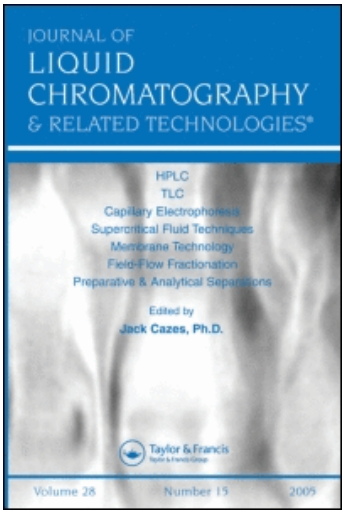

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Theodore Lambros[a]; Pedro Lema Iii[a]; Wen Liu[a]; Jacob Bongers[a]; Arthur M. Felix[a]; Edgar P. Heimer[a]
[a] Department of Peptide Research, Roche Research Center, Hoffmann-La Roche Inc., Nutky, New Jersey

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CAPILLARY ELECTROPHORESIS OF MULTIPLE ANTIGENIC-PEPTIDE (MAPS) OF THE MALARIA CIRCUMSPOROZOITE PROTEIN EPITOPES

THEODORE LAMBROS, PEDRO LEMA III, WEN LIU, JACOB BONGERS, ARTHUR M. FELIX, AND EDGAR P. HEIMER*

Department of Peptide Research
Roche Research Center, Hoffmann-La Roche Inc.
340 Kingsland Street
Nutley, New Jersey 07110

ABSTRACT

Capillary electrophoresis (CE) was evaluated as a means of analyzing the homogeneity of synthetic malaria vaccines consisting of multiple-antigenic-peptides (MAPs). The MAPs consist of a branching oligomeric lysine core (Lys_n, n = 3, 7, 15, ...) with antigenic peptides coupled to each of the α-amino and ε-amino arms (n+1 peptides total). In this report we compare CE with reversed-phase and size-exclusion liquid chromatography (RP-HPLC and SE-HPLC) for the analysis of Lys_3 MAPs corresponding to B-cell and T-cell epitopes of the circumsporozoite protein of the *Plasmodium falciparum* malaria parasite.

INTRODUCTION

The worldwide number of cases of malaria is on the rise, totaling now one hundred million clinical cases. *Plasmodium falciparum*, the parasite causing the majority of these cases, is responsible for over one million deaths each year (1). This resurgence of the disease is due to increasing drug

resistance and resistance of the mosquitos to insecticides. As a control measure, there has also been a wide interest in the development of a synthetic vaccine. The immunodominant epitope of the *P. falciparum* circumsporozoite (CS) protein, which covers the surface of the mature sporozoite, has a repetitive sequence consisting of 40 tandem repeats of the tetrapeptide Asn-Ala-Asn-Pro (NANP) (2). Antipeptide antibodies directed against this sequence have been shown to neutralize CS protein *in vitro* (3). Recently, a synthetic tridecapeptide Ac-Cys-$(NANP)_3$-OH, conjugated to tetanus toxoid (TT) as a carrier protein, was evaluated in a limited trial in human volunteers (4). The results of this study showed that the vaccines exhibited only modest levels of antibody titers and protection possibly due to a dampening of the immune response by TT.

In order to circumvent the use of a carrier protein, Tam has recently developed a novel approach (5) in which an immunogenic peptide was multiply-linked to a branching trifunctional amino acid and prepared by solid phase peptide synthesis (SPPS). The resultant multiple antigenic peptides (MAPs) have been shown to elicit high titers of antipeptide antibodies thus eliminating the need for a protein carrier.

The MAPs system of the CS protein B-cell epitope, $[(NANP)_3]_8Lys_7$-Aca-Cys-NH_2 (Aca = ϵ-amino-*n*-caproic acid) was synthesized in our laboratory by the SPPS procedure and it was shown to elicit comparable antibody titers in mice as that observed for the Ac-Cys-$(NANP)_3$-TT conjugate in two separate immunization studies (6).

It has recently been recognized that in addition to the B-cell epitope, $(NANP)_n$, a universal malaria vaccine should also contain T-cell epitopes (7,8) which are important for cell-mediated immunity to the protective response. Combination of B-cell/T-cell epitopes using MAPs have been described (9-11) and found to elicit high antibody responses. Sinigaglia et al. (12) have recently reported on a T-cell epitope corresponding to the segment 378-398 of the CS protein from *P. falciparum*. This sequence has also been shown to

generate sequence-specific human T-cell clones. This observation prompted us to synthesize a series of MAPs containing the CS protein sequence 378-398 (T-cell) in combination with the NANP (B-cell) repetitive unit as potential vaccine candidates.

The synthesis and characterization of MAPs is a formidable task due to their high molecular weight and tendency to form large aggregates. In this paper we report the use of capillary zone electrophoresis (CZE) in conjunction with analytical HPLC to determine the homogeneity of these malarial B-Cell/T-Cell multiple antigenic peptides.

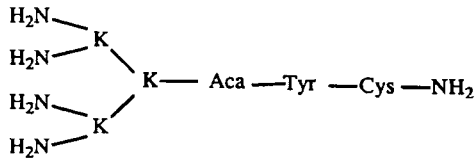
MATERIALS AND METHODS

MAP Peptides

The solid phase syntheses (13) of the tetrameric B-cell MAPs, $[(NANP)_3]_4$-Lys_3-Aca-Tyr-Cys-NH_2 (Aca = ϵ-amino-*n*-caproic acid) ; T-cell MAP peptide, $(EKKIAKMEKASSVFNVV)_4$-Lys_3-Aca-Tyr-Cys-NH_2; and the series- and parallel-(B-cell/T-cell) MAPs were carried out by the multiple antigenic peptide synthesis methodology (5,14,15). The resultant crude peptides were purified by dialysis and size-exclusion (SE) HPLC. Amino acid analysis gave satisfactory results in agreement with the expected composition.

Electrophoresis

All separations were performed on a Spectra PHORESIS 1000 instrument (Spectra Physics, San Jose, CA) equipped with a fast-scanning variable-wavelength UV-vis detector. The fused-silica capillary with polyimide outer-coating (70 cm × 75 μm i.d.) was coiled in a cartridge thermostated by circulating air. Sample injection was by electromigration at 15 kV for 1-2 s and separation was performed at a constant 25 kV, 25°C. The capillary was washed with running buffer (3-5 min) immediately prior to injection and treated with 0.1 M NaOH (3-5 min) followed by water (3-5 min) between runs. The 50 mM phosphate running buffer (pH 1.7) was prepared by mixing equimolar solutions of NaH_2PO_4 and H_3PO_4.



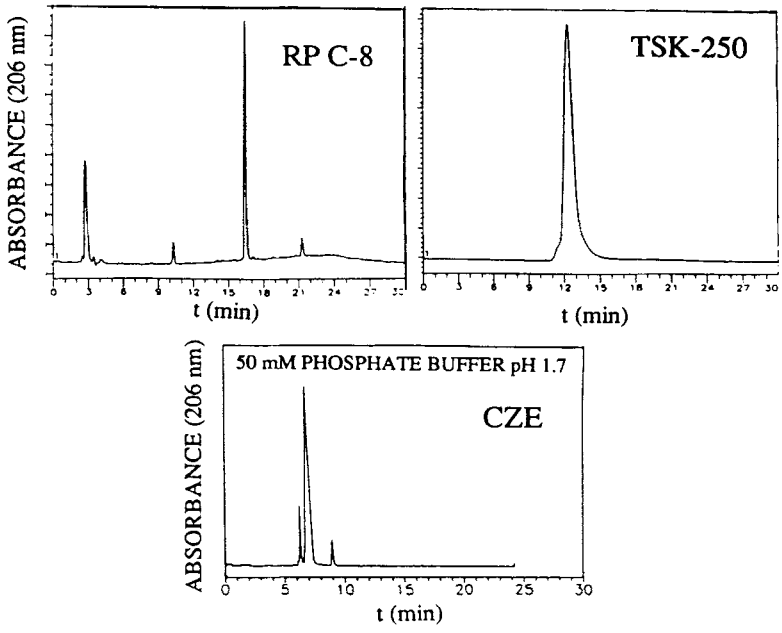


Figure 1. Analysis of $(Lys)_3$-Aca-Tyr-Cys-NH_2.

HPLC

Analytical HPLC was carried out on a Laboratory Data Control Constametric IIG (LDC Analytical, Riviera Beach, FL) equipped with a Gradient Master and Spectromonitor III UV-variable-wavelength detector. Reverse-phase HPLC (RP-HPLC) was carried out on a *Lichrosorb C-8* column (5μ, 0.40 cm × 25 cm) by using the eluants, (A) 0.1 M $NaClO_4$ (pH 2.5) and (B) CH_3CN, with a gradient of 10% to 70% B in 20 minutes, at a

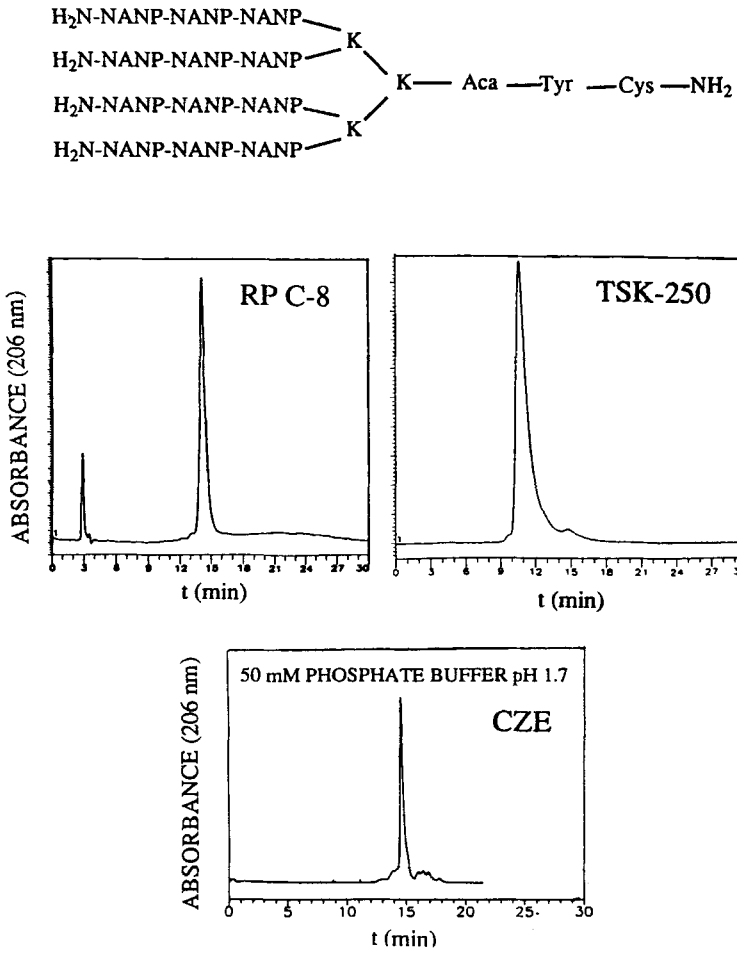


Figure 2. Analysis of B-cell MAPs.

flow rate of 1.0 mL/min, and detection at 206 nm. Size-exclusion HPLC (SE-HPLC) was carried out on a Bio-Sil TSK-250 column (0.75 cm × 30 cm) in 50 mM KH_2PO_4/H_3PO_4 buffer (pH 2.5), at a flow rate of 1.0 mL/min flow rate, and detection at 206 nm.

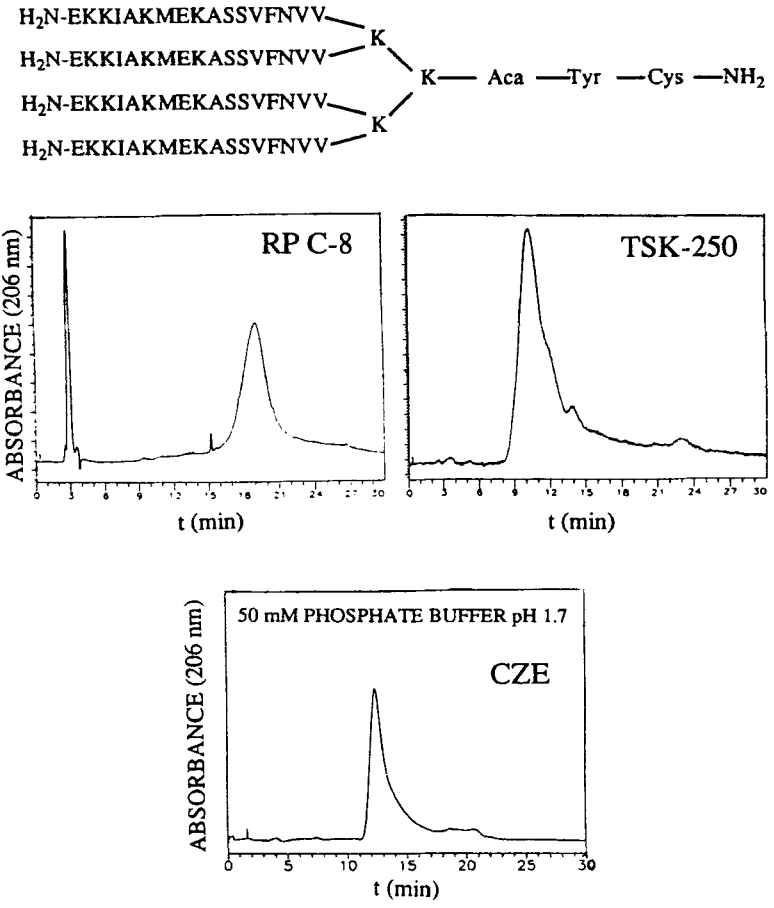
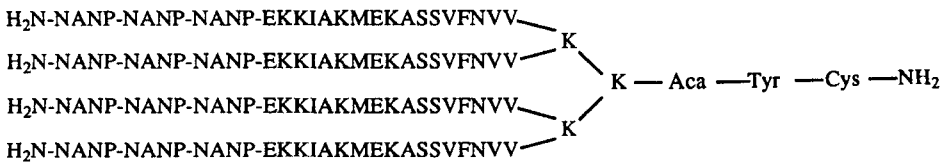


Figure 3. Analysis of T-cell MAPs.

RESULTS AND DISCUSSION

A portion of the $(Lys)_3$-Aca-Tyr-Cys-(benzhydrylamine resin) used in the synthesis of the MAPs was cleaved with anhydrous HF and evaluated as shown in Figure 1. Analytical RP-HPLC and CZE revealed that the material gave sharp peaks and impurities were readily resolved. These impurities



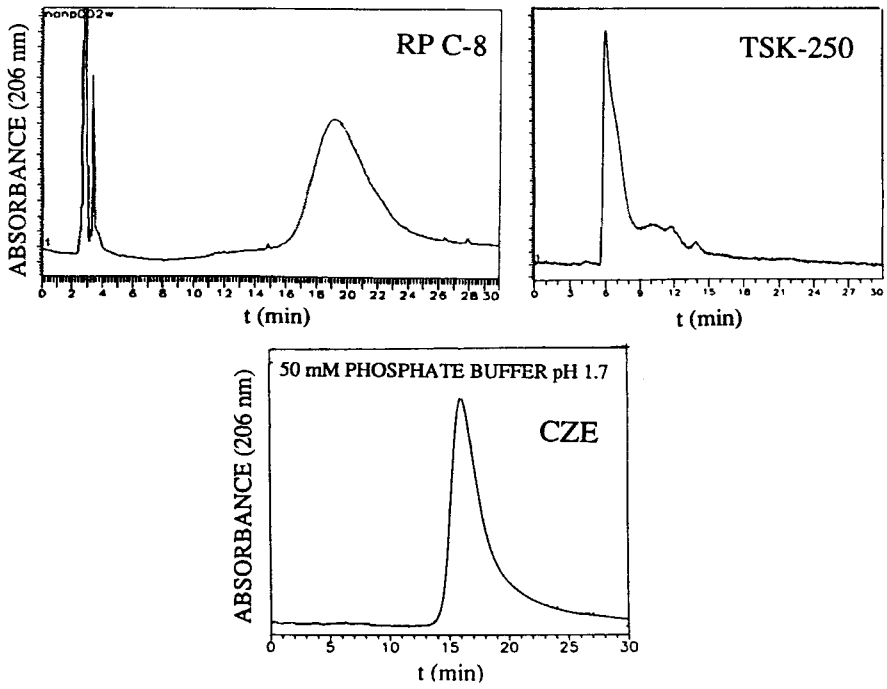


Figure 4. Analysis of series-(B-cell/T-cell) MAPs.

arising from incomplete coupling during solid phase peptide synthesis (SPPS), as expected, are not resolved by size-exclusion chromatography (SE-HPLC).

The B-cell containing MAP, $[(NANP)_3]_4(Lys)_3$-Aca-Tyr-Cys-(benzhydrylamine resin), was cleaved with HF and the resultant product also examined by the three chromatographic methods (Figure 2). RP-HPLC failed to resolve the impurities which eluted as shoulders while CZE clearly showed

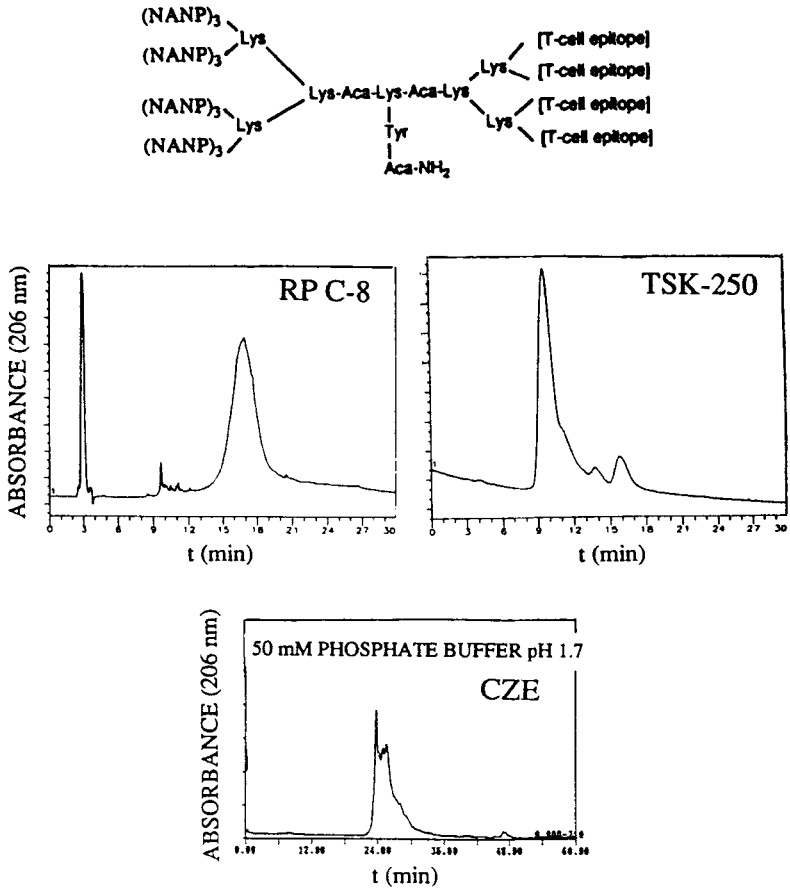


Figure 5. Analysis of parallel-(B-cell/T-cell) MAPs.

their resolution from the product band. The presence of these lower-molecular-weight impurities was shown by SE-HPLC. The T-cell MAPs construct, containing the amphipathic α-helix, [$Ala^{384,389}$]-CS(380-396)-NH_2, was shown to be a broad featureless band by both RP-HPLC and CZE (Figure 3). The series-(B-cell/T-cell) MAPs also showed broadness in both RP-HPLC and CZE and smaller molecular-weight components by SE-HPLC

(Figure 4). Finally, the analytical RP-HPLC of the parallel-(B-cell/T-cell) MAPs showed a broad peak while CZE revealed multiple impurities which were in agreement with the results from the SE-HPLC (Figure 5).

CONCLUSIONS

Multiple antigenic peptides are not easily purified because of their difficulty in synthesis and high aggregation. We have demonstrated that CZE is an effective tool for the analysis of high-molecular-weight MAPs (MW 5000-15000). Good resolution was obtained in the analysis of B-cell MAPs by both RP-HPLC and SE-HPLC. However poor resolution was seen for the T-cell epitope containing MAPs. This poor resolution may be due to the α-helical amphipathic character of this peptide. Analytical HPLC and CZE should be used in conjunction with SE-HPLC for maximum analytical control.

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