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A malaria vaccine evaluation site at Saradidi, Kenya, has been characterized for epidemiological, entomological and demographic parameters over a two-year period. The transmission of falciparum malaria by Anopheles gambiae s.l. and A. funestus was found to be intense year-round. An evaluation of a WRAIR-developed malaria sporozoite vaccine, R32ToxA, was begun in April 1990, with 38 pairs of volunteers.

Leishmaniasis studies have described several parasite-vector-host relationships previously unreported in East Africa which may be important in the diagnosis or control of the disease. In particular, the isolation of two species of leishmania with differing drug sensitivity profiles from a single patient is notable. Collaborative development of a vector-induced model for infection in vervet monkeys has significant potential for vaccine evaluation in a non-human model.

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Malaria, Leishmaniasis & Shistosomiasis Vector Ecology, Transmission, Immunology & Prophylaxis in Kenya

TABLE OF CONTENTS

Malaria Introduction....................1-2
Visual Assessment of Sporozoite and Bloodmeal ELISA Samples in Malaria...3
Malaria Sporozoite Detection....................4-5
Characterization of Malaria Transmission by Anopheles....................6
Plasmodium Species Identification....................7
Effects of P. falciparum on Survival of Naturally Infected Anopheles....................8-9
Species Composition of The Anopheles gambiae Complex....................10-11
Leishmania....................12
Development of Leishmania major in Phlebotomus duboscqi and Sergentomyia schwetzi....................12-13
Experimental Transmission of Leishmania major to Vervet Monkeys....................14
Phlebotomus guggisbergi, A Vector of Leishmania tropica in Kenya....................15
Leishmania infantum sensu lato Isolated From A Giant Rat....................16
Concurrent Infection with Leishmania donovani and Leishmania major....................17-18
Bibliography of Publications....................19-21
Appendix A Safety, Immunogenicity and Efficacy of A Recombinant Falciparum Sporozoite Toxin A Vaccine

FINAL REPORT

KEMRI GRANTS DAMD17-87-G-7016 & DAMD17-89-Z-9032

Malaria, Leishmaniasis, and Shistosomiasis Vector Ecology, Transmission, Immunology, and Prophylaxis in Kenya

The purpose of this final report is to fulfill the requirements of Document No. DAMD17-89-Z-9032 and to report in narrative summary the research findings of tropical diseases investigated. The aim is to summarize in manuscript format all studies accomplished and funded by this grant.

Malaria:

INTRODUCTION:

Nature of The Problem: Malaria is a blood-borne disease, acute, often chronic and sometimes severe occur in most tropical areas of the world to include Kenya. The disease is still one of the major health risks for a major proportion of the Kenya population. All four types of malaria parasites have been observed in Kenya. *Plasmodium falciparum* is by far the most common one accounting for 80-85% of reported cases. Immunity has assumed the greatest role for controlling and preventing the disease, however, much remains to be understood about the basic biochemical factors involved in host resistance manifested by malarial parasites.

Background of Previous Work: Immune responses to malaria parasites can be induced in man and other animals by injection of live, irradiated sporozoites. This immunity is mediated by antibody and immune cells with specificity by species and stage development of the parasite. Dame et al., reported the successful cloning and sequencing of the gene that encodes the circumsporozoite (CS) protein of *Plasmodium falciparum.* Antibodies raised against synthetic peptides and recombinantly produced constructs from the unique repeating sequence of the P. falciparum CS protein have been shown to possess biologic activities possibly predictive of protection against sporozoite challenge, i.e., they mediate the circumsporozoite precipitation reaction and block sporozoite invasion of hepatocytes in vitrol These studies have led to the development of vaccines designated to protect against infection with the sporozoite stage of *P. falciparum* which has been tested in a Phase I safety and immunogenicity trial and a small Phase IIa efficacy

trial in volunteers in the United States. Although this initial vaccine was not sufficiently immunogenic to justify Phase IIb trials, additional vaccines using improved adjuvants are now under development by WRAIR and others.

Planning for a Phase IIb trial will require accurate information on the expected transmission of falciparum malaria at the study site and information regarding the logistical, technical, and community support for conducting such a trial.

Kenya has been chosen by WRAIR as the primary site for the first Phase IIb trial. Studies must now provide that information that will enable accurate estimation of the expected *P. falciparum* attack rate in volunteers staying in these areas for 2-8 weeks and the optimal time to conduct such a trial. Since volunteers will receive their last booster dose of vaccine one month before the study begins, data must be collected which will enable the investigators confidently to predict peak transmission one month in advance of its onset. Epidemiologic and entomologic studies that will provide factual information on these issues will be investigated..

Purpose and Approach of The Present Studies: In hyper- and holoendemic malarious areas the prevalence of malaria infection is highest in children less than 6 years of age, who presumably have not yet developed significant protective immunity against malaria. These young children most closely resemble non-immune adults in regard to their susceptibility to malaria. Determination of the attack rate of falciparum malaria in these children is crucial to confident prediction of the attack rate in non-immune volunteers, a factor critical to the determination of the sample size required to predict the protective efficacy of the vaccine. The previous researh determined these attack rates over a period of approximately two years. While the determination of the entomologic inoculation rate (man biting rate x sporozoite rate) and incidence of malaria in human populations form the core of these data, the ability to predict peak transmission is dependent on continued entomologic and meterologic studies that will help insure an optimal Phase IIb trial of a vaccine.

Saradidi, Siaya District, near Kisumu in Nyanza Province, Kenya, has been chosen as the principal study site because it has met the following criteria:

a. High prevalence of falciparum malaria with low prevalence of other malaria parasites.

b. Large database collected by Kenyan and CDC scientists during the past seven years.

c. Demonstrated community support for malaria studies.

d. Favorable logistics.

e. A low prevalence of other diseases and an absence of filariasis and leishmaniasis which volunteers would be at risk of contracting.

A second site has been selected adjacent to the new KEMRI laboratory 10 KM west of Kisumu. Similar studies to those described for Saradidi will be carried out at this site.

BODY

Because several entomologic studies were conducted to obtain the goals of this malaira research endeavor, methods used and results obtained will be summarized in abstract format as follows:

Visual Assessment of Sporozoite and Bloodmeal ELISA Samples in Malaria Field Studies

John C. Beier and Joseph K. Koros

J. Medical Entomology (unpublished)

Methods: Samples of *Anopheles gambiae* s. l. Giles and *Anopheles funestus* Giles collected in western Kenya (Beier et al 1990) were processed routinely by a P. falciparum sporozoite ELISA (Wirtz et. al., 1988b). In each assay, samples were read by an ELISA plate reader 30 minutes after the addition of substrate. Methods for determining cut-off absorbance values for sample positivity included twice the mean of negative controls for the sporozoite ELISA (Beier et. al., 1988a), and the mean and three standard deviations of negative controls for the bloodmeal ELISA. Accuracy of visual determination was determined in relation to sample positivity assessed by aborbance readings Sensitivity, specificity, and interobserver agreement were calculated by standard methods (Lilienfeld, 1976). The relative percent difference in infection rates or human feeding rates between visual and spectrophotometric determinations was calculated. The percent or misclassified samples was calculated as the number incorrectly scored visually of the total examined , times 100.

Results: The accuracy of visually assessing positivity for samples of field-collected Anopheles tested by enzyme-linked immunosorbent assays (ELISA) for

Plasmodium falciparum sporozoites and human bloodmeals was determined during malaria field studies in Kenya. Six observers evaluated during 5,344 sporozoite ELISA samples and 4 observers avaluated 360 bloodmeal samples as either positive or negative based on the presence and strength of green-colored peroxidase reactions, relative to controls on each microtiter plate. Interobserver agreement ranged from 97.9 to 99.8% for sporozoite samples and from 90.3 to 98.1% for bloodmeal samples. For both assays, te mean sensitivity and specificity of visual readings, compared with spectrophotometric readings, exceeded 98% when absorbance values were >0.4 (on a scale 0.0 - 2.0). Most incorrect visual readings occurred for samples with absorbance values between o.2 and 0.4. The total percent of samples classified correctly by visual examination ranged from 97.7 to 98.5% for the sporozoite ELISA and from 94.5 to 96.7% for the bloodmeal ELISA.

Conclusion: There is minimal potential error associated with visually determining positive reactions for the ELISA assays used in malaria field studies, provided observers have had proper training and experience.

Future Work: Screen new chemicals for non-toxic, non-polluting insecticides. Generate data on the basic biochemical factors involved in resistance both by mosquitoes to insecticides and by malarial parasites to drugs. Explore gene biotechnology to produce non-biting and/or malaria parasite resistance female *Anopheles gambiae* and *A. funestus.*

Malaria Sporozoite Detection by Dissection and ELISA to Assess Infectivity of Afrotropical Anopheles (Diptera: Culicidae)

John C. Beier, Peter V. Perkins, Joseph K. Koros, Fred K. Onyango, Thomas P. Gargan, Robert A. Wirtz, Davy K. Koech, and Clifford R. Roberts

J. Med. Entomol. 27 (3): 377-384 (1990)

Methods: Anopheles were collected by aspiration inside houses from November 1985 to June 1987 near Kisumu in western Kenya. Weekly collections were made from >50 houses in the village of Kisian (10 km west of Kisumu) and in Saradidi (55 km west of Kisumu). *An. gambiae* Giles sensu lato and *An. funestus* Giles constituted >99% of the collected Anopheles and were th only species tested in this study. Study site, collection methods, mosquito identification, and handling procedures are described (J.C. Beier et al.,1987). Hyperendemic malaria

transmission in these two sites was studied by the World Health Organization from 1972 to 1976 (Fontaine et al., 1978). Malaria infection rates in Anopheles were determined by dissection and sporozoite ELISA techniques (J.C. Beier et al., 1987).

Results: Malaria infection rates determined by dissection and *Plasmodium falciparum* enzyme-linked immunosorbent assay (ELISA) were compared for 26,935 *Anopheles gambiae* Giles sensu lato and 17,739 *Anopheles funestus* Giles collected during 20 months in western Kenya. ELISA infection rates were about 43% higher than disection sporozoite rate. In dissection-negative *Anopheles*, circumsporozoites (CS) protein was detected by ELISA in 5.2% of 10,017 salivary gland samples and in 12.2% of 237 thorax samples. The accuracy of dissection and ELISA techniques was compared by the following tests on a group of 352 field-collected *Anopheles* (held 10 d to ensure sporogonic development): salivary gland dissection, examination of Giemsa stained dissection slides, ELISA tests on salivary gland and thorax body parts, and microscopic techniques for determining sporozoite loads. Respective infection rates were 9.9%, 10.8%, and 15.6% for dissection, stained slides, and ELISA. Sporozoite loads were associated significantly with ELISA absorbance values ($r = 0.76$). Compared with Giemsa-stained dissection slide results, the sensitivity was 100.0% for dissection versus 92.0% for ELISA. Immunological detection of CS protein in head-thorax samples of Afrotropical vectors overestimated the proportion of infective *Anopheles* because the comparison of techniques indicated the 45.4% of the ELISA positive Anopheles did not contain salivary gland sporozoites.

Conclusion: The immunological detection of CS protein in head-thorax samples of Afrotropical malaria vectors is significantly less accurate for determining *Anopheles* infectivity than standard dissection techniques. These findings raise important considerations for the use of ELISA or IRMA for measuring vector infectivity in malaria field studies. The accuracy of entomological inoculation rates depends upon standardized measures of the man-biting rate and the sporozoite rate, both of which are difficult to measure and are subject to error. Measures of infectivity provided by immunoassays are useful for measuring relative changes in transmission intensity in different epidemiological areas. However, entomological inoculation rates calculated using ELISA or IMRA infection rates may be overestimated because these infection rates do not represent the actual proportion of *Anopheles* with sporozoites in the salivary glands. In this respect, the use of immunoassays in malaria field studies can complicate the interpretation of malaria sporozoite transmission and when possible results should be standardized against traditional dissection techniques.

Future Work: Use immunoassays in malaria field studies to standardize against traditional dissection techniques for measuring vector infectivity.

Characterization of Malaria Transmission by Anopheles (Diptera: Culicidae) in Western Kenya in preparation for Malaria Vaccine Trials

John C. Beier, Peter V. Perkins, Fred K. Onyango, Thomas P. Gargan, Charles N. Oster, Richard E Whitmire, Davy K. Koech, and Clifford R. Roberts

J. Med. Entomol. 27(4): 570-577 (1990)

Methods: Studies were conducted at Kisian (10 km west of Kisumu) and Saradidi (55 km west of Kisumu), two sites in western Kenya selected for vaccine trials and described previously (Beier et al., 1987). The World Health Organization (1972-1976) used both sites as unsprayed comparison areas in an evaluation of fenitrothion as a residual insecticide for mosquito control (Joshi et al., 1975). Anopheles collection techniques were all-night man-biting collection techniques (World Health Organization, 1975). Weather variables -- rainfall was measured daily using rain guages at two houses per site. Hygrothermographs placed inside the same two houses provide continuous measurements of temperature and relative humidity. Transmission indices and statistical analysis were accomplished as described by Williams, 1937; Sokal & Rohlf, 1969; and MacDonald, 1957.

Results: Malaria transmission was studied for 33 months in the villages of Kisian and Saradidi in western Kenya in preparation of field trials of malaria vaccines. Abundance estimates of *Anopheles gambiae* Giles sensu lato and *Anopheles funestus* Giles, which constituted over 99% of 26,645 anophelines collected, were compared for all-night biting collections inside houses, outdoors, and in tents. The overall numbers of *Anopheles* per man-night were 2.3 times greater in Kisian than in Saradidi. For the three types of collections, mean sporozoite rates by dissection ranged from 2.2 to 5.4% for 13,072 *Anopheles* in Kisian and from 9.9 to 13.6% for 7,058 *Anopheles* in Saradidi. Greater than 90% of the infections were *Plasmodium falciparum*, either alone or mixed with *P. malariae* or *P. ovale*. Heaviest transmission from April to July coincided with the end of the long rainy season. Entomological inoculation rates (EIR) averaged 0.82 infective bites per man per night inside houses in Kisian and 0.65 in Saradidi. Outdoors, EIRs averaged 0.09 in Kisian and 0.52 in Saradidi. In tents, which were evaluated to identify methods for exposing non indigenous volunteers during vaccine efficacy trials, EIRs were 3.3 and 2.5 times less than inside houses for Kisian (EIR = 0.25) and Saradidi (EIR = 0.26) respectively. Exposure in tents averaged one infective bite every 4.0 d in Kisian and every 3.8 d in Saradidi. The use of tents in vaccine efficacy trials should provide adequate exposure for nonindigenous volunteers. Malaria vaccine trials could be conducted efficiently in western Kenya, with timing dependent upon

the intensity of transmission required by vaccine trial objectives.

Conclusions: This study provides long-term baseline information on malaria transmission in western Kenya for planning and conducting vaccine trials. The standard entomological methods used in this study could be employed readily for evaluating the intensity of transmission during malaria vaccind field trials. Estimates of the EIR will be important for the interpretation of vaccine efficacy among sites in different countries or on different continents. The design of entomological components for malaria vaccine trials should be required for phase IIb and Phase III trials.

Future Work: Conduct phase IIb and phase III malaria vaccine trials in Saradidi and Kisian, Kenya.

Plasmodium Species Identification by ELISA for Sporozoites Removed From Dried Dissection Slides

John C. Beier, Robert S. Copeland, Fred K. Onyango, Charles M. Asiago, Mutalib Ramadhan, Davy K. Koech, and Clifford R. Roberts

J. Med Entomol. 28(4): 533-536 (1991)

Methods: Anopheles gambiae s.l. Giles and An. funestus Giles were collected by all-night human-biting catches and by daytime aspiration collections inside houses from the villages of Kisian and Saradidi, western Kenya (Beier et al., 1990b). Sporozoites in the salivary glands were detected during routine dissections on the day of collection and were graded as to the number of sporozoites observed: +1 (1-10), +2 (11-100), +3 (101-1,000) and +4 (>1,000). A P. falciparum sporozoite ELISA (Wertz et al., 1987b) was used to test three 18-ul aliquots which were held either frozen at -20 degrees C in a 2.0-ml vial, dry in a 2.0-ml vial , or dry on a microslide. Dessicant was not used to dry samples. The proportion of P. falciparum sporozoite infections was determined by ELISA for a series of individual mosquitoes (Beier et al., 1988).

Results: The study tested the feasibility of identifying salivary gland sporozoites to species by *Plasmodium faciparum* ELISA by drying them on slides or in vials. The glands were dissected from *Anopheles gambiae* Giles s.l. and *An. funestus* Giles collected in western Kenya. In 119 gland infections containing a geometric mean of 1,222 sporozoites, a mean of 72.5% of sporozoites were

removed in 60 ul saline from slides at the time of dissection. Each of the 119 samples was dried into three 18 ul aliquots. Subsamples were stored at -70 degrees C, dried in vials, or dried on a microslide. When tested by *Plasmodium falciparum* ELISA, positive reactions were observed in 86.6% of frozen samples, 70.6% of samples held dry in vials, and 50.4% of samples held dry on microslides for 1 month. O 90 gland infections where coverslips were removed and slides were left to dry for 1 month before adding blocking buffer, 81.1% were positive for *P. falciparum*. This was not significantly different from either frozen gland samples (where 85.5% of 392 infections were identified or frozen gland plus corresponding thorax samples where 86.2% of 160 samples were identified). In malaria field studies, where it is not always practical to freeze samples, sporozoites from dissected mosquitoes can be preserved adequately for ELISA identification by simply removing coverslips and drying dissection slides.

Conclusion: The implication of these results for malaria field studies is that sporozoites from dissection slides do not have to be removed immediately from slides and held frozen for testing by immunoassays. Circumsporozoite protein remains intact on dried dissection slides for at least 1 month a demonstrated i this study, and much longer as indicated by preliminary tests on dissection slides held 1 year when most sporozoites were removed at the time of dissection (J.C.B., unpublished data). These findings may be especially valuable for situations in which dissections are performed in remote areas where it is not possible to freeze samples.

Effect of Plasmodium falciparum on the Survival of Naturally Infected Afrotropical Anopheles (Diptera: Culicidae)

Geoffrey M. M. Chege and John C. Beier

J. Med. Entomol. 27(4): 454-458 (1990)

Methods: From November 1987 to March 1988, wild *An. gambiae* Giles sensu lato and An. funestus Giles females were collected weekly by aspiration inside houses in Kisian, a locality 10 km west of Kisumu, western Kenya. Malaria transmission dynamics have been studied previously in this area (Fontaine et al., 1978; J.C. Beier et al., 1987). Female anophelines, collected from about 50 houses, were placed into screened 450-ml cartons and transported to Kisumu for processing. In the laboratory, mosquitoes were identified to species. Three to five replicates of 25 females of each species, irrespective of their trophic status, were established each week in screened 3.75-liter cartons. Mosquitoes were provided with 5% sucrose on

cotton that was changed daily. Processing of dead female mosquitoes for ELISA was accomplished by placing each individually into 1.5-ml polypropylene vials, held in a dessicator for 2 d, and then stored at room temperature. Tests for *P. falciparum* circumsporozoite (CS) protein followed the procedure using the 2A10 monoclonal antibody (Wertz et al., 1987b). Determination of daily survival rate was calculated as the antilog of the slope (b) of the regression equation for the ln(y = 1) of the number alive per day (y) for each replicate of caged mosquitoes (52 for An. gambiae s.l. and 50 for An. funestus). For both species , gc was considered to be 2 d (Davidson & Draper, 1953).

Results: The effect of the malarial parasite, *Plasmodium falciparum* Welch, on the daily survival rates and longevity of *Anopheles gambiae* Giles sensu lato and *An. funestus* Giles was determined for wild-caught, naturally infected females from western Kenya. Mosquitoes were collected inside houses and held in cages until death, after which they were assayed for *P. falciparum* circumsporozoite protein by an enzyme-linked immunosorbent assay (ELISA). Survival rates of field populations determined by parity rates were significantly higher than survival rates estimated by regression for *Anopheles* dying in cages. Overall, *An. gambiae* s.l. had a significantly higher daily mortality rate (x = 17.5%) than *An. funestus* (x = 13.2%). *P. falciparum* ELISA infection ratres, which were higher for *An gambiae* s.l. (x = 19.8%; n = 1,221) than for *An funestus* (x = 11.9%; n = 1,128), did not increase as a function of time for caged *Anopheles*. For *An. gambiae* s.l., there was a significant negative correlation between holding time for P. falciparum ELISA absorbance, suggesting that detectable circumsporozoite protein and perhaps the number of sporozoites may decrease with time in the vector. In western Kenya, an area where *Anopheles* populations often have extremely high malaria infection rates, *Plasmodium* infections did not reduce vector survivorship.

Conclusions: Infected An. gambiae tended to lose ELISA detectable CS protein as a function of holding time. This could indicate a progressive decrease in sporozoites, as has been observed in Aedes aegypi L. infected with P. gallinaceum Brumpt (Porter et al.,1954) because ELISA absorbance is correlated positively with levels of CS protein and the number of sporozoites (Baker et al., 1989).

Future Work: This observation should be tested further using quantitation techniques for salivary gland sporozoites (J.C. Beier et al., 1989). A decrease in sporozoite load over time would be significant epidemiologically in terms of vector potential.

Species Composition of the Anopheles gambiae Complex (Diptera: Culicidae) at Two Sites in Western Kenya

V. Petrarca, J.C. Beier, F. Onyango, J. Koros, C. Asiago, D.K. Koech, and C.R. Roberts

J. Med. Entomol. 28(3): 307-313 (1991)

Methods: This study was conducted in the villages of Kisian (5 km west of Kisumu) and Saradidi (45 km west of Kisumu), two sites in western Kenya described previously by Beier et al. (1990b). Collection techniques and processing were accomplished as described (Beier et al., 1988; Wertz et al., 1987).

Results: At two sites in the Kisumu area of western Kenya, the species composition of the *Anopheles gambiae* complex was determined by analysis of ovarian polytene chromosomes. Of 1,915 females, 26.1% were *An. arabiensis* Patton and 73.9% were *An. gambiae* Giles; one *arabiensis* x *gambiae* hybrid was identified. No major differences in the proportions of *An. araabiensis* and *An. gambiae* were observed between sites or between years. The ratio of *An. arabiensis/An. gambiae* was 6.7: 1 (n = 231) in cow-baited traps, 0.2: 1 (n = 1,525) in indoor resting samples, and 0.5: 1 (n = 145) in all-night human bait catches. The proportion of *An. arabiensis* decreased progressively from 50.0% to 8.3% (n = 1,129) during 11 wks from September to November 1987; this change was correlated negatively wirh night temperature and positively wit temperature range. In cow-baited traps, 97.4% (n = 194) of *An. arabiensis* were cow-fed and 95.8% (n = 1,054) of *An. gambiae* from indoor resting collections were human-fed. In indoor collections, 37.2% (n = 215) of *An. arabiensis* were cow-fed and 23.1% (n = 26) of *An. gambiae* from cow traps were human-fed. This demonstrates post-blood-feeding endophily by *An. arabiensis* and suggests post-blood-feeding exophily by *An. gambiae.* Malaria infection rates were higher for *An. gambiae* than *An. arabiensis* by a ratio of 3:1 in 1986 (by *Plasmodium falciparum* ELISA) and 2.3:1 in 1987 (by dissection). Despite the higher proportion of infective *An. gambiae*, both species in this area serve as efficient vectors throuth their remarkably stable contact with the human population as demonstrated by their blood feeding and resting behavior.

Conclusions: As previously observed in east African countries (Gillies & Coetzee, 1987) *Plasmodium falciparum* infection rates were higher in *An. gambiae* than in *An. arabiensis*. In considering Kisian and Saradidi as potential sites for malaria vaccine testing (Beier et al., 1990b), there are several relevant points to be

stressed. At both sites during the study period, the two vector species occur in similar proportions. Though *An. gambiae* shows higher sporozoite rates than *An. arabiensis* in both sites, the proportion of infective *An. gambiae* in Saradidi is almost twice that in Kisian. Because there were no differences in the proportion of *An. gambiae* and *An. arabiensis* in biting collections between tents and housess, volunteers could reasonably be exposed to natural malaria transmission in tents. This is an important consideration for the design of phase IIb vaccine trials. The long-term and short-term stability in blood-feeding patterns and sporozoite rates of the *An. gambiae* complex make these two sites in western Kenya ideal locations for testing malaria vaccines under conditions of intense *P falciparum* transmission.

Future Work: Analyze for differences in blood-feeding patterns, Plasmodium falciparum infection rates, temporal variations in abundance, and biting-resting behavior

Treatment of Chloroquine-resistant Malaria in Monkeys with a Drug Combination That Reverses Resistance In Vitro

`H.L. Williams, Deadre J. Johnson, V.C.N. Nkoye, and S.K. Martin

Methods: Eight healthy malaria-naive *Aotus trivagatus* monkeys were each given a standard intravenous (iv) inoculum of 10/6 chloroquine-resistant Vietnam Smith strain *Plasmodium falciparum* parasites form a donor monkey and their parasitemia were followed daily with Giemsa-stained thin films. Control monkeys were treated with a total dose of 25 mg kg chloroquine intramuscularly. Experimental monkeys received a similar dose of choroquine plus the tiapamil analogue calcium antagonist, N-(3,4 dimethoxy-phenyl-N-methyl-2-naphthyl)-m-dthiane-2-propylamine hydorchloride, Ro-11, that had ' een shown to reverse choroquine resistance in vitro (Kyle et al., 1990).

Results: In both control and experimentally treated monkeys the parasitemias continued to rise above 10%, resulting in treatment with administration of intravenous intramuscular mefloquine on day 9. Ro-11 had no effect on the rate of rise of parasitemia.

Conclusions: In none of the treatment combinations were lowering of the chloroquine-resistant P. falciparum parasitemia observed with Ro-11.

Future Work: Even though inhibition of chloroquine efflux in vitro renders resistant *P. falciparum* parasites sensitive, more information on the pharmacokinetics and host toxicity of the respective drug combinations is needed before clinical trials can be safely conducted.

Leishmaniasis:

A group of diseases caused by parasites of the genus Leishmania The epidemiology of the disease is mainly characterized by the habits of the sandfly which transmits the disease. The sandfly, genera: Phlebotomus, is mainly found in areas below 3,000 feet elevation (Baringo and West Pokot, Kenya). It is found in hot, dry areas with a vegetation of Acacia thornbush and little grass. In Kenya, these are the areas where the termite Macrotermes bellicosus builds its huge termite hills and it is in the ventilation shafts of such termite hills where Phlebotomus prefers to hide. As the flight range of these small sandflies are limited, generally a few hundred meters from their resting sites, individuals usually contract the disease in the neighborhood of such termite hills. Most forms of leishmaniasis also occur in animals, like dogs and rodents. The only reliable diagnosis is by finding the parasite in tissue specimens and, less reliably, by culturing peripheral blood on suitable media for visceral leishmaniasis.

Development of Leishmania Major in Phlebotomus Duboscqi and Sergentomyia schwetzi (Diptera: Psychodidae)

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Methods: The parasite *L. major* (IDUB/KE/83/NLB-172), was originally isolated in 1983 from a *P. duboscqi* female collected near Marigat, Baringo District, Rift Valley Province, Kenya. *P. duboscqi* and *S. schwetzi* established from laboratory colonies were used in these experiments. Both sandfly colonies originated from wild stocks collected in 1983 at Marigat, Baringo District, Rift Valley Province, within an endemic focus of human cutaneous leishmaniasis. Flies were dissected in a large drop of Schneider's Drosophila medium at o, 6, 12, 18, 24, 36, 48, and 60 hours post-feeding to determine when transformation of promastigotes occurred, and then at @ 24 hour intevals from day 3 to day 14 post-feeding. To determine if non-infective, early developmental forms of *Leishmania* could be isolated from a non-vector as well as froma vector *P. duboscqi* and *S. schwetzi* females were fed as before on leishmanial lesions of hamsters. Ten potentially infected flies of each species were then dissected at 1 day intevals for up to 10 days and their gut contents inoculated into culture tubes containing NNN diphasic medium overlaid with Schneider's Drosophila medium supplemented with 20% fetal bovine serum, 250 ug/ml streptomycin, 250 U/ml penicillin, and 500

ug/ml 5-fluorocytosine. The tubes were examined daily thereafter for 2 weeks for the presence of flagellates.

Results: The extrinsic development of *Leishmania major* was observed in 2 man-biting sandflies, *Phlebotomus duboscqi,* a known vector, and *Sergentomyia schwetzi,* an assumed non-vector. Flies fed on a leishmanial lesion on the nose of a hamster were examined for infection at 0,6,12,18,24,36,48, and 60 hr and at @24 intevals from day 3 to day 14 post-feeding. Infection rates, determined by light microscopy, were 47% (n = 258) in *P. duboscqi* and 5% (n = 162) in *S. schwetzi.* Transformation from amastigotes to "procyclic" promastigotes occurred in both species at 6-18 hr post-feeding. In *P. dubosqi,* the parasites multiplied rapidly and developed through as many as 10 forms, including at least 3 dividing-promastigote forms. Metacyclic promastigotes, the "infective" form, appeared at 6 days post-feeding, first in the region of the stomodeal valve, then in the pharynx, cibarium,and proboscis. In a single attempt 14 days post-feeding, a *P. duboscqi* transmitted *L major* to a mouse by bite. In contrast, the parasites multiplied slowly ... *schwetzi,* and did not develop beyond "procyclic" promastigotes. The parasites ... not migrate anteriorly nor survive beyond 90 hr. post-feeding, indicating that *S. schwetzi* is not a vector of L. major. Classical stratgies for vector incrimination may be confounded by the isolation of non-infective early developmental forms of *Leishmania* from wild-caught non-vectors.

Conclusions: This study clearly confirms that the gut environment of *P. duboscqi* is suitable for the multiplication and development of *L. major*. These observations are important to field studies of vector biology. The presence of blood or eggs and the shape of the abdomen may give an indication of the age of a natural infection. The presence of stage-specific forms in combination with the presence or absence of a bloodmeal and/or eggs cab be used to establish the approximate age of an infection and may indicate whether th insect is a suitable host. Recognition of metacyclics anterior to the stomodeal valve shows that the fly is a probable vector. The repeated isolation of early developmental forms of human L. major form S. schwetzi, a non-vector, underscores the need for field workers to interpret with caution the presence of *Leishmania* in wild-caught sandflies.

Future Work: Demonstrate that sandflies must be able to naturally and experimentally maintain *Leishmania* infection throughout the extrinsic life cycle of the parasite in order to be a transmission vector.

Experimental Transmission of Leishmania major to Vervet Monkeys (Cercopithecus aethiops) by Bites of phlebotomus duboscqi (Diptera: Psychodidae)

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Methods: The parasite used was *L. major* (strain IDUB/KE/83=NLB-144) isolated from a *P. duboscqi* female collected in Marigut, Baringo District, Rift Valley Province, Kenya (Beach et al., 1986), within an endemic focus of zoonotic and human cutaneous leishmaniasis. Vervet monkeys (*C. aethiops*) were wild-caught from areas known to be free of human leishmaniasis and quarantined for 3 months during which they were examined clinically and screened for tuberculosis, simian immunodeficiency virus, intestinal parasites and *Leishmania*. Infection of sandflies was accomplished by feeding them on leishmanial lesions on the noses of anesthetized hamsters placed in the colony cage. Before the transmission trials, uninfected sandflies were offered bloodmeals on 2 vervet monkeys to determine if they would feed and to select the optimal exposure site. The flies were allowed to feed for 20 minutes. The flies fed readily on the base of the tail and eyebrow, selected sites. Dissection of sandflies was performed according to the technique of Johnson et al., (1963).

Results: Experimental transmission of *Leishmania major* to vervet monkeys (*Cercopithecus aethiops*) was accomplished by bites of *phlebotomus duboscqi* sandflies. Three-day old, laboratory-reared *P. duboscqi* were fed on leishmanial lesions on hamsters infected wirh *L. major.* The flies were re-fed on monkeys 10 d after infection. Five adult male vervet monkeys were used in concurrent transmission trials. Two of the monkeys received subcutaneous inoculations with stationary-phase promastigotes (2x 10/6) promastigotes in 0.1 ml of medium) on the base of the tail. Putatively infected *P. duboscqi* were allowed to feed on the remaining 3 monkeys at sites on the base of the tail and on the right eyebrow. Challenges by sandfly bites resulted in multiple leishmanial lesions at all bite sites and, consequently, more lesion areas than were produced by needle challenges. Post-feeding dissection of sandflies indicated that multiple lesions could be caused by bites of a single fly, and that probing alone, without imbibing blood. was sufficient for transmission.

Conclusions: These first experimental transmissions of *L. major* to vervets by bites of *P. duboscqi* demonstrate that sandfly challenge is an efficient alternative to needle challenge, making available a unique *Leishmania*-sandfly non-human primate model for use in vaccine development.

Future Work: In evaluating the efficacy of a potential anti-leishmanial vaccine, it seems prudent to use a natural parasite-vector challenge. These first experimental transmission of *L. major* to *C. aethiops* by bites of a natural vector, *P. duboscqi*, demonstrate that a natural challenge is feasible, making available a unique *Leishmania* sandfly non-human primate model for use in future vaccine development.

Phlebotomus guggisbergi (Diptera: Psychodidae), A Vector of Leishmania tropica in Kenya

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Trans. Royal Soc. Trop. Med. Hyg. (in press)

Methods/results: Sandflies were collected in light traps and on oiled papers at 4 active case sites of human cutaneous leishmaniasis due to *Leishmania tropica* at Muruku Sublocation, Laikipia District, Kenya. Nearly 5,200 females 6 species, including 2 Phlebotomus species (*P. guggisbergi* and *P. duboscqi*), were dissected and examined for flagellates. Of 3,867 *P. guggisbergi* females collected at a multiple case site, 167 (4.3%) harbored mature leishmanial infections, to include metacyclic promastigotes, while all other flies were negative. Of the infected flies, 164 were collected in a cave near the patients' home, 2 from crevices on the escarpment immediately behind the house, and 1 from the bedroom of one of the patients. One hundred sixty-four of the isolates were successfully grown in Schneider's Drosophila medium and harvested for typing by cellulose acetate electrophoresis. Isoenzyme profiles of the first 22 of these were compared with those of WHO reference strains and well charactereized local strains using 12 enzyme loci. The isolates yielded isoenzyme migration patterns that were indistinguishable from those of 2 *L. tropica* reference atrains and 6 *L. tropica* patient isolates from the same locality. This is the first reported isolation of *Leishmania* parasites from *P. guggisbergi*, the first reported isolation of *L. tropica* from sandflies in Kenya, and the first confirmed isolation of such a large number of *P. guggisbergi* naturally harboring mature infections of *L. tropica* at an active case site of cutaneous leishmaniasis due to this agent

Conclusions: Although cutaneous lesions are sometimes found in kala-azar as a prodromal sign or after treatment, cutaneous leishmaniasis caused by L. tropica had not been identifed in East Africa. Since 1968, however, a few cases of cutaneous, mostly diffuse skin leishmaniasis were found far outside the kala-azar areas on the southern slope of Mount Elgon. It is likely that those patients suffer from a special form of the disease which normally occurs in rock hyrax, transmitted

by a sandfly which inhabits caves (Mutinga, 1972). The identification of L. tropica in Laikipia District, Kenya may explain the cases observed since 1968, however, much more research is needed.

Future Work: Search for drugs which are more easily administrated and which have a higher cure rate than the pentavalent antimonial compounds. Continuation of the search for an animal reservoir in L. tropica infections. Studies on the life cycles of P. guggisbergi and allied species, particularly their larval stages, breeding sites, additional resting sites, and host preference.

Leishmania infantum sensu lato Isolated From a Giant Rat *(Cricetomys gambianus)* Captured at A Case Site of Human Cutaneous Leishmaniasis in Kenya

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Unpublished

Methods/Results: Investigations were conducted at a multiple-case site within a recently discovered rural focus of human cutaneous leishmaniasis. Six cutaneous *Leishmania* isolates previously aspirated from patients at this and neighboring case sites were cultured and subsequently typed by cellulose acetate electrophoresis as *L. tropica.* An isolate from 1 of 6 giant rats *(Cricetomys gambianus)* captured near the patients' house was grown on NNN diphasic medium overlayed with Schneider's Drosophila medium. Isoenzyme profiles of this isolate, of *L. tropica* patient isolates, and WHO reference strains were compared by cellulose acetate electrophoresis using 12 enzyme loci (Mdh, Me, Icd, 6Gpd, G6pd, Hh1,Nh2, Nh3, Mpi, Gpi, Pgm1, and Pgm2). Unexpectedly, the isolate from the giant rat was found to be indistinguishable form an *L. infantum* reference strain (MH)M/TN/80/LEM235, NLB-317) for all enzymes tested. This is the first isolation of *L. infantum* from any host in Kenya. A preliminary survey for potential vector sandflies in the immediate vicinity of the house and in a nearby cave produced several sandfly species including *Phebotomus guggisbergi* and *P. duboscqi.* Either of these fly species could be involved in the transmission of cutaneous leishmaniasis caused by *L. infantum* and/or *L. tropica.*

Conclusions: The isolation of *L. infantum* from 1 of 6 giant rats (*Cricetomys gambianus*) captured at a case site of human cutaneous leishmaniasis in Kenya, provide insight on the serious problem of leishmaniasis localized in certain areas in Kenya where the ecologic conditions are favorable to transmission. Most

forms of leishmaniasis also occur in animals like dogs and rodents -- such demonstrates the difficulty of eradica 'ng the parasites or separating host and vector.

Future Work: Search and develop drugs which are more easily administered and which have a higher cure rate than current treatment schemes and find ways for elimination of the vector with environmentally safe insecticides.

Concurrent Infection with Leishmania donovani and Leishmania major in a Kenyan Patient: Clinical Description and Parasite Characterization

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Am J. Trop. Med Hyg.

Method/results: An indigenous adult male from Baringo District, Kenya developed kala-azar. His village lies within foci of kala-azar and cutaneous leishmaniasis due to *L. donovani* and *L. major*. A splenic aspirate grew *Leishmania* promastigotes and the patient was treated for kala-azar and discharged. Four months later, after relapse, he returned for a second course of treatment. During this second visit the patient was among a group of randomly sampled participants in a study of diagnosis of leishmaniasis using urine and nasopharyngeal cultures. Urine, nasopharyngeal, and splenic samples were found positive for *Leishmania*. Splenic and urine cultures were cryopreserved. Cellulose acetate electrophoresis using 17 enzymes (LDH, MDH, ME, ICD, 6PGD, GAPDH1, G6PD, HK, PFK, AK, ACP, NH1, NH2, MOI, GPI, PGM1. and PGM2) revealed a mixed infection with *L. donovani* and *L. major*. The original isolate from the splenic aspirate was then characterized and showed mixed infection. Stationary-phase promastigotes from these cultures were inoculated subcutaneously, intraperitoneally, and intracardially into Syrian hamsters and Balb/c mice. Six and a half months later two hamsters and two mice developed both kala-azar and cutaneous leishmaniasis. Parasites from liver, spleen, bone marrow, lymph node, and skin were identified by CAE as *L donovani* and *L. major*.

Conclusions: Endemic visceral leishmaniasis, or kala-azar, which is usually caused by Leishmania donovani, and cutaneous leishmaniasis, caused by L. aethiopica, L. major, L. tropica, and L. donovani (post kala-azar dermal leishmaniasis) are major public health problems in Kenya. Demonstration that naturally occurring mixed infection do occur provide insight for resistant cases which fail to respond to treatment.

Future Work: Identify and develop inproved means for diagnosing the different Leishmania species responsible for human leishmaniasis.

Bibliography of Publications:

Buesing, M., P. Guerry, C. DeiSanti, E. Franke, G. Watt, M.A. Rab, C.N. Oster, J.P. Burans, and P.L. Perine. An Oglionucleotide Probe for Detecting Plasmodium falcaparum: An Analysis of Clinical Specimen From Six Countries. Journal of Infectious Diseases. 155(1): 1315-1318.(1987).

Hoffman, S.L., C.N. Oster, C.V. Plowe, G.R. Woollett, J.C. Beier, J.D. Chulay, R.A. Wertz, M.R. Hollingdale, and M. Mugambi. Naturally Acquired Antibodies to Sporozoites Do Not Prevent Malaria: Vaccine Development Implications. Science. 237: 639-642 (1987).

Oster, C.N., N.I. Githeko, M.L. Adungo, F.K. Owaga, P.V. Perkins, L.D. Hendricks, and R.E. Whitmire. Attack Rates of Malaria in School Children in The Siaya and Kisumu Districts, Nyanza Province, Kenya. In "Advances in The Diagnosis, Treatment, and prevention of Immunizable Diseases in Africa". Eds. Kinoti, S.N., Koech, D.K., and Tukei, P.M., Proceedings of The Seventh Annual Medical Scientific Conference (KEMRI-KTRI) Nairobi, Kenya.. pp. 226-229 (1987).

Watkins, W.M., J.D. Chulay, D.G. Sixsmith, J.C. Spencer, R.E. Howells. A Preliminary Pharmacokinetic Study of The Antimalarial Drugs Proguanil and Chlorproguanil. Journal of Pharmacology. 39: 261-265 (1987).

Beier, J.C., P.V. Perkins, R.A. Wirtz, J. Koros, C. Diggs, T.P. Gargan, and D.K. Koech. Bloodmeal Identification by Direct Enzyme-linked Immunosorbent Assay (ELISA), Tested on Anopheles (Diptera: Culicidae) in Kenya. Journal of Medical Entomology. 25(1): 9-16 (1988).

Hoffman, S.L., C.N. Oster, C. Mason, J.C. Beier, J.A. Sherwood, W.R. Ballou, M. Mugambi, and J.D. Chulay. Identification of A Potentially Protective T cell Epitope on The Plasmodium falciparum CS Protein. In "Technological Advances in Vaccine Development" UCLA Symposia Molecular and Cellular Biology, A.R. Liss, New York, Vol 84, pp. 625-639 (1988).

Beier, M.S., I.K. Schwartz, J.C. Beier, P.V. Perkins, F. Onyango, J.K. Koros, G.H. Campbell, P.M. Andrysiak, and A.D. Brandling-Bennett. Identification of Malaria Species by ELISA in Sporozoite and Oocyst Infected Anopheles From Western Kenya. American Journal of Tropical Medicine and Hygiene. 39(4): 323-327 (1988).

Beier, J.C., C.N. Oster, J.K. Koros, F.K. Onyango, A.K. Githeko, E. Roeton, D.K. Koech, and C.R. Roberts. Effect of Human Circumsporozoite Antibodies in Malaria Infected Anopheles (Diptera: Culicidae). Journal of Medical Entomology. 27: 547-553 (1989).

Hoffman, S.L., C.N. Oster, C. Mason, J.C. Beier, J.A. Sherwood, W.R. Ballou, M. Mugambi, and J.D. Chulay. Human Lymphocyte Response To A Sporozoite T cell Epitope Correlates with Resistance To Malaria. Journal of Immunology. 142: 1299-1303 (1989).

Wirtz, R.A., J.F. Duncan, E.K. Njelesani, I. Schneider, A.E. Brown, C.N. Oster, J.B.O. Were, and H.K. Webster. An Enzyme-linked Immunosorbent Assay (ELISA) For Detection of Plasmodium falcaparum Circumsporozoite Antibody. Bulletin of The World Health Organization. 67(5): 535-542 (1989).

Beier, J.C., P.V. Perkins, F.K. Onyango, T.P. Gargan, C.N. Oster, R.E. Whitmire, D.K. Koech, and C.R. Roberts. Characterization of Malaria Transmission by Anopheles (Diptera: Culicidae) in Western Kenya in Preparation For Malaria Vaccine Trials. Journal of Medical Entomology. 27: 570-577 (1990).

Beier, J.C., P.V. Perkins, J.K. Koros, F.K. Onyango, T.P. Gargan, R.A. Wirtz, D.K. Koech, and C.R. Roberts. Malaria Sporozoite Detection by Dissection and ELISA To Assess Infectivity of Afrotropical Anopheles (Diptera: Culicidae). Journal of Medical Entomology. 27(3): 377-384 (1990).

Geoffrey, M., M. Chege, and J.C. Beier. Effect of Plasmodium falciparum on The Survival of Naturally Infected Afrotropical Anopheles (Diptera: Culicidae). Journal of Medical Entomology. 27(4): 454-458 (1990).

Beier, J.C., P.V. Perkins, J.K. Koros, F.K. Onyango, T.P. Gargan, R.A. Wirtz, D.K. Koech, and C.R. Roberts. Malaria Sporozoite Detection by Dissection and Elisa To Assess Infectivity of Afrotropical Anopheles (Diptera: Culicidae). Journal of Medical Entomology. 27(3): 377-384 (1990).

Lawyer, P.G., J.I. Githure, C.O. Anjili, J.O. Olobo, D.K. Koech, and G.D.F. Reid. Experimental Transmission of Leishmania major to Vervet Monkeys (Cercopithecus aethiops) by Bites of Phlebotomus duboscqi (Diptera: Psychodidae). Transactions of The Royal Society of Tropical Medicine and Hygiene. 85: 229-232 (1990).

Lawyer, P.G., P.M. Ngumbi, C.O. Anjili, S.O. Odongo, Y.B. Mebrahtu, J.I. Githure, D.K. Koech, and C.R. Roberts. Development of Leishmania major in Phlebotomus duboscqi and Sergentomyia schwetzi (Diptera: Psychodidae). American Journal of Tropical Medicine and Hygiene. 43(1): 31-43 (1990).

Petrarca, V., J.C. Beier, F.K. Onyango, J.K. Koros, C. Asiago, D.K. Koech, and C.R. Roberts. Species Composition of The Anopheles gambiae in Western Kenya. Journal of Medical Entomology. 28(3): 307-313 (1991).

Beier, J.C., R.S. Copeland, F.K. Onyango, C.M. Asiago, M. Ramadhan, D.K. Koech, and C.R. Roberts. Plasmodium Species Identification by ELISA for Sporozoites Removal From Dried Dissection Slides. Journal of Medical Entomology. 28(4): 533-536 (1991).

Appendix A

Safety, Immunogenicity and Efficacy of A Recombinant Falciparum Sporozoite-toxin Vaccine (R32TOX-A) in Semi-immune Volunteers in Saradidi, Kenya

TITLE: Safety, Immunogenicity and Efficacy of a Recombinant Falciparum Sporozoite-Toxin A Vaccine (R32ToxA) in Semi-immune Volunteers in Saradidi, Kenya.

BACKGROUND AND OBJECTIVES

INTRODUCTION:

a. Medical application: To develop a malaria vaccine.

b. Objective: To test a recombinant falciparum sporozoite-toxin A malaria vaccine (R32ToxA) for safety and immunogenicity in a prospective, randomized, placebo controlled double blind study in normal indigenous volunteers in an area endemic for malaria in Kenya.

c. Status: In a Phase I study, the Kenyan semi-immune antibody response to R32tet32 appeared anamnestic, higher and longer lasting than the non-immune response observed in a US Phase I study (Ballou et al. 1987). The semi-immune response to R32ToxA may also be better that the non-immune response, which was better than the non-immune response to R32tet32 (Ballou et al. 1987). Current laboratory challenge models to evaluate efficacy of malaria vaccines may be too stringent (100,000 sporozoites/mosquito x 5 infective mosquitoes at once) versus natural field challenge (100-1000 sporozoites/mosquito x 2 infective mosquitoes/week), depending on geography and season. An at least indirect comparison of non-immune vaccinees with laboratory challenge could be made with semi-immune vaccinees and natural field challenge.

The boosting of natural immunity by a vaccine, augmenting existing resistance, may be sufficient to produce a detectable reduction in malaria incidence in persons previously exposed to malaria but not in the malaria naive. A vaccine inadequate for the non-immune may be adequate for the semi-immune.

Evaluating the proposed field testing procedure would be useful for the design of further studies.

The gene which codes for the circumsporozoite protein of P. falciparum has been cloned (Dame et al. 1985). Part of this protein consists of 41 tandem tetrapeptide repeats, 37 of which are Asn-Ala-Asn-Pro and 4 of which are Asn-Val-Asp-Pro.

The recombinant falciparum sporozoite vaccine R32tet32, consisting of 32 tetrapeptide repeats with an alum adjuvant, was more immunogenic in semi-immune volunteers than in non-immune volunteers, producing an anamnestic boosting antibody response with higher and longer lasting levels (Sherwood et al. 1988). This vaccine contained antibody epitopes, but for malaria-naive subjects lacked adequate T cell epitopes for boosting, longer lasting antibody levels, and induction of cellular immunity.

R32Tox-A has been chosen for this study based on superior immunogenicity and safety profiles when compared to three recombinant and eight other conjugated circumsporozoite (CS) protein repeat based vaccines. A dose of 400 mcg of R32ToxA

produced higher titers than 200, 100, or 50 mcg of R32ToxA. A vaccination interval of 0 and 8 weeks produced higher titers than one of 0 and 4 weeks.

The malaria sporozoite vaccine preparation to be administered to volunteers, R32-Toxin A is manufactured by SmithKline Beecham Pharmaceuticals and the Swiss Serum and Vaccine Institute. This vaccine was prepared by the covalent coupling of the recombinant protein R32LR with Toxin A, an exotoxin from Pseudomonas aeruoinosa. R32LR is a purified recombinant protein synthesized in E. coli containing 30 NANP and 2 NVDP repeats of the immunodominant tetrapeptide repeat of the P. falciparum CS protein. Toxin A is purified from the supernatant of fermentor-grown cultures of P. aeruoinosa. In the vaccine preparation, Toxin A is not in its active form, as demonstrated by absence of adenosine diphosphate ribosyl transferase activity.

R32ToxA has been tested by the Swiss Serum and Vaccine Institute in two trials for safety and.immunogenicity in normal human volunteers without prior exposure to malaria or hepatitis. It was administered to 5 volunteers at a dose of 100 mcg on days 0 and 56. The vaccine was found to be mildly reactogenic; all vaccinees experienced slight local reactions which resolved spontaneously within 48 hours.

In the second trial, doses of 50, 100, 200, or 500 mcg of R32ToxA were given to groups of 5 or 6 volunteers on days 0 and 42. The vaccine was safe and well tolerated at all four doses; the 400 mcg dose produced the highest antibody levels by ELISA and IFA. Five of five volunteers immunized with the 400 mcg dose developed significant antibody responses.

R32ToxA has been tested for safety and immunogenicity in non-immune volunteers at the Johns Hopkins University. It was administered to 20 volunteers in a dose of 400 mcg at 0 and 8 weeks. Volunteers reported mild to moderate local or regional soreness. All volunteers developed significant antibody responses. Anti-sporozoite antibody was demonstrated by immunofluorescence.

Given the favorable response of the Kenyan semi-immune volunteers to the R32tet32 vaccine, we believe the immunologic response to R32ToxA may be similarly favorable.

We consider R32ToxA to be a safe, immunogenic and potentially effective candidate vaccine to prevent falciparum malaria. An investigational new drug exemption (BB-IND #3117) has been granted by the U.S. Food and Drug Administration (FDA) to test this vaccine.

d. Study design: Prospective, randomized, placebo controlled double blinded. Laboratory models of malaria challenge using colony raised infected mosquitoes may not be realistic challenges. Laboratory mosquitoes may have as many as 100,000 sporozoites each, with two to five mosquitoes biting per challenge. Wild mosquitoes may have as few as 100 to 1000 each, with the bite of one mosquito every several days. The comparison of current laboratory and field challenge needs to be made in order to validate or modify the laboratory model.

PLAN:

a. Selection of subjects:

(1) Number of subjects: 60. Assuming a vaccine efficacy of at least 70% and an incidence of malaria in controls of 80% over 6 months, (70% incidence over 89 days was found in a previous study in the same area (Hoffman et al. 1987), 19 volunteers would be required in each of the vaccine and control groups for a significance of 0.05 and a power of 0.90 (Fleiss 1981 and Appendix). Thirty per group allow for a 20% drop-out rate and a possibly reduced incidence of malaria due to less rain.

(2) Age range: 18 to 50 years, optimally 18 to 24.

(3) Sex: Male.

(4) Inclusion criteria: Normal volunteers who are residents of the 100 square km area around Saradidi. They must be available for six months.

(5) Diagnostic criteria for entry: May have had malaria. Normal volunteers.

(6) Evaluations prior to entry: Medical history and physical examination will be performed, with attention to previous history of hepatitis, cardiac disease, renal disease, immunologic disease, and splenectomy. The following laboratory tests will be performed: complete blood count (CBC/hemogram), urinalysis, serum chemistries including, creatinine, urea nitrogen, bilirubin, aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), hepatitis serology including hepatitis B surface antigen, antibody, and core antibody, human immunodeficiency virus (HIV) antibody, hemoglobin electrophoresis (HbEP) for sickle trait and thalassemia, and glucose-6phosphatase deficiency (G6PD). History of acute hemolytic episode will be grounds for exclusion from the study. If asymptomatic G6PD deficiency is detected, the volunteer will be enrolled and observed carefully after administration of sulfadoxine-pyrimethamine. Blood type will be determined. Tests for anti-malarial immunity will include anti-sporozoite antibodies by ELISA, anti-blood stage antibodies by immunofluorescent staining (IFA) (Young et al 1985, Hommel et al. 1983) and anti-sporozoite antibodies by IFA (Nardin et al. 1979). Lymphocyte proliferation and cytokine production in response to circumsporozoite peptides and will be performed on selected cases by published methods (Ballou et al. 1987, Hoffman et al. 1989).

(7) Exclusion criteria: Volunteers will be excluded if they have had a splenectomy, have evidence of cardiac, hepatic, renal, or immunologic abnormality, are taking any immunosuppressive drug, have a history of symptomatic hemolysis due to G6PD deficiency, or have any exclusionary criteria.

(8) Source of volunteers: Subjects will be recruited from the 100 square km area around the village of Saradidi.

(9) Volunteer identification: Subjects will be identified by name, identity card number, and study number comprised of village, house and occupant numbers.

(10) Volunteer assignment: Subjects will be assigned randomly to receive either vaccine or placebo. Pairs will be matched as closely as possible by age and each pair will share the same house to equalize exposure.

PROJECT MEDICATIONS:

a. Complete name of all medications including placebo:

(1) Recombinant malaria vaccine, R32 ToxA (Sporovac PF (R)).

(2) Recombinant hepatitis B vaccine, ENGERIX-B, as placebo.

(3) Medicines to treat allergy or anaphylaxis.

(i) Epinephrine (Adrenalin) 1:1000 injection.

(ii) Diphenhydramine (Benadryl) 50 mg capsules or injection.

(iii) Methyl prednisolone (Solu-medrol) 10 mg injection.

(4) Medicines to treat malaria:

(i) Chloroquine phosphate 500 mg tablets.

(ii) Sulfadoxine 500 mg - pyrimethamine 25 mg (Fansidar) tablets.

(iii) Quinine sulfate 300 mg capsules.

(iv) Doxycycline hydrochloride 100 mg capsules.

b. Source of medications and placebo:

(1) The vaccine is manufactured by SmithKline Beecham according to approved Standard Operating Procedures, Good Laboratory Practices, and Good Manufacturing Procedures.

Each lot will be required to pass standard U.S. Food and Drug Administration-required tests for safety in mice and guinea pigs, and to be free from endotoxin contamination using the limulus lysate and rabbit pyrogenicity tests. Each lot will be tested for immunogenicity in mice and rabbits and will be required to induce a positive ELISA test, with absorbance of greater than 1 optical density (O.D.) unit at a serum dilution of 1:100 after two injections.

(2) ENGERIX-B will be obtained from SmithKline Biologicals. This is the same vaccine used in Kenya Hepatitis-B vaccination programs.

(3) Medicines to treat allergy or anaphylaxis: Epinephrine, diphenhydramine, and methyl prednisolone will be obtained from commercial chemists in Kenya.

(4) Medicines to treat malaria will be obtained from commercial chemists in Kenya.

c. Place where study medicines are to be stored during study: Vaccines and skin test antigens will be stored in the laboratory at the Kenya Medical Research Institute, Nairobi. Routine medications will be stored in the Clinic in Saradidi.

d. Dose range: Vaccine dose will have 400 mcg of protein in 0.5 ml. Placebo will have 40 mcg of hepatitis protein.

e. Dose schedule: Each volunteer will receive three doses of vaccine at 0, 2 and 6 months.

f. Administration: Vaccines will be administered at the Walter Reed Clinic at the Saradidi Rural Health Programme under the supervision of a physician trained in the management of anaphylactic reactions. Each dose will be given in the presence of a physician, by intramuscular injection into the deltoid or triceps muscle.

g. Washout periods: Not applicable.

h. Duration of vaccination schedule: 6 months.

i. Accompanying medications: Volunteers will be treated for malaria during the vaccination period. Volunteers will be treated for malaria with sulfadoxine-pyrimethamine on the same day as each of the three vaccinations. All other medicines taken by volunteers should be obtained with the prescription of one of the study physicians, and not without the knowledge of these physicians. Volunteers will not take any medications which may alter the inflammatory response. Since it is possible that chloroquine reduces immune response in vaccination, chloroquine will not be used to treat those who develop malaria during the study.

j. Antidotes: Not applicable.

k. Labelling of study medications: Vials of experimental and placebo vaccines will be labeled by Swiss Serum and Vaccine Institute with individual but indistinguishable outer labels. Each label will have a peel-back section covering the identity of the contents, so the principal investigator may break the code in the event of an emergency. The principal investigator will not know which subjects receive vaccine and which receive placebo.

Subjects, clinical workers and slide readers will not know who received vaccine or placebo.

EVALUATIONS MADE DURING AND FOLLOWING PROJECT:

a. Specimens to be collected. A total of 190 ml of blood will be drawn. Blood will be drawn by a physician, clinical officer, or certified technician. Finger pricks for blood slides will be done by clinical field workers

(1) Schedule:

(i) Blood for complete blood count (2 ml) and biochemistry (3 ml) and serology (5 ml) tests will be taken at initial screening. Blood for biochemistry tests will be taken just before each vaccination, and 4 weeks after each vaccination.

(ii) Blood (5 ml) for anti-malarial antibody research tests will be collected at initial screening, just before vaccination, four weeks after each vaccination and then monthly until 12 months. If subjects are available beyond the first 12 months, they may be asked to give an additional 5 ml of blood at 12, 18, and 24 months. Blood will be allowed to clot at ambient temperature for one hour. Serum will be separated and frozen at -20 C or below.

(iii) Blood (20 ml) for lymphocyte blastogenesis and cytokines (Ballou et al. 1987) will be collected before each vaccination, and at 12 months. Lymphocyte blastogenesis will not be done if and when malaria develops.

(iv) Urine (10 ml) at initial screening for urinalysis.

(v) Blood (finger prick) for thick and thin blood slides, in duplicate will be taken once every two weeks for 12 months, and whenever symptomatic malaria is suspected. Slides will be read blinded by two microscopists with positive and negative controls. All slides will be saved.

(vi) Mosquitoes will be collected at least weekly from the houses of the volunteers receiving vaccine or placebo by the routine day resting method (Beier et al. Manuscript)

(2) Evaluations to be made on specimens: Laboratory research studies on specimens may be performed at both Kenya Medical Research Institute and Walter Reed Army Institute of Research to establish reproducibility and to confirm results. Specimens may be sent to collaborators at other Institutes, to perform specialized tests or to confirm results.

(i) Blood will be used for routine complete blood count and biochemistry tests.

(ii) Blood will be used for serology: hepatitis B surface antigen, antibody, and core antibody; human immunodeficiency virus (HIV) antibody (if malaria develops), and for antibody responses to vaccines by ELISA (Young et al. 1985; Wirtz et al. 1987) using recombinant proteins as capture antigens. Antibody will be compared to pre-immunization levels, reported as absorbance or as optical density (O.D.) units [the highest dilution with an optical density of 1.00]. Vaccine will be considered immunogenic if at two weeks after the last dose there is a mean absorbance or O.D. units two standard deviations greater than pre-immunization levels. Serum will be tested by published methods for antibody by indirect immunofluorescent staining of glutaraldehyde fixed (Young et al. 1985) and fresh (Hommel et al. 1983) blood stage parasites, by circumsporozoite precipitation test using live sporozoites (Vanderberg et al. 1969), and by inhibition of sporozoite invasion (ISI) into cultured hepatoma cells (Hollingdale et al. 1984). If additional in vitro tests are developed which may be useful in evaluating the immune response, they may be performed on the remaining sera or cells.

(iii) Blood will be used for lymphocyte blastogenesis to assay cellular immunity and cytokines by published methods (Ballou at al 1987). Some of these cells may be frozen for later use.

(iv) Urine will be used for routine urinalysis.

(v) Finger prick blood slides in duplicate will be read for malaria parasites, independently by two microscopists. Known positive and negative slides will be included as blind standards, for quality control. All slides will be saved for later review.

(vi) Mosquitoes will be dissected for sporozoites and the species of sporozoite determined by ELISA (Beier et al. MS) and examined for blood type of blood meals to estimate the challenge presented to the volunteers and house members, knowing their blood types.

(3) Storage (where and how): Sera will be stored at -20 C or below at the Kenya Medical Research Institute and the Walter Reed Army Institute of Research. Peripheral blood mononuclear cells and parasites will be cryopreserved using published methods (Barnwell et al. 1983) and stored at the Kenya Medical Research Institute.

(4) Labeling and disposition: Serum or cells will be labeled with the volunteers name, study number, and date.

b. Clinical assessments (including record of adverse effects).

(1) Clinical evaluations of subjects will be made over 6 months to evaluate clinical illness and frequency of parasitemia.

(2) Clinical side effects will be evaluated at 20 minutes, 1 day, 2 days, 2 weeks and 4 weeks after each vaccination, including

fever, tenderness, swelling, warmth, or erythema at the injection site, or lymphadenopathy. The next dose will be administered only if serious side effects have not occurred. The following side effects will be considered serious: fever above 40 C, local or regional inflammation which requires drug therapy, or any other side effect which in the judgement of the responsible physician suggests the vaccine may not be safe. Symptoms and physical findings will be recorded in tables (See Appendix).

(3) Evaluation of vaccine efficacy against P. falcioarum:

(i) Clinical observation: All volunteers will be asked to participate in clinical observation after vaccination for symptoms and signs of malaria and in parasitological observation for malaria parasites in the blood. Subjects will be seen every day by a clinical field worker to answer whether they feel well or ill. Once every 2 weeks for six months, or if symptomatic malaria is suspected, volunteers will have a finger prick for thick and thin blood slides. Blood slides will be examined by two microscopists. All blood slides, both positive and negative, will be saved. Malaria may also be diagnosed using acridine orange immunofluorescent staining (Rickman et al. 1989).

(ii) Entomological evaluation: From daytime resting house collections of mosquitoes and individual mosquito dissection and sporozoite species determination and blood meal blood type, the individualized and average falciparum challenge will be estimated during the study.

c. Vital signs: Vital signs will be performed at initial screening, just before vaccination, 20 minutes after vaccination and when clinically indicated.

d. Follow-up procedures:

(i) Management of volunteers who contract malaria during the study: If fever or other clinical manifestations develop, blood slides will be obtained as frequently as clinical judgement indicates. The volunteer will be evaluated by the study physician or clinical officer. Volunteers who develop symptomatic malaria will be treated. Volunteers may be treated with sulfadoxine 500 mg-pyrimethamine 25 mg (Fansidar) tablets, three tablets at once, or quinine sulfate 600 mg three times per day for three days plus doxycycline hydrochloride 100 mg twice per day for 10 days. The choice of anti-malaria drug to be given will be determined by the best judgement of the pnysician present. Volunteers with malaria will be seen daily and have blood slides daily until three successive negative blood slides are obtained. Volunteers will be examined weekly for 8 weeks after curative treatment.

e. Disposition of data: Data will be recorded in tabular form . Copies of data sheets will be kept filed at the Kenya Medical Research Institute for 10 years following the completion of the

study. The Human Use and Regulatory Affairs Office at Fort Detrick will be notified of the status of the data kept on file.

f. Who will perform statistical reviews: Statistical consultation will be obtained as necessary. We assume the acquisition of falciparum malaria, as defined by positive blood slide following treatment with sulfadoxine-pyrimethamine, in the control group will be 90% by 126 days (4 months) (Hoffman et al. 1989). We assume a vaccine efficacy of at least 70%. We will need 21 volunteers in each group to obtain significance of 0.05 and power of 0.90 (Fleiss 1981 and see appendix). The number of volunteers with positive malaria slides and the number of volunteers with a change in antibody level in the vaccine and placebo groups will be compared using chi square or Fisher's exact test. The mean change in antibody level in the vaccine and placebo groups will be compared using Student's t-test.

g. Facilities: Clinical work will be carried out at the Saradidi Rural Health Programme; and if necessary the Nyanza General Hospital, Kisumu. Laboratory work will be carried out at the Kenya Medical Research Institute, Kisumu and Nairobi; and the Walter Reed Army Institute of Research, Washington, D.C.