

A Modified Hepatitis B Virus Core Particle Containing Multiple Epitopes of the *Plasmodium falciparum* Circumsporozoite Protein Provides a Highly Immunogenic Malaria Vaccine in Preclinical Analyses in Rodent and Primate Hosts

A. Birkett,¹ K. Lyons,¹ A. Schmidt,¹ D. Boyd,¹ G. A. Oliveira,² A. Siddique,² R. Nussenzweig,² J. M. Calvo-Calle,² and E. Nardin^{2*}

Apovia Inc., San Diego, California 92121,¹ and Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, New York 10010²

Received 5 June 2002/Returned for modification 13 July 2002/Accepted 24 August 2002

Despite extensive public health efforts, there are presently 200 to 400 million malaria infections and 1 to 2 million deaths each year due to the *Plasmodium* parasite. A prime target for malaria vaccine development is the circumsporozoite (CS) protein, which is expressed on the extracellular sporozoite and the intracellular hepatic stages of the parasite. Previous studies in rodent malaria models have shown that CS repeat B-cell epitopes expressed in a recombinant hepatitis B virus core (HBc) protein can elicit protective immunity. To design a vaccine for human use, a series of recombinant HBc proteins containing epitopes of *Plasmodium falciparum* CS protein were assayed for immunogenicity in mice [A. Birkett, B. Thornton, D. Milich, G. A. Oliveira, A. Siddique, R. Nussenzweig, J. M. Calvo-Calle, and E. H. Nardin, abstract from the 50th Annual Meeting of the American Society of Tropical Medicine and Hygiene 2001, Am. J. Trop. Med. Hyg. 65(Suppl. 3):258, 2001; D. R. Milich, J. Hughes, J. Jones, M. Sallberg, and T. R. Phillips, Vaccine 20:771-788, 2001]. The present paper summarizes preclinical analyses of the optimal *P. falciparum* HBc vaccine candidate, termed ICC-1132, which contains T- and B-cell epitopes from the repeat region and a universal T-cell epitope from the C terminus of the CS protein. The vaccine was highly immunogenic in mice and in *Macaca fascicularis* (cynomolgus) monkeys. When formulated in adjuvants suitable for human use, the vaccine elicited antisporezoite antibody titers that were logs higher than those obtained in previous studies. Human malaria-specific CD4⁺-T-cell clones and T cells of ICC-1132-immunized mice specifically recognized malaria T-cell epitopes contained in the vaccine. In addition to inducing strong malaria-specific immune responses in naïve hosts, ICC-1132 elicited potent anamnestic antibody responses in mice primed with *P. falciparum* sporozoites, suggesting potential efficacy in enhancing the sporozoite-primed immune responses of individuals living in areas where malaria is endemic.

The complex life cycle of the malaria parasite is initiated by infective sporozoites that are injected into the mammalian host by the mosquito vector. Immunization with irradiated sporozoites can protect mice, monkeys, and human volunteers against sporozoite challenge (reviewed in references 34 and 37). Individuals and experimental animals immunized with irradiated sporozoites develop protective humoral and cellular immune effector mechanisms that specifically target the preerythrocytic stages of the parasite. Since sporozoites cannot be cultivated in vitro, intensive research efforts have focused on the development of malaria subunit vaccines that can simulate sporozoite-induced protective immunity.

The circumsporozoite (CS) protein is a primary target of protective immune responses in sporozoite-immunized experimental hosts (31, 37). Antibodies specific for an immunodominant B-cell epitope in the central repeat region of the CS protein can immobilize sporozoites and block invasion of host hepatocytes. The *Plasmodium falciparum* protective B-cell epitope consists of multiple tandem repeats of the tetramer

NANP sequence (35, 52). The first phase I and II trials of a peptide-protein conjugate containing only *P. falciparum* (NANP)₃ repeats demonstrated the potential of CS subunit vaccines to protect against sporozoite challenge (16).

Additional studies in the rodent malaria model demonstrated that irradiated sporozoites elicited not only neutralizing anti-CS antibodies but also potent cellular immunity that targeted the hepatic exoerythrocytic forms (EEF) of the parasite. However, the CS NANP repeat region lacks strong Th cell epitopes, and little or no parasite-specific T-cell response was elicited following immunization with CS repeat vaccines (10, 11, 15, 16). Second-generation CS peptide vaccines have included parasite-specific CD4⁺-T-cell epitopes to insure that memory Th cells are elicited. These are important both for anamnestic antibody responses and for lymphokines, primarily gamma interferon (IFN- γ), to inhibit development of hepatic EEFs (reviewed in reference 34).

Recently, promising results were obtained in phase I and II trials of a truncated *P. falciparum* CS protein expressed in a recombinant hepatitis B virus (HBV) surface antigen, termed RTS,S. Immunized volunteers developed high levels of antibodies and Th1-type cellular responses (2, 17, 48). More importantly, when administered in a complex adjuvant formulation, this vaccine protected approximately 50% of immunized volunteers

* Corresponding author. Mailing address: New York University School of Medicine, Department of Medical and Molecular Parasitology, 341 East 25th St., New York, NY 10010. Phone: (212) 263-6819. Fax: (212) 263-8116. E-mail: nardie01@popmail.med.nyu.edu.

MATERIALS AND METHODS

Construction of expression vectors. The ICC-1132 recombinant protein was expressed in *Escherichia coli* transfected with the expression plasmid V12.Pf3.1. This plasmid encodes a truncated Hbc gene (amino acids 1 to 149) with the malaria CS T* epitope fused after the C-terminal Val₁₄₉ and the CS repeat epitopes inserted in the Hbc loop region between amino acids 78 and 79 [23; Birkett et al., *Am. J. Trop. Med. Hyg.* **65**(Suppl. 3):258, 2001]. Hbc particles engineered to present heterologous epitopes have historically been truncated at, or around, amino acid 149 to avoid incorporation of host RNA by the protamine-rich C-terminal tail (amino acids 150 to 183).

To construct this hybrid vaccine, a plasmid (V2) containing the truncated Hbc gene was first digested at the *EcoRI* and *SacI* restriction sites, which had been strategically introduced between the codons for D₇₈ and P₇₉ of the loop region. A synthetic double-stranded DNA (dsDNA) fragment encoding the repeat sequence NANPNVDP(NANP)₃, modified by the addition of 5' *EcoRI* and 3' *SacI* overhangs, was then inserted to yield plasmid V2.Pf3.1 (Fig. 1B). V2.Pf3.1 was used to direct the expression of a particle containing only the CS repeats (ICC-1557).

A second plasmid, V7.PfCS(326-345), was used to direct the expression of a particle containing only the T* sequence fused to the C-terminal Val₁₄₉ of the truncated Hbc (ICC-1258). The plasmid was constructed in a similar manner, using a plasmid (V7) containing a Hbc gene that had been modified to accept C-terminal fusions after amino acid Val₁₄₉ at *EcoRI-SacI* restriction sites. The plasmid was first digested with *EcoRI* and *SacI* restriction enzymes, and a synthetic dsDNA fragment encoding the T* sequence, modified by the addition of *EcoRI-SacI* restriction site overhangs, was inserted to produce plasmid V7.PfCS(326-345) (Fig. 1B).

An expression vector, V12.Pf3.1, was then constructed to encode a hybrid Hbc protein containing CS repeats in the immunodominant loop and T* at the C terminus (ICC-1132). To do this, the carboxyl terminus of the V7.PfCS(326-345) plasmid was amplified using two PCR primers to produce a dsDNA fragment corresponding to Hbc amino acids 79 to 149 plus the T* epitope, flanked with *SacI* and *HindIII* restriction sites. The PCR primers used for amplification were Hbc-P79/*SacI*-F (5'-CGCGAGCTCCAGCGTCTAGAGACCTAG) and PfCS(326-345)-H3-R (5'-GCCAAGCTTACTAGGTAACGGAGCACGGA); PfCS(326-345)-H3-R was designed to facilitate deletion of the 2 amino acids (Glu-Leu) coded by the *EcoRI* restriction site following the T* epitope. To yield the final expression vector V12.Pf3.1, the PCR product was cut with *SacI* and *HindIII* and then cloned into the V2.Pf3.1 vector, which had been prepared by cutting with the same two restriction enzymes. The nucleotide sequences of all plasmids were verified by DNA sequencing.

Expression and purification of recombinant particles. *E. coli* strain TB1 was transformed with either the V12.Pf3.1 (ICC-1132), V2.Pf3.1 (ICC-1557), or V7.PfCS(326-345) (ICC-1258) vector and selected on Luria-Bertani plates containing ampicillin (50 µg/ml). After 16 to 24 h of incubation at 37°C, a single colony was picked, expanded overnight, and used to inoculate a 500-ml culture (tryptone-yeast extract-NaCl [TYN] medium supplemented with 1 g of glucose/liter and 50 µg of ampicillin/ml). After 16 to 20 h, cells were harvested by centrifugation, resuspended in 50 to 100 ml of Tris-EDTA buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0), and lysed by a single passage at 16,000 lb/in² through a French pressure cell (ThermoSpectronic, Cambridge, United Kingdom). After centrifugation, the proteins in the supernatant were precipitated with ammonium sulfate and the precipitate was resuspended in a minimal volume of Tris-EDTA buffer. The resuspended protein pellet was dialyzed extensively against the same buffer and centrifuged, and the supernatant was recovered.

All particles were purified by gel filtration chromatography on a Sepharose CL-4B column (Pharmacia, Piscataway, N.J.). Fractions eluted from the column were pooled and loaded onto an hydroxyapatite column (Clarkson, South Williamsport, Pa.). The column was washed with 50 mM sodium phosphate, pH 6.8, and the nonbinding hydroxyapatite eluent was applied to a Mono-Q anion-exchange column (Pharmacia) and eluted with a linear gradient of 0 to 1.2 M NaCl. The protein was concentrated by a second ammonium sulfate precipitation and dialyzed against 20 mM sodium phosphate, pH 6.8, and the concentration was determined using a standard bicinchoninic acid assay.

Immunogenicity. (i) Murine studies. BALB/c and (B6 × 129)F₁ mice were obtained from Jackson Laboratory, Bar Harbor, Maine. MHC class II-deficient mice (I-Aβ^{b-/-}) were a kind gift of Albert Bendelac, Princeton University. Mice were immunized intraperitoneally on days 0, 21, and 42 with 10 µg of ICC-1132 or Hbc149. Endotoxin units were <0.5 µg as determined by *Limulus* amoebocyte lystate assay. Mice were primed with antigen emulsified in Freund's complete

adjuvant followed by boosters with antigen in Freund's incomplete adjuvant. Serum samples were collected 21 days after each immunizing dose.

Cellular studies were carried out using lymph node cells obtained from BALB/c mice primed subcutaneously with ICC-1132 in Freund's adjuvant. Additional cytokine studies used spleen cells from mice primed with 10 µg of ICC-1132 formulated in 70% Montanide ISA 720, a metabolizable water-in-oil adjuvant (SEPPIC, Paris, France), followed by booster injection at 42 days with ICC-1132 either in saline, adsorbed to alum (Alhydrogel, Superfos, Frederikssund, Denmark), or emulsified in Montanide ISA 720. Untreated mice served as control. Five days after the booster inoculation, mice were sacrificed and spleen and lymph nodes were removed for T-cell analysis.

(ii) *M. fascicularis* (cynomolgus) monkeys. The immunogenicity of ICC-1132 formulated in different adjuvants was also assayed in *Macaca fascicularis* (cynomolgus) monkeys (maintained by Sierra Biomedical, Sparks, Nev.). Two monkeys (one male and one female) per group were immunized intramuscularly on days 0 and 42 with 20 µg of ICC-1132 in saline or formulated in different adjuvants. Two alum preparations were tested, aluminum phosphate gel (Adju-Phos) with low binding affinity for ICC-1132 and aluminum hydroxide gel (Alhydrogel) with high binding affinity (Superfos). Two additional groups of monkeys received ICC-1132 in an oil-in-water emulsion (SE vehicle; Corixa, Seattle, Wash.) or in the water-in-oil adjuvant, Montanide ISA 720.

Serological assays. (i) ELISA. Antibodies were measured by enzyme-linked immunosorbent assay (ELISA) using Hbc proteins or malaria peptides as coating antigens [29, 30; Birkett et al., *Am. J. Trop. Med. Hyg.* **65**(Suppl. 3):258, 2001]. Antirepeat antibodies were assayed using the branched peptide, (T1B)₄ MAP, or linear (NANP)₅ peptide; similar results were obtained with both antigens. Coated wells were blocked and incubated with twofold dilutions of sera in phosphate-buffered saline-0.05% Tween 20-bovine serum albumin (BSA). Binding of antibodies was detected by ELISA using enzyme-labeled antibodies specific for murine immunoglobulin G (IgG) (Kirkegaard & Perry, Gaithersburg, Md.) or rabbit anti-monkey Ig (Accurate, Westbury, N.Y.) and peroxidase-labeled anti-rabbit antibodies. Following addition of the enzyme substrate ABTS (2,2'-azino[bis-3-ethylbenzthiazolinesulfonic acid])-H₂O₂, the endpoint titer was determined as the dilution of immune sera giving an optical density greater than the mean + 2 standard deviations obtained with normal sera or two times greater than the optical density obtained with BSA-coated wells.

(ii) IFA. The indirect immunofluorescence assay (IFA) was carried out using *P. falciparum* (NF54) sporozoites dissected from the salivary glands of *Anopheles* mosquitoes infected by feeding on gametocyte cultures. Multiwell slides containing air-dried sporozoites were incubated with twofold dilutions of sera, and the bound antibody was detected by reaction with fluorescein isothiocyanate-labeled antibodies specific for murine IgG (Kirkegaard & Perry) or with rabbit anti-monkey IgG (Accurate) followed by fluorescein isothiocyanate-labeled anti-rabbit antibody (29, 30).

Cellular proliferation and cytokine assays. (i) Murine. Triplicate wells containing lymph node or spleen cells of naive or immunized BALB/c mice were stimulated with ICC-1132 or Hbc149 particles (10 µg/ml) or malaria CS peptide (20 µg/ml). Proliferation in 6-day cultures was measured by [³H]thymidine incorporation, and results were expressed as stimulation indices (SI) obtained by dividing mean counts per minute in wells with antigen by mean counts per minute in wells without antigen.

Culture supernatants were collected at 48 to 72 h for cytokine analysis. Interleukin 2 (IL-2) was measured by bioassay using an IL-2-dependent cell line, and results were expressed as supernatant-induced SI (32). Additional cytokines were measured by using commercial kits for IFN-γ, IL-4, and IL-10, and results were expressed as picograms per milliliter (BD Pharmingen, San Diego, Calif.).

(ii) Human. CD4⁺-T-cell lines (TCL) were established from PBL of volunteers immunized with (T1B)₄ MAP or (T1BT*)₄ polyoxime (32, 36). TCL (2 × 10⁴) were cultured with 5 × 10⁴ irradiated PBL antigen-presenting cells (APC) in triplicate wells. The cells were stimulated with 10-fold dilutions of ICC-1132 or Hbc149 particles starting at 10 µg/ml or CS peptides (20 µg/ml), recombinant CS proteins (20 µg/ml), or extracts of 10⁵ *P. falciparum* sporozoites as positive controls. Proliferation in 4-day cultures and IL-2 levels in 24-h supernatants were measured as described for murine assays. Human IFN-γ in 72-h supernatants was measured by using a commercial kit (BD Diagnostics).

RESULTS

Humoral responses induced by *P. falciparum* ICC-1132 CS-Hbc particles. (i) Immunogenicity in mice. The ICC-1132 particles, containing the *P. falciparum* repeat (NANP)₃ B-cell epitope and two CS-derived T-helper cell epitopes, T1 and T*

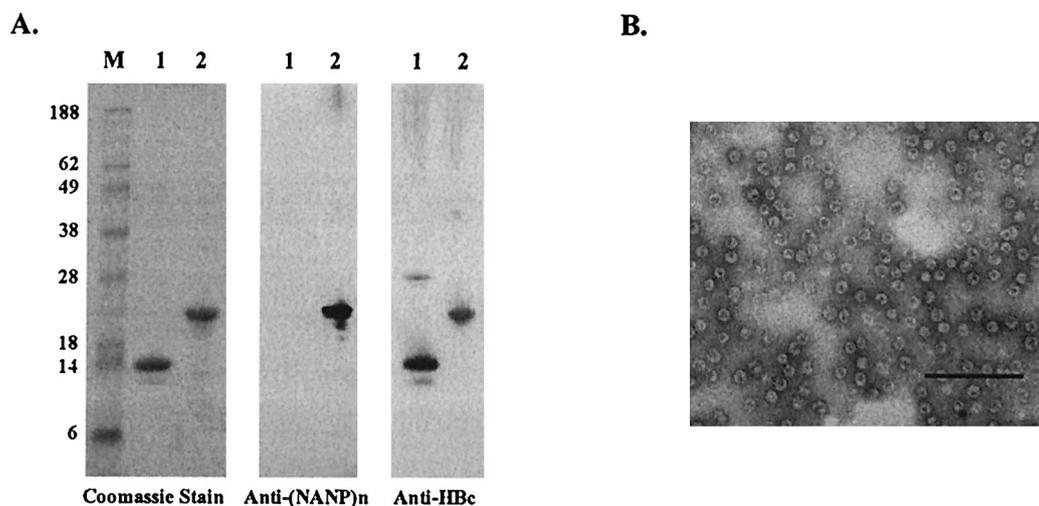


FIG. 2. (A) Physicochemical characterization of truncated HBc149 protein (lane 1) and purified ICC-1132 (lane 2) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Coomassie blue-stained gels and Western blots, using anti-CS or anti-HBc antibodies, demonstrate migration at the expected molecular mass of the reduced ICC-1132, 22,000 Da, and of the C-terminally truncated HBc149, 17,000 Da (M, molecular mass markers). In the Western blots, a monoclonal antirepeat antibody (MAB 2A10) stained ICC-1132 (lane 2) but not HBc149, which lacks the CS repeats (lane 1). (B) Electron microscopy of purified ICC-1132 particles. Images were generated using 1% ammonium acetate as a negative stain. Magnification, $\times 40,000$; scale bar = 250 nm.

(Fig. 1C), can be produced under good manufacturing practices (GMP) conditions and purified to clinical levels by column chromatography. The purified ICC-1132 and the control HBc149 particle that lacks CS inserts were of the expected molecular weight when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (Fig. 2A). The presence of intact particles of an approximate diameter of 32 nm, with a dense central core typical of virus capsids, was demonstrated by electron microscopy (Fig. 2B).

Consistent with previous studies with other murine strains [23; Birkett et al., *Am. J. Trop. Med. Hyg.* **65**(Suppl. 3):258, 2001], administration of two doses of ICC-1132 in Freund's adjuvant elicited extremely high antirepeat antibody titers (geometric mean titer [GMT] = 1.3×10^6) in BALB/c mice (Fig. 3A). Antibody responses were not further increased by a third immunization. The antirepeat peptide titers directly correlated with strong reactivity with *P. falciparum* sporozoites, reaching peak IFA titers of 655,360. Serum from mice immunized with HBc149 did not react with *P. falciparum* sporozoites or malaria peptides in either the IFA or ELISA (data not shown).

In contrast to the repeat epitope, only low levels of antibody specific for the T* epitope were detected in mice immunized with ICC-1132 (Fig. 3A). This is consistent with the poor antibody responses elicited by foreign epitopes inserted at the C terminus of HBc protein (41). The T* epitope in the context of the native CS also does not elicit significant antibody responses in sporozoite-immunized mice and human volunteers (5).

In addition to high levels of antirepeat antibodies, anti-HBc titers exceeding 10^6 were obtained in the mice immunized with ICC-1132 (Fig. 3A) [23; Birkett et al., *Am. J. Trop. Med. Hyg.* **65**(Suppl. 3):258, 2001]. These titers were an order of magnitude lower than those obtained in mice immunized with HBc149 (data not shown). The lower anti-HBc titers result from disruption of the HBc immunodominant B-cell epitope

when foreign epitopes are inserted in the loop region [23, 41; Birkett et al., *Am. J. Trop. Med. Hyg.* **65**(Suppl. 3):258, 2001].

Immunization with ICC-1132 elicited repeat-specific antibodies of all IgG subtypes, with a strong Th2-associated IgG1 response (Fig. 3B). In addition, a significant IgG3 response, a subtype frequently elicited in response to T-independent antigens (9, 26, 46), was also noted in the immunized mice (Fig. 3B). Similar results were obtained in (B6 \times 129) F_1 mice immunized with ICC-1132 in Freund's adjuvant (data not shown). The predominance of IgG1 and IgG3 responses has also been noted in mice immunized with hybrid recombinant core particles formulated in other adjuvants or containing viral epitopes (12, 23).

(ii) T-cell dependency of antibody response to ICC-1132.

The murine IgG3 subtype is frequently elicited by immunization with T-independent antigens, such as polysaccharides with repeating sugar units (26, 46). The HBc protein, unlike the HBV surface antigen, can elicit T-independent, as well as T-dependent, antibody responses to HBc epitopes and to foreign epitopes contained in recombinant hybrid core proteins (12, 24).

To determine if the strong antimalaria response induced by ICC-1132 was T dependent, transgenic mice lacking class II molecules were immunized. The class II $^{-/-}$ mice failed to develop an IgG response specific for CS repeats at any time point during immunization with ICC-1132 (Fig. 4A, open circles). Parental B6 \times 129 controls, expressing normal class II and CD4 $^{+}$ -T-cell levels, developed high antirepeat antibody titers following immunization with ICC-1132 (Fig. 4A, closed circles). Mice immunized with HBc149 did not develop antibodies reactive with CS repeats (Fig. 4A, squares).

The parental B6 \times 129 mice also developed high anti-HBc titers following immunization with ICC-1132 or HBc149 (Fig. 4B, closed symbols). In contrast to antirepeat antibody response, however, anti-HBc antibodies could be detected in the

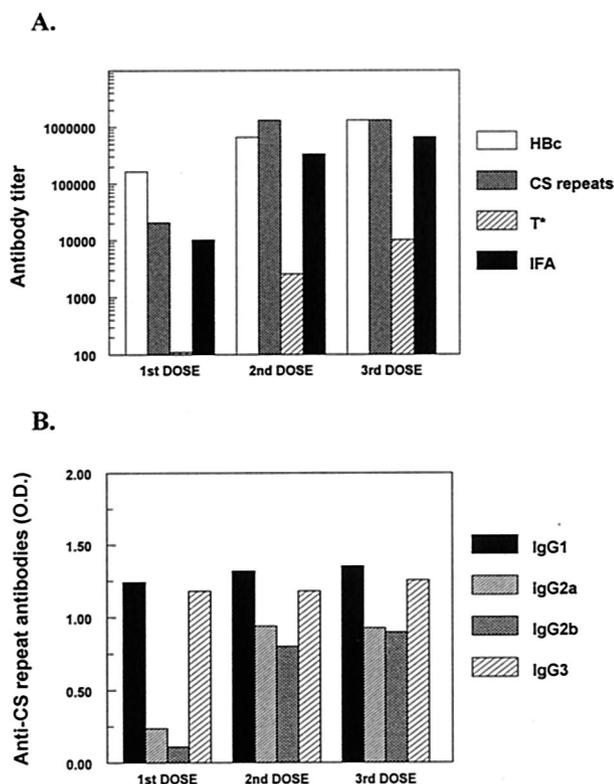


FIG. 3. Antibody titers in BALB/c mouse sera obtained 3 weeks after each dose of ICC-1132–Freund’s adjuvant. (A) Kinetics and fine specificity of IgG responses measured by IFA using *P. falciparum* sporozoites and by ELISA using HBc immunogen or malaria peptides as antigen. (B) IgG subtypes of antirepeat antibody response following each immunization. O.D., optical density.

class II^{-/-} mice, although the magnitude of this response was reduced (Fig. 4B, open symbols). The anti-HBc titers peaked after the first immunization and did not increase following booster inoculations, as is typical of antibody responses to T-independent antigens.

Cellular responses induced by ICC-1132. (i) Induction of malaria-specific T cells in ICC-1132-immunized mice. The specificity of Th elicited by malaria vaccines is a critical determinant of vaccine efficacy. In mice immunized with ICC-1132, T cells specific for either HBc or CS could provide helper factors for the T-dependent antirepeat antibody response. However, only the T cells specific for CS epitopes can provide malaria-specific memory T cells for anamnestic antibody responses and inhibitory lymphokines that can function in resistance to sporozoite challenge.

To determine whether CS-specific T cells were elicited by immunization with ICC-1132, lymph node cells of BALB/c mice primed with ICC-1132 were assayed *in vitro*. Stimulation with HBc particles, with or without CS epitope inserts, elicited strong proliferative and cytokine responses, consistent with the presence of multiple T epitopes in the core antigen (Fig. 5). Importantly, cells from ICC-1132-immunized mice also proliferated and released IL-2 and IFN- γ in response to *in vitro* challenge with the malaria T* peptide. The cells of the ICC-1132-immunized mice did not respond when stimulated with

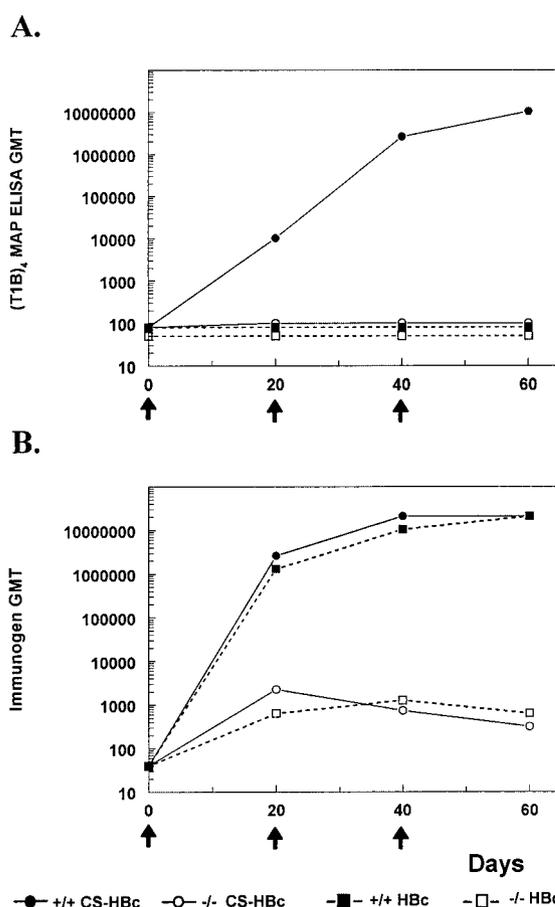


FIG. 4. Antibody titers of class II^{-/-} knockout mice (open symbols) or wild-type mice (closed symbols) as measured by ELISA using *P. falciparum* CS repeats (A) or HBc protein (B) as antigen. Samples were obtained following immunization (arrows) with ICC-1132 (circles) or HBc149 (squares) in Freund’s adjuvant.

repeat peptides, consistent with the nonresponder phenotype of H-2^d mice to CS repeats (4, 10, 15, 30).

The magnitude of the cellular responses to malaria epitopes differed from the response to HBc epitopes. Core particles, with or without CS epitopes, elicited levels of IL-2 response that were 7- to 10-fold higher than those obtained following stimulation of immune cells with T* peptide. In contrast, IFN- γ levels elicited by T* peptide were comparable to those induced by ICC-1132 stimulation, suggesting that the Th1 cellular responses may be predominantly directed to the T* epitope.

Spleen cells of mice primed with ICC-1132 formulated in Montanide ISA 720 and boosted with vaccine formulated in various adjuvants suitable for human use were also tested (Fig. 6). In all vaccine formulations, T cells specific for T* were elicited, indicating that the type of adjuvant did not alter the fine specificity of the CS-specific response. Consistent with the broad range of antibody subtypes detected in ICC-1132-immunized mice, Th1-type as well as Th2-type cytokines could be detected in the culture supernatants. T*-specific cytokine responses were increased following a booster inoculation of the mice primed with ICC-1132–Montanide ISA 720. Enhanced IFN- γ responses could be obtained by booster with ICC-1132

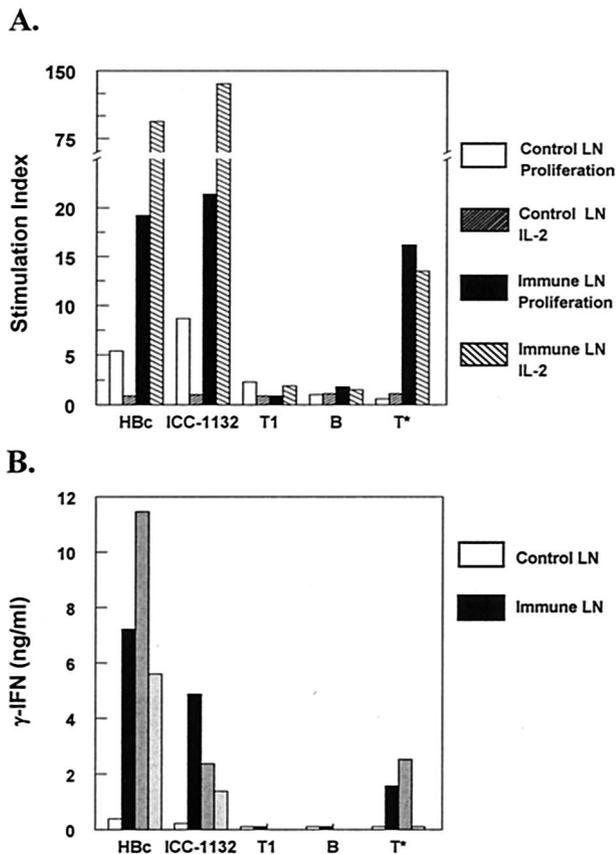


FIG. 5. (A) Lymph node (LN) cells of BALB/c mice immunized with ICC-1132 in Freund's adjuvant or adjuvant-only controls were assayed for proliferation (solid bars) and IL-2 responses (hatched bars). Results are shown as the SI obtained following stimulation with HBc or ICC-1132 core particles (10 μg/ml) or malaria CS peptides T1, B, and T* (20 μg/ml). (B) IFN-γ in supernatants of immune lymph node cells (dark bars) stimulated with 10-fold dilutions of antigen, starting at 10 μg/ml for core particles and 20 μg/ml for malaria CS peptides. Results for only the highest antigen concentration are shown for control nonimmune lymph nodes (open bars).

in saline, as well as with particles formulated in alum or Montanide ISA 720. Consistent with the known Th2 adjuvant properties of alum, higher IL-4 levels were obtained in mice boosted with ICC-1132 adsorbed to alum.

(ii) **Anamnestic responses in *P. falciparum* sporozoite-primed mice.** Importantly for field applications, the CS T-cell epitopes contained in the ICC-1132 stimulated strong anamnestic antibody responses in mice primed with *P. falciparum* sporozoites. A 100-fold increase of antirepeat antibodies and a 250-fold increase in IFA titers were observed in sporozoite-primed mice 10 days after a single injection of ICC-1132 (Table 1). These antibody titers were over 1,000-fold higher than titers in unprimed mice, with a GMT of 103,213 versus one of 101, respectively. The responses were malaria specific, as HBc-lacking CS epitopes did not elicit anamnestic antibody responses in *P. falciparum* sporozoite-primed mice.

(iii) **Immunogenicity of ICC-1132 in cynomolgus monkeys.** While there have been numerous murine studies, the immunogenicity of HBc particles, either with or without foreign epitope inserts, has not been examined in nonhuman primates.

The antibody response to ICC-1132 formulated in different adjuvants was therefore tested in Old World cynomolgus monkeys.

Significant differences were noted in the primate antibody responses to ICC-1132 compared to those in previous murine studies using the same vaccine formulations [23; Birkett et al., Am. J. Trop. Med. Hyg. 65(Suppl. 3):258, 2001]. ICC-1132 without adjuvant was substantially less immunogenic in the cynomolgus monkeys (Fig. 7A) than in mice. Similarly, alum formulations that bound ICC-1132 with high affinity (Alhydrogel) or low affinity (Adju-Phos) were poorly immunogenic in the monkeys and elicited antibody titers that were < 10⁴ by ELISA and IFA. The primate responses to ICC-1132 emulsified in oil in water (Corixa vehicle) were similar to those obtained with alum formulations.

Optimal antibody responses in the cynomolgus monkeys, as in mice, were obtained using the water-in-oil emulsion Montanide ISA 720 (Fig. 7A). Antirepeat antibodies were detectable 20 days after a single dose of ICC-1132 in Montanide ISA 720, and both IFA and ELISA titers reached 10⁵ by 40 days postinjection (Fig. 7B). A booster inoculation of the same formulation significantly increased antibody responses, reaching peak titers of 9 × 10⁵ in ELISA and 1.3 × 10⁶ by IFA. In all adjuvant formulations, the antirepeat antibody responses directly correlated with antisporezoite antibody titers.

Antigenicity of hybrid HBc particles for human cells. The results obtained in the murine and primate host have supported progression to human trials to assay safety and immunogenicity. In preparation for these studies, ICC-1132 particles were assayed in vitro using naïve human PBL and human malaria-specific T-cell clones.

(i) **Naïve PBL.** To determine the nonspecific inhibitory properties of HBc particles, naïve PBL were cultured with HBc particles and stimulated with phytohemagglutinin mitogen. Similar levels of proliferation and IL-2 production were elicited by mitogen in cultures with or without ICC-1132 and HBc149, indicating that the particles were not directly toxic for human PBL (data not shown).

Naïve PBL cultured with ICC-1132 or HBc particles in medium alone, without mitogen, demonstrated significant dose-dependent nonspecific proliferation, with SI ranging from 2 to 47. Despite the presence of high levels of proliferation in some individuals, IL-2 could not be detected in the culture supernatants, suggesting proliferation of B rather than T cells (data not shown).

Consistent with B-cell stimulatory properties, lymph node cells from nonimmune mice proliferated when cocultured with high concentrations of either ICC-1132 or HBc particles (Fig. 5A), but T-cell cytokines IL-2, IFN, IL-4, and IL-10 were not detected in the culture supernatants (Fig. 5B and 6). Previous studies have shown that HBc particles without foreign epitope inserts can stimulate naïve murine B cells, as well as naïve human B cells transferred to transgenic SCID mice (6, 19, 22). This property is believed to play a role in the T-independent antibody responses elicited by core particles and to contribute to their potent immunogenicity as vaccine carriers.

(ii) **Human malaria-specific CD4⁺-T-cell clones.** Human CD4⁺-T-cell clones, specific for T* or repeat epitopes (32; J. M. Calvo-Calle, unpublished data), were used to determine whether the malaria epitopes, expressed either in the loop

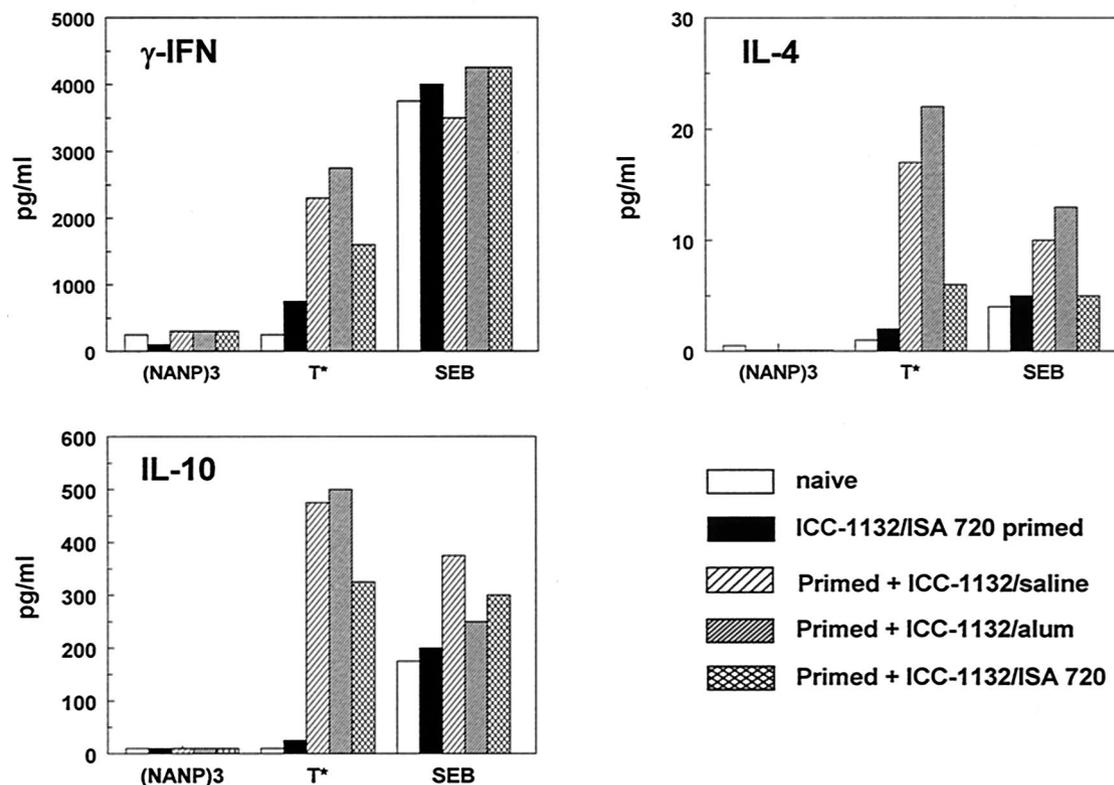


FIG. 6. Cytokine production by spleen cells of mice primed with a single injection of ICC-1132–Montanide ISA 720 or followed by a booster injection of ICC-1132 either without adjuvant (in saline), adsorbed to alum, or emulsified in Montanide ISA 720. The spleen cells obtained from immunized mice or naive mice were stimulated with malaria CS peptides or superantigen staphylococcal enterotoxin B (SEB), and levels of cytokine (in picograms per milliliter) were measured in culture supernatants.

region or C terminus of the Hbc core protein, were antigenic. The CD4⁺ T cells specifically recognized the ICC-1132 particles but not Hbc particles, indicating that these T-cell epitopes were correctly processed and presented by HLA class II molecules (Table 2). Stimulation levels were comparable to those obtained with extracts of *P. falciparum* sporozoites (clone 9-2D4) or a recombinant *P. falciparum* CS protein. The T-cell responses were malaria specific as recombinant vivax CS protein expressed in yeast, murine dihydrofolate reductase expressed in *E. coli*, or extracts of *P. berghei* sporozoites did not elicit proliferation or IL-2 production. The Hbc particles, with

or without CS epitopes, did not stimulate irradiated APC or human CD4⁺-TCL of other specificities (data not shown).

In addition, human CD4⁺-TCL specific for the T1 epitope, derived from volunteers immunized with MAP vaccine (36), also recognized Hbc particles containing the CS repeat epitopes (ICC-1557) but not Hbc149 (data not shown). These CD4⁺ T cells are restricted by class II molecules encoded by DRB1*0401, DRB1*1101, or DQB1*0603 (36), indicating that the T1 epitope contained in core particles can be processed for presentation by multiple HLA class II isotypes. ICC-1132 elicited proliferation, IL-2, and IFN- γ levels similar to levels obtained when T1-specific TCL were stimulated with MAP or recombinant *P. falciparum* CS protein (data not shown). TCL stimulated with Hbc149, or with a recombinant CS protein lacking the repeat region, did not proliferate or produce cytokines.

DISCUSSION

Recombinant Hbc particles containing rodent malaria B-cell epitopes had earlier been found to elicit protective immunity in rodent malaria models; however, similar constructs containing *P. falciparum* CS repeats were significantly less immunogenic (43). A series of hybrid core particles containing *P. falciparum* CS repeats with or without CS-derived T-cell epitopes were screened to identify a more potent immunogen [23; Birkett et al., *Am. J. Trop. Med. Hyg.* 65(Suppl. 3):258,

TABLE 1. ICC-1132 elicits anamnestic antibody responses in *P. falciparum* sporozoite-primed mice^a

Priming	Boost	IgG titer for:	
		ELISA	IFA
<i>P. falciparum</i> sporozoites	ICC-1132	103,213	327,680
<i>P. falciparum</i> sporozoites	Hbc149	1,280	2,560
None	ICC-1132	101	320

^a BALB/c mice were primed with a single injection of 10⁵ *P. falciparum* sporozoites. Approximately 3 months later, when ELISA GMTs were 970 (range, 320 to 2,560) and pooled sera IFA titer was 1,280, the sporozoite-primed mice were injected with 5 μ g of ICC-1132 or Hbc. Naive mice received a single injection of ICC-1132 as control. IgG ELISA and IFA titers were determined 10 days after the boost.

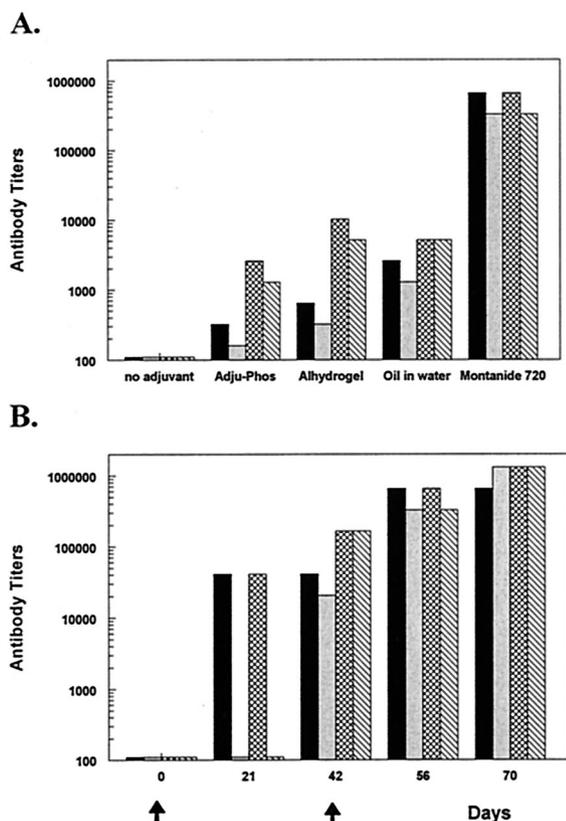


FIG. 7. Cynomolgous monkeys were immunized with ICC-1132 formulated with adjuvants acceptable for human use: Adju-Phos, Alhydrogel, oil-in-water emulsion, or Montanide ISA 720. Two monkeys were immunized per group. (A) ELISA and IFA titers for the first monkey of each group are shown as black and grey bars, respectively, and for the second monkey as hatched and diagonal bars. (B) Kinetics of ELISA and IFA responses in the two cynomolgous monkeys immunized with ICC-1132 in Montanide ISA 720.

2001]. In the present studies, immunogenicity of the optimal construct, designated ICC-1132, was assayed *in vivo* in rodents and nonhuman primates and *in vitro* using naïve and malaria-immune human CD4⁺ T cells.

The ICC-1132 particle contains the CS repeat epitope in-

serted into the immunodominant located HBc loop region that forms spikes on the particle surface and the universal T-cell epitope fused to the C terminus of the HBc protein (Fig. 1C). Mice immunized with the ICC-1132 particle developed high antirepeat ELISA titers (1.3×10^6) and, more important, equally high antisporezoite IFA titers of 655,360. These antibody titers represent a 30-fold increase over ELISA titers and a 400-fold increase over antisporezoite IFA titers, obtained in earlier studies with HBc particles containing only (NANP)₄ CS (43).

In contrast to the malaria repeat epitopes, the universal T* epitope elicited only weak antibody responses, consistent with the poor immunogenicity of foreign B-cell epitopes inserted at the C terminus of the core protein (41). The immunodominance of the CS repeat B-cell epitope and the low immunogenicity of the nonrepeat T* epitope in the mice immunized with ICC-1132 are consistent with the fine specificity of antibody responses in volunteers immunized with *P. falciparum* sporozoites (5) or synthetic peptide vaccines (32). The multimeric presentation of CS repeat epitopes on the surface of the hybrid HBc particle may effectively mimic native CS protein on the sporozoite surface.

The multiple tandem repeats of the CS protein have been hypothesized to function as a T-independent antigen, similar to the repetitive subunits of bacterial polysaccharides (45). More recent studies, however, have demonstrated that the antirepeat antibody response in sporozoite-immunized mice, and in human volunteers immunized with malaria synthetic peptide vaccines, is T cell dependent (25, 36). Following immunization with ICC-1132, transgenic class II^{-/-} mice failed to develop an antirepeat IgG response, indicating that the repeat epitopes in the context of the core particle also function as a T-dependent antigen (Fig. 4A). Both T-independent and T-dependent anticore antibody responses were elicited in mice immunized with HBc particles with or without CS inserts (Fig. 4B), consistent with previous studies (24).

Although numerous Th cell epitopes are present in the HBc protein, the ability to elicit anamnestic antibody and cytokine responses following sporozoite challenge requires the presence of malaria-specific T cells. ICC-1132 contains two CS-derived T-cell epitopes, T1 and T*, which are known to be immunogenic in human volunteers immunized with irradiated *P. falciparum*

TABLE 2. Human CD4⁺ T-cell clones recognize *P. falciparum* CS repeat and universal T* epitopes contained in ICC-1132^a

Antigen	Assay results for:					
	T-cell clone 9-2D4			T-cell clone 9-2E10		
	Proliferation (SI)	IL-2 (SI)	IFN- γ (pg/ml)	Proliferation (SI)	IL-2 (SI)	IFN- γ (pg/ml)
T* peptide	1	1	<5	124	402	2,374
Repeat peptide	21	195	106	1	1	<5
ICC-1132	10	44	22	128	364	2,073
HBc149	1	1	<5	2	3	<5
<i>P. falciparum</i> rCS	12	107	ND	83	202	1045
Control protein	1	1	<5	1	2	<5
<i>P. falciparum</i> sporozoites	6	116	122	ND	ND	ND
<i>P. berghei</i> sporozoites	1	1	<5	ND	ND	ND

^a T-cell clones were derived from a volunteer immunized with a malaria polyoxime vaccine (32; Calvo-Calle, unpublished). The T cells were stimulated with 10 to 20 μ g of CS peptides, core particles, or recombinant CS protein (rCS) per ml or freeze-thawed extracts of 10⁵ sporozoites, using irradiated PBL as APC. Proliferation and IL-2 assay results are expressed as SI. ND, not determined.

parum sporozoites or with synthetic peptide malaria vaccines (28, 32, 33, 36). The present studies demonstrate that these malaria T-cell epitopes were also immunogenic in the context of the HBc particle. High levels of IFN- γ and IL-2 production were observed following T* peptide stimulation of cells from mice immunized with ICC-1132 in Freund's adjuvant (Fig. 5A and B). Consistent with the broad spectrum of antibody subtypes observed in immune sera, Th2-type cytokines IL-4 and IL-10 were also detected in the sera of mice immunized with ICC-1132 formulated in alum or Montanide ISA 720 (Fig. 6).

The parasite-specific T-cell epitopes present in ICC-1132 elicited strong anamnestic responses in sporozoite primed mice (Table 1). A single booster inoculation of ICC-1132 increased antibody titers 100- to 150-fold in the sporozoite-primed mice. The presence of strong Th1-type CD4⁺ T cells, in addition to providing T-cell help for B-cell antibody responses, also provides the potential to target the intracellular hepatic stages through production of IFN- γ (13, 44). Mice immunized with malaria synthetic peptides have been shown to develop CD4⁺ T cells that can mediate IFN- γ -dependent protective immunity by inhibiting EEF development within hepatic cells (21, 51).

Cynomolgus monkeys immunized with two injections of ICC-1132 in Montanide ISA 720 developed antirepeat and ant sporozoite antibody titers in excess of 10⁶, the highest titers obtained to date in simian hosts immunized with a recombinant malaria protein (Fig. 7A and B). Although ICC-1132 in saline or adsorbed to alum was highly immunogenic in mice, these formulations were significantly less immunogenic in the cynomolgus monkeys. Whether these differences relate to the use of rodent versus simian hosts, different immunization protocols, or dose-to-body weight ratios is unknown. No adverse effect or reactogenicity was noted in cynomolgus monkeys immunized with ICC-1132 in any of the adjuvant formulations.

In both mice and monkeys, optimal responses were obtained with Montanide ISA 720 formulations. Malaria CS synthetic peptide vaccines or recombinant blood stage vaccines adjuvanted with Montanide ISA 720 have shown promising results in nonhuman primates and phase I clinical studies (1, 7, 14, 20, 40). Immunization protocols in which primary doses with vaccine formulated in Montanide ISA 720 are followed with a booster inoculation containing the same antigen in a different adjuvant have reduced reactogenicity noted with some antigen-Montanide ISA 720 formulations (39, 49). It is encouraging that the murine immune responses elicited with ICC-1132-Montanide ISA 720 could be efficiently boosted by a second dose of ICC-1132 either without adjuvant or with alum. These findings suggest that highly immunogenic vaccine formulations can be designed that minimize potential reactogenicity in humans.

Important for human vaccine development, the *P. falciparum* T-cell epitopes contained in ICC-1132 were processed and presented by human APC to malaria specific HLA-restricted T cells. Human CD4⁺-T-cell clones specific for either the *P. falciparum* CS repeat or T* epitope proliferated and produced cytokines (IL-2 and IFN- γ) when stimulated with ICC-1132 (Table 2). DR- and DQ-restricted CD4⁺-TCL that were specific for the T1 repeat epitope also recognized recombinant core particles containing CS repeats (data not shown). Therefore, *P. falciparum* T-cell epitopes expressed in either the im-

munodominant loop region or the C terminus of the HBc particle can be recognized by malaria-specific human cells in the context of multiple class II isotypes. Stimulation of these human CD4⁺ T cells with hybrid CS-HBc particles elicited IFN- γ levels comparable to those elicited by recombinant full-length CS protein or *P. falciparum* sporozoite extract (Table 2). These findings indicate that the hybrid particles can elicit epitope-specific responses relevant to sporozoite immunity.

The ability of ICC-1132 to stimulate malaria-specific human CD4⁺ T cells and to elicit high levels of *P. falciparum*-specific immunity in rodent and primate hosts supports phase I testing to assess safety and immunogenicity in human volunteers. Recent clinical trials of the RTS,S recombinant HBsAg malaria vaccine suggest that protective immunity to *P. falciparum* sporozoites can be mediated by high levels of antirepeat antibodies and CS-specific CD4⁺ T cells in the absence of CD8⁺ T cells (18, 48). Although protection was short-lived in these volunteers, as well as in immunized African adults, studies with RTS,S clearly demonstrate the feasibility of CS-based subunit vaccines (2, 47). Based on the persistence of anticore responses in hepatitis infections, it is hoped that ICC-1132 will elicit long-lived ant sporozoite immune responses in humans, as found in murine and simian hosts. The presence of minimal CS epitopes in ICC-1132 may also enhance humoral and cellular immunity by focusing the host immune response on rationally defined T- and B-cell epitopes of the *P. falciparum* CS protein.

Progression to human clinical trials requires advancing the manufacture and batch release of the recombinant particles, as well as of the adjuvant formulation, to more highly regulated and controlled processes. To this end, master and working cell banks for an ICC-1132-producing *E. coli* clone have been produced under GMP conditions and a scalable fermentation process has been developed with culture media that are devoid of animal products. Likewise, a scalable purification process, which utilized only reagents with associated drug master files, has been developed and used to direct the manufacture of multiple batches of GMP-grade material. These scale-up and purification processes should facilitate application of modified HBc particles in the development of vaccines for other infectious diseases, in addition to malaria.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Rita Altszuler and Ivette Caro-Aguilar. Electron microscopy of ICC-1132 particles was carried out by R. Nessler, Central Microscopy Research Facility, University of Iowa. We thank Victor Nussenzeig for critical review of the manuscript.

Studies at New York University were supported by NIH grants AI 25085 and AI 45138 to E.N. and by an Apovia unrestricted research grant. Studies at Apovia were supported by a grant from the Malaria Vaccine Initiative at PATH and by NIH grant AI43830 to A.B.

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Editor: W. A. Petri, Jr.