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SAFETY AND IMMUNOGENICITY OF RTS,S+TRAP MALARIA VACCINE, FORMULATED IN THE AS02A ADJUVANT SYSTEM, IN INFANT RHESUS MONKEYS

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Abstract. Malaria vaccine RTS,S combined with thrombospondin-related anonymous protein (TRAP) and formulated with AS02A (RTS,S+TRAP/AS02A) is safe and immunogenic in adult humans and rhesus monkeys (Macaca mulatta). Here, RTS,S+TRAP/AS02A was administered on a 0-, 1-, and 3-month schedule to three cohorts of infant monkeys, along with adult comparators. Cohort 1 evaluated 1/5, 1/2, and full adult doses, with the first dose administration at one month of age; cohort 2 monkeys received full adult doses, with the first dose administration at one versus three months of age; and cohort 3 compared infants gestated in mothers with or without previous RTS,S/AS02A immunization. Immunization site reactogenicity was mild. Some infants, including the phosphate-buffered saline only recipient, developed transient iron-deficiency anemia, which is considered a result of repeated phlebotomies. All RTS,S+TRAP/AS02A regimens induced vigorous antibody responses that persisted through 12 weeks after the last vaccine dose. Modest lymphoproliferative and ELISPOT (interferon-γ and interleukin-5) responses, particularly to TRAP, approximated adult comparators. RTS,S+TRAP/AS02A was safe and well tolerated. Vigorous antibody production and modest, selective cell-mediated immune responses suggest that RTS,S+TRAP/AS02A may be immunogenic in human infants.

INTRODUCTION

Malaria is the leading parasitic cause of morbidity and mortality with 200 million cases and two million deaths worldwide each year.1,2 Malaria control efforts are impeded by the spread of multiple drug-resistant Plasmodium falciparum and the development of insecticide resistance by the anopheline vector, making the development of a safe and effective vaccine a public health priority.1-6 In particular, a vaccine that is safe and protects infants and children against P. falciparum malaria would be particularly useful because this group is especially prone to develop severe and often fatal malaria.7,8

RTS,S/AS02A is a promising malaria vaccine candidate being developed by GlaxoSmithKline Biologicals (Rixensart, Belgium) with the Walter Reed Army Institute of Research. The RTS,S antigen is recombinantly expressed in yeast, incorporating a large portion of the circumsporozoite (CS) protein of P. falciparum fused to the S antigen of hepatitis B virus in a particle that also includes the unfused S antigen.9,10 When RTS,S is formulated with the potent oil-in-water proprietary adjuvant AS02A (formerly SBAS2; GlaxoSmithKline) containing the immunostimulators 3-deacylated monophosphoryl lipid A (3D-MPL) and QS21, the vaccine is highly immunogenic and confers partial protection against infection by P. falciparum sporozoites delivered via laboratory-reared, infected mosquitoes in malaria-naive subjects, and against natural exposure in semi-immune subjects.2,13 In an effort to increase the efficacy of RTS,S/AS02A, baculo-virus-expressed thrombospondin-related anonymous protein (TRAP), a protein that confers gliding motility and may enhance infectivity, was combined with the RTS,S antigen.15,17

In 1997, RTS,S+TRAP/AS02A administered on a 0-, 1-, and 3-month schedule to adult rhesus monkeys generated robust humoral and cell-mediated immune responses (Heppner DG, unpublished data), leading to this expanded assessment in infant monkeys in 1998. Subsequently, Phase I/IIa clinical studies conducted in 1999 and 2000 showed that a two-dose regimen of RTS,S+TRAP/AS02 administered one month apart was safe and immunogenic in malaria-naive adult humans, but failed to provide protection in 10 of 11 vaccine recipients after experimental P. falciparum sporozoite challenge (Kester KE, Holland CA, unpublished data). In this study, a variety of dose regimens of RTS,S+TRAP/AS02A administered on a 0-, 1-, and 3-month schedule were safe and well tolerated. Vigorous antibody production and modest, selective cell-mediated immune responses suggest that RTS,S+TRAP/AS02A may be immunogenic in human infants.

MATERIALS AND METHODS

Protocol. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee, Armed Forces Research Institute of Medical Sciences (AFRIMS) (Bangkok, Thailand) and the U.S. Army Medical Research and Materiel Command Animal Use Review Division (Fort Detrick, Frederick, MD). The entire study, which was conducted at the Department of Veterinary Medicine at AFRIMS, a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, was in compliance with the Animal Welfare Act and other federal and U.S. Department of Defense regulations relating to animal experiments and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council Publication (1996). The study was conducted from July 1998 through October 2000.

Screening and enrollment of adult monkeys. Female rhesus monkeys (Macaca mulatta) producing offspring for this study and adult comparators had normal results on physical examinations and laboratory assessments, intact spleens, no previous immunizations with any RTS,S-containing vaccine (except Cohort 3, Group 9), were seronegative within last 12
months for simian herpes B virus, simian retrovirus, and simian immunodeficiency virus, and had negative tuberculin screenings within the last 12 months. Nursing mothers and their offspring were housed singly to facilitate frequent handling and observation and to avoid injury from fighting with cage mates. Animal restraint was accomplished by administering 5–20 mg/kg of ketamine hydrochloride intramuscularly (IM) for physical examinations, phlebotomies, and vaccinations. No paralytics were used.

**Study design.** The study was an open-label, safety, and immunogenicity study of RTS,S+TRAP/AS02A in infant rhesus monkeys administered IM on a 0-, 1-, and 3-month schedule. Three sequential cohorts were evaluated (Table 1).

**Cohort 1, dose escalation (groups 1–4, n = four per group).** Groups 1, 2, and 3 assessed 1/5, 1/2, and full adult RTS,S+TRAP/AS02A doses in infant monkeys, respectively. Dose 1 was administered at one month of age (± 1 week). A 1/5 dose was chosen as the lowest test dose based on best estimates of the lower end of an immunogenic dose of RTS,S/AS02A in adult humans and rhesus monkeys.10 Immunizations were administered sequentially by group to assess toxicity before starting the next higher dose. Group 4 controls were adult male and non-pregnant female RTS,S-naive comparators that received full RTS,S+TRAP/AS02A doses. Safety data from Cohort 1 guided dose selection for Cohorts 2 and 3.

**Cohort 2, dose 1 administered at one versus three months of age (groups 5–7, n = four per group).** Group 5 and 6 infants received dose 1 of RTS,S+TRAP/AS02A at one or three months of age, respectively. Full doses were administered. Group 7 controls were adult, male and non-pregnant female RTS,S-naive comparators that received full RTS,S+TRAP/AS02A doses.

**Cohort 3, infants gestated in RTS,S/AS02A-naive versus immunized mothers (groups 8–10).** Group 8 and 9 infants (n = four per group) were gestated in RTS,S/AS02A-naive versus immunized mothers, respectively (three doses pre-gestation: 0-, 1-, and 3-month schedule). The interval between the last RTS,S/AS02A immunization and delivery in Group 9 mothers ranged from two to five years. Group 8 and 9 infants received three full RTS,S+TRAP/AS02A doses, starting at one month of age. Group 10, a single infant gestated in an RTS,S/AS02A-immunized mother, received three immunizations of phosphate-buffered saline (PBS) only.

**Vaccine and administration.** The vaccine, manufactured at GlaxoSmithKline Biologicals (formerly SmithKline Beecham, Rixensart, Belgium) was formulated by combining RTS,S+TRAP with the proprietary adjuvant AS02A. RTS,S+TRAP was a combined lyophilized product reconstituted using AS02A just prior to use and administered at 1/5, 1/2, or full adult doses [RTS,S:TRAP microgram ratio = 10:4 (0.1 mL), 25:10 (0.25 mL), and 50:20 (0.5 mL)], respectively. All IM immunizations were given on alternate legs by perpendicular injection with a 1/2-inch 26 gauge needle into the rectus femoris at the mid-anterior thigh.

**Safety assessments.** Monkeys were observed daily for general health. Immunization sites were examined for induration, erythema, skin swelling, warmth, ulceration, and regional lymphadenopathy by direct examination 1, 3, 7, and 14 days after each immunization by investigators unaware of group assignment. Hematology and micro-volume biochemical analyses (1.5–2 mL per phlebotomy) were conducted 7 and 14 days after each immunization, and then every 2–4 weeks until study completion. Measurements were made for white blood cells, hematocrit, hemoglobin, mean corpuscular volume, platelets, creatinine, alanine aminotransferase, and aspartate aminotransferase.

**Immunogenicity.** Heparinized blood (2 mL per phlebotomy) for analysis of humoral responses was obtained at baseline and then monthly for six months. For RTS,S, specific IgG levels were measured by enzyme-linked immunosorbent assay (ELISA) with recombinant R32LR (R32) as capture antigen for antibodies against tandem repeat epitopes, recorded as optical density units and expressed as titers.10 R32 is a recombinant yeast-expressed central repeat region of the parental CS protein.10 Antibody levels to TRAP, quantified by ELISA using purified recombinant TRAP (GlaxoSmithKline baculovirus expression system) as the capture antigen, were also recorded as optical density units and expressed as titers. The malarial plate antigens used for the ELISA were all based on the P. falciparum 3D7 sequence. Antibodies against the hepatitis B surface antigen (HBsAg) carrier matrix were measured using a commercially available kit (AUSAB enzyme immunoassay; Abbott Laboratories, Abbott Park, IL) expressed in mIU/mL. Seroconversion to RTS,S, TRAP, or hepatitis B was considered to have occurred if post-immunization antibody titers assessed in triplicate against CS tandem-repeat epitopes (R32), TRAP, or hepatitis B virus exceeded the specific vaccine group mean baseline values + 2 SD, respectively.

<p>| Table 1 | Immunization schedule and doses* |
|---|---|---|---|---|</p>
<table>
<thead>
<tr>
<th>Cohort</th>
<th>Group</th>
<th>Offspring monkeys (n)</th>
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<td>1</td>
<td>RTS,S naive</td>
<td>1 month</td>
<td>2 months</td>
<td>4 months</td>
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<tr>
<td>4</td>
<td>–</td>
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<td>Adults (n = 4)</td>
<td>+ 1 month</td>
<td>+ 3 months</td>
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<tr>
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<td>1 month (PBS)</td>
<td>2 months (PBS)</td>
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</tr>
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*PBS = phosphate-buffered saline.
†Immunized with three doses of RTS,S/AS02A 2–5 years prior to delivery.
**Panel 1.** Infant hematocrits of cohorts 1–3. **Arrows** depict immunizations and **hatched areas** are a reference range for infant rhesus monkeys. Other hematology values are shown to the right of some graphs, with normal reference ranges in parentheses. TRAP = thrombospondin-related anonymous protein; MCV = mean corpuscular volume; TIBC = total iron binding capacity; PBS = phosphate-buffered saline.
PANEL 2. Antibody responses of cohorts 1–3. Concentrations of antibody to hepatitis B surface antigen > 10 mIU/mL are considered protective against hepatitis B infection for humans. There were no significant intra-group or inter-group differences for any cohort. TRAP = thrombospondin-related anonymous protein; PBS = phosphate-buffered saline.
For cell-mediated immune (CMI) responses, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by gradient centrifugation on Ficoll-Hypaque (Organon Technika, Durham, NC) and stored in liquid nitrogen until use. For lymphoproliferative assays (LPAs), cryopreserved PBMC collected at five weeks after dose 2 (week 9) were thawed and washed twice with RPMI 1640 medium. Cell viability was assessed by trypan blue exclusion. Washed PBMC (2 × 10^6 cells/mL) were cultured in RPMI 1640 medium containing 10% pooled monkey serum (tested for low background mitogenicity) and RTS,S or TRAP in test wells at a final concentration of 10 μg/mL. Negative control wells consisted of medium only and positive control wells had concanavalin A (Con A) added at a concentration of 10 μg/mL. One set of plates, negative control and antigen wells, were cultured for five days; another set of plates, negative control and Con A wells, were cultured for two days. They were then pulsed with 0.5 μCi/well of 3H-thymidine. After 18 hours, cells were harvested onto glass fiber filters using an automated plate harvester (Tomtec, Orange, CT). Proliferative responses were measured by the uptake of 3H-thymidine (beta plate, Wallac, Turku, Finland) and expressed as a stimulation index (SI).

To measure Th1-like and Th2-like responses, individual PBMC samples collected at four weeks after dose 3 (week 16) were assayed for interferon-γ (IFN-γ) and interleukin-5 (IL-5) production using modified ELISPOT assays whereby 200,000 PBMC per well were plated. First, Maxisorp 96-well plates (Nalge Nunc International, Naperville, IL) were coated at 4°C overnight with 50 μg/well of monoclonal antibody to monkey IFN-γ (0.25 μg/well, clone MD-1; U-CyTech BV, Utrecht, The Netherlands) or monoclonal antibody to mouse/human IL-5 (0.5 μg/well, clone TRFK 5; BD PharMingen, San Diego, CA). Plates were then washed three times with PBS and blocked with PBS containing 10% fetal bovine serum (FBS) for two hours at 37°C. The PBMC prepared as described earlier were cultured at a concentration of 1 × 10^6 cells/mL in 48-well plates (Corning, Inc., Corning, NY) with either RTS,S or TRAP added to test wells at a final concentration of 20 μg/mL. Con A was added to control wells for IFN-γ and IL-5 at concentrations of 5 μg/mL and 0.6 μg/mL, respectively (based on assay optimization studies, Pichyangkul S, unpublished data). Cell cultures were incubated at 37°C for 18 hours and mixed gently with a pipette; 200 μL of cell suspension (2 × 10^6 cells) was then transferred into each well of pre-coated ELISPOT plates. The incubation times for IFN-γ and IL-5 were approximately 22 hours and 44 hours, respectively. Plates were then washed six times with PBS/0.1% Tween 20 (PBST). Polyclonal antibody to monkey IFN-γ (biotinylated rabbit, 0.06 μg/well; U-CyTech BV) or biotinylated monoclonal antibody to human IL-5 (0.5 μg/well, clone JESI-5A10; BD PharMingen) was added to each well at 50 μL/well. After incubation for two hours at 37°C, the plates were washed six times with PBST, and 50 μL of color developer solution (U-CyTech BV), composed of equal volumes of “initiator” and “enhancer” solutions, was added to each well for 10–15 minutes. Color development was stopped by passing the plates through running tap water. The plates were air-dried and then placed under an inverted microscope for spot counting (100×). Duplicate plates were counted by two independent experienced readers unaware of group assignment (SP and PT). The four readings were then recorded as spots/200,000 cells, and reported as spots/million cells.

Statistical analyses. Statistical significance was calculated by the Mann-Whitney rank sum test for non-parametric comparisons within a group and Kruskal-Wallis one-way Analysis of variance (ANOVA) based on ranks for comparisons within a cohort. P values ≤ 0.05 were considered significant. Data
sets with two or fewer values were not included in any statistical analysis.

RESULTS

Cohort 1: RTS,S+TRAP/AS02A dose escalation, dose 1 at one month of age. The vaccine was well tolerated at each dose level. Injection site reactions in infant and adult monkeys consisted of muscle induration and cutaneous erythema that always resolved by day 7. Reaction intensity was unrelated to dose or vaccine sequence number. Inguinal lymphadenopathy, ipsilateral with the immunization, was observed in one monkey on day 1 after dose 3. Biochemistry values, with the exception of transient CPK elevations immediately after immunization in some monkeys, were unremarkable. Hematology values in infants receiving 1/5 or 1/2 doses and adult comparators receiving full doses of RTS,S+TRAP/AS02A, were unremarkable (Panel 1). In full dose infants, monkey 896 was anemic two weeks after immunization 2. Giemsa-stained blood smears showed hypochromicity and microcytosis, and the mean corpuscular volume (MCV) was low, consistent with iron-deficiency anemia. There were no important clinical signs surrounding the anemic period and the monkey continued in the study. Iron supplements were administered, the hematocrit slowly normalized, and the animal thrived. Among cohort 1 infant monkeys, phlebotomy frequency and the total volume of blood drawn were similar, as were rates of weight gain.

Seroconversion against R32, TRAP, and HBsAg occurred in all animals after dose 1 (Panel 2). All doses were maximally immunogenic in infant rhesus monkeys, with no dose response patterns. For RTS,S and TRAP, dose 2 provided a mild boost, whereas dose 3 generated plateau-like levels through week 24. For antibodies to hepatitis B virus, doses 2 and 3 provided boosting, generating levels above what correlates with protection in humans.

The LPAs were expressed as an SI pre-immunization and post-immunization following in vitro cultivation with Con A, RTS,S, or TRAP (Panel 3). Robust responses to Con A verified lymphoproliferative capability in all animals. For RTS,S, median pre-immunization and post-immunization SIs in the infant groups remained similar, whereas adults had a significant increase in SI post-immunization. For TRAP, modest post-immunization responses were observed at the lower vaccination doses (1/5 and 1/2), whereas full dose infants had responses similar to adult comparators. Despite the relatively higher post immunization median value by full dose infants, a single low value prevented a significant difference across the infant groups (P = 0.54, by one-way ANOVA based on ranks).

The ELISPOTs for IFN-γ and IL-5, expressed as responses to Con A, and then media alone versus RTS,S or TRAP, are shown in Panel 4. For IFN-γ, Con A responses confirmed cytokine release capability in all 1/2 dose infants and adult comparators, and in three of four animals from the 1/5 and full dose infants. In comparison with media only, median RTS,S responses in the infant groups were similar, and significantly lower than in the adults. Median IFN-γ responses against TRAP for the infants showed a modest dose-response trend, but was significant (versus media only) for the 1/2 dose group only. Non-responders to Con A were also non-responders for IFN-γ production against specific stimuli. Adult IFN-γ production against TRAP was significant in comparison with media.

For the IL-5 ELISPOT, responses to Con A confirmed cytokine production capability in most animals. In comparison with media only, infant median responses against RTS,S were similar, and significantly lower than in the adults. Infant median TRAP responses had sporadic increases, significant for the 1/2 dose group. For the 1/2 and full dose infants, non-responders to Con A were also non-responders for IL-5 production against specific stimuli. Adult IL-5 production was significant in comparison with media.

Cohort 2: Dose 1 of RTS,S+TRAP/AS02A at one versus three months old, full adult doses. The vaccine was well tolerated, regardless of age at dose 1. Injection site reactogenicity in all monkeys consisted of muscle induration and cutaneous erythema that always resolved by day 7. Reaction intensity was independent of age at dose 1 or vaccine sequence number. Inguinal lymphadenopathy, ipsilateral with the immunization, was observed in 1 infant monkey on day 1 after the dose 3. Biochemistry values, with the exception of transient CPK elevations immediately after immunization in some monkeys, were unremarkable. Hematology values were abnormal in two monkeys (Panel 1). Monkey 926 (Group 5) and 921 (Group 6) had anemia at day 90 (dose 3). Giemsa-stained blood smears showed hypochromicity and microcytosis, MCVs and ferritin levels were low, and reticulocyte counts were normal, consistent with iron-deficiency anemia. For each infant, there were no important clinical signs surrounding the anemic period and both continued in the study. Iron supplements were administered, hematocrits normalized, and the monkeys thrived. Among cohort 2 monkeys, phlebotomy frequency and the total volume of blood drawn were similar, as were rates of weight gain.

Seroconversion against R32, TRAP, and hepatitis B virus occurred in all animals after dose 1, with no age-dependent patterns (Panel 2). For RTS,S and TRAP, dose 2 provided a mild boost, whereas dose 3 generated plateau-like levels through week 24. For antibodies to hepatitis B virus, doses 2 and 3 provided boosting, generating levels above what correlates with protection in humans. The generally weaker adult antibody responses (versus infants) in comparison with cohort 1 adults (versus infants) may have related to the higher mean age of the cohort 2 monkeys of 10.8 versus 7.3 years old, respectively.

The LPAs were expressed as an SI pre-immunization and post-immunization following in vitro cultivation with Con A, RTS,S, or TRAP (Panel 3). Robust responses to Con A verified lymphoproliferative capability in all animals. For RTS,S, pre-immunization and post-immunization SIs in the infant groups remained similar, whereas adults had a significant increase in SI post-immunization. For TRAP, modest post-immunization responses were observed at the lower vaccination doses (1/5 and 1/2), whereas full dose infants had responses similar to adult comparators. Despite the relatively higher post immunization median value by full dose infants, a single low value prevented a significant difference across the infant groups (P = 0.54, by one-way ANOVA based on ranks).

The ELISPOTs for IFN-γ and IL-5, expressed as responses to Con A, and then media alone versus RTS,S or TRAP, are shown in Panel 4. For IFN-γ, Con A responses confirmed cytokine release capability in all 1/2 dose infants and adult comparators, and in three of four animals from the 1/5 and full dose infants. In comparison with media only, median RTS,S responses in the infant groups were similar, and significantly lower than in the adults. Median IFN-γ responses against TRAP for the infants showed a modest dose-response trend, but was significant (versus media only) for the 1/2 dose group only. Non-responders to Con A were also non-
and all adults. Median RTS,S responses were unremarkable in the infant and adult groups. Median responses against TRAP in the infant and adult groups, including the Con A unresponsive individual from the 1-month-old group, were significantly elevated. For IL-5, Con A responses confirmed cytokine release capability in all animals. Median responses against RTS,S among the infants were unremarkable, but elevated against TRAP in the 3-month old group. Adult IL-5 responses against RTS,S and TRAP were significantly elevated.

**Cohort 3:** Infants gestated in RTS,S/AS02A-naive versus immunized mothers; dose 1 at one month of age, full vaccine doses (or PBS). The vaccine was well tolerated by all groups. Injection site reactogenicity in all monkeys consisted of
muscle induration and cutaneous erythema that always resolved by day 7. Reaction intensity was independent of maternal RTS,S/AS02A exposure history or vaccine sequence number. There was no lymphadenopathy. Biochemistry values, with the exception of transient CPK elevations immediately after immunization in some monkeys, were unremarkable. Hematology values were abnormal in three infants (Panel 1). Monkeys 925 and 931 had anemia at day 126, and monkey 935, the single control infant receiving PBS only, had anemia at day 90. Giemsa-stained blood smears showed hypochromicity and microcytosis and MCVs were low, consistent with iron-deficiency anemia. There were no important clinical signs during the anemic period and all three monkeys were continued on study. Iron supplements were administered, hematocrits normalized, and the animals thrived.

Among cohort 3 monkeys, phlebotomy frequency and the total volume of blood drawn were similar, as were rates of weight gain.

Baseline antibody values in the infant monkeys gestated in RTS,S/AS02A-immunized mothers indicated that maternal antibodies to R32 had disappeared prior to dose 1 of RTS,S+TRAP/AS02A, whereas antibodies against hepatitis B virus remained at nearly 10,000 mIU/mL, consistent with transplacental or neonatal transmission of maternal antibody (Panel 2). Accordingly, in the absence of detectable preimmunization maternal antibodies against R32 in the infants, no influence of R32 antibody on infant responses against RTS,S+TRAP/AS02A was noted. Seroconversion against R32 and TRAP occurred in all monkeys after dose 1 of the vaccine, with doses 2 and 3 providing a mild boost and plateau-like levels through week 24, respectively. For hepatitis B virus in infants with RTS,S/AS02A-immunized mothers, antibodies levels decreased from birth to week 4, but doses 2 and 3 provided boosts similar to those observed in infants gestated in RTS,S/AS02A-naive mothers. The PBS control infant, gestated in an RTS,S/AS02A-immunized mother, showed a progressive decrease in maternal antibodies to hepatitis B virus that returned to baseline at week 12.

For LPAs, robust responses to Con A verified lymphoproliferative capability in all animals. The RTS,S and TRAP pre-immunization and post-immunization responses in the

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**Cohort 1 ELISPOTs**

**IFN-γ ELISPOT**

- **Concanavalin A**

- **Infants 1/5 doses**
- **Infants 1/2 doses**
- **Infants Full doses**
- **Adults Full doses**

**IL-5 ELISPOT**

- **Concanavalin A**

- **Infants 1/5 doses**
- **Infants 1/2 doses**
- **Infants Full doses**
- **Adults Full doses**

**Panel 4.** ELISPOT assays of cohorts 1 and 2, (-----) = group medians; (---) = upper limit of 95% confidence interval for media only, all groups. IFN-γ = interferon-γ; IL-5 = interleukin-5; TRAP = thrombospondin-related anonymous protein; mo = month. *P < 0.05, versus media only.
three infant groups, including the PBS control, were similar to cohort 1. The ELISPOTs for IFN-γ and IL-5 were expressed as responses to Con A, and then media alone versus RTS,S or TRAP. For the IFN-γ ELISPOT, Con A responses confirmed cytokine release capability in three of four animals from the two immunized infant groups, and in the single animal that received PBS only. For the two immunized infant groups, median RTS,S responses were unremarkable, whereas TRAP responses were elevated, similar to cohort 1. For IL-5, Con A responses confirmed cytokine release capability in all isolates from the two immunized infant groups, but not the PBS negative control. Among the two immunized groups, IL-5 responses against RTS,S were unremarkable, but elevated against TRAP, similar to cohort 1. All responses from the PBS control infant were unchanged.

**DISCUSSION**

Here, in the first documented assessment of a malaria vaccine candidate in infant non-human primates, a three-dose regimen of RTS,S+TRAP/AS02A was safe, acceptably reactogenic, and immunogenic. In particular, all dose regimens generated vigorous antibody responses against each of the three major vaccine components that persisted for at least 12 weeks after the last vaccine dose, similar in magnitude to adult controls, and modest CMI responses, especially against TRAP, approximating adult comparator responses. Taken together, these results bode well for pediatric RTS,S-based malaria vaccine trials.

Most safety and reactogenicity outcomes in the infant rhesus monkeys were similar to those of adult humans and monkeys immunized with RTS,S/AS02A or RTS,S+TRAP/
AS02A (Heppner DG, Kester KE, unpublished data).\textsuperscript{3,10,12} 
All infant monkeys were healthy throughout the study. Immunization site reactogenicity, consisting primarily of transient muscle induration and erythema, was independent of dose or immunization cycle number. Clinical biochemistry values, with the exception of transient CPK elevations after some immunizations, were unremarkable. In contrast to adult monkey and human studies, however, transient iron-deficiency anemia occurred in some infants, including the single PBS recipient. This observation, coupled with improvement upon iron supplementation in all monkeys, argues that the etiology was likely a result of repeated phlebotomies.\textsuperscript{18,19} Indeed, gastrointestinal bleeding, the most common cause of iron-deficiency anemia in adults, was not observed, and red blood cell morphologies and hematology indices were consistent with an iron-deficiency etiology.

Adult humans and rhesus monkeys immunized with RTS,S/AS02A or RTS,S+TRAP/AS02A generate vigorous, antigen-specific humoral responses (Holland CA, Heppner DG, Kester KE, unpublished data).\textsuperscript{3,10,12,13} In contrast, human infants are immunologically immature, especially for humoral responsiveness to most polysaccharide (T cell-independent) antigens.\textsuperscript{20,21} For infant rhesus monkeys, little is known about their immune response capacity or how their vaccine responses compare with adults. Here, infant rhesus monkeys generated brisk humoral responses to all three vaccine components, approximating adult responses. In cohorts 1 and 2, the responses appeared independent of dose or immunization starting age, suggesting that for humoral responses, three 1/5 doses administered on a 0-, 1-, and 3-month schedule at one starting age, suggesting that for humoral responses, three 1/5 doses administered on a 0-, 1-, and 3-month schedule at one month of age or later may be sufficiently immunogenic to produce humoral responses that persist for at least 12 weeks after the last vaccine dose. We propose that the strong humoral responses, independent of dose or age, may reflect inclusion of the potent oil-in-water-based adjuvant AS02A.

Especially interesting were the infant antibody responses against the HBsAg carrier matrix of RTS,S being similar in magnitude to adult responses. This contrasts with a recent human study comparing infant and adult responses to alum-adsorbed HBsAg, whereby infants generated significantly higher antibody responses than adults, correlating with weaker IFN-\(\gamma\) and higher Th2-like memory responses than adults.\textsuperscript{22} Here, infant humoral responses approximating adults, at least for hepatitis B virus, might indicate either faster immune maturation in infant rhesus monkeys than in human infants or the influence of AS02A. For Cohort 3 (Group 9), designed to address the potential field scenario of early life priming with 1/5, 1/2, or full adult doses of RTS,S+TRAP in AS02A starting at one or three months of age, followed by two closely spaced boosts, was safe and induced adult-like antibody responses to each vaccine component. However, CMI responses against RTS,S antigen were relatively weaker than adults, whereas anti-TRAP CMI responses were similar in the infant and adult monkeys. Enhanced CMI responses against malaria vaccine antigens in pediatric populations might be improved by the use of appropriate adjuvant delivery systems or novel vaccine administration approaches,\textsuperscript{27} translating into better protective efficacy against malaria in vaccinated infants and children.

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