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Immunology

Use of a tuberculin purified protein derivative–Asn-Ala-Asn-Pro conjugate in bacillus Calmette–Guérin primed mice overcomes H-2 restriction of the antibody response and avoids the need for adjuvants

(*Mycobacterium tuberculosis* var. *bovis*/vaccination/circumsporozoite protein/malaria)

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ABSTRACT **Because of its immunodominancy, and because it is conserved in different geographical isolates of *Plasmodium falciparum*, the repetitive sequence of the circumsporozoite protein, (Asn-Ala-Asn-Pro)$_n$ [(NANP)$_n$], has been envisaged for the development of an anti-falciparum malaria subunit vaccine. However, the murine immune response to (NANP)$_n$ peptides, either carrier-free or coupled to carrier proteins, was shown to be inducible only by using strong (e.g., Freund's) adjuvants. Furthermore, response to the carrier-free peptide, administered in adjuvant, is genetically restricted to *I-A*^{b} mice. In the present paper, we report that high titers of antibodies against the NANP repetitive epitope were obtained in responder C57BL/6 (*H-2*^{b}) mice when they were primed with live BCG (bacillus Calmette–Guérin *Mycobacterium tuberculosis* var. *bovis*) and immunized once with the synthetic peptide (NANP)$_{40}$ coupled to tuberculin purified protein derivative (PPD) without the use of any adjuvant. This approach also led to the production of high titers of anti-NANP antibodies in ASW (*H-2*^{s}), B10.RIII (*H-2*^{r}), BALB/c (*H-2*^{d}), C3H/He (*H-2*^{k}), and DBA/1 (*H-2*^{q}) nonresponder mice after two injections of the conjugate. In both cases, BCG priming was obligatory for the induction of antibodies reacting with the synthetic peptide. The levels of anti-NANP antibodies in nonresponder BALB/c mice were demonstrated to be comparable to the levels induced after PPD–(NANP)$_{40}$ immunization in Freund's complete or incomplete adjuvant. The antibodies induced were also capable of recognizing *P. falciparum* sporozoites in immunofluorescence assays and, furthermore, these antibodies inhibited the penetration of live sporozoites into human hepatocytes *in vitro*. This system functioned independently of the subjects' resistance or susceptibility to BCG infection. Given the widespread natural exposure to mycobacterial antigens and the extensive use of BCG and PPD in the human population, this approach might be envisaged for vaccination with malaria peptides.**

Despite their short persistence in the bloodstream after injection by anopheline vectors, malaria sporozoites elicit a species- and stage-specific immune response, which has been shown to be able to confer immune protection in experimental models (1). Sporozoite-mediated immune protection is a complex phenomenon that involves the intervention of T cells (2–5), lymphokines (6, 7), and antibodies (8–11). Anti-sporozoite antibodies have been shown to predominantly recognize the repetitive sequence present within the major surface antigen of the parasite's circumsporozoite (CS) protein (8, 9). Because of its immunodominance, and because it is conserved in different geographical isolates of *Plasmodium falciparum* (12), the repetitive sequence of the CS protein, (Asn-Ala-Asn-Pro)$_n$ [(NANP)$_n$], has been envisaged for the development of an anti-falciparum malaria subunit vaccine. However, the murine immune response to (NANP)$_n$ peptides was shown to be genetically restricted to *I-A*^{b} mice (13, 14). The use of carrier proteins was required to induce an anti-NANP immune response in otherwise nonresponsive mice (13, 14). In both cases, the anti-NANP response was only inducible by using strong (e.g., Freund's) adjuvants, the immunization with (NANP)$_n$ constructs in physiological solutions being ineffective (15). Vaccination trials have been conducted in human volunteers by using recombinant and synthetic NANP constructs. The low level of responsiveness among the human subjects immunized in this manner indicated that further study was necessary before using CS protein analogues as components of an eventual malaria vaccine (16–18).

Mycobacteria induce strong T-cell responses and can potentiate immune responses. BCG (bacillus Calmette–Guérin *Mycobacterium tuberculosis* var. *bovis*) is widely used to vaccinate people against tuberculosis (19), and the tuberculin purified protein derivative (PPD) is currently used to measure the T-cell reactivity in people exposed to tuberculosis or vaccinated with BCG. Previous work by Lachmann *et al.* (20) has shown that immunization with antigens conjugated to PPD into mice primed with BCG could induce a strong immune response to those antigens. We therefore questioned whether priming with BCG followed by immunizations with PPD conjugated to (NANP)$_n$ peptides might overcome the genetic restriction previously observed without recourse to conventional adjuvants. In this paper, we report the results of experiments with the aim of developing a strategy that induces anti-NANP antibodies while avoiding the use of adjuvants and circumventing the problem of genetic restriction by using an appropriate carrier system.

MATERIALS AND METHODS

Bacterial Strain, Peptides, and Conjugation. The *M. tuberculosis* var. *bovis* variant used was the BCG-F strain obtained

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Abbreviations: BCG, bacillus Calmette–Guérin *Mycobacterium tuberculosis* var. *bovis*; PPD, tuberculin purified protein derivative; CS, circumsporozoite; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant.

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from the BCG laboratory of the Institut Pasteur (Paris). PPD was purchased from the Statens Seruminstitut (Copenhagen). $(NANP)_{40}$, a synthetic peptide consisting of 40 NANP repeats, was produced at the Polypeptide Synthesis Department, Enricherche (Monterotondo, Italy). The original synthesis of the peptide has been described in the Italian patent application (no. 21718, June 25, 1985). The PPD–$(NANP)_{40}$ conjugate was prepared by coupling 1 mg of PPD with 1 mg of $(NANP)_{40}$ in the presence of 0.002% glutaraldehyde for 2 hr and then dialyzing against phosphate-buffered saline (PBS) overnight.

Mice. Female mice (6–8 weeks old; five mice per group) were used in all cases unless otherwise stated. BALB/c and C3H/He mice were bred in our animal facilities. C57BL/6 mice were purchased from K.F.M. (Basel, Switzerland). B10.RIII, DBA/1, and ASW mice were purchased from Harlan Olac (Bichester, U.K.). The original pairs of these mice were obtained from The Jackson Laboratory.

Immunizations. The priming dose was 10^6 colony-forming units of BCG per mouse delivered intraperitoneally (i.p.) in 500 μl of PBS. Each mouse received 20 μg of the PPD–$(NANP)_{40}$ conjugate i.p. in 500 μl of PBS on each immunization date.

$(NANP)_{40}$ ELISA. Mice were bled weekly from the periorbital plexus, the serum was separated, and the titers of anti-$(NANP)_{40}$ antibody were measured in ELISA using the $(NANP)_{40}$ peptide in the solid phase, as described in detail elsewhere (14). Samples having titers of <200 in ELISA were considered negative. The results are expressed as the mean ± SEM $\log_{10}$ reciprocal of the last serum dilution positive ($A_{495} \geq 0.2$) in the $(NANP)_{40}$ ELISA.

Immunofluorescence. *P. falciparum* sporozoites (NF54 strain) were handled as described (21). Briefly, fresh isolated parasites were deposited on glass slides coated with poly-L-lysine and kept at 4°C for 24 hr in a moist chamber to allow attachment. Antibodies were titrated by standard techniques (21).

Inhibition of Sporozoite Penetration *in Vitro*. Human hepatocytes were seeded in eight-chamber Lab-Tek plastic culture wells (Miles) and incubated for 24 hr before inoculation with sporozoites. Salivary glands from *Anopheles stephensi* mosquitoes, infected with the NF54 strain of *P. falciparum*, were aseptically dissected, pooled, and disrupted in a glass tissue grinder. Sporozoite number was evaluated in a Neubauer chamber and 4000 parasites per hepatocyte were added to each culture. The percentage inhibition of sporozoite penetration in the presence of test and control mouse sera was then evaluated by a double-staining technique (21).

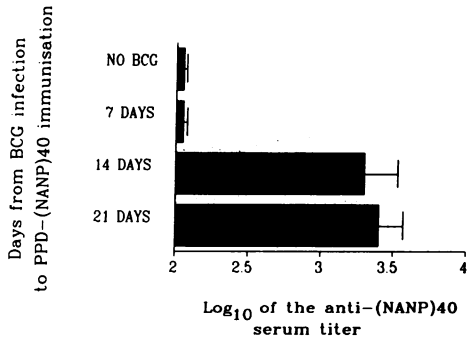

FIG. 1. Detection of anti-$(NANP)_{40}$ antibodies in responder ($H\text{-}2^b$) mice after BCG priming and one injection of PPD–$(NANP)_{40}$. Three groups of five C57BL/6 mice were immunized with 20 μg of PPD–$(NANP)_{40}$ after 7, 14, or 21 days of infection with 10^6 live BCG. One group of five mice was not primed with BCG but received the PPD–$(NANP)_{40}$ immunization. The titers of anti-$(NANP)_{40}$ antibodies were measured by ELISA as described in detail elsewhere (14). Results from sera taken 42 days after infection are presented here. The results are expressed as the mean ± SEM of the $\log_{10}$ reciprocal of the last serum dilution positive ($A_{495} \geq 0.200$) in the anti-$(NANP)_{40}$ ELISA.

RESULTS

Anti-$(NANP)_{40}$ Antibody Induction in Responder Mice Without Adjuvant. A first series of experiments was designed to determine the potential for BCG priming followed by PPD–$(NANP)_{40}$ immunization to induce anti-$(NANP)_{40}$ antibodies without adjuvant in mice responsive [i.e., capable of producing anti-$(NANP)_{40}$ antibodies when the carrier-free peptide was injected with adjuvant] to the malaria epitope. C57BL/6 mice ($H\text{-}2^b$) genetically responsive to the *P. falciparum* CS protein NANP repetitive sequence (13, 14) were primed i.p. with BCG and immunized either 7, 14, or 21 days later with the PPD–$(NANP)_{40}$ conjugate prepared in PBS. A control group that received no BCG priming but did receive the conjugate immunization in PBS never developed detectable anti-NANP antibodies. Also, the mice immunized after only 7 days sensitization with BCG did not develop a detectable anti-$(NANP)_{40}$ antibody response. However, the mice immunized with the PPD–$(NANP)_{40}$ after 14 or more days of priming with BCG, without the use of adjuvants, developed a strong anti-$(NANP)_{40}$ antibody response as measured in ELISA (Fig. 1).

Anti-$(NANP)_{40}$ Antibody Induction in Nonresponder Mice. Having determined the applicability of our system to the induction of anti-$(NANP)_{40}$ antibodies in responder mice, we then questioned whether the same strategy would overcome the genetic restriction to the peptide in nonresponder mice. We also wished to determine the comparative efficacy of our approach to the induction of anti-NANP antibodies with respect to Freund's complete (FCA) and incomplete (FIA) adjuvants.

Hence, the same experimental approach was applied to BCG primed mice of the ASW ($H\text{-}2^s$), B10.RIII ($H\text{-}2^r$), BALB/c ($H\text{-}2^d$), C3H/He ($H\text{-}2^k$), DBA/1 ($H\text{-}2^q$), and the responder C57BL/6 ($H\text{-}2^b$) strains. Interestingly, after the first conjugate immunization, only the responder C57BL/6 mice

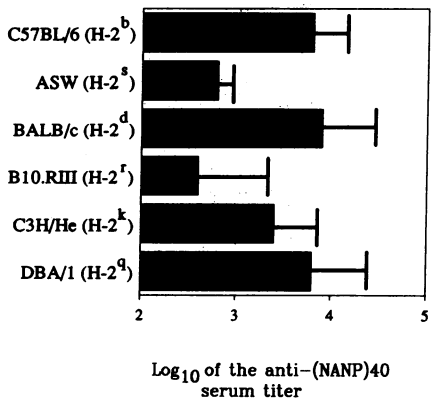

FIG. 2. Induction of anti-NANP antibodies in responder mice as compared with nonresponder mice. Seven or eight mice from each of the C57BL/6 ($H\text{-}2^b$), ASW ($H\text{-}2^s$), B10.RIII ($H\text{-}2^r$), BALB/c ($H\text{-}2^d$), C3H/He ($H\text{-}2^k$), or DBA/1 ($H\text{-}2^q$) strains were given 10^6 colony-forming units of BCG in 500 μl of PBS i.p. on day 0. Fourteen days later, all the mice received 20 μg of the PPD–$(NANP)_{40}$ conjugate in 500 μl of PBS i.p. On day 35 after BCG infection, the mice were bled from the periorbital plexus and then reimmunized with the PPD–$(NANP)_{40}$ conjugate. On day 49, the mice were bled again and all the sera were tested for the presence of anti-$(NANP)_{40}$ antibodies in ELISA as described (14). Results from the sera collected on day 49 are presented here. The results are expressed as the mean ± SEM of the $\log_{10}$ reciprocal of the last serum dilution positive ($A_{495} \geq 0.200$) in the anti-$(NANP)_{40}$ ELISA.

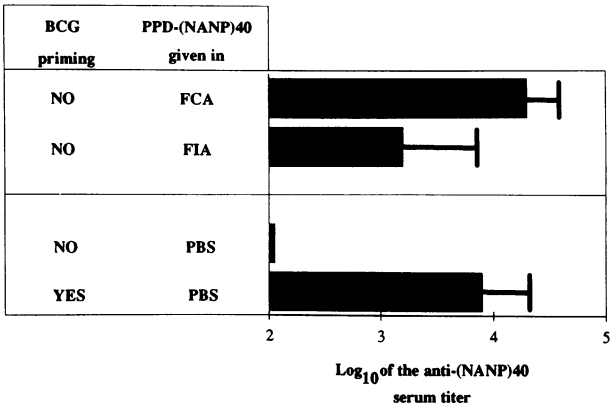

FIG. 3. Comparison of the levels of anti-NANP IgG induced in BCG-primed or not primed nonresponder mice immunized with PPD–$(NANP)_{40}$ without adjuvant and those immunized with the conjugate prepared in FCA or FIA. One group of five BALB/c mice was infected with 10^6 colony-forming units of BCG. On day 14 after infection, this group and a corresponding control group that was not primed with BCG received 20 μg per mouse of the PPD–$(NANP)_{40}$ conjugate prepared in 500 μl of PBS and administered i.p. At the same time, two other groups of mice received the same dose of conjugate prepared in either FCA or FIA administered at the base of the tail. On day 35 after infection, the mice were bled from the periorbital plexus, then injected with the corresponding PPD–$(NANP)_{40}$ preparation. On day 49, the mice were bled again and all the sera were tested for the presence of anti-$(NANP)_{40}$ antibodies in ELISA as described (14). Results from the sera collected on day 49 are presented here. The results are expressed as the mean ± SEM of the $\log_{10}$ reciprocal of the last serum dilution positive ($A_{495} \geq 0.200$) in the anti-$(NANP)_{40}$ ELISA. There was no statistical difference between the BCG/PPD–$(NANP)_{40}$ immunized group and either the FCA or FIA groups ($P \geq 0.05$ by Wilcoxon test).

produced anti-$(NANP)_{40}$ antibodies. After the second conjugate immunization, all of the mouse strains tested produced antibodies reacting with the synthetic peptide (Fig. 2).

To expand upon this observation that anti-$(NANP)_{40}$ antibodies were inducible without adjuvant in nonresponder strains, BALB/c (*H-2*^{d}) mice were immunized with PPD–$(NANP)_{40}$ prepared in FCA, FIA or PBS following BCG priming for 2 weeks. None of the mice produced detectable anti-$(NANP)_{40}$ antibodies after the first injection. However, 2 weeks after a second immunization with the conjugate, an anti-$(NANP)_{40}$ antibody response was detectable in all the mice (Fig. 3). The control group, which did not receive the BCG priming but was immunized twice with the conjugate in PBS, did not develop a detectable anti-NANP response. There was no statistical difference between the antibody titers obtained in the mice immunized with the BCG/PPD–$(NANP)_{40}$ strategy and those obtained from the mice immunized with the conjugate prepared in either FCA or FIA ($P > 0.05$ by Wilcoxon test).

In another similar experiment also conducted with BALB/c mice, the antibody titers persisted for 5–6 weeks in groups primed with BCG and immunized twice with the conjugate in PBS or immunized twice with the conjugate prepared in FIA, after which time the antibody concentrations fell to barely detectable levels (Table 1). A third injection of the PPD–$(NANP)_{40}$ conjugate caused the reappearance of anti-$(NANP)_{40}$ antibodies at levels higher than those observed after the second injection. These antibody titers persisted for 20 weeks after the third injection.

It is worthy of note that anti-$(NANP)_{40}$ antibodies raised in all the strains of mice immunized with the PPD–$(NANP)_{40}$ conjugate in PBS belonged to the IgG isotype; IgM antibodies were never detectable (data not shown). It should also be stressed that in corresponding control groups that did not receive the prior BCG sensitization but were immunized twice with the PPD–$(NANP)_{40}$ conjugate prepared in PBS and delivered i.p., anti-NANP antibodies were never detectable.

Specificity and *in Vitro* Activity of the Anti-$(NANP)_{40}$ Antibodies. The capability of the anti-$(NANP)_{40}$ antibodies induced in the BCG-primed, PPD–$(NANP)_{40}$ immunized BALB/c mice to recognize native forms of the tetrapeptide repeat in *P. falciparum* CS protein was then investigated. In the first instance, the pooled sera gave positive results in an immunofluorescence assay performed on a preparation of whole *P. falciparum* sporozoites. In the second, which also evaluated the biologic activity of the antibodies *in vitro*, the sera tested inhibited the penetration of *P. falciparum* sporozoites into cultured human liver cells (Table 1) to an extent similar to that obtained with anti-$(NANP)_{40}$ antibodies from mice immunized with the synthetic peptide in FIA.

Influence of Resistance to Infection with BCG on Antibody Induction. We addressed the question of whether susceptibility or resistance to BCG [i.e., the inability to control the growth of the bacteria in the lymphoid organs for at least 3

Table 1. Anti-NANP antibodies induced in BCG-primed, PPD–$(NANP)_{40}$ immunized BALB/c (*H-2*^{d}) mice recognize $(NANP)_{40}$ in ELISA and intact *P. falciparum* sporozoites in immunofluorescence and inhibit the penetration of sporozoites into human hepatocytes *in vitro*

Days post-BCG infection	PPD–$(NANP)_{40}$ mixed in	ELISA, $\log_{10}$ titer of $(NANP)_{40}$	IFA, $\log_{10}$ titer of sporozoites	Inhibition of sporozoite penetration, %
35	FIA	3.6 ± 0.3	ND	ND
	PBS	<2.0	ND	ND
91	FIA	3.0 ± 0.5	3.8	71 ± 2
	PBS	3.3 ± 0.3	≥5.3	89 ± 3
140	FIA	<2.0	≥5.3	75 ± 3
	PBS	<2.0	4.7	68 ± 6
198	FIA	3.4 ± 0.6	≥5.3	71 ± 0
	PBS	≥4.0	5.0	87 ± 5

Six nonresponder BALB/c mice were infected with 10^6 live BCG and immunized i.p. with 20 μg of PPD–$(NANP)_{40}$ prepared in PBS on days 21, 70, and 147. An additional four BALB/c mice received no BCG priming and were immunized with the same dose of the PPD–$(NANP)_{40}$ conjugate prepared in FIA. The sera were tested for the presence of anti-$(NANP)_{40}$ antibodies in ELISA (14), and the results are presented as the mean ± SEM $\log_{10}$ titer. The immunofluorescence (IFA) results with intact sporozoites are presented as the $\log_{10}$ reciprocal of the last positive titer obtained using pooled serum from the day indicated to identify intact parasites. Inhibition of sporozoite penetration is expressed as the mean ± SD of the percent of the sporozoite inoculum prevented from entering the liver cells in quadruplicate wells, as compared to an equal number of control wells that received normal mouse serum. ND, not done.

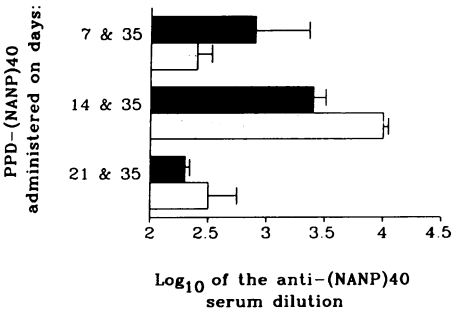

FIG. 4. Influence of resistance to BCG infection on the induction of anti-NANP antibodies. BCG sensitive (■, BALB/c) and BCG resistant (□, C3H/He) mice (five per group), both strains being nonresponsive to the synthetic peptide, were infected with 10^6 live BCG and then immunized with 20 μg of the PPD–$(NANP)_{40}$ conjugate on days 7 and 35, days 14 and 35, or days 21 and 35 after BCG infection. The mice were bled from the periorbital plexus, and the sera were tested in the anti-NANP ELISA (14). Results from the sera taken 49 days after BCG infection are presented here as the mean ± SEM of the $\log_{10}$ reciprocal of the last serum dilution positive in the anti-NANP ELISA.

weeks after infection with BCG is classified as susceptibility to the infection (22)] could influence the carrier effect provided by PPD for anti-$(NANP)_{40}$ antibody production. The experiments carried out with C3H/He mice ($H\text{-}2^k$), which are genetically unresponsive to the $(NANP)_{40}$ peptide (14) and are resistant to BCG infection (i.e., capable of controlling the growth of the bacterium within 24 hr after injection) (22), and BALB/c mice, which are nonresponders and are susceptible to BCG infection, clearly show that resistance to BCG did not influence the ability of BCG-primed C3H/He mice to produce anti-$(NANP)_{40}$ antibodies when the injection of the PPD–$(NANP)_{40}$ conjugate was given 1, 2, or 3 weeks after BCG immunization (Fig. 4). The anti-$(NANP)_{40}$ antibody titers obtained in the BCG-resistant mice were comparable to those obtained in the BCG-sensitive mouse strains. There was no statistical difference between the serum titers obtained from the sensitive or resistant mice regardless of the immunization dates ($P \geq 0.05$ by Wilcoxon test).

DISCUSSION

The present work describes a simple method of immunization leading to the induction of antibodies specific for the $(NANP)_n$ tetrapeptide repeat of the *P. falciparum* CS protein without the use of any adjuvant. This method utilizes presensitization with BCG followed by immunizations of the synthetic peptide coupled to mycobacterial antigens to induce the antibody response. Repeated immunizations with such PPD–$(NANP)_{40}$ conjugates in nonsensitized mice without adjuvant are completely ineffective in inducing anti-NANP antibody production. This method of immunization leads to the production of high titers of anti-$(NANP)_{40}$ antibodies in responder ($H\text{-}2^b$) mice and overcomes the *I-A*-linked control of the immune response to the epitope in at least five nonresponder strains of mice, all of differing *H-2* haplotypes. The antibody response induced in nonresponder BALB/c ($H\text{-}2^d$) mice when they are primed with BCG and subsequently stimulated with two immunizations of PPD–$(NANP)_{40}$ is comparable to that obtained when the two PPD–$(NANP)_{40}$ immunizations are delivered in either FCA or FIA. The antibodies produced after BCG priming and PPD–$(NANP)_{40}$ immunization are capable of recognizing intact sporozoites in immunofluorescence tests and of inhibiting the penetration of *P. falciparum* sporozoites into human liver cells *in vitro*. Furthermore, this system functions independently of the subjects' resistance or susceptibility to BCG infection.

T-cell epitopes capable of binding to the majority of immune response gene product motifs are called for in the choice of carrier proteins suitable for use in vaccines as long as many adjuvants are not suitable for human use and others, such as alum, are not usable with certain peptides for technical reasons. Strategies to overcome the genetic restriction to the $(NANP)_{40}$ peptide have concentrated either on the identification of T-cell epitopes on the CS protein not in the repeat region itself (23–25) or on the identification of T-cell epitopes on proteins important in the asexual blood stage forms of the parasite (26, 27). Much work on the former strategy has shown that one of the T-helper epitopes of the CS protein maps to polymorphic regions of the molecule (28) and thus would protect the vaccinated individual only if he were infected with a homologous form of the parasite. Nevertheless, an epitope from the *P. falciparum* CS protein capable of binding many of the known DR variants has been identified and may be useful in vaccine development (25).

The addition of T-cell epitopes is not the only means to increase the immunogenicity of a given peptide. Delivery in liposomes with lipid A (29) or proteosomes as vectors that are potent B-cell mitogens (30) has been shown to ameliorate the immune response to malaria peptides. Augmentation of the immune response to NANP has also been obtained with interleukin 2, suggesting that this cytokine may locally stimulate the few T cells in a nonresponder mouse recognizing the malaria epitope, thus giving rise to a more generalized immune response against the sequence (31).

Sensitization with BCG followed by immunization with PPD–$(NANP)_{40}$ presents several advantages in supplying the T-cell epitopes necessary to overcome genetic restriction of the immune response to the synthetic peptide. PPD is composed of a plethora of mycobacterial epitopes (20). Thus, a wide variety of T-cell epitopes are linked to $(NANP)_{40}$. Supplying many varied epitopes at once may, for the present, represent the best method for ensuring adequate T-cell stimulation in a population with a heterogeneous major histocompatibility complex make-up. In addition, we have never observed the phenomenon of epitope-specific suppression with respect to PPD purported to play a role in the nonreactivity toward the NANP epitope cited in other immunization strategies in which other carrier proteins are used (17). Our system may not lead to a secondary immune response to NANP upon natural exposure to sporozoites, but it presents the advantage of inducing substantial levels of IgG lasting several weeks, thereby directly reaping many of the benefits such a secondary response would entail. Furthermore, the constituents of our system, BCG/PPD, have been sufficiently used, either in tuberculosis vaccination or Mantoux testing, to make one speculate about their use in increasing the immunogenicity of peptides in humans. Certainly in depth analysis of the constituents of PPD would be required before trials in humans could be undertaken.

In the long term, cloning the CS protein genes directly into bacteria used for immunization may prove the most efficient method of exposing individuals to the NANP antigen. Already work has shown that mice can be vaccinated with an attenuated strain of *Salmonella typhimurium* into which the CS gene of *Plasmodium berghei* has been introduced and are consequently protected from sporozoite challenge (32). However, these mice failed to produce anti-NANP antibodies. The shuttle plasmid cloning system is currently being applied to the mycobacteria in the hope of using these potent immunomodulators as vectors for introducing antigens in a new generation of vaccines (33, 34).

In light of these varied other methods for inducing a strong immune response to NANP, our approach appears simpler. Many individuals in malaria-endemic regions already harbor

T cells reactive with BCG and/or PPD. It is interesting to note that in the study by Good and co-workers (28) lymphocytes from all of the subjects tested in the Gambia proliferated in response to PPD. Such widespread immunological recognition of the mycobacterial constituents in the PPD–$(NANP)_{40}$ could substantially enhance the reactivity to a conjugate vaccine. One can envisage replacing the $(NANP)_{40}$ with other vaccine relevant epitopes, thus making the BCG/PPD epitope system a polyvalent method for overcoming immune response restriction and inducing strong immune responses without recourse to conventional adjuvants.

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