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PROTECTIVE EFFICACY OF THE RTS,S/AS02 *PLASMODIUM FALCIPARUM* MALARIA VACCINE IS NOT STRAIN SPECIFIC

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Abstract. RTS,S/AS02 is a recombinant protein malaria vaccine that contains a large portion of the C-terminal of the circumsporozoite protein (CSP) sequence of the NF54 isolate of *Plasmodium falciparum* fused to the hepatitis B virus surface antigen. It has been shown to induce significant protection to challenge infection with a homologous parasite strain in American volunteers. In a recently completed trial in semi-immune Gambian adults, vaccine efficacy against natural infection was 34% (95% confidence interval = 8–53%, $P = 0.014$) during the malaria season following vaccination. Breakthrough *P. falciparum* parasites sampled from vaccinated subjects and from controls were genotyped at two polymorphic regions of the *csp* gene encoding T cell epitopes (*csp-th2r* and *csp-th3r*) to determine if the vaccine conferred a strain-specific effect. The overall distribution of *csp* allelic variants was similar in infections occurring in vaccine and control groups. Also, the mean number of genotypes per infection in the RTS,S/AS02 group was not reduced compared with the controls.

INTRODUCTION

Several prototype *Plasmodium falciparum* vaccines have undergone preliminary testing in naturally exposed human populations, including circumsporozoite protein (CSP) vaccines against the pre-erythrocytic stage of the infection.¹ Trials of CSP vaccines have generally not shown good levels of protection.^{2–4} However, encouraging results have been obtained with a recombinant protein vaccine known as RTS,S/AS02. This comprises most of the *P. falciparum* CSP sequence fused to hepatitis B virus surface antigen (HBsAg) co-expressed as a fusion protein in yeast and formulated with the adjuvant AS02.⁵ An initial trial in naive individuals showed that RTS,S/AS02 conferred a high degree of protection against experimental *P. falciparum* sporozoite challenge with parasites of the NF54 strain (3D7 clone) used to produce the vaccine.^{5,6} Subsequent trials in naive individuals have given similar degrees of protection.^{6–8} Evaluation of the efficacy of RTS,S/AS02 against natural parasite challenge has recently been undertaken in phase IIb trials in Gambian semi-immune adults with rabies vaccine as the comparator.⁹ Overall, vaccine efficacy against infection during the malaria transmission season following vaccination was 34% (95% confidence interval [CI] = 8–53%, $P = 0.014$).⁹ Vaccine efficacy was 71% (95% CI = 46–85%) during the first nine weeks of surveillance, but subsequently decreased to 0% (95% CI = –52–34%) in the last six weeks.

To evaluate whether RTS,S/AS02 has a protective effect only against parasites with a *csp* sequence similar to that of NF54, parasites from breakthrough infections in control and vaccine groups have been characterized at two highly polymorphic epitope sequences encoded within the *csp* gene, *csp-th2r* and *csp-th3r*. In addition, we investigated whether vaccination with RTS,S/AS02 led to a reduction in the mean number of *P. falciparum* genotypes per infection, as has been described previously in trials with the SPf66 malaria vaccine conducted in Tanzania and in The Gambia.^{10,11}

MATERIALS AND METHODS

Study area, population, and design. The aim of the vaccine trial was to evaluate the efficacy, safety, and immunogenicity

of RTS,S/AS02; the primary endpoint of efficacy was first infection with *P. falciparum*. Details of the study area and study design have been previously reported.¹² Briefly, adult male volunteers 18–45 years of age were recruited from six villages in Basse in the Upper River Division of The Gambia in 1998. Volunteers were given either three doses of RTS,S/AS02 or rabies vaccine over a five-month period (February to August) before the malaria transmission season (August to December). Pyrimethamine/sulfadoxine (Fansidar;® F. Hoffmann LaRoche, Basel, Switzerland) was given two weeks before the third dose of vaccine to clear blood stage infections. Starting two weeks after administration of the third dose of vaccine, volunteers were visited daily by field workers who were posted to each of the six villages for 15 weeks during the transmission season. Each week and whenever a volunteer had symptoms compatible with malaria, two thick blood films were made and three drops of blood were spotted onto glass fiber membranes for parasite genotyping. Only the first parasite-positive samples for each subject were used in genotyping for allelic specificity of RTS,S/AS02 at the *csp-th2r* and *csp-th3r* loci and for measurement of the multiplicity of infection (MOI) at three unrelated loci.

Written informed consent was obtained from adult males participating in the trial. The study was approved by the Joint Gambia Government/Medical Research Council Ethics Committee, an Independent Data Safety Monitoring Committee, and collaborating partners' Institutional Review Boards. The trial, which was conducted according to International Conference of Harmonisation (ICH) Good Clinical Practice guidelines, was monitored by the World Health Organization and GlaxoSmithKline Biologicals.

Genotyping of *csp* polymorphic epitope sequences. Extraction of DNA and amplification by a polymerase chain reaction (PCR) of a 319-base pair fragment of the *csp* gene were performed as described previously.¹² Samples of DNA were amplified routinely in a single round PCR. However, samples with low parasitemia were amplified in a second round PCR using fresh primers, *Taq* DNA polymerase (BIOTAQ;™ Bioline, London, United Kingdom), and 1 μ l of PCR *csp* gene product from the first PCR. PCR products were subsequently tested by sequence-specific oligonucleotide probing, a tech-

nique that was designed specifically for these investigations. The overall allele frequencies at both regions of the *msp* sequence (*th2r* and *th3r*) were determined by counting each allelic type detected in an isolate and dividing it by the total number of alleles detected in the samples analyzed. When more than one allele was present in an isolate, it was necessary to score the results based on the relative intensity of hybridization of the probes to each allele. In this way, it was possible to determine the major allele, i.e., the allele that gave the strongest hybridization signal (minor alleles hybridized weakly but specifically to their respective probe). Allele frequencies at *msp-th2r* and *msp-th3r* loci at the trial site had been characterized in 1997 before the start of the study.¹²

Genotyping of other markers to assess multiplicity of infection. The same set of DNA samples was analyzed for diversity at other genetic markers that contain polymorphic repeat sequences. The markers tested were within the merozoite surface protein 1 (*msp1*), *msp2*, and glutamate-rich protein (*glurp*) genes. An allele-specific, nested PCR was used for the discrimination of *msp1* block 2 and *msp2* allelic families, and the allelic variants present within the RII repeat region of *glurp* were detected by semi-nested PCR.¹³ PCR products were resolved by electrophoresis through Metaphore™ (FMC BioProducts, Rockland, ME) agarose gels in 0.5× TBE buffer, stained with ethidium bromide, and visualized by ultraviolet transillumination. Bands corresponding to parasite allelic forms were distinguished by their size and counted and the number of genotypes for *msp1*, *msp2*, and *glurp* loci determined for each isolate. In addition, the overall MOI for each isolate was then determined as the highest number of alleles observed at any of the loci.

Statistical analyses. To test if RTS,S/AS02 vaccine efficacy was allele-specific, we compared the frequency of the *msp-th2r*03* and *msp-th3r*03* alleles (vaccine type) in breakthrough infections in the vaccine and control groups using the chi-square test. The Wilcoxon test¹⁴ was used for two-group comparisons and the Cuzick¹⁵ non-parametric linear trend test was used when the groups were ordered (parasite density and time). Poisson regression was used to produce estimates of the vaccine effect on MOI adjusted for the effect of time, village, and parasite density. The MOI values are presented as means with SD values in Table 1.

RESULTS

Of 306 volunteers who were enrolled in the trial, 250 received all three doses of vaccine (131 RTS,S/AS02 and 119 controls) and were followed-up for 15 weeks. One hundred sixty-one volunteers had at least one episode of asexual parasitemia and DNA from 157 (98%) of these individuals was successfully amplified by PCR and characterized at several loci (77 from subjects in the RTS,S/AS02 group and 80 from those in the controls). Forty-nine samples had low parasitemias (< 10 parasites/μL), and for some of these it was necessary to perform two rounds of PCR amplification before genotyping at the *msp-th2r* and *msp-th3r* loci could be performed. Age distribution, pre-vaccination anti-CSP and anti-HBsAg antibody concentrations were similar in the two vaccine groups at enrolment and at the start of surveillance.

Genotyping at the *msp-th2r* and *msp-th3r* loci. The overall allele frequencies were similar to those described previously for a population sample in the trial site before vaccination.¹² The primary analysis concentrated on the effects of vaccination with RTS,S/AS02 on the frequency of the NF54-like allele in breakthrough infections when present either alone or with other alleles. Fifteen alleles were detected at *msp-th2r* locus (Figure 1a). No new types were detected. At the *msp-th3r* locus, 10 alleles were detected (Figure 1b), and a small number of new alleles were detected with low frequencies in both groups (< 5%), but these were not characterized further. The *msp-th2r*03* allele (vaccine type for *th2r* sequence) was present in nine of 77 infections in subjects in the RTS,S/AS02 group (12%) and in 13 of 80 infections in subjects in the control group (16%) ($P = 0.5$), whereas *msp-th3r*03* (vaccine type for *th3r* sequence) was present in 27 of 77 infections in subjects in the RTS,S/AS02 group (35%) and in 28 of 80 infections in the control group (35%) ($P = 0.9$) (Figure 1). These proportions are higher than the allele frequencies because some infections contained more than one parasite genotype.

Analysis of MOI. One hundred fifty-seven DNA samples were genotyped at the polymorphic loci *msp1*, *msp2*, and *glurp* (all samples were successfully amplified at the three loci). A high degree of genetic diversity was observed. Overall MOI was significantly higher in the RTS,S/AS02 group than in the controls, 4.90 and 4.23, respectively ($P = 0.05$) (Table 1). The ratio of control to RTS,S/AS02, which was estimated using

TABLE 1
Multiplicity of infections (MOI) at *msp1*, *msp2*, and *glurp* loci*

		MOI			
		<i>msp1</i>	<i>msp2</i>	<i>glurp</i>	Overall
Vaccine group†	RTS,S/AS02 (n = 77)	3.86 (1.85)	4.25 (2.11)	2.61 (1.50)	4.90 (2.13)
	Controls (n = 80)	3.54 (1.86)	3.45 (2.01)	2.11 (1.44)	4.23 (1.94)
		$P = 0.31$	$P = 0.006$	$P = 0.03$	$P = 0.05$
Time to first infection‡	0–59 days (n = 49)	3.20 (1.54)	3.31 (1.62)	2.33 (1.48)	4.04 (1.49)
	60–76 days (n = 54)	3.69 (1.83)	3.76 (2.01)	2.13 (1.54)	4.35 (2.03)
	77–111 days (n = 54)	4.15 (2.05)	4.41 (2.41)	2.61 (1.42)	5.22 (2.36)
		$P = 0.03$	$P = 0.03$	$P = 0.31$	$P = 0.01$
Parasite density/μl	<10 (n = 46)	3.43 (1.87)	3.13 (1.76)	1.91 (1.38)	4.02 (1.91)
	10–99 (n = 60)	3.88 (1.83)	4.12 (2.06)	2.55 (1.53)	4.78 (2.02)
	≥100 (n = 51)	3.71 (1.88)	4.16 (2.27)	2.53 (1.46)	4.76 (2.17)
		$P = 0.48$	$P = 0.02$	$P = 0.05$	$P = 0.06$

* *msp* = merozoite surface protein; *glurp* = glutamate-rich protein. Standard deviations for the MOI are in parentheses.

† 161 subjects who received 3 doses of vaccine developed parasitemia; 157 of 161 primary infections were genotyped.

‡ Time to infection and parasite density were grouped to give three groups with approximately equal numbers of infections in each group. P values were obtained using the non-parametric trend test. Median time to infection was 82 days in the RTS,S/AS02 group and 73 days in the rabies group.

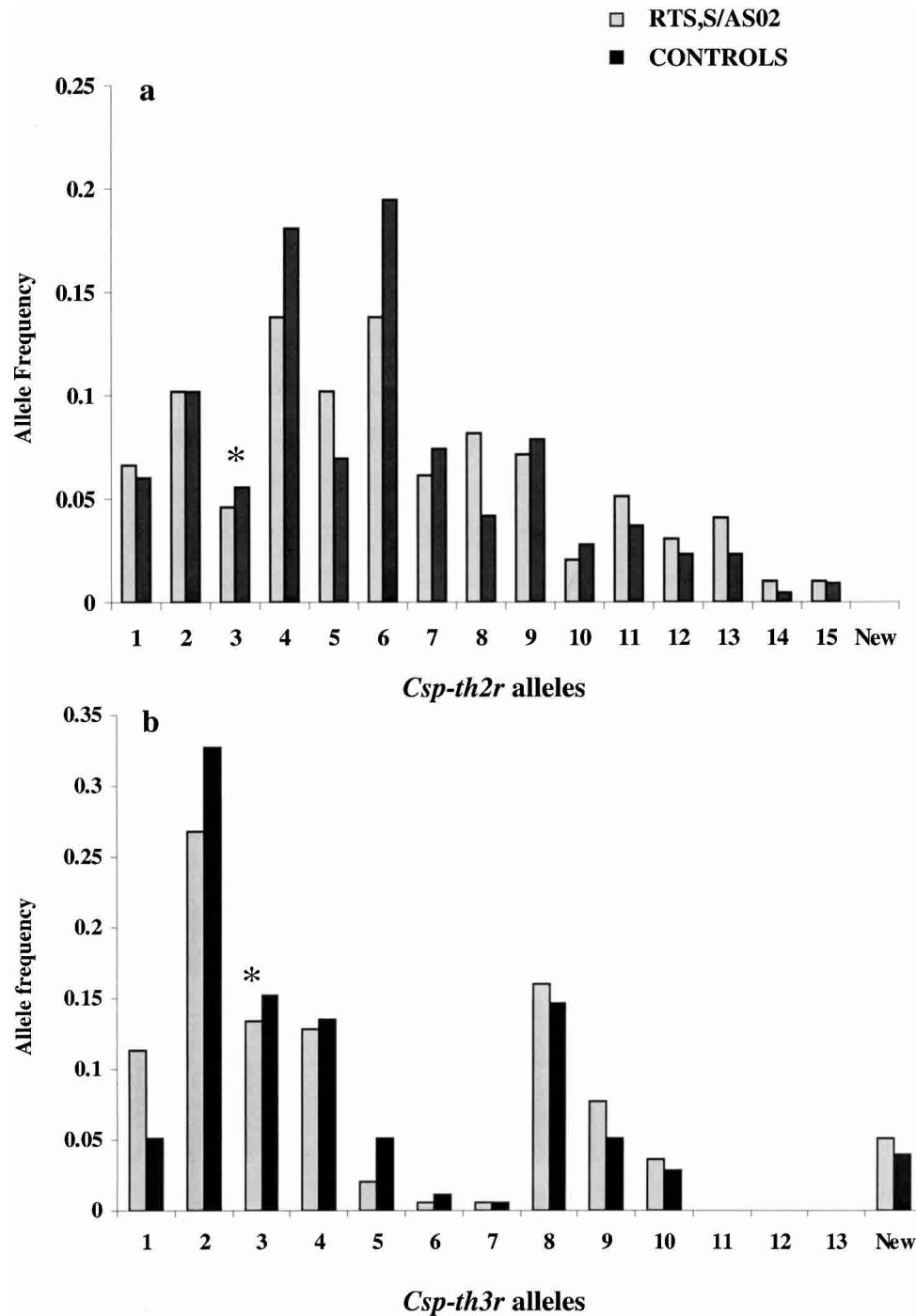


FIGURE 1. Frequency distribution of the *Plasmodium falciparum* circumsporozoite surface protein (CSP) *csp-th2r* and *csp-th3r* alleles in the RTS,S/AS02 vaccine (n = 77) and control (n = 80) groups. **a**, At the *csp-th2r* epitope, 196 alleles were detected in the RTS,S/AS02 group and 216 were detected in the control group. **b**, At the *csp-th3r* epitope, 197 alleles were detected in the RTS,S/AS02 group and 177 were detected in the control group. Allele *03 at both *csp-th2*03* and *csp-th3r*03* corresponds to the vaccine type (NF54) and is indicated with an asterisk.

Poisson regression and adjusting for effects of time period and village by including these variables as factors in the model, was 0.96 (95% CI = 0.81–1.13) for *msp1*, 0.84 (95% CI 0.71–0.99) for *msp2*, 0.80 (95% CI = 0.65–0.99) for *glurp*, and 0.88 (95% CI = 0.76–1.03) overall. The MOI increased during the transmission season and was higher at the end of the season than at the beginning (Table 1); however, there was no interaction between the effect of the vaccine group and the time to infection.

The MOI varied significantly between villages in the study area and correlated positively with parasite density. No association was seen between MOI and age.

DISCUSSION

This study demonstrates that RTS,S/AS02 protected Gambian semi-immune adults against *P. falciparum* infections in a

non-allele-specific manner. Genotyping of breakthrough parasites at the *csp-th2r* and *csp-th3r* epitopes showed that allelic frequency of the vaccine-type allele sequences were similar in both vaccine and control groups, although the vaccine had a marked effect on the incidence of infection. Frequencies of all other *csp* allelic types were similar in the two groups.

If RTS,S/AS02 had an allele-specific effect, a reduction in the prevalence of the *csp-th2r*03* and *csp-th3r-03* alleles should have been observed. Given the prevalence of the *csp-th3*03* allele (35%) and the sample size in each group, the study had 99% power to detect a two-fold allele-specific effect of RTS,S/AS02. Since the prevalence of the vaccine type at *th2r* (*csp-thr2*03*) was 16%, the study had 60% power to detect a two-fold effect at that locus. Thus, the statistical power was very high for *th3r* and reasonably high for *th2r*, so the lack of an allele-specific effect is well supported.

Studies of immune responses elicited by vaccination with RTS,S/AS02 suggest that protection may be mediated, at least in part, by antibodies against the NANP(n) repeat region of CSP, a conserved region among all *P. falciparum* parasite strains.¹⁶ In the RTS,S/AS02 group, anti-CSP antibody levels to the NANP(n) repeat region were 20 times higher after the third dose than those in the control group and these levels correlated with efficacy among the vaccinees.⁹

The vaccine also induced strong T cell responses as determined by proliferation and cultured ELISPOT (Millipore, Watford, England) to many peptides in the *th2r* and *th3r* epitopes of *csp*, although, given the small numbers studied for these responses it has yet to be examined whether these responses were associated with protection.⁹ Our decision to analyze *csp-th2r* and *csp-th3r* epitopes for correlation with protection was based partly on the absence of real T-cell protective epitopes, and partly on existing suggestive data^{8,17,18} that these two epitopes might be protective.

The T cell responses to peptides within the conserved CST3 region apparently correlated with protection.⁹ This and the conserved NANP(n) antibody epitope might explain why there was no specific effect on the frequency of any allelic type of *csp* in vaccinees compared with controls.

Having established that RTS,S/AS02 vaccine did not have any effect on the distribution of alleles at the *csp-th2r* and *csp-th3r* loci, the possibility that it reduced the genetic complexity of infection (MOI) as assessed by typing of unrelated polymorphic loci was investigated. This approach was prompted by reports that children vaccinated with another malaria vaccine (SPf66) in Tanzania and The Gambia had a slightly lower number of genotypes compared with the controls.^{10,11}

Differences in multiplicity were found between villages, suggesting local variations in the level of transmission, and these correlated with parasite density. Although the vaccine reduced the incidence of infection, it did not reduce the multiplicity per infection compared with the controls. A liver-stage vaccine would be expected to induce a reduction or no change in the number of genotypes depending on whether it was strain-specific, but this was not observed in this study. However, overall vaccine efficacy was still maintained at 34% at the end of the follow up-period.⁹

The molecular characterization of parasites in breakthrough cases following vaccination with RTS,S/AS02 has added another dimension to the understanding of the molecu-

lar mechanisms involved in conferring protection against a specific parasite strain. This study has clearly demonstrated that the effect of RTS,S/AS02 vaccine is not allele-specific. The non-allelic protective efficacy of RTS,S/AS02 vaccine should encourage the testing of this vaccine in transmission settings where the NF54 strain is not the predominant type. Molecular typing should become an integral part of the evaluation of future malaria vaccine trials in which vaccines that might be anticipated to have a strain specific effect is used.

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