)_4$–SDKHIEQYLKKIKNSIST
3	HA–$(NANP)_4$–TH	SSFERFEIFPK–[Sp]–$(NANP)_4$–SDKHIEQYLKKIKNSIST
4	HA′–$(NANP)_4$–TH	SPKYVKQNTLKLATGMRNVPEKQT–[Sp]–$(NANP)_4$–SDKHIEQYLKKIKNSIST

[Sp]: –NH–$(CH_2)_5$–CO–, aminohexanoic acid unit used as spacer.

the former instance, Gly also played a role as an internal standard.

These peptides were synthesized by the Boc-based solid-phase method,[16] followed by deprotection with 1 M trimethylsilyl trifluoromethanesulfonate (TMSOTf)–thioanisole in TFA[17] and purified to apparent homogeneity by high-performance liquid chromatography (HPLC). Each peptide was administered to mice (three groups of congenic strains, *i.e.*, H-2^b, H-2^k, and H-2^d) in the presence or absence of IL-2 and antibodies raised were examined by enzyme-linked immunosorbent assay (ELISA).[18]

Materials and Methods

All reagents and chemicals, including Boc-amino acid derivatives and 4-oxymethylphenylacetamidomethyl (PAM)-resins, were purchased from Applied Biosystems. Synthetic peptides were hydrolyzed with redistilled 6 N HCl for 1 h at 150 °C and amino acid analysis was carried out on a Beckman system 6300 analyzer. HPLC was conducted with a Waters 204 compact model. Sequence analysis was carried out by using an Applied Biosystems 470A gas-phase protein sequencer equipped with a 120A PTH analyzer. H-2 congenic mice were purchased from Jackson Laboratory. Recombinant interleukin 2 (IL-2, lot 003C) was purchased from Dupont.

Solid-Phase Peptide Synthesis Each peptide was synthesized by the Boc-based solid-phase method using the model 430A peptide synthesizer (Applied Biosystems). The following Boc-protected amino acid derivatives were used, *i.e.*, Asp (OBzl), Glu (OBzl), Ser (Bzl), Thr (Bzl), Tyr (Br–Z),[19] Lys (Cl–Z),[20] His (Tos),[21] and Arg (Mts).[22] Each amino acid in DMF was condensed in a stepwise manner by the symmetrical anhydride method,[23] except for Arg, Gln, and Asn. For these amino acids, double couplings of the respective preformed *N*-hydroxybenzotriazole esters in DMF were employed. Every reaction was continued until the resin became negative to the Kaiser test.[24] Thus, other double couplings were occasionally performed.

Deprotection and Purification After assembling every amino acid for peptide 1, 2, 3, and 4, each peptide-resin (0.40 g) was treated with 1 M TMSOTf–thioanisole/TFA (13 ml) in the presence of *m*-cresol (200 eq) in an ice-bath for 120 min, then the resin was removed by filtration. The deprotected peptide was precipitated from the filtrate by addition of dried ether (approximately 200 to 300 ml) and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in water (*ca.* 10 ml) and the pH of the solution was adjusted to 9 by addition of aqueous 10% Et_3N at ice-bath temperature. After being stirred for 20 min in an ice bath, the solution was adjusted to pH 5 with 1 N AcOH. The solution was applied to a Sephadex G-25 column (2.8 × 90 cm), which was eluted with 1 N AcOH. The fractions corresponding to the front main peak [monitored by ultraviolet (UV) absorption measurement at 275 nm] were combined and the solvent was removed by lyophilization to give a white fluffy powder. For further purification of peptide 1 and peptide 2, each gel-filtered sample was subjected to reversed-phase HPLC on a μ-Bondapak C18 column (7.8 × 300 mm). The column was eluted with 13% acetonitrile in aqueous 0.1% TFA for peptide 1, and with a gradient of acetonitrile (26% to 35%, 60 min) in aqueous 0.1% TFA for peptide 2 at a flow rate of 1.8 ml per min. The desired peak fractions were combined and the solvents were removed by repeated lyophilization. The retention time of peptide 1 was 6.7 min in HPLC on an analytical μ-Bondapak C18 column (3.9 × 150 mm), eluted with 13% acetonitrile in aqueous 0.1% TFA at a flow rate of 0.5 ml per min (Fig. 1A), and the retention time of peptide 2 was 10.0 min in HPLC on the same analytical column, eluted with a gradient of acetonitrile (20% to 40%, 20 min) in aqueous 0.1% TFA at a flow rate of 0.8 ml per min (Fig. 1B). For further purifications of peptide 3 and peptide 4, the above respective gel-filtered samples were first fractionated by ion-exchange chromatography. Each sample (100 mg) was dissolved in pH 6.0, 0.01 M ammonium acetate (10 ml), and the solution was applied to a carboxymethyl (CM)-cellulose column (2 × 5 cm), which was eluted with a linear gradient formed from pH 6.0, 0.2 M ammonium acetate (150 ml) through a mixing flask containing pH 6.0, 0.01 M ammonium acetate (150 ml). The respective fractions containing the desired peptide were combined and the solvents and salts were removed by repeated lyophilization. Each CM-purified peptide was finally purified by HPLC on an Ultrapore TM RPSC column (4.6 × 75 mm). The column was eluted with a gradient of acetonitrile (10% to 40%, 40 min) in aqueous 0.1% TFA for peptide 3, and with a gradient of acetonitrile (5% to 35%, 40 min) in aqueous 0.1% TFA for peptide 4 at a flow rate of 0.6 ml per min. The retention time of peptide 3 was 16.3 min (Fig. 1C) and the retention time of peptide 4 was 19.5 min (Fig. 1D) in HPLC on the same column, eluted with a gradient as stated above.

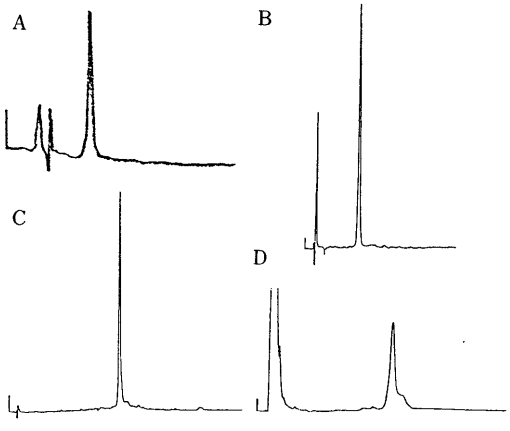

Fig. 1. Analytical HPLC of Synthetic Peptides

Immunoassay For antibody production, as a primary intraperitoneal injection, each synthetic peptide (80 μg) emulsified in complete Freund's adjuvant with or without IL-2 (50 k units) was given to three groups of mice. After 28 d, as a secondary intraperitoneal injection, the synthetic peptide (40 μg each) emulsified similarly in incomplete Freund's adjuvant with or without IL-2 (50 k units) was given. At 10 d after the 2nd injection, the mice were bled from the tail vein and each antibody produced in the sera was estimated by ELISA at various dilutions. For ELISA, the well of a flexible assay plate was first coated with $(NANP)_4$–Gly (2 μg/ml, 90 μl) and then the well was blocked with bovine serum albumin. Next, serum was added, then a second antibody to mouse immunoglobulin G (IgG) and IgM conjugated with alkaline phosphatase was added. Finally, a phosphatase substrate (*p*-nitrophenyl phosphate) was added and, after 30 min, the absorbance was read at 405 nm.

Results and Discussion

Four malaria related peptides (Table I) were synthesized by the Boc-based solid-phase method. In the final step, 1 M TMSOTf–thioanisole/TFA[17] was used to cleave the peptide from the resin, and at the same time to remove all protecting groups employed. For purification of the peptides 1 and 2, consisting of less than 40 amino acids, single preparative HPLC purification was enough to obtain homogeneous products. The purities of the peptides 1 and 2 were both judged to be greater than 97% from the results of analytical HPLC (Fig. 1A and B) and the amino acid analyses (Table II). For purification of longer peptides (peptides 3 and 4), ion-exchange chromatography was necessary before HPLC purification, since the gel-filtered peptides showed a complex elution pattern on HPLC, probably due to contamination with deleted peptides. The purities of the peptides 3 and 4, were evaluated by amino acid analyses (Table II) and analytical HPLC (Fig. 1C and D). Sequence analyses were also performed. Thus, the purity of peptide 3 (46 residues) was judged to be greater than 97% by HPLC and sequencing evaluation, but the purity of peptide 4 (59 residues) was estimated to be 82% from the data of cummulated preview on sequencing. Total yields based on the first amino acid loaded on the resin are listed in Table III. Peptides 1 and 2 were obtained in relatively good yields (30% each), but the yields decreased with chain elongation. Considering the scope and limitations of the present solid-phase peptide synthesis, the yields of peptides 3 and 4 (8% and 3%, respectively) were

Table II. Amino Acid Composition of the Synthetic Peptides

	Peptide			
	1	2	3	4
Asp	7.2 (8)[a]	9.2 (10)	9.9 (10)	11.1 (12)
Thr		1.0 (1)	1.0 (1)	3.6 (4)
Ser		2.6 (3)	4.3 (5)	3.2 (4)
Glu		2.1 (2)	4.1 (4)	4.7 (5)
Pro	3.8 (4)	4.2 (4)	3.4 (5)	6.1 (6)
Gly	1.0 (1)			1.4 (1)
Ala	3.7 (4)	4.0 (4)	4.4 (4)	5.4 (5)
Val				1.8 (2)
Met				0.4 (1)
Ile		2.6 (3)	3.7 (4)	3.0 (3)
Leu		1.0 (1)	1.0 (1)	3.6 (3)
Tyr		0.5 (1)	0.8 (1)	1.0 (2)
Phe			2.9 (3)	
His		0.8 (1)	1.0 (1)	0.8 (1)
Lys		3.5 (4)	4.6 (5)	6.4 (8)
Arg			1.0 (1)	0.7 (1)
Hex[b]			1.2 (1)	0.8 (1)

a) Values are not corrected for acid decomposition at high temperature (150 °C) used for acid hydrolysis. *b*) Aminohexanoic acid.

Table III. Yields of the Synthetic Peptides

No.	Peptide	Residues	Final yield[a] (%)
1	$(NANP)_4$–G	17	30
2	$(NANP)_4$–TH	34	30
3	HA–$(NANP)_4$–TH	46	8
4	HA′–$(NANP)_4$–TH	59	3

a) Final yield, after deblocking and purification.

Table IV. Anti-$(NANP)_4$ Titers Measured by ELISA

Peptide	Mouse strain (H-2)			
	B10·D2 (H-2^d)		B10·BR (H-2^k)	B10 (H-2^b)
	+IL-2	—	—	—
$(NANP)_4$–G	268 (1.23)[a] $n=4$[b]	54 (1.05) $n=3$	95 (1.37) $n=4$	239 (1.28) $n=3$
$(NANP)_4$–TH	243 (1.27) $n=4$	65 (1.60) $n=3$	198 (1.29) $n=5$	258 (1.11) $n=4$
HA–$(NANP)_4$–TH	232 (2.15) $n=3$	45 (1.21) $n=8$	72 (1.19) $n=8$	653 (1.62) $n=5$
HA′–$(NANP)_4$–TH	335 (2.18) $n=5$	45 (1.26) $n=4$	74 (1.56) $n=3$	183 (1.44) $n=3$

a) Numbers in parentheses indicate the standard deviations. *b*) Number of mice examined.

judged to be reasonable. Thus, we were able to demonstrate the usefulness of 1 M TMSOTf–thioanisole/TFA as a deprotecting reagent for solid-phase peptide synthesis.

Using ELISA, we examined antibodies raised against each synthesized peptide in three strains of mice (H-2^b, H-2^k, and H-2^d). As shown in Table IV, despite the addition of T-cell epitopes to the tetramer, the ability to produce antibodies differed depending on the strain of mice. The mice of H-2^b strain produced specific antibody against every peptide administered, but the mice of H-2^k strain produced antibody with a relatively high titer only against peptide 2. Thus, the addition of TH peptide to the tetramer exhibited a positive effect on specific antibody production in the H-2^k strain mice, but the addition of HA or HA′ peptides had no effect. The mice of H-2^d strain could not produce specific antibodies. However, by the administration of each peptide together with IL-2 as an adjuvant, antibodies with relatively high titers were obtained in the H-2^d strain mice.

As reported by Kawamura *et al.*,[11] and Good *et al.*,[25] IL-2 may have an ability to enhance suboptimal T-cell help in low responders, but the mechanism of this effect is still unclear. In our experiments, the non-responder mice to the repeated sequence behaved as responder mice when IL-2 was used as an adjuvant. The role of IL-2 in antibody production is under investigation by using a T-cell proliferation assay method.

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References and Notes

1) Part CLXVI. Y. Hayashi, S. Katakura, M. Nomizu, A. Tashiro, S. Kuwata, N. Miaki, T.-Y. Liu, N. Fujii, and H. Yajima, *Chem. Pharm. Bull.*, **36**, 4993 (1988).
2) Amino acids used here are of the L-configuration and single letter abbreviations for amino acids are used: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr. The following abbreviations are also used: Boc = *tert*-butoxycarbonyl, Bzl = benzyl, Z = benzyloxycarbonyl, Mts = mesitylenesulfonyl, Tos = *p*-toluenesulfonyl, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DMF = dimethylformamide.
3) W. Trager and J. B. Jensen, *Science*, **193**, 673 (1976).
4) R. S. Nussenzweig and V. Nussenzweig, *Phil. Trans. R. Soc. Lond.*, **B307**, 117 (1984).
5) L. H. Miller, R. J. Howard, R. Carter, M. F. Good, M. Nussenzweig, and R. S. Nussenzweig, *Science*, **234**, 1349 (1986).
6) J. B. Dame, J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, I. Schneider, D. Roberts, G. S. Sanders, E. P. Reddy, C. L. Diggs, and L. H. Miller, *Science*, **225**, 593 (1984).
7) *a*) W. R. Ballou, J. Rothbard, R. A. Wirtz, D. M. Gordon, J. S. Williams, R. W. Gore, I. Schneider, M. R. Hollingdale, R. L. Beaudoin, W. L. Maloy, L. H. Miller, and W. T. Hockmeyer, *Science*, **228**, 996 (1985); *b*) F. Zavala, J. P. Tam, M. R. Hollingdale, A. H. Cochrane, I. Quakji, R. S. Nussenzweig, and V. Nussenzweig, *ibid.*, **228**, 1436 (1985).
8) M. F. Good, J. A. Berzofsky, W. L. Maloy, Y. Hayashi, N. Fujii, W. T. Hockmeyer, and L. H. Miller, *J. Exp. Med.*, **164**, 655 (1986).
9) G. D. Giudice, J. A. Cooper, J. Merino, A. S. Verdini, A. Pessi, A. R. Togna, H. D. Engers, G. Corradin, and P.-H. Lambert, *J. Immunol.*, **137**, 2952 (1986).
10) M. J. Francis, G. Z. Hastings, A. D. Syred, B. McGinn, F. Brown, and D. J. Rowlands, *Nature* (London), **300**, 168 (1987).
11) H. Kawamura, S. A. Rosenberg, and J. A. Berzofsky, *J. Exp. Med.*, **162**, 381 (1985); I. A. Ramshaw, M. E. Andrew, S. M. Phillips, D. B. Boyle, and B. E. H. Coupar, *Nature* (London), **329**, 545 (1987).
12) C. Delisi and J. A. Berzofsky, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7048 (1985); J. L. Spouge, H. R. Guy, J. L. Cornette, H. Margalit, K. Cease, J. A. Berzofsky, and C. Delisi, *J. Immunol.*, **138**, 204 (1987).
13) M. F. Good, W. L. Maloy, M. N. Lunde, H. Margalit, J. L. Cornette, G. L, Smith, B. Moss, L. H. Miller, and J. A. Berzofsky, *Science*, **235**, 1059 (1987).
14) C. J. Hackett, B. Dietzschold, W. Gerhard, B. Ghrist, R. Knorr, D. Gillessen, and F. Melchers, *J. Exp. Med.*, **158**, 294 (1983).
15) J. R. Lamb and N. Green, *Immunology*, **50**, 659 (1983).
16) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
17) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakoshi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 274.
18) E. Engvall and P. Perlmann, *Immunochemistry*, **8**, 871 (1971).
19) D. Yamashiro and C. H. Li, *J. Org. Chem.*, **38**, 591 (1973).

20) B. W. Erickson and R. B. Merrifield, *J. Am. Chem. Soc.*, **95**, 3757 (1973).
21) S. Sakakibara and T. Fujii, *Bull. Chem. Soc. Jpn.*, **42**, 1466 (1969).
22) H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3752 (1978).
23) J. Blake and C. H. Li, *Int. J. Peptide Protein Res.*, **7**, 495 (1975).
24) E. Kaiser, R. L. Colescott, C. D. Bossinger, and P. I. Cook, *Anal. Biochem.*, **34**, 595 (1970).
25) M. F. Good, D. Pombo, M. N. Lunde, W. L. Maloy, R. Halenbeck, K. Koths, L. H. Miller, and J. A. Berzofsky, *J. Immunol.*, **141**, 972 (1988).