INFECTION AND IMMUNITY, Nov. 1987, p. 2658–2661
0019-9567/87/112658-04$02.00/0

Vol. 55, No. 11

Enhanced Epitopic Response to a Synthetic Human Malarial Peptide by Preimmunization with Tetanus Toxoid Carrier

L. D. LISE,[1] D. MAZIER,[2] M. JOLIVET,[3] F. AUDIBERT,[1] L. CHEDID,[1,3]* AND D. SCHLESINGER[4]

Departments of Pharmacology and Therapeutics[1] and Immunology and Microbiology,[3] University of South Florida College of Medicine, Tampa, Florida 33612-4799; Service de Parasitologie, Hôpital Pitié-Salpétrière, 75013 Paris, France[2]; and Department of Medicine and Cell Biology, New York University Medical Center, New York, New York 10016[4]

Received 20 May 1987/Accepted 16 July 1987

Successful human vaccination by synthetic malarial sporozoite peptides may depend on the choice of an appropriate carrier. Tetanus toxoid (TT) has been proposed because of its safe and widespread use in humans. Paradoxically, however, prior exposure to this toxoid vaccine could produce specific epitopic suppression against synthetic malarial peptides conjugated to this same protein as carrier. Indeed, we have previously reported that such a phenomenon can occur in the case of a synthetic vaccine made with a streptococcal peptide conjugated to TT. Our present study shows that similar results can be observed in mice preimmunized with TT 1 month before the administration of a conjugate containing TT and a *Plasmodium knowlesi* peptide. Analysis of the isotypic pattern of the antipeptide response showed that the immunoglobulin G1 (IgG1) subclass and especially the IgG2a and IgG2b subclasses were suppressed. In contrast, when a sporozoite peptide from *Plasmodium falciparum* was coupled to TT, the total antipeptide antibodies and particularly the IgG1 subclass were enhanced by preimmunization by TT. This increase of antipeptide antibodies was correlated with a greater ability of the sera to neutralize sporozoite infectivity. These results indicate that prior exposure to TT does not systematically impair the antibody response against a peptide administered as a peptide-TT conjugate.

Protective immunity against malaria in animals and humans was first obtained by immunization with irradiated sporozoites. The use of a natural structure to obtain a vaccine is not being considered because sufficient amounts of antigens cannot be produced. Two substitutes for natural vaccines are presently being studied: recombinant DNA technology and peptide synthesis.

Protective antibodies produced in animals and human volunteers immunized with irradiated sporozoites are directed against a surface protein called circumsporozoite protein (CSP) (6, 12, 19). Such antibodies recognized a single immunodominant region of the protein (25) formed by tandem repeated sequences of 12 amino acids (QAQGDGAN AGQP) in *Plasmodium knowlesi* (10, 22) and of 4 amino acids (NANP) in *Plasmodium falciparum* (7, 11). Synthetic peptides representing these repetitive regions conjugated to carriers are capable of producing biologically active antibodies (2, 5, 13, 18). In most cases, tetanus toxoid (TT) was chosen as a carrier because of its extensive use in humans. However, preexposure to this toxoid could induce a specific epitopic suppression against the hapten in subjects immunized later with hapten-TT conjugate vaccines. Indeed this specific epitopic suppression, first described by Herzenberg et al. (15), was also observed in the case of synthetic vaccine models (24) such as a streptococcal SCB_7 (for synthetic cyanogen bromide fragment no. 7) peptide (4) conjugated to TT.

It was therefore of interest to investigate in an animal model the influence of TT preimmunization on the immunogenicity of a *P. falciparum* peptide [$(NANP)_4$] coupled to TT. Moreover, since this conjugate could potentially be used as a human vaccine, it is important to know whether epitopic suppression is always obtained when TT is used as the carrier. Control mice were immunized with *P. knowlesi* (PK26) or SCB_7 peptides also conjugated to TT. It was observed that preimmunization by TT enhanced the antibody response to the *P. falciparum* peptide. In contrast, the same treatment induced specific epitopic suppression in the case of the streptococcal and *P. knowlesi* peptides.

MATERIALS AND METHODS

Mice. Female Swiss mice were used at 8 to 12 weeks of age; they were purchased from Iffa Credo (L'Arbresle, France).

Synthetic peptides. Four synthetic peptides were used in our experiments: two peptides representing four [$(NANP)_4$] and eight [$(NANP)_8$] repeats of the tetrapeptide sequence found in the CSP of *P. falciparum*; a peptide named PK26 containing the 24 amino acids of the dodecapeptide repeating sequence found in the CSP of *P. knowlesi* with 2 additional amino acids (tyrosine at the N-terminal end and cysteine at the C-terminal end); and a synthetic peptide of 34 amino acids named SCB_7 representing a repetitive fragment of type 24 streptococcal M protein. This last peptide was used as a reference since it has been shown to be very susceptible to epitopic suppression (24). All these peptides were synthetized by a solid-phase method (16).

Preparation of peptide-carrier conjugates. Conjugates were prepared by a method described previously (1). Briefly, each peptide was coupled by glutaraldehyde via its NH_2 group to TT. The coupling reaction was carried out at 20°C in phosphate-buffered saline, pH 7.35, by mixing the peptide to the carrier protein at a molar ratio of 40:1 and adding 1 volume of glutaraldehyde at 2.63 mM. The reaction was allowed to proceed for 6 days with constant stirring, followed by dialysis against phosphate-buffered saline.

Analysis of amino acid composition of the conjugates. Amino acid analysis was performed after acid hydrolysis with a Beckman amino acid analyzer.

* Corresponding author.

TABLE 1. Effect of TT priming on antipeptide responses induced by peptide-TT conjugates

Pretreatment	Treatment	Antipeptide response (titer)[a]			
		Pool		Individuals, day 37	P
		Day 21	Day 37		
None	SCB_7-TT + $Al(OH)_3$	1,300	17,600	4.04 ± 0.38	
TT	SCB_7-TT + $Al(OH)_3$	600 (51)	5,900 (66)	3.31 ± 0.56	<0.02
None	PK26-TT + $Al(OH)_3$	8,600	83,400	4.89 ± 0.32	
TT	PK26-TT + $Al(OH)_3$	5,700 (33)	35,300 (57)	4.30 ± 0.21	<0.01
None	$(NANP)_4$-TT + $Al(OH)_3$	130	4,300	3.22 ± 0.55	
TT	$(NANP)_4$-TT + $Al(OH)_3$	380	14,500	4.02 ± 0.55	<0.05
None	$(NANP)_4$-TT + FCA[b]	2,500	8,300	NT[c]	
TT	$(NANP)_4$-TT + FCA	8,500	20,300	NT	

[a] Titers of sera pooled on days 21 and 37 are shown. Numbers in parentheses indicate the percent suppression versus control groups. Averages of $\log_{10}$ individual titers measured on day 37 were used to evaluate statistical significance by Student's *t* test.
[b] FCA, Freund complete adjuvant.
[c] NT, Not tested.

Preparation of specific antibodies. All sera were prepared in Swiss mice. Antipeptide antibodies were obtained by immunizing animals in Freund complete adjuvant with 100 μg of a polyvalent synthetic vaccine containing SCB_7, PK26, or $(NANP)_4$ but no TT carrier (17).

Immunization by peptide-TT conjugate. Female Swiss mice (eight per group) were pretreated subcutaneously with either phosphate-buffered saline or TT (100 μg) in saline solution 30 days before being immunized with the conjugates. All mice received 50 μg of one of the three peptide conjugates in aluminum hydroxide, except in one case where the $(NANP)_4$-TT conjugate was administered with Freund complete adjuvant. On day 60 the mice received a second injection of 50 μg of the same conjugate. Sera were collected by retro-orbital bleeding at weekly intervals after the first injection of conjugate and stored at −20°C before titration.

Antipeptide antibody titration. Antibody titers against the synthetic peptides or against the carrier were determined by enzyme-linked immunosorbent assay (ELISA). Wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 10 μg of SCB_7, PK26, or $(NANP)_8$ or 4 μg of TT per ml. After incubation for 2 h at 37°C, the plates were washed and incubated for 1 h with serial dilutions of sera at the same temperature. The wells were then washed and treated with a rabbit anti-mouse immunoglobulin G (IgG)-peroxidase conjugate (Institut Pasteur) for an additional hour at 37°C. ELISA titers of IgG subclasses were determined by the use of peroxidase-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b (Nordic) and IgG3 (Bionetics) 12 or 8 min after addition of a substrate solution; the reaction was stopped with 50 μl of 12% H_2SO_4. Optical density was determined with a spectrophotometer reader (Titertek; Flow Laboratories, McLean, Va.). ELISA titers were expressed as the maximal dilution giving a twofold-higher absorbance than that of the negative control serum diluted at 1:100.

Recognition of the natural structure and biological activity of antibodies. Antibodies reacting with *P. falciparum* sporozoite surface antigens were measured by an immunofluorescence assay, with, as previously described (9), wet preparations of sporozoites attached to poly-L-treated glass slides. Anti-$(NANP)_4$ antibodies were also measured by inhibition of *P. falciparum* sporozoite penetration in cultured cells. Quantitative tests were performed as previously described (18). Human hepatocytes obtained from liver biopsies were seeded at a concentration of 10^5 cells per chamber in eight-chambered plastic Lab-Tek slides (Miles Laboratories) and cultured for 24 h before sporozoite inoculation. A 25-μg amount of a 1:5 dilution of the serum was added to each chamber. Sporozoites were obtained from *Anopheles stepthensi* after feeding on gametocytes from cultures of the NF54 strain through an artificial membrane. Sporozoites (4×10^4) were added to each chamber in a volume of 25 μl of medium.

Percent inhibition was estimated by counting the number of intracellular trophozoites at 48 h compared with the number in the corresponding control culture.

Statistical significance was evaluated by Student's *t* test. Differences were considered significant when *P* values of <0.05 were obtained.

RESULTS

Physicochemical and immunochemical characteristics of peptide-carrier conjugates. Determination of the molecular ratio of peptide to carrier of the conjugates after amino acid analysis gave the following data: 10 mol of SCB_7, 16 mol of PK26, and 21 mol of $(NANP)_4$ per mol of TT carrier. This corresponds to 11 μg of SCB_7, 11 μg of PK26, and 9 μg of $(NANP)_4$ in 50 μg of the respective TT conjugates. Immunochemical analysis of these conjugates showed that antigenic determinants of free or coupled peptides or TT were recognized by their homologous antibodies and did not cross-react. In addition, coupling procedures did not decrease the immunogenicity of the peptides or of TT.

Effect of preimmunization with carrier on antibody response to peptide-carrier conjugates. Antipeptide antibodies measured by ELISA titration are shown in Table 1. Mice preimmunized with carrier before administration of SCB_7-TT or PK26-TT had antipeptide titers markedly lower than those of their respective controls. In these experiments TT was administered in saline solution for preimmunization. Preliminary experiments performed with the same peptide-TT conjugates showed that the addition of aluminum hydroxide to the first treatment did not affect the establishment of epitopic suppression, as already reported by Herzenberg et al. (14). These differences were shown to be statistically significant by individually titrating the sera collected on day 37. In both cases epitopic suppression was not linked to a reduction of the antibody response against the carrier. Indeed, on day 21 anti-TT antibodies of TT-primed mice were markedly increased (121,400 for SCB_7-TT and 19,800 for PK26-TT) compared with those of nonprimed mice (55,800 and 8,300, respectively). In contrast, preimmunization with carrier did not inhibit the response to $(NANP)_4$.

TABLE 2. TT priming enhances the antisporozoite responses induced by the $(NANP)_4$-TT conjugate[a]

Pretreatment	Treatment	ELISA titer	IFAT titer	% Inhibition
None	$(NANP)_4$-TT + $Al(OH)_3$	4,300	5,000	45
TT	$(NANP)_4$-TT + $Al(OH)_3$	14,500	25,000	88

[a] Day 37 pooled sera of mice immunized successively with TT and $(NANP)_4$-TT conjugate with $Al(OH)_3$ were titrated by ELISA, immunofluorescence (IFAT), and inhibition of sporozoite penetration into hepatocytes.

Indeed, the antipeptide titers were more elevated after preimmunization with 100 μg of TT. Absence of epitopic suppression was also observed when the $(NANP)_4$-TT conjugate was administrated with Freund complete adjuvant in place of aluminum hydroxide. Sera of TT-primed mice immunized with the $(NANP)_4$-TT conjugate contained higher levels of antibodies to sporozoites, as measured by the immunofluorescence antibody test. They were also shown to be more biologically active, as measured by inhibition of penetration of sporozoites into human hepatocytes (Table 2). In most experiments, 100 μg of TT was used for preimmunization. However, when mice were pretreated with 10 μg of TT, suppression of antibody responses to SCB_7-TT and PK26-TT and enhancement of $(NANP)_4$-TT were also observed (data not shown).

Influence of TT priming on IgG subclasses of antipeptide antibodies. Preimmunization by TT may also influence the IgG subclasses of antipeptide antibodies. Results in Table 3 confirm that in the case of SCB_7 and PK26, total IgG was decreased, whereas it was increased in the case of $(NANP)_4$. These data also show that with the first two peptides, all the IgG isotypes measured were suppressed (approximately 40% for the IgG1 subclass and 95 to 100% for IgG2a and IgG2b). In contrast, mice immunized with $(NANP)_4$-TT in aluminum hydroxide or Freund complete adjuvant had a marked enhancement of the IgG1 subclass (threefold increase). Such an effect was weaker and less defined for the IgG2a and IgG2b subclasses, especially in animals in which aluminum hydroxide was used as an adjuvant.

DISCUSSION

Experiments described in this report were performed to investigate whether epitope-specific suppression could compromise the use of TT as a carrier in vaccines containing synthetic malarial peptides. Previous observations had shown that preimmunization with TT inhibited the subsequent antibody response against a streptococcal peptide conjugated to the same carrier (24). Data presented here show that similar results can be obtained with a simian malarial sporozoite peptide (PK26) conjugated to TT. Surprisingly, however, preimmunization of Swiss mice with TT did not inhibit the response but rather enhanced antibody production to a human malarial sporozoite peptide, $(NANP)_4$, conjugated under the same conditions. Antibodies were measured by ELISA, immunofluorescence, and inhibition of penetration of sporozoites into hepatocytes. The last method is considered to be correlated with the presence of protective antibodies (18).

Analysis of the isotypic pattern of anti-SCB_7 and anti-PK26 antibodies showed that the decrease in total IgG was correlated with suppression of the three isotypes measured, especially IgG2a and IgG2b as previously reported by Herzenberg et al. (14). It would be of the utmost importance to ascertain whether lower titers of antibodies against SCB_7 and PK26 correspond to a decrease in biological activity. Peptide-specific IgG and IgG1 were strongly enhanced in mice immunized with the $(NANP)_4$-TT conjugate after pretreatment. This enhancement was correlated with greater inhibition of sporozoite penetration into hepatocytes. Enhancement or suppression of the immune response against the hapten could depend on several experimental factors, such as the conditions of preimmunization, the nature of the carrier or the hapten, and the species. Other investigators have shown that preimmunization with the same carrier can enhance response to the hapten. Schneerson et al. (23), using a carbohydrate antigen from *Haemophilus influenzae* coupled to bovine serum albumin, showed that the response against the saccharide was higher in mice pretreated with bovine serum albumin, and Sarvas et al., using 4-hydroxy-3-iodo-5-nitrophenyl coupled to human gamma globulin, observed similar results in chickens (21). To our knowledge, however, such a phenomenon had never been reported with a peptidic hapten. Since the *P. falciparum* sporozoite peptide is highly repetitive, it would be interesting to perform similar experiments with other highly repetitive peptide haptens. Another possible explanation may be that the presence of proline in each four-amino-acid repeat confers on the peptide a favorable secondary structure. It is not likely that a difference in the ability of mice to respond to the human and simian antigens could explain our data, since PK26 was shown to be a better immunogen in Swiss mice than $(NANP)_4$ in the group not pretreated with TT. For wider use of future synthetic malaria vaccines, carrier proteins could be required to overcome genetic control. Thus, in nonresponder mice, antibodies against a 160-amino-acid-peptide, residue sporozoite $(NANP)_{40}$, could only be in-

TABLE 3. Influence of TT priming on the IgG subclasses of antipeptide antibodies[a]

Pretreatment	Treatment	Antipeptide response (titer)[b]			
		IgG	IgG1	IgG2a	IgG2b
None	SCB_7-TT + $Al(OH)_3$	17,600	14,500	3,700	13,500
TT	SCB_7-TT + $Al(OH)_3$	5,900 (66)	8,200 (43)	<100 (100)	<100 (100)
None	PK26-TT + $Al(OH)_3$	83,000	34,000	16,000	25,600
TT	PK26-TT + $Al(OH)_3$	35,000 (57)	19,600 (42)	950 (95)	650 (98)
None	$(NANP)_4$-TT + $Al(OH)_3$	4,300	4,200	1,000	1,200
TT	$(NANP)_4$-TT + $Al(OH)_3$	14,500	17,000	300	1,100
None	$(NANP)_4$-TT + FCA[c]	8,300	3,700	900	2,700
TT	$(NANP)_4$-TT + FCA	20,300	12,500	1,700	5,500

[a] Antipeptide-specific isotypes were measured on day 37 (Table 1). Total IgG and IgG1, IgG2a, and IgG2b isotypes were measured with appropriate isotype-specific sera.

[b] Numbers in parentheses indicate the percent suppression versus control groups.

[c] FCA, Freund complete adjuvant.

duced when this antigen was coupled to a carrier (8). Clinical trials in volunteers (3) with a recombinant DNA *P. falciparum* vaccine showed that 80% of the recipients develop an immune response against the CSP repeat antigen. However, the antibody titers were low even after five consecutive booster injections. The authors suggest that the addition of nonsporozoite T epitopes, for example, by means of carrier proteins, may enhance CSP antibody production.

Although extrapolation to humans should be made cautiously, it is encouraging to observe in Swiss mice an absence of epitopic suppression in case of human malarial sporozoite peptide coupled to TT. However, in view of the large amounts of peptide probably required for a vaccine and the risk of sensitization by repeated injections of TT, more studies are necessary to find a highly efficient and safe carrier.

Immunization with a malarial peptide conjugated to a toxoid carrier might not be boosted by subsequent infection with the homologous pathogen (20). Therefore, it may be necessary to link an additional sporozoite peptide which would be recognized by helper T cells. Immunization with this construct may allow infection to boost the antipeptide response. Furthermore, use of conjugates containing both B and T epitopes may obviate the need for the carrier.

ACKNOWLEDGMENTS

We are very grateful to Christine Dubeau, Helene Gras-Masse, and Andre Tartar (Institut Pasteur, Lille), who synthesized and purified the $(NANP)_4$ and SCB_7 peptides.

This work was supported by the BRSGS SO7 RRO5749, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and in part by the United Nations Development Program/World Bank/World Health Organization Special Program on Training and Research in Tropical Disease.

LITERATURE CITED

1. **Audibert, F., M. Jolivet, L. Chedid, R. Arnon, and M. Sela.** 1982. Successful immunization with a totally synthetic diphtheria vaccine. Proc. Natl. Acad. Sci. USA **79**:5042–5046.
2. **Ballou, W. R., J. Rothbard, R. A. Wirtz, D. M. Gordon, J. S. Williams, R. N. Gore, I. Schneider, M. R. Hollingdale, R. L. Beaudoin, W. L. Maloy, L. H. Miller, and W. T. Hockmeyer.** 1985. Immunogenicity of synthetic peptides from circumsporozoite protein of *Plasmodium falciparum*. Science **228**:996–999.
3. **Ballou, W. R., J. A. Sherwood, F. A. Neva, D. M. Gordon, R. A. Wirtz, G. F. Wasserman, C. L. Diggs, S. L. Hoffman, M. R. Hollingdale, W. T. Hockmeyer, I. Schneider, J. F. Young, P. Reeve, and J. D. Chulay.** 1987. Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. Lancet **i**:1277–1281.
4. **Beachey, E. H., J. M. Seyer, J. B. Dale, W. A. Simpson, and A. H. Kang.** 1981. Type-specific protective immunity evoked by synthetic peptide of streptococcus pyogenes M protein. Nature (London) **289**:457–459.
5. **Clough, E. R., F. M. Audibert, J. W. Barnwell, D. H. Schlesinger, R. Arnon, and L. Chedid.** 1985. Biologically active antibodies elicited by a synthetic circumsporozoite peptide of *Plasmodium knowlesi* administered in saline with a muramyl dipeptide derivative. Infect. Immun. **48**:839–842.
6. **Cochrane, A. H., R. S. Nussenzweig, and E. H. Nardin.** 1980. Immunization against sporozoites, p. 163–202. *In* J. P. Krier (ed.), Malaria in man and experimental animals. Academic Press, Inc., New York.
7. **Dame, J. B., 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.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225**:593–599.
8. **Del Giudice, G., J. A. Cooper, J. Merino, A. S. Veridini, A. Pessi, R. Togna, H. D. Enders, G. Corradin, and P. H. Lambert.** 1986. The antibody response in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is a $I\text{-}A^b$ restricted: possible implications for malaria vaccines. J. Immunol. **137**:2952–2955.
9. **Druilhe, P., O. Pradier, J. P. Marc, F. Miltgen, D. Mazier, and G. Parent.** 1986. Levels of antibodies to *Plasmodium falciparum* sporozoite surface antigens reflect malaria transmission rates and are persistent in the absence of reinfection. Infect. Immun. **53**:393–397.
10. **Ellis, J., L. S. Osaki, R. W. Gwadz, A. H. Cochrane, V. Nussenzweig, R. S. Nussenzweig, and G. N. Godson.** 1986. Cloning and expression of the *Plasmodium knowlesi* sporozoite surface antigen in *E. coli*. Nature (London) **302**:536–538.
11. **Enea, V., J. Ellis, F. Zavala, D. E. Arnot, A. Asavanich, A. Masuda, I. Quakyi, and R. S. Nussenzweig.** 1984. DNA cloning of *Plasmodium falciparum* circumsporozoite gene amino acid sequence of repetitive epitope. Science **225**:628–629.
12. **Gwadz, R. W., A. H. Cochrane, V. Nussenzweig, and R. S. Nussenzweig.** 1979. Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of *Plasmodium knowlesi* and characterisation of surface antigens of these parasites. Bull. W.H.O. **57**(Suppl. I):165–173.
13. **Gysin, J., J. Barnwell, D. H. Schlessinger, V. Nussenzweig, and A. S. Nussenzweig.** 1984. Neutralization of the infectivity of the sporozoites of *Plasmodium knowlesi* by antibodies to a synthetic peptide. J. Exp. Med. **160**:935–940.
14. **Herzenberg, L. A., T. Tokuhisa, and K. Hayakawa.** 1983. Epitope specific regulation. Annu. Rev. Immunol. **1**:609–632.
15. **Herzenberg, L. A., T. Tokuhisa, and L. A. Herzenberg.** 1980. Carrier-priming leads to hapten specific suppression. Nature (London) **285**:664–667.
16. **Jolivet, M., F. Audibert, E. H. Beachey, A. Tartar, H. Gras-Masse, and L. Chedid.** 1983. Epitope specific immunity elicited by a synthetic streptococcal antigen without carrier or adjuvant. Biochem. Biophys. Res. Commun. **117**:359–366.
17. **Jolivet, M., F. M. Audibert, H. Gras-Masse, A. L. Tartar, D. H. Schlesinger, R. Wirtz, and L. Chedid.** 1987. Induction of biologically active antibodies by a polyvalent synthetic vaccine constructed without carrier. Infect. Immun. **55**:1498–1502.
18. **Mazier, D., S. Mellouk, R. L. Beaudoin, B. Texier, P. Druilhe, W. Hockmeyer, J. Trosper, C. Paul, Y. Charoenvit, J. Young, F. Miltgen, L. Chedid, J. P. Chigot, B. Galley, O. Brandicourt, and M. Gentilini.** 1986. Effect of antibodies to recombinant and synthetic peptides on *P. falciparum* sporozoites *in vitro*. Science **231**:156–159.
19. **McCarthy, V., and D. Clyde.** 1977. Plasmodium vivax: correlation of circumsporozoite precipitation (CSP) reaction with sporozoite-induced protective immunity in man. Exp. Parasitol. **41**: 167–171.
20. **Miller, L. H., R. J. Howard, R. Carter, M. F. Good, V. Nussenzweig, and R. S. Nussenzweig.** 1986. Research toward malaria vaccines. Science **234**:1349–1356.
21. **Sarvas, H., O. Makela, P. Toivanen, and A. Toivanen.** 1979. Effect of carrier preimmunization on the anti-hapten response in the chicken. Scand. J. Immunol. **31**:455–460.
22. **Schlesinger, D. H., A. H. Cochrane, R. W. Gwadz, G. N. Godson, R. Melton, R. S. Nussenzweig, and V. Nussenzweig.** 1984. Structure of an immunodominant epitope of the circumsporozoite surface protein of *Plasmodium knowlesi*. Biochemistry **23**:5665–5670.
23. **Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins.** 1980. Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152**:360–376.
24. **Schutze, M. P., C. Leclerc, M. Jolivet, F. Audibert, and L. Chedid.** 1985. Carrier-induced epitopic suppression, a major issue for synthetic vaccines. J. Immunol. **135**:2319–2322.
25. **Zavala, F., E. H. Nardin, A. H. Cochrane, R. S. Nussenzweig, and V. Nussenzweig.** 1983. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. J. Exp. Med. **157**:1947–1957.