

ORIGINAL ARTICLE

Protection against a Malaria Challenge by Sporozoite Inoculation

Meta Roestenberg, M.D., Matthew McCall, M.D., Joost Hopman, M.D., Jorien Wiersma, Adrian J.F. Luty, Ph.D., Geert Jan van Gemert, B.Sc., Marga van de Vegte-Bolmer, B.Sc., Ben van Schaijk, M.Sc., Karina Teelen, Theo Arens, Lopke Spaarman, B.Sc., Quirijn de Mast, M.D., Will Roeffen, Ph.D., Georges Snounou, Ph.D., Laurent Rénia, Ph.D., Andre van der Ven, M.D., Cornelus C. Hermesen, Ph.D., and Robert Sauerwein, M.D.

ABSTRACT

BACKGROUND

An effective vaccine for malaria is urgently needed. Naturally acquired immunity to malaria develops slowly, and induction of protection in humans can be achieved artificially by the inoculation of radiation-attenuated sporozoites by means of more than 1000 infective mosquito bites.

METHODS

We exposed 15 healthy volunteers — with 10 assigned to a vaccine group and 5 assigned to a control group — to bites of mosquitoes once a month for 3 months while they were receiving a prophylactic regimen of chloroquine. The vaccine group was exposed to mosquitoes that were infected with *Plasmodium falciparum*, and the control group was exposed to mosquitoes that were not infected with the malaria parasite. One month after the discontinuation of chloroquine, protection was assessed by homologous challenge with five mosquitoes infected with *P. falciparum*. We assessed humoral and cellular responses before vaccination and before the challenge to investigate correlates of protection.

RESULTS

All 10 subjects in the vaccine group were protected against a malaria challenge with the infected mosquitoes. In contrast, patent parasitemia (i.e., parasites found in the blood on microscopical examination) developed in all five control subjects. Adverse events were mainly reported by vaccinees after the first immunization and by control subjects after the challenge; no serious adverse events occurred. In this model, we identified the induction of parasite-specific pluripotent effector memory T cells producing interferon- γ , tumor necrosis factor α , and interleukin-2 as a promising immunologic marker of protection.

CONCLUSIONS

Protection against a homologous malaria challenge can be induced by the inoculation of intact sporozoites. (ClinicalTrials.gov number, NCT00442377.)

From the Departments of Medical Microbiology (M.R., M.M., J.H., J.W., A.J.F.L., G.J.G., M.V.-B., B.S., K.T., T.A., L.S., W.R., C.C.H., R.S.) and General Internal Medicine (Q.M., A.V.), Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands; the Department of Parasitology, INSERM Unité 511, Hôpital Pitié-Salpêtrière, and the Université Pierre et Marie Curie — both in Paris (G.S.); and the Laboratory of Malaria Immunobiology, Singapore Immunology Network, Agency for Technology and Research, Biopolis, Singapore (L.R.). Address reprint requests to Dr. Sauerwein at the Department of Medical Microbiology 268, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB Nijmegen, the Netherlands, or at r.sauerwein@mmb.umcn.nl.

Drs. Roestenberg and McCall contributed equally to this article.

N Engl J Med 2009;361:468-77.
Copyright © 2009 Massachusetts Medical Society.

MALARIA IS RESPONSIBLE FOR A significant burden of morbidity and mortality in the developing world, and an effective vaccine against this disease is urgently needed.¹ Despite decades of research, a licensed vaccine is still not available, largely because immunity to *Plasmodium falciparum* malaria is considered difficult to acquire, whether through natural exposure or artificially through vaccination. A further critical factor is our incomplete understanding of precisely what constitutes protective antimalarial immunity in humans.

The possibility of vaccinating humans against *P. falciparum* malaria was raised originally by the success of the radiation-attenuated sporozoite model developed several decades ago.^{2,3} Irradiation of infectious mosquitoes disrupts the gene expression of sporozoites, which remain capable of hepatocyte invasion but are no longer capable of complete liver-stage maturation or progression to the pathogenic blood stage.⁴ Infection of human volunteers with irradiated sporozoites thus exposes them to liver-stage antigens and generates pre-erythrocytic immunity. However, the requirement of a minimum of 1000 bites by irradiated mosquitoes during five or more immunization sessions in order to successfully induce sterile immunity in humans⁵ precludes this method for routine immunization.

A subunit vaccine can be developed on the basis of antigens expressed by pre-erythrocytic, intra-erythrocytic, or sexual stages of the parasite. Unfortunately, results of many such subunit vaccines in humans have been disappointing. To date, only one candidate vaccine, which is based on the circumsporozoite protein and known as RTS,S, has progressed to phase 3 field trials. The protection induced by this vaccine is encouraging, but the ultimate success of this approach remains to be determined.⁶⁻⁹

In rodent models, sterile protection against malaria can be achieved by the inoculation of intact sporozoites while treating the animals concomitantly with chloroquine,¹⁰ a drug that kills parasites in the asexual blood stage but not in the pre-erythrocytic stage.¹¹ The efficacy of this treatment is significantly higher than that of the radiation-attenuated sporozoite model.¹² We therefore designed a proof-of-concept study in volunteers who had not been previously exposed to malaria to investigate whether protection can be

induced by this approach in humans and to explore the immune responses elicited.

METHODS

STUDY SUBJECTS

We recruited 15 healthy volunteers between the ages of 18 and 45 years who had no history of malaria or of living in an area in which malaria is endemic in the 6 months before study entry. Only one volunteer had ever been in an endemic area, several years earlier. All volunteers underwent routine physical examination and hematologic and biochemical screening at the Clinical Research Center at Radboud University Nijmegen Medical Centre. The results of serologic analysis for the human immunodeficiency virus (HIV), hepatitis B and C, and asexual *P. falciparum* parasites were negative in all subjects.

STUDY OVERSIGHT

All subjects provided written informed consent. The trial was approved by the institutional review board at the Radboud University Nijmegen Medical Centre. The study sponsor, the Dioraphte Foundation, was not involved in the design of the study, in the gathering or analysis of the data, or in the writing of the manuscript. All authors vouch for the accuracy and completeness of the data.

STUDY DESIGN

We randomly assigned the subjects in a double-blind fashion to two study groups: 10 to a vaccine group and 5 to a control group (Fig. 1). The mean ($\pm$ SD) age of the subjects was 22.0 ± 1.5 years in the vaccine group and 24.0 ± 1.4 years in the control group; seven subjects in the vaccine group were women, as were four subjects in the control group.

Chloroquine was provided to all subjects in a standard prophylactic regimen of a loading dose of 300 mg on each of the first 2 days and then 300 mg once a week, starting on day 7, for a total duration of 13 weeks. While receiving chloroquine, subjects in the vaccine group were exposed on three occasions at monthly intervals to bites of 12 to 15 mosquitoes that had been infected with *P. falciparum*, for a total exposure of bites from 36 to 45 infected mosquitoes per subject. Control subjects received bites from an equal number of uninfected mosquitoes on the same occasions.

Anopheles stephensi mosquitoes were reared ac-

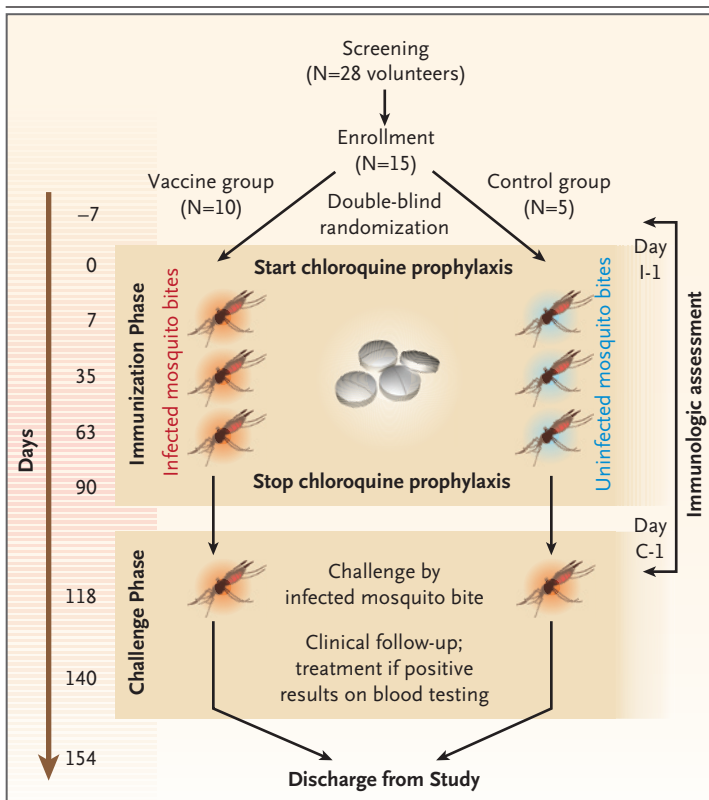


Figure 1. Study Design and Enrollment.

Immunologic assessment was performed 1 day before the first immunization (day I-1) and 1 day before challenge infection (day C-1). A final challenge with infectious mosquito bites was performed 28 days after the discontinuation of chloroquine prophylaxis.

cording to standard procedures at our insectary. Infected mosquitoes were obtained by feeding on gametocytes of NF54, a chloroquine-sensitive strain of *P. falciparum*, as described previously.¹³ NF54 is genetically homogeneous but has not been formally cloned. Only the technicians who prepared the mosquitoes were aware of their infectivity status, and these staff members had no clinical involvement with the subjects or the investigators. Blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. If necessary, feeding sessions were repeated until precisely the predefined number of infected mosquitoes had fed. However, a single feeding session was sufficient in 49 of 60 instances of immunization or challenge, whereas a second session was required in just 10 instances and a third session in only 1 instance.

On days 6 to 10 after each immunization by mosquito exposure, all subjects were followed on

an outpatient basis, and blood was drawn for standard whole-blood counts and daily peripheral-blood smears. Any signs and symptoms were recorded by the attending physician as follows: mild events (easily tolerated), moderate events (interferes with normal activity), or severe events (prevents normal activity).

Eight weeks after the last immunization dose and 4 weeks after the discontinuation of chloroquine prophylaxis, all 15 subjects were challenged by exposure to the bites of five mosquitoes that were infected with the homologous NF54 strain of *P. falciparum*. This period was considered to be sufficient for chloroquine levels to drop below those that might be inhibitory to parasite multiplication.¹⁴ All subjects were checked twice daily on an outpatient basis from day 5 to day 21 for symptoms and signs of malaria, and hematologic tests and peripheral-blood smears were performed.

If results of peripheral-blood testing were positive, subjects were treated with a standard curative combination regimen of 80 mg of artemether and 480 mg of lumefantrine, followed by five identical doses at 8, 24, 36, 48, and 60 hours. The subjects were then followed closely for 3 days. Complete cure was confirmed on the basis of peripheral-blood smears. All subjects who continued to have negative results on the peripheral-blood smear from the day of infection until day 21 after the challenge were presumptively treated with artemether-lumefantrine.

Hematologic and biochemical measures were determined in routine fashion at the hospital's central clinical laboratory. The use of nucleic acid sequence-based amplification and real-time polymerase-chain-reaction (PCR) assays to determine the densities of *P. falciparum* parasites have been described previously.^{15,16} Chloroquine levels were measured by liquid chromatography.^{17,18} Minimum therapeutic concentrations for plasma chloroquine levels maintained by the laboratory were 30 μ g per liter.¹⁴

IMMUNOLOGIC ANALYSIS

Venous whole blood was collected in Vacutainer cell-preparation tubes (CPT, Becton Dickinson) before the first immunization and again before the malaria challenge. Plasma was collected and stored at -70°C . Peripheral-blood mononuclear cells were isolated by density gradient centrifugation, frozen in fetal-calf serum containing 10% dimethyl-

sulfoxide, and stored in liquid nitrogen. Antibody titers were assessed by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay, according to standard protocols.¹⁹⁻²¹ Cellular responses to cryopreserved asexual parasites were assessed by 24-hour in vitro peripheral-blood-stimulation assays,²² followed by intracellular cytokine staining with the use of a Fix and Perm Kit (Caltag Laboratories) and flow cytometry. A more detailed description of these immunologic assays is provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

STATISTICAL ANALYSIS

We performed flow cytometric analysis using Cell-Quest software, and all analyses were performed with the use of SPSS software. Differences in responses among subjects at various time points and between subjects in the vaccine group and those in the control group were analyzed by non-parametric measures (Wilcoxon and Mann-Whitney tests, respectively). A two-sided P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

STUDY SUBJECTS

All 15 subjects completed the immunization phase of the study. All subjects received chloroquine prophylaxis and subsequently underwent a malaria challenge (Fig. 1).

No parasites were seen in the peripheral-blood smears of any of the 10 subjects in the vaccine group after each of the three immunization sessions during chloroquine prophylaxis. However, after the first immunization, a brief submicroscopic parasitemic episode was detected in all vaccinees (Fig. 2A). This finding was not unexpected, since chloroquine has no effect against either sporozoites, liver-stage parasites, or the early ring forms of the first generation of blood-stage parasites that are caused by merozoites released from mature hepatic schizonts.¹¹ After each of the subsequent two immunizations, a progressively reduced incidence and burden of submicroscopic parasitemia was seen.

In line with these findings, all vaccinees reported solicited or unsolicited symptoms that were recorded as adverse events at least once during the immunization phase. With the exclusion of local itching after the mosquito bites, adverse

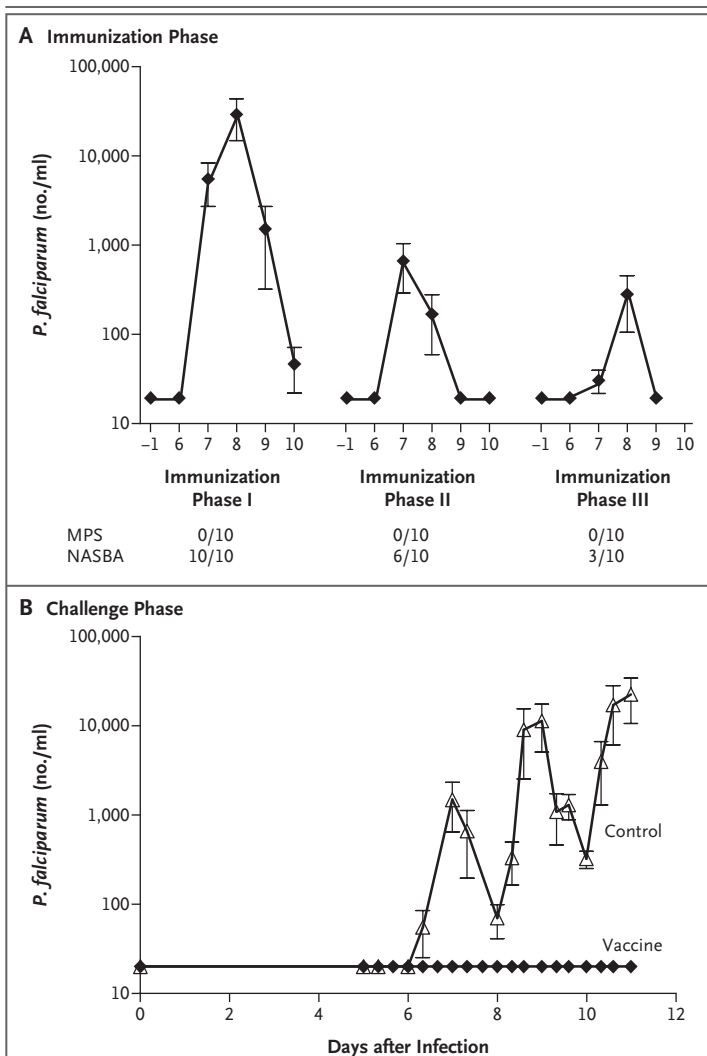


Figure 2. Parasitemia in the Vaccine Group and the Control Group.

Panel A shows the mean number of *Plasmodium falciparum* parasites per milliliter, as measured by nucleic acid sequence–based amplification (NASBA), after each of three immunizations on days before infection and during expected blood-stage parasitemia (days 6 to 10) in the vaccine group. The numbers of subjects who had positive results on peripheral-blood smears for malarial parasites (MPS) and NASBA are shown below the graph. Panel B shows the mean number of *P. falciparum* parasites in 5 subjects in the control group and 10 subjects in the vaccine group, as determined by real-time polymerase-chain-reaction assay before treatment with artemether–lumefantrine. The I bars denote standard errors.

events were most commonly reported after the first immunization (in 9 of 10 subjects), with headache being the most frequent symptom (reported by 7 subjects) (Table 1). Only a few adverse events were reported in the vaccine group subsequently (in two subjects after the third immunization). Severe adverse events were reported by

Table 1. Adverse Events after the First, Second, and Third Exposures to Immunizing Mosquito Bites and after Challenge with Infectious Mosquito Bites.*

Adverse Event	Vaccine Group (N=10)			Control Group (N=5)		
	After Immunization			After Immunization		
	Exposure I	Exposure II	Exposure III	Exposure I	Exposure II	Exposure III
Abdominal pain — no.						
Mild			2			
Moderate						
Severe						
Fatigue — no.						
Mild	1		1			1
Moderate	2			1	1	
Severe	1					
Fever — no.						
Mild	2					
Moderate	1					
Severe	2					5
Headache — no.						
Mild	4		7	2	3	2
Moderate	2		1	1	1	1
Severe	1					1
Loss of appetite — no.						
Mild			1			
Moderate						
Severe						
Malaise — no.						
Mild	1		1			1
Moderate	2					1
Severe	1		1			5
Myalgia — no.						
Mild	1		2			3
Moderate	2				1	4
Severe	1					
Nausea — no.						
Mild	3		1		1	1
Moderate	1		1	1		
Severe						1
Vomiting — no.						
Mild	1					
Moderate			1			
Severe						
Total — no. (%)†						
Mild	4 (40)		1 (10)	8 (80)	1 (20)	3 (60)
Moderate	3 (30)			1 (10)	1 (20)	2 (40)
Severe	2 (20)		1 (10)			1 (20)

* Subjects could have more than one adverse event, and reports of events could have been either solicited or unsolicited. Only adverse events that were possibly or probably related to the study are listed.

† The highest-grade event is listed per subject per infection.

three vaccinees: two had a fever above 39°C after the first immunization, and one reported severe malaise after the last immunization.

After challenge with the homologous NF54 strain of *P. falciparum*, asexual blood-stage parasites were detected in peripheral-blood smears of all five control subjects between days 7 and 11 after exposure (mean prepatent period, 9.2 days). Real-time PCR analyses revealed the expected cyclical multiplication of blood-stage parasites (Fig. 2B). The clinical course and kinetics of parasite multiplication were similar to those in previous studies involving subjects who had not been exposed to malaria,^{23,24} with all control subjects reporting severe events, in particular fever above 39°C and malaise (Table 1). In contrast, there was no evidence of blood-stage parasites in any of the vaccinees at any time during the post-challenge follow-up period until day 21, either by repeated microscopy of peripheral-blood smears or by real-time PCR analyses (Fig. 2B). Interestingly, in the week after the malaria challenge, nine vaccinees reported mild-to-moderate events. No serious adverse events occurred during any part of the trial, and all 15 subjects completed follow-up according to protocol.

Mean peak plasma levels of chloroquine and desethylchloroquine were 76 μg per liter (range, 58 to 104) and 13 μg per liter (range, 5 to 33), respectively, 24 hours after administration; levels did not differ significantly between vaccinees and control subjects. The day before the malaria challenge, mean plasma levels of chloroquine and desethylchloroquine had dropped to 8 μg per liter

(range, <5 to 14) and less than 5 μg per liter, respectively, which were deemed to be below the minimum therapeutic concentrations in vivo.¹⁴ Furthermore, blood-stage parasite multiplication kinetics in the control subjects were identical to those in previous studies,²⁴ which suggested that any residual chloroquine levels had no measurable parasitocidal effect.

After immunization, antibody responses to both sporozoites and blood-stage parasites developed in vaccinees but not in control subjects, as shown by ELISA (Table 2) and immunofluorescence assay (Fig. 1 in the Supplementary Appendix). Seroconversion to the circumsporozoite protein, an immunodominant sporozoite-stage and liver-stage antigen, occurred in eight vaccinees. In contrast, seroconversion to crude asexual-stage antigen occurred in only three vaccinees. Antibodies to two predominantly asexual antigens, apical membrane antigen 1 (AMA-1) and glutamate-rich protein (GLURP), both of which are leading vaccine candidates, were undetectable. These data are consistent with the relatively low-dose exposure to asexual blood-stage antigens in the vaccinees.

Cellular immune responses were assessed by counting cytokine-producing cells in peripheral-blood specimens from the subjects, with the use of intracellular cytokine staining and flow cytometry after 24 hours of in vitro stimulation with erythrocytes infected with the homologous strain of *P. falciparum* or with uninfected erythrocytes (Fig. 3, and Fig. 2 and 3 in the Supplementary Appendix). Whereas cellular responses to uninfected erythrocytes did not differ in any experi-

Table 2. Antibody Reactivity.*

Test	Day I-1				Day C-1			
	Vaccine Group (N=10)		Control Group (N=5)		Vaccine Group (N=10)		Control Group (N=5)	
	No. of Subjects	Median Antibody Titer	No. of Subjects	Median Antibody Titer	No. of Subjects	Median Antibody Titer	No. of Subjects	Median Antibody Titer
		AU		AU		AU		AU
CSP	0	<12	0	<12	8	33 (21–210)	0	<12
AMA-1		ND		ND	0	<5.8	0	<5.8
GLURP		ND		ND	0	<42	0	<42
Asexual blood-stage antigen	0	<0.6	0	<0.6	3	1.7 (1.5–2.0)	0	<0.6

* Data are for subjects who had a detectable response. Numbers in parentheses are ranges. All analyses were performed with the use of an enzyme-linked immunosorbent assay. Plasma was collected from all subjects before immunization (day I-1) and before the malaria challenge (day C-1). A plasma pool obtained from 100 Tanzanian adults living in an area in which malaria was endemic was used as a reference positive control, set at 100 arbitrary units (AU). Thresholds for circumsporozoite protein (CSP), apical membrane antigen 1 (AMA-1), glutamate-rich protein (GLURP), and asexual blood-stage positivity were 12, 5.8, 42, and 0.6 AU, respectively. ND denotes not done.

ment from responses to culture medium alone, stimulation with infected erythrocytes elicited small percentages of lymphocytes producing interferon- γ or TNF- α , but not interleukin-2, in the two groups before immunization (see day I-1 in Figure 2 in the Supplementary Appendix).

Although there was no significant difference in the overall proportion of cells producing individual cytokines (interferon- γ or TNF- α) in either group after immunization (day C-1), a significant increase was observed in the proportion of cells producing multiple cytokines in response to infected erythrocytes in vaccinees, as compared with baseline: $P=0.03$ for the within-group comparison for interferon- γ and interleukin-2; $P=0.046$ for TNF- α and interleukin-2; and $P=0.03$ for interferon- γ , TNF- α , and interleukin-2 (Fig. 3A, 3B, and 3C). The importance of these pluripotent lymphocytes in acquired immune protection is suggested by their higher cytokine content and may reflect better effector function (Fig. 3D, 3E, and 3F). The major contributors to this increase in pluripotent lymphocytes with a response to infected erythrocytes were CD3+CD45RO+ memory-like T cells ($P=0.02$ for the comparison with day I-1) (Fig. 3G, and Fig. 3 in the Supplementary Appendix). CD4+CD8- cells showed a particularly marked response ($P=0.005$ for the comparison with day I-1) (Fig. 3H). Most noticeably, these new pluripotent lymphocytes were predominantly of the effector memory CD62L-CD45RO+ phenotype ($P=0.005$ for the comparison with day I-1), although there was also a small but significant increase in the numbers of responding central memory CD62L+CD45RO+ cells in vaccinees ($P=0.02$ for the comparison with day I-1) (Fig. 3I).

DISCUSSION

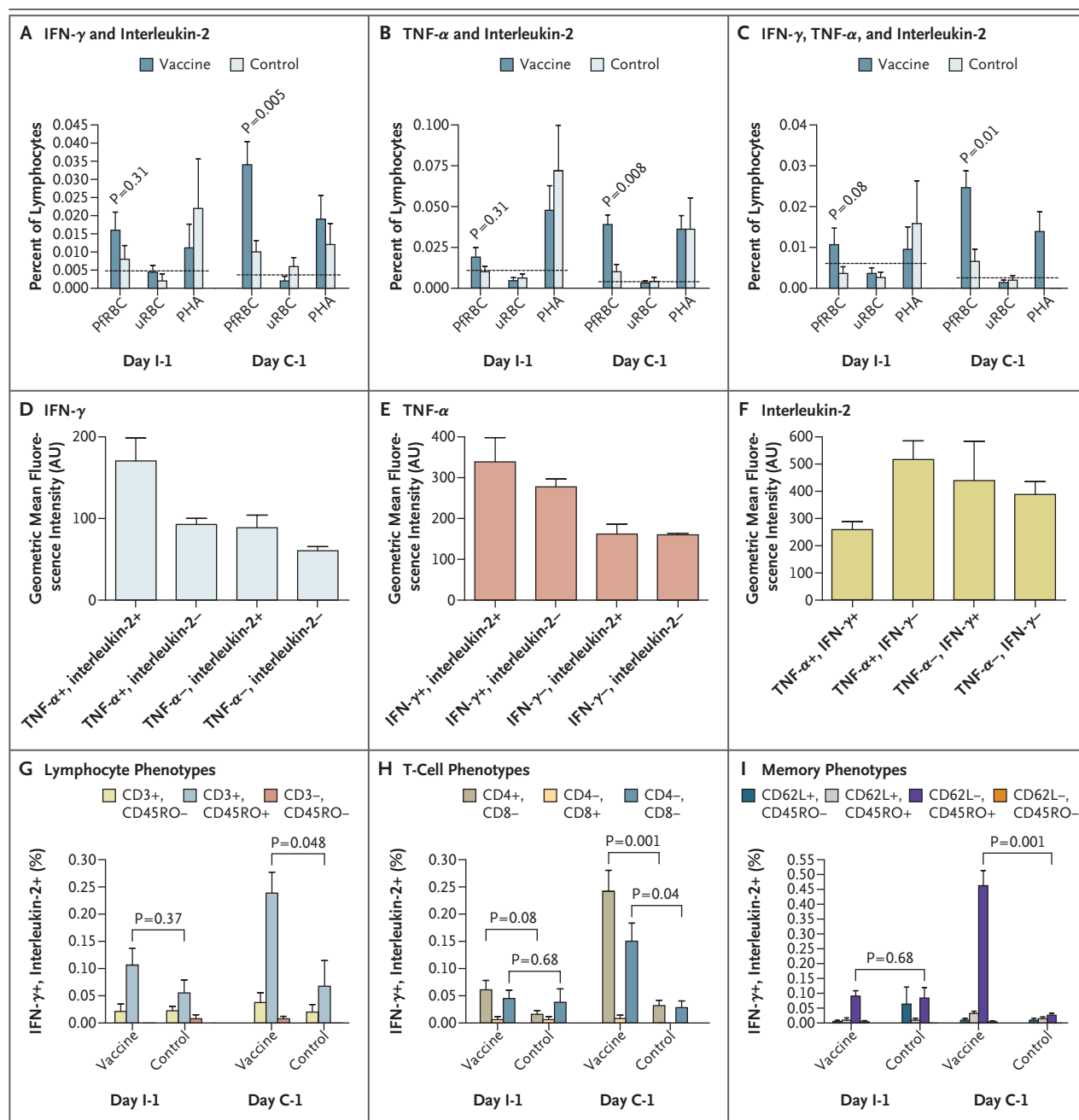
Our study shows that the inoculation of intact sporozoites induces more effective protection against a homologous challenge with *P. falciparum* malaria than does irradiation-attenuated sporozoite immunization. In the endemic situation, however, nonsterile semi-immunity is acquired only after years of repeated natural exposure. We believe that the improved efficiency of our approach was due to a critical balance of exposure to pre-erythrocytic and intraerythrocytic antigens. In contrast to irradiated sporozoites that arrest early during liver-stage development,⁴ intact sporozoites under chloroquine cover mature fully and

Figure 3 (facing page). In Vitro Pluripotent Cytokine Responses to *Plasmodium falciparum* Parasites on Flow Cytometry.

The proportion of lymphocytes that produced interferon- γ (IFN- γ) and interleukin-2 (Panel A), tumor necrosis factor α (TNF- α) and interleukin-2 (Panel B), or IFN- γ , TNF- α , and interleukin-2 (Panel C) after in vitro stimulation with erythrocytes infected with the homologous strain of *P. falciparum* (PfRBC), with uninfected erythrocytes (uRBC), or with phytohemagglutinin (PHA) as a positive control are shown before immunization (day I-1) and before malaria challenge (day C-1). Dashed lines represent the proportion of positive cells in unstimulated wells (culture medium only). The geometric mean fluorescence intensity of cells producing IFN- γ (Panel D), TNF- α (Panel E), and interleukin-2 (Panel F) that were isolated from vaccinees on day C-1 is shown after stimulation in vitro with infected erythrocytes. Cells are grouped according to their positivity or negativity for each of the other two cytokines. In Panels G, H, and I, the proportion of lymphocytes that produced IFN- γ and interleukin-2 in response to infected erythrocytes are shown on day I-1 and day C-1 for lymphocyte phenotypes, including naive T cells (CD3+CD45RO-), memory T cells (CD3+CD45RO+), and non-T lymphocytes (CD3-CD45RO-) (Panel G); for T-cell phenotypes, including helper T cells (CD4+CD8-), cytotoxic T cells (CD4-CD8+), and other lymphocytes (CD4-CD8-) (Panel H); and for memory phenotypes, including naive T cells (CD62L+CD45RO-), central memory T cells (CD62L+CD45RO+), effector memory T cells (CD62L-CD45RO+), and other lymphocytes (CD62L-CD45RO-) (Panel I). The proportions of lymphocytes that produced IFN- γ and interleukin-2 after stimulation with uninfected erythrocytes were below 0.005% (not shown). All P values are for the comparison between the vaccine group and the control group and were calculated with the use of the Mann-Whitney test. The T bars represent standard errors.

develop into a first generation of blood-stage parasites,¹¹ thus presenting to the host's immune system a broader array of pre-erythrocytic antigens, as well as erythrocytic-stage antigens (albeit at relatively low dose).

The contribution of intraerythrocytic antigens to the development of protective immunity is suggested by Pombo et al.,²⁵ who reported that repeated intravenous injection of ultra-low densities of blood-stage parasites, followed by drug cure with atovaquone-proguanil, induced protection in human volunteers against a similarly low-dose blood-stage challenge. However, caution needs to be exercised when interpreting the latter results, since residual concentrations of antimalarial drugs may partially or even fully have accounted for the observed protection.²⁶



In the field, in contrast, patent parasitemia typically develops before patients seek treatment. In such patients, acute blood-stage infection may suppress the induction of protective pre-erythrocytic immunity, as has been shown in rodent models.²⁷ Indeed, parasitemic episodes or attacks of febrile malaria in Kenyan children are prospectively associated with a poorer induction and more rapid attrition of cellular ex vivo and memory responses to a pre-erythrocytic *P. falciparum* antigen.²⁸

Thus, patent parasitemia and probably chronic subpatent parasitemia, which occur regularly in children in endemic areas, appear to induce inhibitory mechanisms that delay the generation of protective antiparasite immunity. Meta-analysis of studies of intermittent preventive therapy in infants has decreased the concern about a rebound effect of prophylaxis and in some cases even indicates sustained protection after discontinuation of prophylaxis,²⁹ thus further indicating that the

acquisition and maintenance of protective immunity do not depend on chronic blood-stage exposure.

Thus, the salient feature of our approach seems to be the exposure of the immune system to a greater array of pre-erythrocytic and intraerythrocytic antigens, while restricting the development of symptomatic and potentially immunosuppressive parasitemia.³⁰ Since NF54 is known to be a chloroquine-sensitive strain *in vitro*,³¹ we cannot formally exclude a synergistic effect of residual subtherapeutic chloroquine levels on immunologic parasite clearance. However, chloroquine levels before the malaria challenge approached or fell below the limit of detection and had no measureable parasitocidal effect in control subjects. Of more importance, the longevity of immunologic responses, both naturally acquired and vaccine-induced, remains a critical issue in malaria, and follow-up studies are planned to address this issue.

In this model, we have identified responses of pluripotent effector memory T cells as being associated with protection. In one study,³² undefined subgroups of lymphocytes with the same cytokine profile were associated with the induction and maintenance of antigen-specific T-cell memory in subjects who were immunized with pre-erythrocytic candidate malaria vaccines, but associations with protection were not explored. However, the potent effector function of pluripotent cells, as suggested by their high cytokine content, has been noted in other investigations that showed their protective role in other infectious diseases.^{33,34} Further detailed investigations will be necessary to determine the longevity of this immunologic response, its association with central memory-type T-cell activity, and its ability to serve as a true correlate of protection.

Since the magnitude of the first wave of parasitemia is thought to directly reflect the burden of erupting mature liver schizonts, the stepwise decrease of such organisms after each subsequent immunizing infection and the absence of PCR-detectable parasitemia after the malaria challenge

would suggest that the protection in our model was primarily due to pre-erythrocytic immunity. However, a component of blood-stage immunity (i.e., the inhibition of erythrocyte invasion and maturation of minute liver-derived merozoite inocula that cannot be detected on PCR) is also possible. Indeed, we found that cellular responses to asexual blood-stage parasites before challenge were a discriminative marker of exposure and protection in our subjects, and similar immune responses may have contributed to protection in the rodent model.¹² However, many of the best-studied *P. falciparum* antigens conferring protective immunity are shared among sporozoite, liver-stage, and blood-stage parasites.^{35,36} Thus, it is plausible that our findings represent the response to a broad antigenic repertoire that transcends parasitic developmental stages,³⁷ making a division between pre-erythrocytic immunity and intraerythrocytic immunity inappropriate. At present, the stage specificity of the protective immune response must remain formally unresolved, although one way to further address this issue in future studies would be a blood-stage challenge.

Although the methods described here do not represent a widely implementable vaccine strategy, the induction of sterile protection against a homologous malaria challenge suggests that the concept of a whole-parasite malaria vaccine warrants further consideration. In addition, this model allows the nature of protective immune responses against malaria, both stage-specific and antigen-specific, to be further investigated.

Supported by the Dioraphte Foundation, by a fellowship from the European Union FP6 Network of Excellence (to Dr. McCall), and by grants from the NutsOhra Foundation (to Dr. van der Ven) and Agence Nationale de la Recherche in France (to Dr. Snounou).

Dr. Snounou reports receiving consulting fees from Sigma Tau and GlaxoSmithKline. No other potential conflict of interest relevant to this article was reported.

We thank the study volunteers for their participation; K. Nganou Makamdop for help with the RT-PCR assays; J. Bakkers and W. Melchers for parasite genotyping; W. Arts, N. Huibers, and P. Beckers for reading blood slides; P. Houze and D. Mazier for chloroquine measurements; J. Klaassen, L. Pelsers-Posthumus, J. Kuhn, and A. Pouwelsen for technical assistance generating infected mosquitoes; the safety monitors for the study, B. Schouwenberg and P. Smits; and independent physician A. Brouwer.

REFERENCES

- Greenwood BM, Fidock DA, Kyle DE, et al. Malaria: progress, perils, and prospects for eradication. *J Clin Invest* 2008; 118:1266-76.
- Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozoite-induced falciparum malaria. *Am J Med Sci* 1973;266:169-77.
- Nussenzweig RS, Vanderberg J, Most H, Orton C. Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. *Nature* 1967;216:160-2.
- Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Drulhe P. Protection against

- malaria induced by irradiated sporozoites. *Lancet* 1990;335:721.
5. Hoffman SL, Goh LM, Luke TC, et al. Protection of humans against malaria by immunization with radiation-attenuated *Plasmodium falciparum* sporozoites. *J Infect Dis* 2002;185:1155-64.
 6. Alonso PL, Sacarlal J, Aponte JJ, et al. Duration of protection with RTS,S/AS02A malaria vaccine in prevention of *Plasmodium falciparum* disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. *Lancet* 2005;366:2012-8.
 7. Abdulla S, Oberholzer R, Juma O, et al. Safety and immunogenicity of RTS,S/AS02D malaria vaccine in infants. *N Engl J Med* 2008;359:2533-44.
 8. Bejon P, Lusingu J, Olotu A, et al. Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. *N Engl J Med* 2008;359:2521-32.
 9. Aponte JJ, Aide P, Renom M, et al. Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *Lancet* 2007;370:1543-51.
 10. Beaudoin RL, Strome CP, Mitchell F, Tubergen TA. *Plasmodium berghei*: immunization of mice against the ANKA strain using the unaltered sporozoite as an antigen. *Exp Parasitol* 1977;42:1-5.
 11. Yayon A, Vande Waa JA, Yayon M, Geary TG, Jensen JB. Stage-dependent effects of chloroquine on *Plasmodium falciparum* in vitro. *J Protozool* 1983;30:642-7.
 12. Belnoue E, Costa FT, Frankenberg T, et al. Protective T cell immunity against malaria liver stage after vaccination with live sporozoites under chloroquine treatment. *J Immunol* 2004;172:2487-95.
 13. Ponnudurai T, Lensen AH, van Gemert GJ, Bensink MP, Bolmer M, Meuwissen JH. Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* 1989;98:165-73.
 14. Rombo L, Bergqvist Y, Hellgren U. Chloroquine and desethylchloroquine concentrations during regular long-term malaria prophylaxis. *Bull World Health Organ* 1987;65:879-83.
 15. Hermesen CC, Telgt DS, Linders EH, et al. Detection of *Plasmodium falciparum* malaria parasites in vivo by real-time quantitative PCR. *Mol Biochem Parasitol* 2001;118:247-51.
 16. Schneider P, Wolters L, Schoone G, et al. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. *J Clin Microbiol* 2005;43:402-5.
 17. Houzé P, de Keynies A, Baud FJ, Benatar MF, Pays M. Simultaneous determination of chloroquine and its three metabolites in human plasma, whole blood and urine by ion-pair high-performance liquid chromatography. *J Chromatogr* 1992;574:305-12.
 18. Lejeune D, Souletie I, Houzé S, et al. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *J Pharm Biomed Anal* 2007;43:1106-15.
 19. Bousema JT, Roeffen W, van der Kolk M, et al. Rapid onset of transmission-reducing antibodies in Javanese migrants exposed to malaria in Papua, Indonesia. *Am J Trop Med Hyg* 2006;74:425-31.
 20. Hermesen CC, Verhage DE, Telgt DS, et al. Glutamate-rich protein (GLURP) induces antibodies that inhibit in vitro growth of *Plasmodium falciparum* in a phase 1 malaria vaccine trial. *Vaccine* 2007;25:2930-40.
 21. Remarque EJ, Faber BW, Kocken CH, Thomas AW. A diversity-covering approach to immunization with *Plasmodium falciparum* apical membrane antigen 1 induces broader allelic recognition and growth inhibition responses in rabbits. *Infect Immun* 2008;76:2660-70.
 22. McCall MB, Netea MG, Hermesen CC, et al. *Plasmodium falciparum* infection causes proinflammatory priming of human TLR responses. *J Immunol* 2007;179:162-71.
 23. Verhage DE, Telgt DS, Bousema JT, et al. Clinical outcome of experimental human malaria induced by *Plasmodium falciparum*-infected mosquitoes. *Neth J Med* 2005;63:52-8.
 24. Hermesen CC, de Vlas SJ, van Gemert GJ, Telgt DS, Verhage DE, Sauerwein RW. Testing vaccines in human experimental malaria: statistical analysis of parasitemia measured by a quantitative real-time polymerase chain reaction. *Am J Trop Med Hyg* 2004;71:196-201.
 25. Pombo DJ, Lawrence G, Hirunpetcharat C, et al. Immunity to malaria after administration of ultra-low doses of red cells infected with *Plasmodium falciparum*. *Lancet* 2002;360:610-7.
 26. Edstein MD, Kotecka BM, Anderson KL, et al. Lengthy antimalarial activity of atovaquone in human plasma following atovaquone-proguanil administration. *Antimicrob Agents Chemother* 2005;49:4421-2.
 27. Orjih AU. Acute malaria prolongs susceptibility of mice to *Plasmodium berghei* sporozoite infection. *Clin Exp Immunol* 1985;61:67-71.
 28. Bejon P, Mwacharo J, Kai O, et al. The induction and persistence of T cell IFN-gamma responses after vaccination or natural exposure is suppressed by *Plasmodium falciparum*. *J Immunol* 2007;179:4193-201.
 29. Grobusch MP, Egan A, Gosling RD, Newman RD. Intermittent preventive therapy for malaria: progress and future directions. *Curr Opin Infect Dis* 2007;20:613-20.
 30. Sutherland CJ, Drakeley CJ, Schellenberg D. How is childhood development of immunity to *Plasmodium falciparum* enhanced by certain antimalarial interventions? *Malar J* 2007;6:161.
 31. Davis JR, Cortese JF, Herrington DA, et al. *Plasmodium falciparum*: in vitro characterization and human infectivity of a cloned line. *Exp Parasitol* 1992;74:159-68.
 32. Bejon P, Keating S, Mwacharo J, et al. Early gamma interferon and interleukin-2 responses to vaccination predict the late resting memory in malaria-naïve and malaria-exposed individuals. *Infect Immun* 2006;74:6331-8.
 33. Darrah PA, Patel DT, De Luca PM, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against *Leishmania major*. *Nat Med* 2007;13:843-50.
 34. Precopio ML, Betts MR, Parrino J, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. *J Exp Med* 2007;204:1405-16.
 35. Hogg B, Thompson R, Zakiuddin IS, Boudin C, Borre M. Glutamate rich *Plasmodium falciparum* antigen (GLURP). *Parasitologia* 1993;35:Suppl:47-50.
 36. Silvie O, Franetich JF, Charrin S, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J Biol Chem* 2004;279:9490-6.
 37. Krzych U, Lyon JA, Jareed T, et al. T lymphocytes from volunteers immunized with irradiated *Plasmodium falciparum* sporozoites recognize liver and blood stage malaria antigens. *J Immunol* 1995;155:4072-7.

Copyright © 2009 Massachusetts Medical Society.