PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Towards a Synthetic Malaria Vaccine: Cyclization of a Peptide Eliminates the Production of Parasite-Unreactive Antibody

Howard M. Etlinger and Arnold Trzeciak

Phil. Trans. R. Soc. Lond. B 1993 **340**, 69-72
doi: 10.1098/rstb.1993.0049

Towards a synthetic malaria vaccine: cyclization of a peptide eliminates the production of parasite-unreactive antibody

HOWARD M. ETLINGER AND ARNOLD TRZECIAK

Pharmaceutical Research New Technologies, F. Hoffmann-La Roche & Co. Ltd, CH-4002, Basel, Switzerland

SUMMARY

In a previous study, human beings were vaccinated with a *P. falciparum* malaria vaccine candidate consisting of tetanus toxoid coupled to linear (Asn-Ala-Asn-Pro)$_3$ ((NANP)$_3$). The vaccine initiated protection in some people, but some individuals mainly produced anti-peptide antibodies that did not react with the pathogen. A likely contributor to the formation of epitopes that give rise to pathogen-unreactive antibodies is the free terminal proline which is not a terminal residue in the native protein. To avoid the elicitation of antibodies against terminal epitopes, (NANP)$_3$ was cyclized. In contrast to monoclonal antibodies to the linear peptide where 35% were unreactive with the parasite, all monoclonal antibodies to the cyclized peptide were found to react with the parasite.

1. INTRODUCTION

The structures of a pathogen that are recognized by protective antibodies or T lymphocytes can be termed 'protectopes.' Protectopes can be made using synthetic peptides corresponding to a partial protein sequence. (Asn-Ala-Asn-Pro)$_3$ (NANP)$_3$ (Herrington *et al.* 1987) is such a protectope. It represents (Zavala *et al.* 1985) the central tandem repeat area of the major sporozoite surface protein, the circumsporozoite (CS) protein, of *P. falciparum* (Dame *et al.* 1984; Enea *et al.* 1984). People who were injected with (NANP)$_3$-coupled tetanus toxoid (TT) produced pathogen-reactive antibody. However, some individuals produced mainly anti-peptide antibodies that did not react with the pathogen (Etlinger *et al.* 1988). One approach to representing protein structure more precisely is to conformationally restrict peptides by hydrogen bond mimicry with covalent hydrogen bond mimics (Satterthwait *et al.* 1988; Satterthwait *et al.* 1989). An alternative is the use of cyclized peptides which may be superior to linear peptides in inducing antibodies that are reactive with the native protein when the peptide is not a terminal sequence in the protein (Arnon & Sela 1969; Arnon *et al.* 1971; Steward & Howard 1987). Here we show that in mice the use of cyclic (NANP)$_3$ (c(NANP)$_3$) eliminates the generation of non pathogen-reactive, i.e. 'useless' antibodies.

2. MATERIALS AND METHODS

(*a*) *Preparation of c(NANP)*$_3$

c(NANP)$_3$ was synthesized by the solid-phase technique using a *p*-benzyloxybenzylalcohol polystyrene resin (Atherton & Sheppard 1987). The synthesis was started with an N^{α}-unprotected Dab(Z)-resin that was elongated by three repeated couplings of Fmoc-NANP using the *O*-benzotriazolyl-*N*,*N*,*N*,*N*-tetramethyl uronium tetrafluoroborate (TBTU) procedure (Knorr *et al.* 1989). (NANP)$_3$-Dab(Z) was cleaved from the resin by trifluoroacetic acid and cyclized with TBTU. The Z-group of the gamma diaminobutyric acid was removed by hydrogenolysis. The free amino group of the cyclized peptide was acylated with Boc-Cys(Trt)-OH in the presence of TBTU. For conjugation the peptide was treated with trifluoroacetic acid–triethylsilane to obtain the free thiol function.

(*b*) *Production of monoclonal antibodies*

Hybridomas were then prepared by fusing spleen cells of BALB/c mice immunized with (Ac-Cys-(NANP)$_3$)$_{19\text{ or }35}$-TT (three fusions yielding 14 monoclonals) or (c(NANP)$_3$)$_{19}$-TT (eight fusions yielding 17 monoclonals) with PAI, a non-Ig secreting myeloma (Stocker *et al.* 1982). TT conjugates with linear or c(NANP)$_3$ side chains were prepared as described (Etlinger *et al.* 1988). Monoclonals were judged to be independent if they were derived from different fusions or if their isoelectric focusing spectrotype of Ig light chains or fine specificity of binding to variant (Etlinger *et al.* 1991) (NANP)$_3$ peptides were distinct (data not shown).

(*c*) *Reactivity of monoclonal antibodies*

Supernates from cloned hybridomas were tested for reactivity with sporozoites by fluorescent antibody

Phil. Trans. R. Soc. Lond. B (1993) **340,** 69–72
Printed in Great Britain

Table 1. *Reactivity of monoclonal anti-linear (NANP)$_3$ antibodies*

Ac-Cys-(NANP)$_3$ reactive			Titer on							
antibody	isotype	Sporo. reactive	Ac-Cys-(NANP)$_3$	Ac-Cys-(PNAN)$_3$	Ac-Cys-(NANP)$_3$ BSA	c(NANP)$_3$	c(NANP)$_3$ BSA	(NANP)$_{50}$	HEL46-61BSA	TT73-99
A24(10)	IgG1	yes	6400	200	3200	6400	6400	6400	12	< 3
A5(1)	IgG1	yes	3200	25	1600	1600	3200	6400	3	< 3
C20(1)	IgG1	yes	13	< 3	800	< 3	13	13	3	< 3
D21(8)	IgG1	yes	25	< 3	12 800	< 3	6	20	< 3	< 3
A8(1)	IgG1	yes	100	25	6400	1600	12 600	1600	6	3
1A(3)	IgM	yes	1600	800	800	1600	1600	1600	13	6
A11(2)	IgM	yes	1600	400	1600	1600	1600	6400	50	13
MB4	IgM	yes	3200	3200	1600	1600	3200	6400	50	25
A3(2)	IgM	yes	400	1600	1600	800	800	6400	25	13
C12(1)	IgG2b	no	1600	6	6400	6	13	50	13	6
C6(4)	IgG2b	no	1600	< 3	3200	3	3	25	3	3
B2(8)	IgG2b	no	50	3	3200	3	6	13	3	3
A13(3)	IgM	no	1600	13	1600	25	25	100	25	6
MA10	IgM	no	800	13	1600	25	12	25	25	6
non-Ac-Cys-(NANP)$_3$ reactive										
2A(5)	IgM	no	25	25	13	25	25	25	50	13
3C(3)	IgM	no	50	50	25	100	25	100	50	50

techniques (Etlinger *et al.* 1988) and for reactivity to the antigens shown in tables 1 and 2 using an ELISA (Etlinger et al. 1991). c(NANP)$_3$ and peptide consisting of amino acid residues 46–61 of hen egg-white lysozyme (HEL) were coupled to bovine serum albumin (BSA) as described for the coupling of Ac-Cys-(NANP)$_3$ to TT (Etlinger et al. 1988). Ac-Cys-(PNAN)$_3$, (NANP)$_{50}$, peptide consisting of amino acid residues 73–99 of tetanus toxoid (TT73–99) and HEL46–61 were prepared as described (Etlinger *et al.* 1990, 1991; Etlinger & Knorr 1991). For ELISA, antigens were coated at a concentration of 2 μg ml^{-1}. Titers are reciprocals of the last dilution of supernate for which $0.1 \leqslant A_{455} < 0.2$, where A_{455} is absorbance at wavelength 455 nm. Titers greater than twice that on HEL46–61BSA (for conjugates) or TT73–99 (for peptides) are considered significant. Monoclonals not reacting with Ac-Cys-(NANP)$_3$ are included in order to show the specificity of binding to the various antigens.

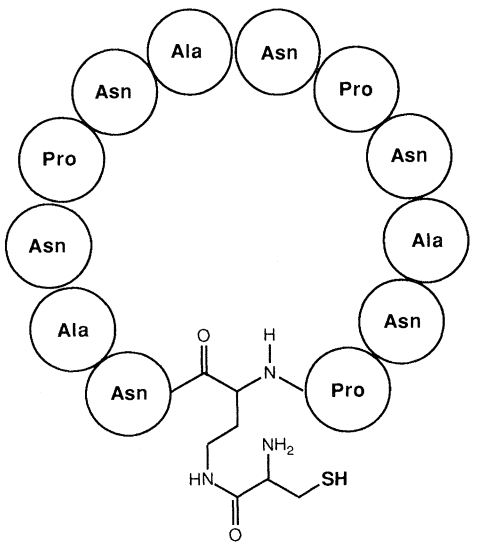

Figure 1. Representation of c(NANP)$_3$-Dab-Cys.

3. RESULTS

Each linear (NANP)$_3$ molecule conjugated to TT has a free terminus. Since there is no such structure in the native protein, these free termini are likely contributors to the formation of epitopes that elicit useless antibodies. We cyclized (NANP)$_3$, so that the cyclic peptide would lack 'terminal epitopes'. For cyclization, the amino group of the terminal asparagine and the carboxyl group of the terminal proline were joined to diamino butyric acid (Dab); cysteine was coupled to the gamma amino group of Dab to permit linkage to the carrier protein (figure 1).

To evaluate whether c(NANP)$_3$ elicited a higher proportion of sporozoite-reactive antibodies than linear (NANP)$_3$, monoclonal antibodies were prepared from lymphocytes of mice injected with linear (NANP)$_3$-TT or c(NANP)$_3$-TT. Of 14 monoclonals obtained against linear (NANP)$_3$, nine reacted with sporozoites while five did not (table 1). In contrast, all 17 monoclonals obtained against c(NANP)$_3$ reacted with sporozoites (table 2).

Ac-Cys-(PNAN)$_3$ shares linear sequence 'inner domains' but not 'end domains' with Ac-Cys-(NANP)$_3$ (figure 2). We expected that sporozoite-

Ac-Cys-NANPNANPNA-NP
Ac-Cys-P-NANPNANPNA-N

Figure 2. 'Inner domains' of Ac-Cys-(NANP)$_3$ and Ac-Cys-(PNAN)$_3$ with identical primary structures marked by square box.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Table 2. *Reactivity of monoclonal anti-c(NANP)$_3$ antibodies*

			Titer on							
Antibody	Isotype	Sporo. reactive	Ac-Cys-(NANP)$_3$	Ac-Cys-(PNAN)$_3$	Ac-Cys-(NANP)$_3$ BSA	c(NANP)$_3$	c(NANP)$_3$ BSA	(NANP)$_{50}$	HEL46-61BSA	TT73-99
1A1(1)	IgG1	yes	< 3	400	3200	400	3200	3200	< 3	< 3
A1/4(1)	IgG2$_a$	yes	25 600	51 200	51 200	102 400	204 800	204 800	100	200
A5/4(1)	IgG1	yes	3	25	12 800	3200	25 600	12 800	3	3
A2/1(1)	IgG1	yes	12 800	12 800	12 800	12800	6400	25 600	25	6
A1/6(1)	IgG2$_a$	yes	< 3	< 3	100	3200	12 800	6400	< 3	< 3
A1/1(2)	IgG2$_b$	yes	25 600	6400	12 800	6400	6400	25 600	6	6
A5/1(1)	IgG1	yes	25	13	6400	25 600	25 600	25 600	13	< 3
A4/7(1)	IgG1	yes	100	50	12 800	25 600	51 200	1600	3	< 3
A2/6(1)	IgG1	yes	1600	50	12 800	6400	25 600	51 200	200	< 3
C4/6(1)	IgG1	yes	1600	200	6400	6400	51 200	3200	100	50
A5/5(1)	IgG1	yes	6400	800	25 600	12800	51 200	12 800	100	50
A3/3(1)	IgG1	yes	< 3	< 3	6	400	6400	12	< 3	< 3
A6/3(3)	IgG1	yes	50	25	6400	6400	25 600	1600	< 3	< 3
A6/6(1)	IgG1	yes	800	50	6400	6400	12 800	12 800	200	< 3
C1/6(1)	IgG1	yes	6	50	6400	6400	12 800	25 600	< 3	< 3
A1/5(1)	IgG1	yes	25	25	800	6400	102 400	1600	25	13
B3/4(1)	IgG1	yes	25	25	1600	6400	102 400	25 600	3	< 3

unreactive antibodies would react with an epitope which includes the terminal proline and, thus, not react with Ac-Cys-(PNAN)$_3$. Only the sporozoite-reactive antibodies elicited by Ac-Cys-(NANP)$_3$-TT reacted with Ac-Cys-(PNAN)$_3$ (table 1).

Finally, antibodies from individuals living in an area in Nigeria where *P. falciparum* malaria is endemic as well as antibodies elicited by *P. falciparum* sporozoites in mice reacted with c(NANP)$_3$ (data not shown) and mouse antisera to c(NANP)$_3$ inhibited *P. falciparum* sporozoites from invading fresh human liver cells in an *in vitro* assay (D. Mazier & H. Etlinger, unpublished observations).

4. DISCUSSION

The NANP repeat region in the native protein has been predicted to have helical conformations (Brooks *et al.* 1987; Gibson & Scheraga 1986). Proton nuclear magnetic resonance and ultraviolet circular dichroism spectroscopy measurements indicate that in water solution linear repeat sequences have local and/or turnlike or helical structures in dynamic equilibrium with extended chain forms (Dyson *et al.* 1990). Because neither the structures of the cyclized (NANP)$_3$ peptide nor the (NANP) repeat area in the native CS protein are known, it is unclear whether the increased frequency of anti-peptide antibodies that react with the pathogen results from elimination of the free termini and/or a better mimicry of the native structure.

The capacity of antibodies elicited by c(NANP)$_3$ to prevent sporozoites from invading liver cells indicates the peptide's potential for eliciting protective immunity. However, it should be noted that monkeys are protected by some but not all antibodies against *P. vivax* CS repeat sequences, even though each antibody inhibits sporozoites from invading liver cells in an in vitro assay (Charoenvit *et al.* 1991) . It appears that the fine specificity of anti-repeat antibodies is crucial for protection. Very recently, ectopic human hepatocyte transplantation has been used as a mouse model for the exoerythrocytic stages of *P. falciparum* (Sacci *et al.* 1992). This model requires further evaluation with regard to its relevance for human beings. Despite this promising development there is no practical animal model for evaluating vaccines against the sporozoite stage of the parasite. Our aim is to evaluate the efficacy of a vaccine based on c(NANP)$_3$ in a clinical study.

We are grateful to D. Kronenberger, A. Woreth, M. Felder, B. Hennequin, I. Bolliger and C. Kocyba for excellent technical assistance, H. Matile for determination of sporozoite reactivity and gift of sporozoites, M. Mannenberg for performing amino acid analyses, F. Oliveri and K. Karjalainen for advice and help with isoelectric focusing, and W. Haas and C. Steinberg for critical review of the manuscript.

REFERENCES

Arnon, R. & Sela, M. 1969 Antibodies to a unique region in lysozyme provoked by a synthetic antigen conjugate. *Proc. natn. Acad. Sci. U.S.A.* **62**, 163–170.

Arnon, R., Maron, E., Sela, M. & Anfinson, C.B. 1971 Antibodies reactive with native lysozyme elicited by a completely synthetic antigen. *Proc. natn. Acad. Sci. U.S.A.* **68**, 1450–1455.

Atherton, A. & Sheppard, R.C. 1987 In *The peptides: analysis, synthesis, biology* vol. 9. (ed. S. Udenfriend, & J. Meienhofer), pp. 1–38. New York: Academic Press.

Brooks, B.R., Pastor, R.W. & Casson, F.W. 1987 Theoretically determined three-dimensional structure for the repeating tetrapeptide unit of the circumsporozoite coat

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

protein of the malaria parasite *Plasmodium falciparum*. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4470–4474.

Charoenvit, Y., Collins, W.E., Jones, T.R., Millet, P., Yuan, L., Campbell, G.H., Beaudoin, R.L., Broderson, J.R. & Hoffman, S.L. 1991 *Science, Wash.* **251**, 668–671.

Dame, J.B., Williams, J.L., McCutchan, T.F., Weber, J.L., Wirtz, R.A., Hockmeyer, W.T., Maloy, W.L., Haynes, J.D., Schneider, I., Roberts, D., Sanders, G.S., Reddy, E. P., Diggs, C.L. & Miller, L.H. 1984 Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. *Science, Wash.* **225**, 593–599.

Dyson, H.J., Satterthwait, A.C., Lerner, R.A. & Wright, P.E. 1990 Conformational preferences of synthetic peptides derived from the immunodominant site of the circumsporozoite protein of *Plasmodium falciparum* by ^{1}H NMR. *Biochemistry* **29**, 7828–7837.

Enea, V., Ellis, J., Zavala, F., Arnot, D.E., Asavanich, A., Masuda, A., Quakyi, I. & Nussenzweig, R.S. 1984 DNA cloning of *Plasmodium falciparum* circumsporozoite gene: amino acid sequence of repetitive epitope. *Science, Wash.* **225**, 628–634.

Etlinger, H.M., Felix, A.M., Gillessen, D., Heimer, E.P., Just, M., Pink, J.R.L., Sinigaglia, F., Stürchler, D., Takacs, B., Trzeciak, A. & Matile, H. 1988 Assessment in humans of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, *Plasmodium falciparum*. *J. Immunol.* **140**, 626–633.

Etlinger, H.M., Gillessen, D., Lahm, H.-W., Matile, H., Schönfeld, H.-J. & Trzeciak, A. 1990 Use of prior vaccinations for the development of new vaccines. *Science, Wash.* **249**, 423–425.

Etlinger, H.M., Renia, L., Matile, H., Manneberg, M., Mazier, D., Trzeciak, A. & Gillessen, D. 1991 Antibody responses to a synthetic peptide based malaria vaccine candidate: influence of sequence variants of the peptide. *Eur. J. Immunol.* **21**, 1505–1511.

Etlinger, H.M. & Knorr, R. 1991 Model using a peptide with carrier function for vaccination against different pathogens. *Vaccine* **9**, 512–514.

Gibson, K.D. & Scheraga, H.A. 1986 Predicted conformations for the immunodominant region of the circumsporozoite protein of the human malaria parasite *Plasmodium falciparum*. *Proc. natn. Acad. Sci. U.S.A.* **83**, 5649–5653.

Herrington, D.A., Clyde, D.F., Losonsky, G., Cortesia, M., Davis, J., Murphy, J.R., Felix, A.M., Heimer, E.P., Gillessen, D., Nardin, E., Nussenzweig, R.S., Nussenzweig, V., Hollingdale, M.R. & Levine, M.M. 1987 Safety and immunogenicity in man of a synthetic peptide tetanus toxoid conjugate malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature, Lond.* **328**, 257–259.

Knorr, R., Trzeciak, A., Bannwarth, W. & Gillessen, D. 1989 New coupling reagents in peptide chemistry. *Tetrahedron Lett.* **30**, 1927–1930.

Sacci, J.B., Schriefer, M.E., Resau, J.H., Wirtz, R.A., Detolla, Jr., L.J., Markham, R.B. & Azad, A.F. 1992 Mouse model for exoerythrocytic stages of *Plasmodium falciparum* malaria parasite. *Proc. natn. Acad. Sci. U.S.A.* **89**, 358–362.

Satterthwait, A.C., Arrhenius, T., Hagopian, R.A., Zavala, F., Nussenzweig, V. & Lerner, R.A. 1988 Conformational restriction of peptidyl immunogens with covalent replacements for the hydrogen bond. *Vaccine* **6**, 99–103.

Satterthwait, A.C., Arrhenius, T., Hagopian, R.A., Zavala, F., Nussenzweig, V. & Lerner, R.A. 1989 The conformational restriction of synthetic peptides, including a malaria peptide, for use as immunogens. *Phil. Trans. R. Soc. Lond.* B **323**, 565–572.

Steward, M.W. & Howard, C.R. 1987 Synthetic peptides: a next generation of vaccines? *Immunol. Today* **8**, 51–58.

Stocker, J.W., Forster, H.K., Miggiano, V., Stähli, C., Staiger, G., Takacs, B. & Staehelin, T. 1982 Generation of 2 new mouse myeloma cell lines PAI and PAI-O for hybridoma production. *Res. Disclosure* 21713. **217**, 155–156.

Zavala, F., Tam, J.P., Cochrane, A.H., Quakyi, I., Nussenzweig, R.S. & Nussenzweig. 1985 Rationale for development of a synthetic vaccine against *Plasmodium falciparum* malaria. *Science, Wash.* **228**, 1436–1440.

Received 17 September 1992; accepted 22 October 1992

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES