*Immunology* 1991 **72** 109–113 ADONIS 001928059100020A

# The antibody response in BALB/c mice to the *Plasmodium falciparum* circumsporozoite repetitive epitope covalently coupled to synthetic lipopeptide adjuvant

K.-H. WIESMÜLLER,* G. JUNG,* D. GILLESSEN,† C. LOFFL, W. G. BESSLER & T. BÖLTZ

**Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle, Tübingen, Institute of Immunobiology, University of Freiburg, Freiburg, Germany, †Central Research Department, F. Hoffmann-La Roche and Co. Ltd., Basel, Switzerland*

*Accepted for publication 11 September 1990*

## SUMMARY

The repetitive epitope of *Plasmodium falciparum* circumsporozoite protein (Asn-Ala-Asn-Pro)$_3$ [(NANP)$_3$] was coupled to tripalmitoyl-S-glyceryl-cysteine ($P_3C$) and tripalmitoyl-S-glyceryl-cysteinyl-serine ($P_3CS$). The lipopeptide $P_3CS$ is a potent B-cell and macrophage activator. The resulting immunogenic lipopeptides were used for immunization of the low responder mouse strain BALB/c. These low molecular weight conjugates induced specific anti-(NANP)$_3$ IgG and IgM levels without any carrier proteins or admixed adjuvants after a single administration.

## INTRODUCTION

Stage-specific protection from *Plasmodium falciparum* malaria can be induced by immunization with irradiated sporozoites.[1,2] The immunodominant epitope of the protective antigen consists of NANP (Asn-Ala-Asn-Pro) repeats present in the circumsporozoite (CS) protein of the sporozoite surface.[3,4] Mice and rabbits immunized with synthetic (NANP)$_n$ peptides conjugated to bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH) and emulsified in complete Freund's adjuvant (CFA) showed high antibody titres.[5] Recent studies demonstrated that (NANP)$_3$ conjugated to tetanus toxoid and administered together with Al(OH)$_3$, as well as a recombinant DNA vaccine containing multiple NANP repeats, elicited anti-sporozoite antibody levels in humans.[6,7] However, protection after challenge with *P. falciparum* sporozoites was inconsistent. The poor immunogenicity of these molecules emphasizes the need for carrier proteins and potent adjuvants suitable for use in humans. Furthermore, it is obvious that the humoral immune response against the repetitive units might not be sufficient for full protection.

In this study, we tested the immunogenic potency of low molecular weight conjugates composed of (NANP)$_3$ and synthetic analogues of the N-terminal part of the lipoprotein of *Escherichia coli*, N-palmitoyl-S-[2,3-(bispalmitoyloxy)-(2RS)-propyl]-cysteine ($P_3C$).[8] Lipooligopeptides, such as $P_3$ CSSNA, $P_3CSS$ or even $P_3CS$, have been shown to act as potent B-cell and macrophage activators.[9,10] Weakly immunogenic antigens can be covalently linked to $P_3C$, thus creating efficient immunogens with built-in adjuvanticity. The conjugates anchor with three fatty acids in the lipid bilayer of the cell membrane, whereas the more polar antigen can be presented to immunocompetent cells.[11,12] The lipotripeptide $P_3CSS$ was used as built-in adjuvant in a novel type of vaccine, $P_3CSS$-[FMDV-VP1 (135–154)], which induces a long-lasting protection against challenge with foot-and-mouth disease virus and serotype-specific virus-neutralizing antibodies in guinea-pigs after a single administration without any additional adjuvant or carrier.[13]

Recent studies have shown for the first time that the lipotripeptide moiety $P_3CSS$ could induce virus-specific cytotoxic T lymphocytes (CTL) *in vivo* after linkage to the influenza nucleoprotein epitopes NP365–380 and NP147–158.[14] Therefore, the synthetic lipopeptide vaccines are highly interesting tools to induce both humoral and cell-mediated immune responses. This approach would require the application of a mixture of the relevant B- and T-cell epitopes conjugated to lipopeptides.

Abbreviations: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; i.p., intraperitoneal; LPS, lipopolysaccharide; (NANP)$_3$, (Asn-Ala-Asn-Pro)$_3$; $P_3C$, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteine; $P_3CS$, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-cysteinyl-serine; PBS, phosphate-buffered saline; p.i., post-infection.

Correspondence: Dr G. Jung, Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen, Germany.

## MATERIALS AND METHODS

*Adjuvant and immunogen preparation*

(NANP)$_3$ was prepared by solution synthesis and condensation of the segment NANP using the dicyclohexylcarbodiimide/1-hydroxybenzotriazole method and the *tert.*-butyloxycarbonyl

(Boc) protecting group. $P_3C$ and $P_3CS$ were prepared according to previously described procedures.[8] $P_3C$-*N*-hydroxysuccinimide ester ($P_3C$-OSu), respectively $P_3CS$(tBu)-OSu and $(NANP)_3$, were stirred at room temperature for 20 hr in dimethylformamide. After the addition of methanol the lipopeptide conjugate precipitated at 0°. $P_3CS$(tBu)–$(NANP)_3$ was stirred in trifluoroacetic acid for 45 min to remove the *tert.*-butyl protecting group. $(NANP)_3$ was covalently linked to bovine serum albumin (BSA) using 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide (EDC) as coupling reagent for 1 hr (peptide/protein/EDC = 1:1:2; w/w/w; pH 5·0, dialysis against water). Quantitative amino acid analysis of BSA and peptide–BSA conjugate was carried out to determine the loading of BSA using gas chromatography of pentafluoropropionyl–amino acid methyl esters on glass capillaries coated with Chirasil-Val.[15] $(NANP)_3$-BSA contains 20 covalently bound peptides per molecule BSA.

*Reagents*

Peroxidase-conjugated rabbit anti-mouse IgG and IgM sera were purchased from Dianova, Hamburg, Germany; peroxidase-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 sera from Nordic Laboratory, Tilburg, The Netherlands; purified myeloma proteins of each isotype (IgM, TEPC 183; IgG1, MOPC 21; IgG2a, UPC 10; IgG2b, MOPC 141; IgG3, FLOPC 21) from Litton Bionetics, Charleston, SC. Lecithin (L-phosphatidyl-cholin-dipalmitoyl) and ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) were obtained from Sigma Chemie, Taufkirchen, Germany; bovine serum albumin (BSA) from Paesel, Karlsruhe, Germany; complete Freund's adjuvant (CFA) from Difco, Detroit, MI. Lipopolysaccharide (LPS) from *S. abortus equi* was kindly provided by Dr Kleine, Institute of Immunobiology, University of Freiburg, Germany.

*Incorporation of $^3$[H]thymidine*

Experiments were performed in flat-bottomed Falcon 3040 microtitre plates (Falcon Plastics, Los Angeles, CA). Speen cells were cultured at a cell density of $3{\cdot}3 \times 10^6$/ml in a volume of 180 μl culture medium [RPMI-1640 supplemented with 5% heat-inactivated foetal calf serum (FCS; Seromed, Biochrom, Berlin, FRG), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and $5 \times 10^{-5}$ M 2-mercaptoethanol]. The cultures were pulsed for 24 hr by adding 0·625 μCi $^3$[H]thymidine to each well (Amersham, Braunschweig, Germany; specific activity 5 Ci/mmol). The cultures were harvested with a Skatron harvester (Lier, Norway), collected on glass fibre filters and measured by liquid scintillation (Beckman, Fullerton, CA).

*Immunization experiments*

Six to 8-week-old female BALB/c mice were kindly provided by Professor Rossbach, Institute for Biomedical Research, Füllinsdorf, Switzerland. The animals (four to six per group) were injected intraperitoneally (i.p.) with a single dose of antigen (injection volume 200 μl). The mice were bled before immunization (pre-immune sera) and at Days 7, 14, 28, and 56 after injection from the retro-orbital plexus. Sera were stored at −20° and tested by ELISA for the presence of anti-$(NANP)_3$ antibodies.

$P_3C$, $P_3C$–$(NANP)_3$ or $P_3CS$–$(NANP)_3$ were emulsified in phosphate-buffered saline (PBS) with an equal amount of lecithin by sonication. $(NANP)_3$ and BSA–$[(NANP)_3]_{20}$ were dissolved in PBS. CFA was emulsified with an equal volume of antigen solution by sonication.

*ELISA*

96-well polystyrene microtitre plates (Dynatech, M129B, Denkendorf, Germany) were coated with sonicated $P_3CS$–$(NANP)_3$ conjugate in PBS at a concentration of 1 μg/well and air-dried at 37° overnight. The following incubations were performed at room temperature. Any remaining binding sites were saturated with 1% bovine serum albumin (BSA) in PBS for 1 hr followed by washing of the wells with PBS. Serum samples were diluted in PBS, 1% BSA, 0·05% Tween 20 and added in 50-μl aliquots overnight. The wells were washed three times with PBS/0·05% Tween 20, and incubated for 2 hr with affinity-purified, horseradish peroxidase-conjugated antisera at a dilution of 1:1000 in PBS/1% BSA/0·05% Tween 20. After washing, 50 μl of substrate solution (5 mg ABTS and 0·03% $H_2O_2$ in 10 ml of 0·1 M citrate buffer, pH 4·2) were added for 15 min. The optical densities were measured at 405 nm by using an automated ELISA reader (SLT EAR 400; SLT, Overath, FRG). The specificity of anti-isotype conjugates were performed in an ELISA, using purified myeloma proteins of each isotype as test antigens. Binding on irrelevant myeloma proteins gave absorbance values included in the average ± 3 SD of background absorbance, while binding on the relevant isotype resulted in values between 0·4 and 0·9 OD units.

## RESULTS

### Stimulation of B lymphocytes

As shown by incorporation of $^3$[H]thymidine, the lipopeptide analogue $P_3CS$ and the conjugate $P_3C$–$(NANP)_3$ induced mitogenic lymphocyte proliferation in BALB/c spleen cells comparable the B-cell mitogen LPS (Fig. 1). Optimal stimulation could be achieved with 18 μg $P_3C$/ml.

### Induction of $(NANP)_3$-specific antibodies

Immunization of BALB/c mice with a single dose of the lipopeptide conjugates $P_3C$–$(NANP)_3$ and $P_3CS$–$(NANP)_3$ induced specific IgG and IgM anti-$(NANP)_3$ levels (Fig. 2) with a peak at Day 7 followed by decreasing values thereafter. This pattern of the immune response, with an early peak in the antibody levels, could be demonstrated for other lipopeptide–antigen conjugates as well (T. Böltz, J. Metzger, W. G. Beuler and G. Jung, manuscript in preparation), but may depend on the antigen. Wiesmüller, Jung & Hess[13] detected increasing neutralization titres 3 months after immunization with a single dose of a synthetic vaccine against foot-and-mouth disease, which contains $P_3CSS$ covalently coupled to the synthetic B- and T-cell epitope VP1(135–154) homologous to a viral protein. The free peptide $(NANP)_3$ as well as the mixture $P_3C + (NANP)_3$ were ineffective in producing a measurable anti-$(NANP)_3$ response. Injection of $(NANP)_3$ coupled to the carrier protein BSA resulted in low levels of specific antibodies. However, after inoculation of BSA-$[(NANP)_3]_{20}$ emulsified in CFA, the specific antibody titres markedly increased during the following weeks. This augmentation of the immune response could also be observed after application of $P_3CS$–$(NANP)_3$ emulsified in CFA (Fig. 2). When the plates were coated with the lipopeptides $P_3C$

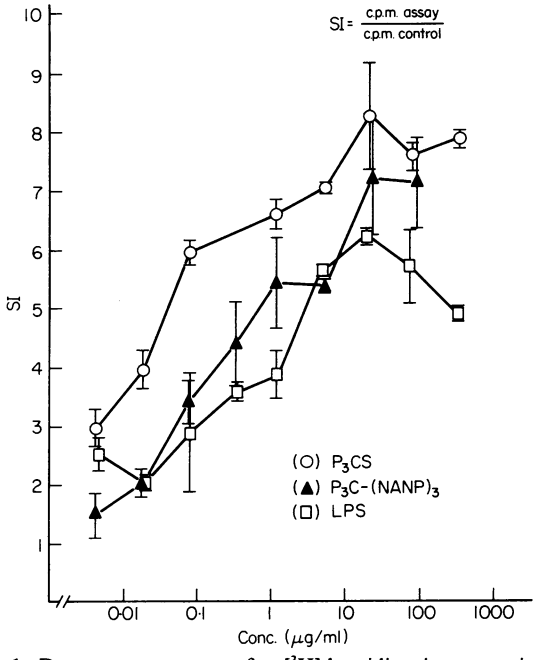


**Figure 1.** Dose–response curve for [$^3$H]thymidine incorporation in splenocytes of BALB/c mice after addition of $P_3CS$, $P_3C$–$(NANP)_3$ and LPS.

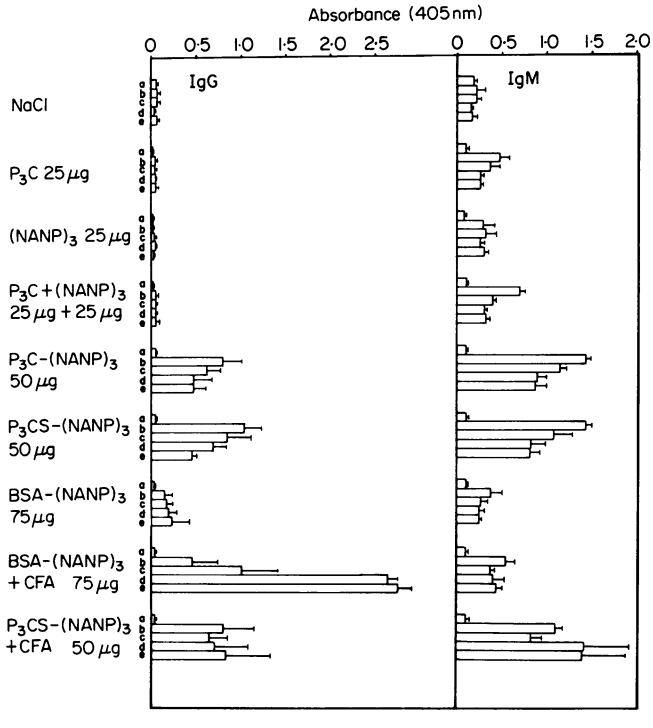


**Figure 2.** IgG and IgM antibody levels after immunization of BALB/c mice ($n=4$) with NaCl ($n=6$), $P_3C$ ($n=4$), $(NANP)_3$ ($n=4$), $P_3C+(NANP)_3$ ($n=4$), $P_3C$–$(NANP)_3$ ($n=4$), $P_3CS$–$(NANP)_3$ ($n=8$), BSA–$[(NANP)_3]_{10}$ ($n=4$), BSA–$[(NANP)_3]_{10}$ ($n=4$)+CFA, and $P_3CS$–$(NANP)_3$ ($n=8$)+CFA. Sera diluted 1/100 were tested individually by ELISA for anti-$(NANP)_3$ antibodies. Results are expressed as mean values ± SE. (a) Pre-immune serum; (b) Day 7 p.i.; (c) Day 14 p.i.; (d) Day 28 p.i., (e) Day 56 p.i.

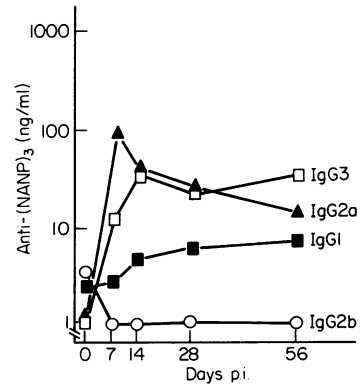


**Figure 3.** IgG isotype pattern after immunization of BALB/c mice ($n=4$) with a single dose of 50 μg of $P_3CS$–$(NANP)_3$.

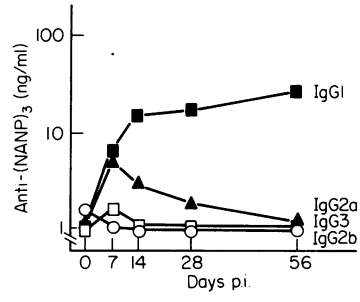


**Figure 4.** IgG isotype pattern after application of 75 μg of BSA–$[(NANP)_3]_{10}$ to BALB/c mice ($n=4$).

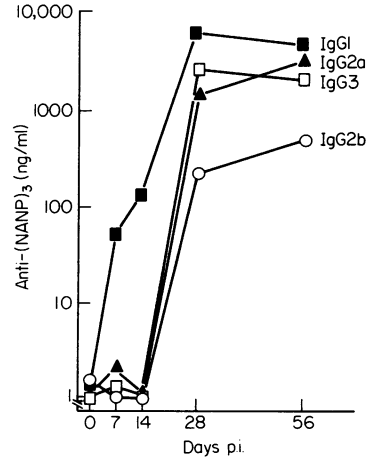


**Figure 5.** IgG isotype pattern after immunization of BALB/c mice with 75 μg of BSA–$[(NANP)_3]_{10}$ emulsified in CFA ($n=4$).

and $P_3CS$, respectively, no specific binding could be detected (data not shown).

### IgG isotype pattern

To investigate the influence of the different adjuvant/carrier systems we determined the IgG isotype pattern of the anti-$(NANP)_3$ antibodies. The predominant isotypes were IgG2a and IgG3, and to a lesser extent IgG1 (Fig. 3). A similar isotype distribution was found after immunization with $P_3C$–$(NANP)_3$ or $P_3CS$–$(NANP)_3$. Application of BSA–$[(NANP)_3]_{20}$ leads to production of IgG1 and IgG2a (Fig. 4), whereas after immunization with BSA–$[(NANP)_3]_{20}$ emulsified in CFA all IgG isotypes could be detected (Fig. 5).

## DISCUSSION

The results of our study demonstrate that the immunodominant CS epitope $(NANP)_3$ covalently coupled to the lipodipeptide tripalmitoyl-*S*-glyceryl-cysteinyl-serine ($P_3CS$) or to the lipoamino acid tripalmitoyl-*S*-glyceryl-cysteine ($P_3C$) induces specific anti-$(NANP)_3$ IgG and IgM levels in BALB/c mice (H-$2^d$) without any carrier proteins or admixed adjuvants.

The antibody response to $(NANP)_3$ has been shown to be H-$2^b$ restricted,[16,17] indicating binding to the I-$A^b$ molecule and the presence of specific T cells.[18] Del Giudice *et al.*[19] (1986) found specific antibodies in C57BL/6 mice (H-$2^b$) after immunization with the carrier-free synthetic polymers $(NANP)_{40}$ and $(NANP)_{20}$, and a variable weak response with $(NANP)_4$ when injected with CFA. BALB/c mice (H-$2^d$) and other haplotypes did not mount an antibody response to $(NANP)_{40}$, even at high doses. However, coupling of $(NANP)_{40}$ to the carrier protein KLH induced a specific antibody response in the low responder strains BALB/c and CBA/Ca (H-$2^k$). An approach to overcome the Ir gene controlled low responsiveness in mice has been reported by Good *et al.*[16] They emulsified a fusion protein consisting of 32 *P. falciparum* CS protein repeat tetramers and human recombinant interleukine-2 (rIL-2) in CFA, incomplete IFA, or peanut oil plus alum, for the induction of an anti-$(NANP)_n$ humoral immune response in mice of the low responder H-$2^k$ haplotype.[16,20]

Our results show that the genetic restriction could be overcome when the $P_3C$–$(NANP)_3$ or $P_3CS$–$(NANP)_3$ conjugates were used for immunization of the low responder BALB/c strain. Several mechanisms may be involved in this phenomenon. The lipophilic molecules form stable aggregates acting as depot forms diminishing enzymatic degradation.[21] In addition, the hydrophobic moiety could be anchored in the cell membrane, leading to an improved targeting of the antigen to immunocompetent cells. The polyclonal B-cell mitogen $P_3CS$ may activate resting B cells by binding to mitogen receptors[22,23] and the lipopeptide-induced release of mediators like IL-1 from monocytes/macrophages would facilitate the maturation of B cells and immunoglobulin secretion.[10] The activation of specific T cells is currently under investigation.

The use of synthetic lipopeptides as immunogens provides several advantages. Multiple B- and T-cell epitopes for different MHC haplotypes could be combined in a polyvalent vaccine.[24,25] Immunization with synthetic hybrid molecules containing epitopes from different infection stages of *P. falciparum* and polymerized into synthetic proteins has been shown to inhibit the development of parasitaemia in humans and animals.[26,27] The addition of potent adjuvants might further enhance the protective capacity of these molecules.

With our approach, we can demonstrate that the CS protein repeat $(NANP)_3$ covalently coupled to the lipopeptide $P_3CS$ and the lipoamino acid $P_3C$, respectively, constitute potent immunogens with built-in adjuvanticity which could overcome genetic low responsiveness. To our knowledge the lipopeptide conjugates $P_3C$–$(NANP)_3$ and $P_3CS$–$(NANP)_3$ are the smallest molecules reported so far to induce antibodies against $(NANP)_3$ without any further carrier or adjuvant.

## ACKNOWLEDGMENTS

This work has been supported by the Bundesministerium für Forschung und Technologie (FK 0318768).

## REFERENCES

1. Clyde D.F., McCarthy V.C., Miller R.M. & Hornick R.M. (1973) Specificity of protection of man immunized against sporozoite-induced falciparum malaria. *Am. J. Med. Sci.* **266,** 398.
2. Kawamura H., Rosenberg S.A. & Berzofsky J.A. (1985) Immunization with antigen and interleukin 2 *in vivo* overcomes *Ir* gene low responsiveness. *J. exp. Med.* **162,** 381.
3. Dame J.B., Williams S.L., McCuthchan T.F., Weber J.L., Wirt R.A., Hockmeyer W.T. *et al.* (1984) Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum. Science*, **225,** 539.
4. Enea V., Ellis J., Zavala F., Arnot D.E., Asavanich A., Masuda A., Quakyi I. & Nussenzweig R.S. (1984) DNA closing of *Plasmodium falciparum* gene: amino acid sequence of repetitive epitope. *Science*, **255,** 628.
5. Ballou W.R., Rothbard J., Wirtz R.A., Gordon D.M., Williams J.S., Gore R.W. *et al.* (1985) Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum. Science*, **228,** 996.
6. Etlinger H.M., Heimer E.P., Trzeciak A., Felix A.M. & Gillessen D. (1988) Assessment in mice of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, *P. falciparum. Immunology*, **64,** 551.
7. Herrington D.A., Clyde D.F., Losonsky G., Cortesia M, Murphy F.R., Davis J. *et al.* (1987) Safety and immunogenicity in man of a synthetic peptide malaria vaccine against *Plasmodium falciparum* sporozoites. *Nature (Lond.)*, **328,** 257.
8. Wiesmüller K.-H., Bessler W. & Jung G. (1983) Synthesis of the mitogenic *S*-[2,3-bis(palmitoyloxy)propyl]-*N*-palmitoylpentapeptide from *Escherichia coli* lipoprotein. *Hoppe-Seyler's Z. Physiol. Chem.* **364,** 593.
9. Bessler W.G., Cox M., Lex A., Suhr B., Wiesmüller K.-H. & Jung G. (1985) Synthetic lipopeptides analogues of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. *J. Immunol.* **135,** 1900.
10. Hoffmann P., Heinle S., Schade U.F., Loppnow L., Ulmer A.J., Flad H.D. & Bessler W.G. (1988) Stimulation of human and murine adherent cells by bacterial lipoprotein and synthetic lipopeptide analogues. *Immunobiol.* **177,** 158.
11. Jung G. (1984) Synthesis and perspectives of new adjuvants and carrier systems for potential application for synthetic vaccines. *Ann. Sclavo*, **2,** 191.
12. Bessler W.G., Suhr B., Bühring H.-J., Muller C.P., Wiesmüller K.-H., Becker G. & Jung, G. (1985) Specific antibodies elicited by antigen covalently linked to a synthetic adjuvant. *Immunobiol*, **170,** 239.
13. Wiesmüller K.-H., Jung G. & Hess G. (1989) Novel low-molecular-weight synthetic vaccine against foot-and-mouth-disease

containing a potent B-cell and macrophage activator. *Vaccine*, **7,** 29.

14. DERES K., SCHILD H., WIESMÜLLER K.-H., JUNG G. & RAMMENSEE H.-G. (1989) *In vivo* priming of virus-specific cytotoxic T-lymphocytes with synthetic lipopeptide vaccine. *Nature (Lond.)*, **342,** 561.
15. FRANK H., NICHOLSON G.J. & BAYER E. (1978) Enantiomer labelling, a method for the quantitative analysis of amino acids. *J. Chromatogr.* **167,** 187.
16. GOOD M.F., POMBO D., LUNDE M.N., MALOY W.L., HALENBECK R., KOTHS K., MILLER L.H. & BERZOFSKY J.A. (1988) Recombinant human IL-2 overcomes genetic nonresponsiveness to malaria sporozoite peptides. *J. Immunol.* **141,** 972.
17. TOGNA A.R., DEL GIUDICE G., VERDINI A.S., BONELLI F., PESSI A., ENGERS H.D. & CORRADIN G. (1986) Synthetic *P. faciparum* circumsporozoite peptides elicit heterogenous L3T4$^+$ T cell proliferative responses in H-2b mice. *J. Immunol.* **137,** 2956.
18. RUSSO D.M., SUNDY J.S., YOUNG J.F., MAGUIRE H.C., WEIDANZ W.P. (1989) Cell-mediated immune responses to vaccine peptides derived from the circumsporozoite protein of *Plasmodium falciparum*. *J. Immunol.* **143,** 655.
19. DEL GIUDICE G., COOPER J.A., MERINO J., VERDINI A.S., PESSI A., TOGNA A.R., ENGERS H.D., CORRADIN G. & LAMBERT P.-H. (1986) The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is 1-A$^b$-restricted: possible implications for malaria vaccines. *J. Immunol.* **137,** 2952.
20. KAWAMURA H., ROSENBERG S.A. & BERZOFSKY J.A. (1985) Immunization with antigen and interleukin 2 *in vivo* overcomes *Ir* gene low responsiveness. *J. exp. Med.* **162,** 381.
21. PRASS W., RINGSDORF H., BESSLER W.G., WIESMÜLLER K.-H. & JUNG G. (1987) Lipopeptides of the *N*-terminus of *Escherichia coli* lipoprotein: synthesis, mitogenicity and properties in monolayer experiments. *Biochim. Biophys. Acta*, **900,** 116.
22. COUTINHO A. & MÖLLER G. (1974) Immune activation of B cells: evidence for one nonspecific triggering signal not delivered by Ig receptors. *Scand. J. Immunol.* **3,** 133.
23. MELCHERS F. & ANDERSSON J. (1984) B cell activation: three steps and their variations. *Cell*, **37,** 715.
24. SINIGAGLIA F., GUTTINGER M., KILGUS J., DORAN D.M., MATILE H., ETLINGER H., TRZECIAK A., GILLESSEN D. & PINK J.R.L. (1988) A malaria T-cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature (Lond.)*, **336,** 778.
25. GUTTINGER M., CASPERS P., TAKACS B., IRZECIAK A., GILLESSEN D., PINK J.R. & SINIGAGLIA F. (1988) Human T cells recognize polymorphic and non-polymorphic regions of the *Plasmodium falciparum* circumsporozoite protein. *EMBO J.* **7,** 2555.
26. PATARROYO M.E., ROMERO P., TORRES M.L., CLAVIJO P., MORENO A., MARTINEZ A., RODRIGUEZ R., GUZMAN F. & CABEZAS E. (1987) Induction of protective immunity against experimental infection with malaria using synthetic peptides. *Nature (Lond.)*, **328,** 629.
27. PATARROYO M.E., AMADOR R., CLARIJO P., MORENO A., GUZMAN F., ROMERO P. *et al.* (1988) A synthetic vaccine protects humans against challenge with asexual blood stages of *Plasmodium falciparum* malaria. *Nature (Lond.)*, **332,** 158.